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Address: Veterinary Faculty, Gerbičeva 60, 1000 Ljubljana, Slovenia
Naslov: Veterinarska fakulteta, Gerbičeva 60, 1000 Ljubljana, Slovenija
Tel.: +386 (0)1 47 79 100, 47 79 129, Fax: +386 (0)1 28 32 243
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Slov Vet Res 2019; 56 (Suppl 22)

Original Research Articles

Atiba A S, Ghazy A, Farrag F A, Abu El-Magd M, Almadaly E A. Ultrasound-guided brachial plexus nerve block in donkeys....7

Gad El-Karim D RS, GA El-Amrawi. Cyclophosphamide hepatotoxicity: the role of 4-hydroxynonenal and cytochrome C oxidase and the possible protective effect of *Ganoderma lucidum* extract....15

Ismail T, Nassef ED, Hegazi ES, Bakr AN, Moustafa E M, Abdo W, Elbialy Z I. The modulatory effect of dietary betaine on intesti-nal absorptive capacity, lipogenesis and expression of lipid metabolism- and growth-related genes in Nile tilapia fed on soybean meal-based diet....25

Abou-ElnagaA F, Rehan I F, Thompson R RJ, Abou-Ismail U A, Mahmoud M E, Mohamed R A, Mohammed H H, Sabek A A, Elhussiny M Z. The effects of group versus single housing on home cage behaviour in two strains of laboratory mice....39

El-Sheshtawy S, El-Gobary G, Omar N, Shawky N. Ameliorating the toxic effects of cypermethrin by sesame oil in male rabbits....51

Abdel-Razek M A, Abozeid A M, Eltholth M M, Abouelenien F A, El-Midany S A, Moustafa N Y, Mohamed R A. Bioremediation of a pesticide and selected heavy metals in wastewater from various sources using a consortium of microalgae and cyanobacteria....61

Elmadawya M A, El-Kassasb S, Abdoc S E, Nagaid A, Bunaid Y. Genetic diversity among two common populations of *Canis lupus familiaris* in Egypt by using mitochondrial DNA HVR1 sequence....75

Magouz F, Salem M, Moustafa E, Elkhamy S. Impact of Biomos and Agrimos dietary supplementation on growth performance, feed utilization and immunological parameters of Nile tilapia (*Oreochromis niloticus*) fingerlings....87

Dawood M A, Shukry M, Zayed M M, Omar A A, Zaineldin A I, El Basuini M F. Digestive enzymes, immunity and oxidative status of Nile tilapia (*Oreochromis niloticus*) reared in intensive conditions....99

Ali M, Eweedah N, Gaafar H, Abd El-Salam M. Utilization of sugar beet by products in feeding growing rahmani lambs....111

Elkomy H E, Taha A E, Basha H A, Abo-Samaha M I, Sharaf M M. Growth and reproduction performance of japanese quails (*Coturnix coturnix japonica*) under various environ-ments of light colors....119

Shewita R S, El-Naggar K, Abd El Naby W SH. Influence of dietary vitamin C supplementation on growth performance, blood biochemical parameters and transcript levels of heat shock proteins in high stocking density reared broiler chickens....129

Mohammed H H, Rehan I F, Abou-Elnaga A F, Mohamed R A. Effects of feeder shape on behavioral patterns, performance and egg quality traits of Japanese quail....139

Mohammed H H, Abdelaty A I, Saleem A Y, Youssef M I, Abdel-Hamid S EL. Effect of bedding materials on duck's welfare and growth performance....149

Meligy A MA, Al-Taher A Y, Ismail M Al-Naeem, A A, El-Bahr S M, El-Ghareeb W R. Pesticides and toxic metals residues in muscle and liver tissues of sheep, cattle and dromedary camel in Saudi Arabia....157

Salah HM, Moussa EA, SM El. Bakr, Atta MS, Farrag FA, Hafez AS. Green tea mitigates streptozotocin-induced diabetic mice through antifibrotic activity and modulation of proinflammatory cytokines....167

Donia MA, Gomaa NA, Abdelmegeid M, Nassif MN. Biomarkers versus duplex ultrasonography for early detection of acute kidney injury in dogs: an experimental study....179

Kamal M A, Khairy M H, ELSadek N A, Hussein M MA. Therapeutic efficacy of zinc oxide nanoparticles in diabetic rats....187

El-Shawadfi A, El-Khyat F, El-Gohary A. Identification of virulent Newcastle disease viruses in broilers in kafrelsheikh governorate through the year 2017....195

Mansour A, Mahfouz NB, Husien MM, El-Magd MA. Molecular identification of *Aeromonas hydrophila* strains recovered from kafrelsheikh fish farms....201

El-Sharawy M E, Mashaly I M, Atta M S, Kotb M, El-Shamaa I S. Influence of body condition score on blood metabolites and oxidative stress in pre- and post-calving of Friesian dairy cows in Egypt....209

Abdelfattah-Hassan A, El-Ghazali H M. Effects of diet's acidifying additives on the intestinal histomorphology in ducks....219

Magouz F I, Khalafalla M M, Moustafa E M, Sadek A S. The impact of different stocking denesities and dietary protein levels on the performance of African catfish....229

Ramoun A, E Almadaly, Hattab H, Darwish S, EL- Kon I. Transrectal ultrasonography and rectal palpation for judging uterine and cervical involutions in buffalo: a comparative study....239

El-Ghareeb W R, Almathen, Fayez M M, Alsultan R A. Methicillin resistant *Staphylococcus aureus* (MRSA) in camel meat: prevalence F S and antibiotic susceptibility....249

Alattar R, Abdel Alim A, Abdelmetal S, Abdel Aziz S. Corticosteroid-induced osteoporosis and osteonecrosis: role of oxidative stress....257

Al-Sultan S I, Abdel-Raheem S M, Abd-Allah S M S, Edris A M. Alleviation of chronic heat stress in broilers by dietary supplementation of novel feed additive combinations....269

Elkhenany H, Nabil S, Abu-Ahmed H, Mahmoud H, Korritum A, Khalifa H. Treatment and outcome of horses with cutaneous pythiosis, and meta-analysis of similar reports....281

Khalafalla M, Mohamed A, Zayed M, Metwally A. Using of animal by products as an alternative protein source in Nile tilapia (*Oreochromis niloticus*) diets....293

Kholife M, Moawad A, Diab A, El-keredy M. Mycological examination of fish feed stuff with special reference to mycotoxin production....303

Saleh E A, Al-Hawary I, Elnajar M M. Antibacterial and anti-oxidant activities of laurel oil against *Staphylococcus aureus* and *Pseudomonas fluorescence* in *Oreochromis niloticus* fillets....313

Mahfouz N, Moustafa E, Kassab M, Marzouk W. Seasonal Screening of the Mycotic Infections of Cultured Freshwater Fishes in Kafr El-Sheikh Governorate....321

Abdelhakam O, Elsebaie E, Ghazi A, Gouda M. Quality characteristics of beef hamburger enriched with red grape pomace powder during freezing storage....333

El-Katcha M, Soltan M, El-Naggar K, El-Shobokshy S, El-Erian M. Laying performance, fat digestibility and liver condition of laying hens supplemented with vitamin B_{12} or biotin and/ or bile acids in diet....341

Abdel-Raheem S, Mahmoud G, Senosy W, El-Sherry T. Influence of vitamin E and selenium supplementation on the performance, reproductive indices and metabolic status of Ossimi ewes....353

Moselhy A, Mahdy E. Comparative three dimensional computed tomography (CT) scans and anatomical investigation of rabbit (*Oryctolagus cuniculus*) and cat (*Felis domestica*) skull....365

Nassef E, Hegazi E, Sherif A, Elshamy M, Bakr A, Abouzed T. Impact of altering dietary omega 6 to omega 3 fatty acids ratio on growth performance, carcass composition, hemato-biochemical parameters and absorptive capacity of the intestine of Nile tilapia....381

Al-Mallah K, Hussien M. Monitoring of pathological lesions in non-gravid ovine uterus....391

El-Nagar S, Helal M, Mahmoud S, Dillard L. Comparative histopathological changes of liver, kidney and appendix of rabbits treated with inorganic nano chromium to ameliorate heat stress effect....399

Dawood M, Mohsen M, El-dakar A, Abdelraouf E, Moustafa E, Ahmed H. Effectiveness of exogenous digestive enzymes supplementation on the performance of rabbitfish (*Siganus rivulatus*)....409

Abdelaal A, Elgioushy M, Gouda S, El-Adl M, Hashish E, Elgaml S, Ali M, El-Gedawy A. Hemato-biochemical and molecular markers (IS900) of cattle infected with Johne's disease in Egypt....421

Omar A, Gharib H, Said E. Effect of feeding different concentrate roughage ratio on growth, reproductive performance and behavior of sheep....433

Abd-Elfatah E, El-Mekkawi M, Aboul-Soud E, Fawzi E, El-Soally S. Immunological response of a new trivalent Capripoxvirus vaccine in pregnant ewes and does....445

Hafez Y M, Moustafa N Y, Magouz A F, Al-Maria N F. Isolation of white spot syndrome virus (WSSV) in Egyptian shrimp using conventional PCR and real time PCR (QPCR) techniques....457

El-shafaey E, Sayed-Ahmed M, Shoeib S, El-khodery S. Occurrence and risk factors of digital dermatitis in Egyptian dairies....465

Gaffer W, Gwida M, Abo Samra R, Al- Ashmawy M. Occurrence and molecular characterization of extended spectrum beta-lactamase producing *Enterobacteriaceae* in milk and some dairy products....475

Mokh A., Abdelhady D., Ghazy E., Aboumosalem H., Goda W. Sesame oil mitigates initiation stage of diethynitrosamine hepatocarcinogenesis in rats....487

Salama M, Morsy W, Mohamed R, El-Midany S. Effect of some feed-additives on the growth performance, physiological response and histopathological response and histopathological changes of rabbits subjected to ochratoxin-A feed contamination....499

El Zeny T, Essa R Y, Bisar B A, Metwalli S M. Effect of using chicory roots powder as a fat replacer on beef burger quality....509

Mostafa N, Kirrella G, Aideia H, Abo Shaisha J. Assesstment of mould contamination of Tilapia nilotica and *Mugil cephalus* fish and trials to reduce using natamycin....515

Salem A, El–Awady H, Tag EL-Dein M, Eisa D. Effect of aromatic plants oils on immunity, udder health and milk production of Friesian cows....523

Elsheikh H, El-Mekkawi M, Abou-zaid A, Abd El Raof A. Diagnosis and phylogenetic analysis of the circulating peste des petits ruminants virus in Al-Sharqia governate....531

Almadaly E, Tawfik F, El-Kon I, Heleil B, Fattouh El. Effect of cryoprotectants on *in vitro* semen quality and *in vivo* fertility of buffalo (*Bubalus bubalis*) bull semen....541

Ghazy E., Mokhbatly A., Keniber S., Shoghy K. Synergistic ameliorative effect of *Lactobacillus* and *Spirulina platensis* against expermintal colitis in albinorats: antioxidant, histopathological and molecular studies 553

Ebissy E, El-Sayed A, Mohamed R. Hematological and biochemical profile in female camels (*Camelus dromedarius*) during the transition period 571

Ramou A, EL- Kon I, Almadaly E, Aly A, Abo EL- Ghate H. Assessment the synchrony between uterine status and hormonal profiles in modified Ovsync protocols in relation to fertility in buffaloes 579

Shoeib S, Sayed-Ahmed M, El-khodery S. Hypomagnesemic tetany in camel calves (*Camelus dromedarius*): clinical consequences and treatment outcomes 589

Elblehi S S, Gad El-Karim D R. Potential amelorative effect of *Panax ginseng* on hepatorenal damage ocuured in hydroxurea-treated rats 595

Abdel-Hay A M, Hamed S A. Zayd M M, Abouelenien F A, Rehan I F, El-Kassas S, Deyab A M, Mohamed R A. Night feeding regime improves water quality, growth performance and feeding behaviour of Eropean eels, *Anguilla anguilla* 607

Sherif A A, Nada H S, Moawad A A, El- desouky I E, Helmy S M. Using real-time polymerase chain reaction to study the effect of salicylic acid and sodium chloride on the pyocyanin production of *Pseudomonas aeruginosa* 615

ALomaisi S A, El-Ghazali H, Nosseur H, Ahmed S, Konsowa M. Prenatal development of the vomeronasal organ in rabbit 623

Elbaz S, Nassef E, Bakr A, Hegazi E, EL-keredy A. Impact of dietary eicosapentaenoic and docosahexaenoic fatty acids supplementation on inflammatory response of post calving cows during transition to lactation 633

Moawad A, Noor El Deen A, Mahfouz N, Helmy S, Hashem Y Phenotypic and molecular identification of yeasts isolated from cultured tilapia (*Oreochromis niloticus*) in Kafrelsheikh province, Egypt 647

Hafez Y, Sobeih A, Mansour N. Pivotal role of *Lactobacillus* strains in improvement of soft cheese quality and inhibiting the growth of harmful and dangerous bacterial pathogens 657

Kamal T, Dorghamm D, Kahilo K, Elkattawy A, Nassef E, El-sawy H. Impact of L-carnitine supplementation on growth of broiler chicken through determination of changes in the expression of CAT2, MYOD and MYF5 genes 665

Ragab A, Nasr N, Kahilo K. Protective effect of glycyrrhizc acid against carbon tetrachloride-induced liver fibrosis in rats: role of integrin subunit β LIKE 1 (ITG B L1) 673

Shireen S, Nasr E, Khaled A, Hossam E. Hemotoxic and genotoxic effects of lead acetate and chlorpyrifose on freshwater cat fish (*Calarias gariepinus*) 681

Allam S, Mostafa N, Kirrella G, Eleiwa N, El-Magd M. Molecular detection of inva and hila virulent genes in salmonella serovars isolated from fresh water fish 693

Omar M A, Abdel-Hamid T M, Esam S, Omar A E. Growth and economic performance of using dried tomato pomace for Mallard ducks 699

Abdel-hamid T M, Omar M A. Growth and economic performance, some hematological, biochemical and oxidative stress parameters, and carcass traits of summer stressed Californian and crossbred rabbits as affected by dietary supplementation of formic acid 707

Aziz M, Zahra A, Kheder Z, Fikry H. The role of L. methionine, L. carnitine, choline and/or silymarin in hepatoprotection against paracetamol intoxication and oxidative stress in broilers 717

Shabana S, Helmy S, Hegazy A E. Characterization of class 1 integrons and some anti-microbial resistance genes in *Salmonella* species isolated from poultry in Egypt 725

Ghazy E, Mokh A, Abdelhady D, Goda W, Hashem E. The role of thymoquinone in ameliorating the hepatoxic effect of diazinon in male rats 735

Elmonir W, Essa H, El-Tras W. Ecology of *Staphylococcus aureus* and its antibiotic resistance genes in dairy farms: contributing factors and public health implications 747

Elmonir W, Aglan H, Elmahallawy E, El-Tras W. Antimicrobial phenotypes of geographically matched *Staphylococcus aureus* isolated from buffalo's milk and clinical human cases in Egypt: potential zoonotic risks 755

Abu El-Hamd M, Metwally A, Hegazy M, Ghallab Z, Elateeqy O. Effect of supplementation of omega-3 fatty acids on blood parameters and semen quality of Friesian bulls 765

Omara N A, Elsebaie E, Kassab H, Salama A. Production of chitosan from shrimp shells by microwave technique and its use in minced beef preservation 773

Abdelmoez N S, Shawky M M, Abdelhady H A, Lebdah M A, Salama S S. Isolation and identification of some possible causative agents of Swollen Head Syndrome (SHS) in broiler chickens in Egypt 781

Salama S, Abdelhady H, Atia L. Field application for experimental inactivated multivalent *P. multocida* and avian influenza (H9N2) vaccine in poultry 789

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Original Research Article

ULTRASOUND-GUIDED BRACHIAL PLEXUS NERVE BLOCK IN DONKEYS

Ayman S. Atiba[1]*, Alaa Ghazy[1], Foad A. Farrag[2], Mohammed A. El-Magd[2], Essam A. Almadaly[3]

[1]Departments of Surgery, Anesthesiology and Radiology, Faculty of Veterinary Medicine, Kafrelsheikh University, 33516, Egypt, [2]Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Kafrelsheikh University, 33516, Egypt, [3]Department of Theriogenology, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt

*Corresponding author, E-mail: atiba_2003@yahoo.co.uk

Abstract: This study was conducted to describe and evaluate the ultrasound-guided technique and the anatomical relation for brachial plexus block in donkeys. In the first study, three donkey's cadavers were used to determine the neuroanatomical description of the brachial plexus. In the second study, four donkeys were used to evaluate the ultrasound structure of brachial plexus and the distribution of methylene blue solution that was injected around the nerves during subsequent postmortem anatomical dissection. In the third study, six donkeys were used to evaluate the efficient of the ultrasound-guided brachial plexus block after injection of lidocaine. Ultrasound was used to identify brachial plexus and blood vessels. Anatomical scrutiny assured the relationship between the nerves and the blood vessels. The nerves were adequately stained with methylene blue solution in all donkeys. A volume of 25 mL of lidocaine per injection site resulted in effective sensitive and motor block of forelimbs nerves after brachial plexus block. Based on the obtained data we could conclude that brachial plexus nerve block guided by ultrasound can be used in surgery of donkey distal fore limb.

Key words: ultrasound; brachial plexus; nerve block; donkey

Introduction

The donkey (*Equus asinus*) is the most important working animal in many parts of the world (1). Donkeys provide one of the most cost effective sources of transport in the rural agricultural sector. There are an estimated 59 millions of donkeys and mules in the world (2). Although greater populations of donkeys live in developing countries, very little scientific information specific for this species exists in the literature.

Peripheral nerve block techniques are becoming increasingly popular for performing certain surgeries in both animals and humans (3-5). These techniques do not only decrease general anesthesia drawbacks but they also lead to effective analgesia (6-8). In animals many surgical operations especially in the distal parts of the limbs can be performed under peripheral nerve block such as sciatic and femoral nerves block of the pelvic limbs in cattle, sheep and dogs (9-11) and brachial plexus nerve block in the forelimbs in cattle, dogs and cats (3, 5, 7, 9).

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In humans, brachial plexus block is very universal and safe, and allows anesthesia for the upper limb. Therefore, various operations, principally orthopedic, could be carried out under this block (12, 13).

Several techniques exist to produce local anesthetic blockade of the brachial plexus include the use of anatomic landmarks (blind technique), a nerve locator technique, and ultrasound guidance technique. Many studies comparing these techniques and all stated that ultrasound-guided one is the most effective technique to facilitate the peripheral nerve block (8, 14, 15).

Previous literatures lack detailed description for neuroanatomy and block technique of brachial plexus in donkeys. Thus, this study was designed to describe and evaluate the ultrasound-guided technique and the anatomical relation for brachial plexus block in donkeys.

Materials and methods

The experimental protocol was approved by the Animal Care and Use Committee of Faculty of Veterinary Medicine, Kafrelsheikh University.

Study 1: Anatomical study of the brachial plexus

For the anatomical dissection of the brachial plexus, cadavers of three donkeys (23 ± 2 months old, weighing 167 ± 10 kg) were used. The forelimb of the three donkeys were separated carefully from the trunk by cutting the skin, the pectoralis superficialis and pectoralis profundus muscles at their origin on the sternum, brachiocephalicus and trapezius muscles at the cranial border of scapula, serratus ventralis, rhomboideus muscles at their insertion near the dorsal border of scapula and latissmus dorsi muscle at the caudal border of scapula. The forelimb was abducted to expose the axillary artery and brachial plexus. The nerves and main blood vessels were dissected and identified. Finally, the dissected region was photographed.

Study 2: Ultrasound-guided brachial plexus blockade in cadavers

The ultrasound images of the brachial plexus blockade of both forelimbs from four donkeys (18 ± 4 months old, weighing 181 ± 18 kg) were taken using a high frequency 5–10 MHz linear transducer (Mindray Diagnostic Ultrasound System, Z5, Germany). The transducer was placed in front of the cranial border of scapula and the first rib and dorsal to the shoulder joint (Fig. 1C). Color Doppler mode was utilized to aid vessels identification. The location of brachial plexus nerves were injected with 25 mL methylene blue (Sigma-Aldrich, USA) using a 20 gauge, 90 mm spinal needle. The needle was inserted just ventral to the ultrasound transducer and vertical to the nerves then methylene blue was injected when the needle tip reached a depth of 1 mm. After injection, donkeys were euthanized and injection site was dissected to determine staining distributions around nerves.

Study 3: Ultrasound-guided brachial plexus blockade in live donkeys

For the ultrasound-guided brachial plexus blockade, six donkeys (aged 19 ± 7 months and weighing 188 ± 41 kg) were used. The brachial plexus nerves were blocked unilaterally. Each donkey was used for three times with 1 week interval. The donkeys were mild sedated with 0.3 mg/kg IV xylazine (Xyla-Ject, ADWIA Pharmaceuticals Co. Cairo, Egypt). In the standing position, the skin of the craniolateral aspect of the first rib was clipped. The brachial plexus were blocked with 25 ml of a lidocaine (Debocaine 2% Al-Debeiky Pharmaceutical Industries Co., Egypt). To evaluate analgesia, a pin-prick test was applied with a 21 gauge needle inserted through the skin. The sensitive response was scored using a three-point numerical rating scale (Table 1). The areas of sensitive responses were evaluated according to the sensory branches of brachial plexus (Table 2).

The motor effect was evaluated as previously described (3) and was scored using a three-point numerical rating scale (Table 1). The sensitive and motor responses of the forelimb were evaluated and compared before and after lidocaine injection. Twelve injections were given with 10 min intervals. Moreover, each injected forelimb was compared with the other contralateral non-injected forelimb.

Statistical analysis

Descriptive statistical data are expressed as medians and ranges. The effects of local anesthetic on brachial plexus in the various anatomical regions were analyzed by the non-parametric Friedman test with pairwise comparisons using SPSS for Windows Version 16 (SPSS Inc., IL, USA). A value of $P < 0.05$ was considered significant.

Results

Study 1: Anatomical study and dissection of the brachial plexus nerves

The gross dissection of donkeys brachial plexus revealed that it was composed of the anastomosis of the ventral branches of the last three cervical (6^{th}, 7^{th} and 8^{th}) and the first two thoracic spinal nerves. The brachial plexus was located cranial to the first rib and dorsal to scalenius medius muscle. It was covered laterally by subclavius and supraspinatus muscles slightly above the shoulder joint (Figs. 1A and B). The brachial plexus gave many branches which innervated the skin and muscles of forelimb in addition to parts of the lateral wall of thorax and abdomen as well as parts of the shoulder girdle (Fig 1B). The branches of brachial plexus either motor or sensory were summarized in table 2.

Study 2 and 3: Ultrasound-guided nerve blockade

The brachial plexus was identified as a hyperechoic band with several intermittent hypoechoic strips separated by hypoechoic lines (Figs. 2A-C). These hyperechoic structures were confirmed to be brachial plexus after utilizing color Doppler mode, which showed the axillary blood vessels ventral to these hyperechoic structures (Fig. 2B). The ultrasound transducer was placed cranially near the first rib. In this position the needle was advanced above the brachial plexus and axillary blood vessels. After assuring that blood was not withdrawn, methylene blue was injected. The extent of dye was monitored as an anechoic area proximal to the brachial plexus. Staining of brachial plexus was observed in all donkeys. The dye was spread more than 2 cm in 3 donkeys (Fig. 1D). During this procedure, only one animal was intravascularly injected in axillary vessels.

Figs. 2D and E show the scores of the sensitive and motor effects after ultrasound-guided brachial plexus block in 16 forelimbs. One animal was died after 1 week with a reason not related to the current study. The sensory score for the blocked area showed a significant increase at 30, 40 and 50 min in musclocutaneous nerve area, at 30 and 40 min for the area of median nerve, and at 30 min for the area of ulnar nerve ($p < 0.05$). The motor scores increased significantly at time points 20, 30, 40 and 50 min. A motor effect was extended more than the sensory effect in all examined animals.

Discussion

To the best of our knowledge, this may be the first study to investigate the ultrasound-guided brachial plexus blocking in donkeys. With this study we were able to demonstrate the feasibility to block the brachial plexus in donkeys with a highly successful rate.

Table 1: The score of sensitive and motor response (3)

Score	Sensitive response	Motor response
0	Normal response	Normal gait while walking and no abnormal clinical sign while standing.
1	Diminished response	Donkey can walk while bearing mild to moderate weight and shows no abnormal sign while standing.
2	No response	Donkey can walk but is barely able to bear weight while walking, and shows abnormal signs while standing.

Table 2: Summary of branches of brachial plexus and its motor and sensory innervation

Branches of brachial plexus	Innervation	
	Motor	Sensory
Long thoracic nerve	Thoracic part of serratus ventralis muscle.	
Dorsal thoracic nerve	Latissmus dorsi muscle.	
Lateral thoracic nerve	Cutaneous trunci muscle	Skin of lateral thoracic wall
Cranial pectoral nerve	Superficial pectoral and subclavian muscles	
Caudal pectoral nerve	Deep pectoral muscles	
Suprascapular nerve	Supra spinatus and infra spinatus muscles	
Axillary nerve	Deltoid, teres major, teres minor, capsularis humeri and cleidobrachialis muscles	Skin of craniolateral aspect of brachium and cranial aspect of antebrachium
Subscapular nerve	Subscapularis muscle	
Musculocutaneous nerve	Coracobrachialis, brachialis and biceps brachii muscles	Skin of medial part of antebrachium
Radial nerve	Triceps brachi, anconeus, tensor fascia antebrachii, extensor carpi radialis, common digital extensor, lateral digital extensor, extensor carpi ulnaris and extensor carpi obliqus	Skin of the lateral aspect of brachium and antebrachium
Ulnar nerve	Flexor Carpi Ulnaris, superficial digital flexor and ulnar head of Flexor Digitorum Profundus, interosseous muscles	Skin of the caudal aspect of antebrachium and dorsolateral aspect of metacarpus and digit.
Median nerve	Flexor carpi radialis, humoral and radial head of deep digital flexor muscles	Skin of the palmar aspect of metacarpus and digit.

The detailed description of Equine forelimb nerve supply has been found in several standard anatomical textbooks (16-19). Briefly, this nerve supply comes from the brachial plexus which is a complex network of nerves formed by the anastomoses of the ventral roots of some spinal nerves extending from the 6^{th} cervical nerve to the 2^{nd} thoracic nerve. It passes between the two parts of the scalenius medius muscle and the medial aspect of shoulder in the axillary region. The major nerves emanated from the brachial plexus are the suprascapular, subscapular, musculocutaneous, axillary, radial, median, and ulnar nerves. Brachial plexus supplies sensory and motor innervation of the forelimb (20). The previous description of the nerve supply of forelimb in horses coincides with the findings of the current study regarding the brachial plexus position and nerves emanating from it in donkeys. The higher degree of similarity between donkeys and horses might be attributed to close relation between the two species which belong to the same family (Equidae) and genus (Equus).

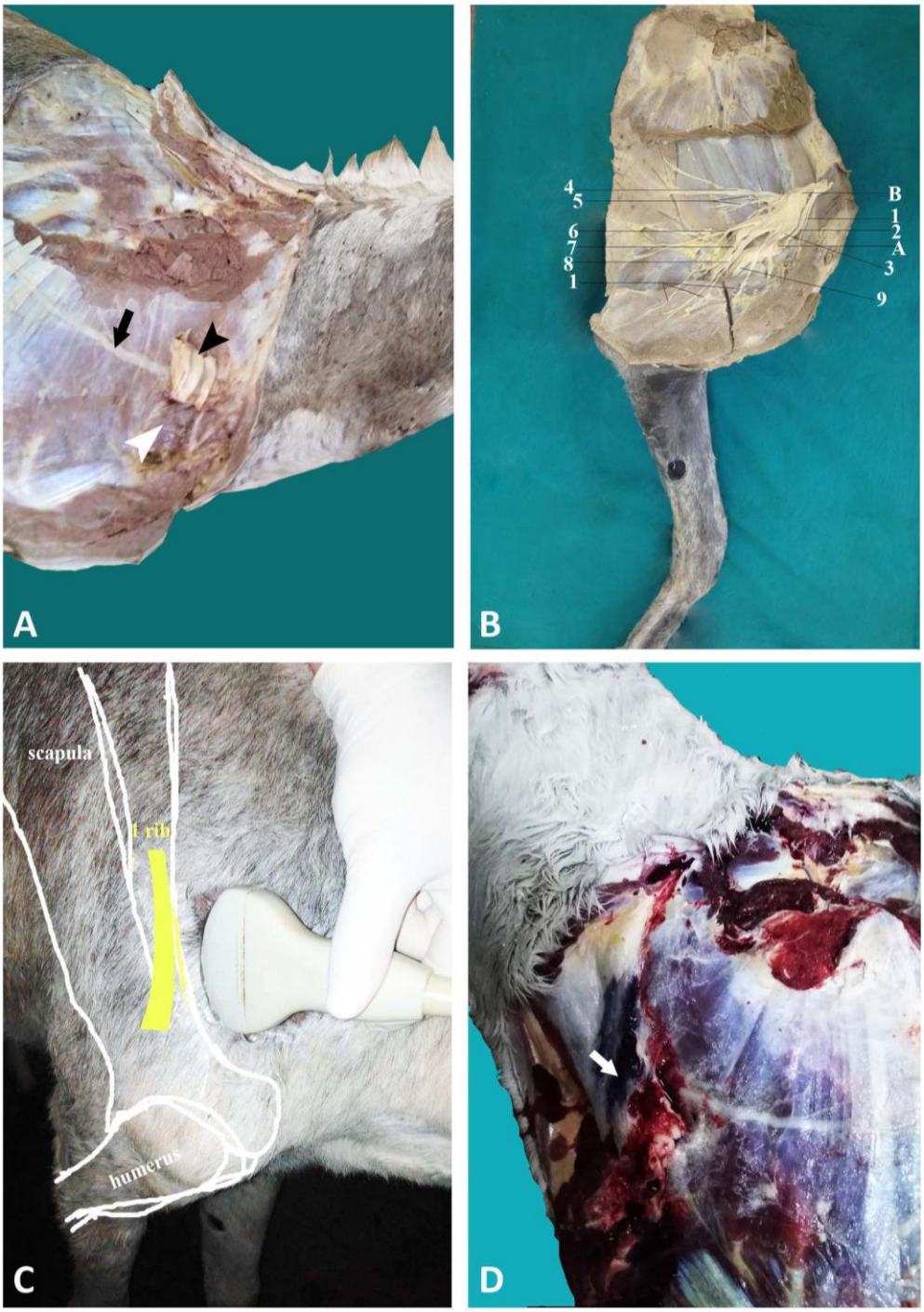

Figure 1: Position, branches of brachial plexus and site of ultrasound transducer. A. Position of brachial plexus (black arrowhead), scalenius medius muscle (white arrowhead) and long thoracic nerve (black arrow). B. Axillary artery (A) surrounded by branches of brachial plexus (B); pectoral nerves (1), musculocutaneous nerve (2), median nerve (3), dorsal thoracic nerve (4), subscapular nerve (5), lateral thoracic nerve (6), radial nerve (7), ulnar nerve (8) and the musculocutaneous with median nerves (9). C. Position of ultrasound transducer in front of cranial border of scapula and first rib and dorsal to the shoulder joint. D. Site of injection of methylene blue dye (white arrow) in the region of brachial plexus

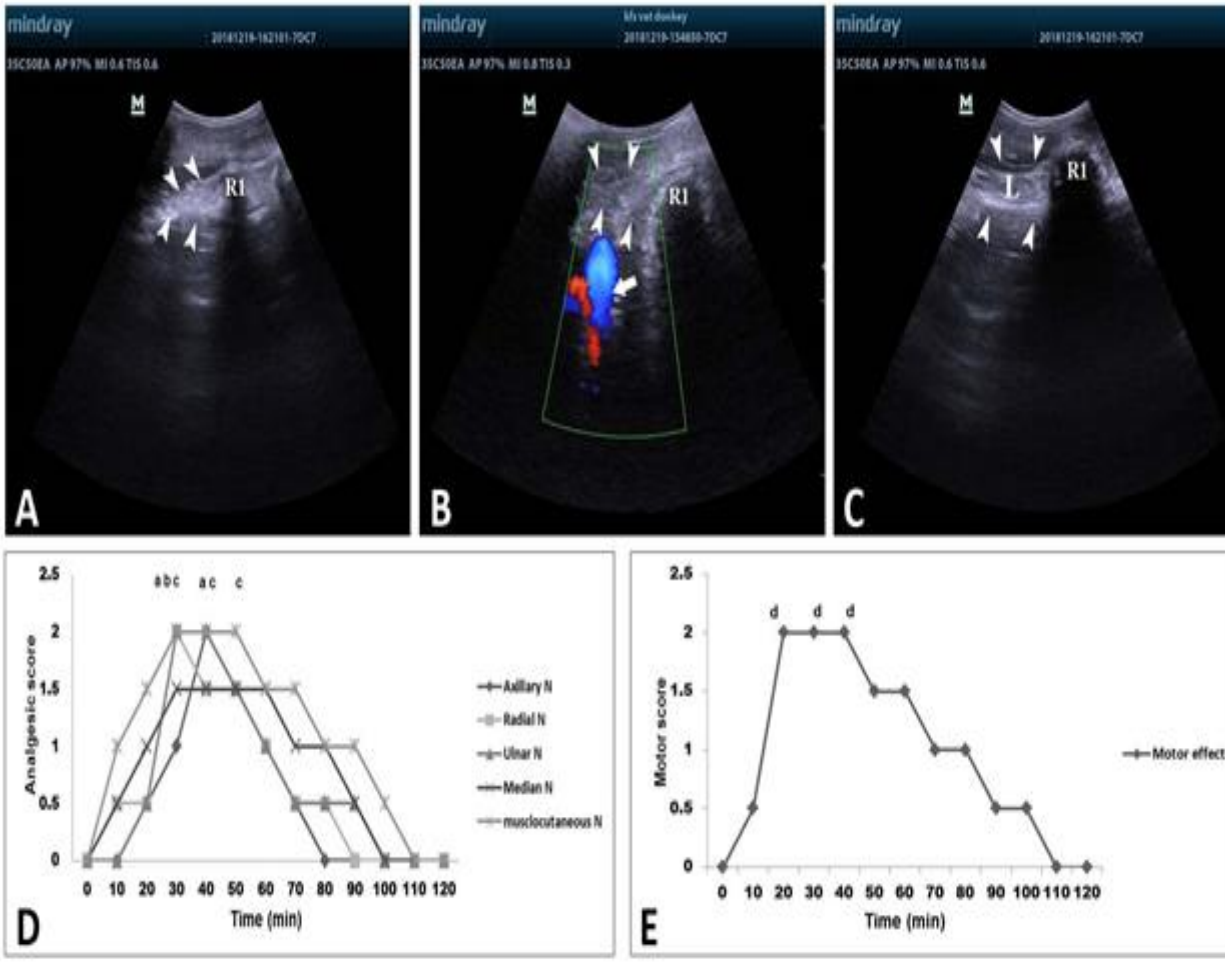

Figure 2: Ultrasound images of the area around the brachial plexus, the sensory and motor score after nerve block. A. Ultrasound image of the brachial plexus (the area surrounded by the arrowheads) and the first rib (R1). B. Color Doppler ultrasound image of the brachial plexus (the area surrounded by the arrowheads) and the axillary blood vessels (arrow). C. Ultrasound image of the brachial plexus after injection of local anesthetic drug (L). D. Sensitive and motor (E) scores after ultrasound-guided brachial plexus block in donkeys. Data are expressed as the median. Symbols indicate significant differences from baseline value within a defined anatomical subarea ($P < 0.05$)

Currently, the use of the ultrasonography has been reported to provide an accurate guide for performance of peripheral nerve block when compared with the use of conventional blind or electrolocation techniques (8, 15). This technique provided the ability to administer a calculated amount of anesthetic drug at the target site under real-time observation (7, 14). Additionally, it minimized complications like maldistribution of local anesthetic or intravascular needle placement (21). In the present study, among all injected cases, only one case exhibited intravascular insertion of the needle.

Peripheral nerves may look hypoechoic or hyperechoic on ultrasound image (22). In the present study, the brachial plexus appeared hyperechoic structures dorsal to the axillary vessels. Similar findings were reported for ultrasound-guided nerve blockade of the brachial plexus in humans, calves, dogs and cats (3, 7, 9, 13, 15).

The brachial plexus provides the sensory and motor supply of the thoracic limb. Therefore, its successful block would provide complete desensitization of the whole forelimb (7, 20). In the present study, the regions dominated by

musculocutaneous, median and ulnar nerves showed a significant sensory scores ($p < 0.05$). While the other regions dominated by radial and axillary nerves showed insignificant increased score as compared to the basal line time. Motor score was increased significantly and extended more than the sensory effect. Our obtained sensory and motor scores were more effective than that obtained after brachial plexus block in calves (3). This may be attributed to the volume of local anesthetic drug used. In calves, the volume was 10 ml 2% lidocaine while we used 25 ml 2% lidocaine. Increasing the dose of local anesthetic associated with efficacy of peripheral nerve block (23).

Conclusion

To the best of our knowledge, this may be the first description to the anatomical and ultrasound-guided brachial plexus block in donkeys. The ultrasound-guided technique was found to be a feasible and safe method to provide adequate analgesia of the forelimb in donkeys but further trials would be needed to assess the usefulness and the applicability of this technique in the clinical cases.

Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

CYCLOPHOSPHAMIDE HEPATOTOXICITY: THE ROLE OF 4-HYDROXYNONENAL AND CYTOCHROME C OXIDASE AND THE POSSIBLE PROTECTIVE EFFECT OF *Ganoderma lucidum* EXTRACT

Dina R. S. Gad El-Karim[1]*, Gamal El-Amrawi[2]

*[1]Department of Pathology, Clinical Pathology branch, Faculty of Veterinary Medicine, Alexandria University, [2]Department of Theriogenology, Faculty of Veterinary, Medicine, Alexandria University, 11511, Egypt

*Corresponding author, E-mail: Gaddidi@yahoo.com

Abstract: Cyclophosphamide (CPA) is a widely used anti-neoplastic drug, but its usage is associated with several side effects including hepatotoxicity. This study was designed to evaluate the ameliorative effect of *Ganoderma lucidium* (GL) extract as a natural antioxidant against CPA hepatotoxicity in male Wistar albino rats. Four groups of rats (n = 7) were used in this study, Group I: served as a control group, group II: was administrated CPA (5 mg/kg), group III: received GL extract (50 mg/kg), and group IV: received CPA and GL extract at the same doses of groups II and III. All the treatments were orally administrated via gastric intubation for 21 consecutive days. Serum activities of the hepatic enzymes (AST, ALT and GGT) in addition to serum proteinogram were evaluated. Moreover, hepatic oxidant-antioxidant markers (4- hydroxynonenal (4-HNE), GSH, CAT and SOD) besides activity of cytochrome C oxidase enzyme were detected. Also, histopathological examination of the hepatic sections (H&E stained) was performed. Serum activity of liver enzymes was elevated, proteinogram was disturbed in CPA-treated animals which accompanied by an increase in hepatic content of 4-HNE, depletion of hepatic antioxidant (GSH, CAT, SOD) and inhibition of hepatic cytochrome C oxidase activity, also hepatic histopathological disruptions were noticed in this group. All these hepatic disturbances were partially ameliorated upon co-administration of GL extract with CPA. In conclusion, production of 4- HNE and inhibition of cytochrome C oxidase enzyme may play a role in occurrence of CPA hepatotoxicity and GL extract may have an ameliorative role against this toxicity.

Key words: cyclophosphamide; hepatotoxicity; *Ganoderma lucidium;* 4-hydroxynonenalon; cytochrome C oxidase

Introduction

Cyclophosphamide (CPA) is a drug with a wide spectrum of clinical uses especially in the treatment of cancer (lymphoma, acute and chronic leukemia, multiple myeloma) and autoimmune diseases such as rheumatoid arthritis and vasculitis (1). As a chemotherapy, CPA is an alkylating agent depending on formation of phosphoramide mustard (major chemically reactive CPA metabolite) which brings about interstrand cross-links between opposite DNA strands and hampers their replication and tran-

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scription process and this characterizes the clinical activity of CPA (2). Bioconversion of CPA into its metabolites (acrolein and phosphoramide mustard) leads to the formation of high level of reactive oxygen species (ROS), which disturb antioxidant activity inducing CPA-related tissue injuries (3-5).

Oxidative stress (disruption of redox balance) which generates biochemical and physiological disturbances, mediates the injury of normal tissues (as hepatic cells damage and mutagencity) and this is considered the major limitation of CPA usage as an anti-neoplastic drug (6,7). Several studies suggest that antioxidant supplementation can influence the response to chemotherapeutic drugs and attenuate the development of their adverse side effects (8).

Ganoderma lucidum (GL) or Reishi is an oriental fungus which has been recognized as a medicinal mushroom for over 2000 years due to its multiple powerful effects (9). It presents in many commercial forms including dietary supplement, tea or powder which are produced from different mushroom parts as fruits, mycelia or spores (10). GL polysaccharides (GLPS) is one of the major active bio-components of GL which have multiple pharmacological effects, such as antitumor (11), and antioxidant activity (12) in a dose dependent manner (13) through direct free radicals scavenging and ability to increase antioxidant enzymes activity (14), immunomodulation (15). It also has a well proved hepato-protective effect against chemical or immune-related hepatic damage (16-19).

This study was an attempt to prove our hypothesis about the inhibitory effect of 4-hydroxynonenalon and cytochrome C oxidase enzyme in relation to the occurrence of cyclophosphamide hepato-toxicity and possible attenuating role of *Ganoderma lucidium* against this toxicity in Wistar albino rats.

Material and methods

Experimental animal

Twenty eight adult male Wistar albino rats weighting about 180-200 g were obtained from Alexandria University Research Institute, Egypt and kept under 12 hr-12 hr light/dark cycles. They were allowed to standard commercial pelleted food with water *ad- libitum*. The animals were left without any treatment for 10 days for acclimatization. All the experimental procedures of this study were performed according to the instructions of "Care and Use of Laboratory Animals" prepared by Faculty of Veterinary Medicine, Alexandria University, Egypt. The protocol of this study was approved by the Research Ethics Committee of the University.

Drugs

Commercially available CPA tablets (Endoxan® 50mg, Baxter Oncology GmbH) and GL whole fruiting body extract (Standardized Reishi, 376 mg capsules standardized to contain10% polysaccharides, Nature's Way®, USA) were used to accomplish this study. The accurate doses of the drugs were dissolved in saline solution daily and shortly before administration.

Experimental protocol

The acclimated animals were randomly divided into four equal groups (n =7) and treated as follow: group I (control group): rats received 1 ml saline; group II (GL-treated group): rats received GL extract at a dose of 50 mg/kg body weight, bw (20); group III (CPA-treated group): rats received CPA at a dose of 5 mg /kg bw (21); and group IV (CPA+GL-treated group): rats received CPA and GL extract at the same doses of groups II and III. All treatment were orally administrated by gastric intubation for 21 consecutive days.

Twenty four hours after the last administration and under the effect of light ether anesthesia, blood aliquots were collected from retro-orbital venous plexus and then, the animals were dispatched by cervical dislocation to harvest the liver tissue samples. Serum samples were separated by centrifugation of coagulated blood aliquots for 10 min at 1000 ×g and kept frozen at -4 °C for later analysis.

Serum biochemical analysis

Determination of serum activity of hepatic damage biomarkers enzymes including AST, ALT (22) and GGT (23), in addition to serum

level of total protein (TP) (24), albumin (25) and globulins (26) were evaluated using commercially available kits (Biosystems, Spain).

Liver homogenate preparation and biochemical assays

The liver of each animal was rinsed with 0.9% NaCl and deionized water. The tissues were perfused with phosphate buffer saline (PBS), pH 7.4 containing 0.1 mMethylenediaminetetraacetate (EDTA) to discard any red blood cell or clots. Small piece of each liver (about 1 g) was homogenized in 10 ml ice-cold PBS using Glas-Col® tissue homogenizer and centrifuged at 10,000 × g for 30 min. Supernatant was separated, filtrated and stored at -80°C in Eppendorf's tubes for detection of catalase enzyme (CAT) activity depending on rate of hydrolysis of hydrogen peroxide (27), superoxide dismutase enzyme (SOD) activity which determined as the rate of inhibition of reaction between superoxide with a WST-1 dye to form a colored product (28) and reduced glutathione (GSH) content which based on reduction of 2-nitrobenzoic acid (DTNB) with glutathione (29) using locally available kits (Biodiagnostic, Egypt and Abnova, Taiwan). Protein content of tissue samples was detected spectrophotometerically using Bradford reagent (Sigma-Aldrich, USA) (30).

Detection of hepatic level of 4-HNE and activity of cytochrome C oxidase

According to manufacturer instruction, about 100 mg of hepatic tissues was rinsed with PBS, homogenized in 1 ml of ice-cold PBS and stored overnight at -20°C. Two freeze-thaw cycles were done to break the cell membranes; the homogenates were centrifuged for 5 min at 5000 x g. The supernatant was removed and kept at 4° C for assessment of 4-hydroxynonenallevel using specific ELISA kits (Mybiosource, USA) in addition to activity of cytochrome C oxidase enzyme using specific assay kits (Sigma-Aldrich, USA).

Histopathological examination

Fixed liver tissues (in 10 % formalin) were sliced into 5 µm thick sections after paraffin embedding and stained with hematoxylin and eosin (H&E) (31) using light microscope.

Semiquantitative grading system for hepatic histopathological alterations

Briefly, five random fields from each animal liver histopathological sections were examined (×100), the grade of the detected lesion severity was assessed depending on the percentage of affected area/entire section and recorded as follow: (-): absence of lesion, (+): for mild degree of lesions (5–25%), (++): for moderate lesions degree (26–50%) and (+++): for severe degree of lesions (≥50%).

Statistical analysis

The statistical analysis was performed using One-way analysis of variance (ANOVA) to study the effect of different treatment on different studied parameters using analysis SAS system software (32).

Results

Serum biochemical results

As shown in Table (1), serum activities of the hepatic enzymes (AST, ALT and GGT) were significantly elevated in CPA-treated animals ($P < 0.05$) when compared to the control group. Concerning serum proteinogram, none of the treated groups showed any significant changes from control group in serum total protein level ($P < 0.05$), but serum albumin level recorded a significant decrease ($P < 0.05$) in CPA and CPA+GL-treated group when compared to the control group. While, serum globulins level showed a significant increase ($P < 0.05$) in CPA-treated group which was accompanied by a significant decrease in A/ G ratio when compared to the control group.

In comparison with CPA-treated group, co-administration of GL with CPA significantly decreased the evoked serum hepatic enzymes activity, decreased serum globulins level and significantly reversed A/G ratio toward normal control level, but, it did not affect the decreased serum albumin level significantly. Administration of GL extract only to the rats significantly decreased serum activity of different hepatic enzymes (AST, ALT and GGT), ($P < 0.05$) but,

it did not have any significant effect on serum proteinogram of these rats as compared to the control group.

Hepatic antioxidant biomarkers

In comparison with the control group, the liver content of GSH along with hepatic activities of CAT and SOD enzymes recorded a significant decrement ($P < 0.05$) in the CPA-treated group. Fortunately, these assessed hepatic antioxidant biomarkers were significantly increased in CPA+GL-treated animals as compared to CPA-treated rats. As compared to the control group, GL extract successfully and significantly increased ($P < 0.05$) these tested antioxidant biomarkers in hepatic tissues of rats when administrated alone (Table 2).

Hepatic level of 4-hydroxynonenal and activity of cytochrome C oxidas enzyme

In CPA-treated group, the hepatic level of 4-hydroxynonenal was increased significantly which was coupled with a significant decrease in hepatic activity of cytochrome C oxidase enzyme ($P < 0.05$) when compared to the control group. These changes were partially and significantly alleviated when GL extract was administrated with CPA. Compared to the control group, administration of GL extract alone to the rats significantly decreased ($P < 0.05$) liver content of 4-hydroxynonenal and enhanced the activity of cytochrome C oxidase enzyme (Table 2).

Histopathological results

Light microscopy examination of representative histological liver sections of various treated animals groups showed normal and well detailed hepatocytes which were arranged in regular cords around portal vein in area in both of control and GL extract administrated groups (Fig.1). However, CPA-treated group exhibited severe hemorrhage (Fig. 2) with broadening of portal area with mononuclear inflammatory cell and fibroblast besides vacuolar degeneration of the majority of hepatocytes (Fig. 3), but CPA+GL extract-treated group showed small focus of necrotic hepatocytes with inflammatory cells infiltration (Fig. 4) with minimal infiltration of these inflammatory cells in portal area (Fig. 5).

Table (3) illustrated the results of histopathological evaluation of the hepatic lesions of different groups using simple semiquantitative scoring system which affirmed the alleviating role of GL extract on the detected hepatic lesions.

Discussion

Despite of CPA effectiveness in treatment of a wide variety of neoplastic and autoimmune diseases (1), damage or injury of healthy tissues may be the major factor of limited usage of CPA as an anti-neoplastic treatment (33). CPA hepatotoxicity was reported in several studies

Table 1: The effect of different treatments on serum hepatic biomarkers enzymes (AST, ALT and GGT) and serum proteinogram

	Control	GL	CPA	CPA+GL
AST (U/L)	35.20±2.91c	31.80±3.43d	79.80±3.85a	61.40±3.61b
ALT(U/L)	25.80±3.12c	22.80±2.63d	69.80±3.81a	40.60±3.16b
GGT(U/L)	22.00±2.35c	19.80±1.32d	51.60±2.66a	34.20±2.52b
TP (g/dl)	6.52±0.19ab	6.50±0.19ab	6.88±0.13a	6.02±0.13b
Albumin(g/dl)	4.18±0.14a	4.22±0.09a	2.98±0.11b	3.56±0.05b
Globulins(g/dl)	2.34±0.14b	2.28±0.11b	3.90±0.14a	2.37±0.08b
A/G ratio	1.82±0.12a	1.87±0.06a	0.76±0.05c	1.51±0.07b

All the values are expressed as mean ±SD. Means within the same raw of different litters are significantly different at ($P < 0.05$). **GL:** *Ganoderma lucidium* extract, **CPA:** Cyclophosphamide, **CPA+GL:** Cyclophosphamide+ *Ganoderma lucidium* extract.

Table 2: The effect of different treatments on hepatic antioxidants content (GSH, CAT and SOD), 4-HNE level and cytochrome C oxidase activity

	Control	GL	CPA	CPA+GL
GSH (mmol/mg protein)	79.00±3.18b	110.60±4.45a	37.20±2.80d	55.80±2.84c
CAT (U/mg protein)	10.10±0.87b	17.00±0.79a	3.58±0.39d	7.40±0.43c
SOD (U/mg protein)	9.72±0.82b	16.04±0.62a	4.02±0.18d	6.76±0.40c
4-HNE (pg/ mg protein)	13.90±1.49c	8.10±0.64d	48.40±3.08a	29.00±2.93b
Cytochrome C oxidase (U/mg protein)	51.20±3.34b	66.40±3.04a	25.80±2.22d	35.40±2.80c

All the values are expressed as mean ±SD. Means within the same raw of different litters are significantly different at ($P < 0.05$).

Table 3: The score of detected hepatic lesions in male Wistar albino rats of different experimental groups

Scored liver lesions	Incidence[1] and Severity[2] of histopathological lesions							
	CPA-treated rats				CPA+ GL treated rats			
	Absent (-)	Mild (+)	Moderate (++)	Severe (+++)	Absent (-)	Mild (+)	Moderate (++)	Severe (+++)
1-Congestion of blood vessels		2	3	2	0	4	3	0
2-Hepatocytes vacuolar degeneration	0	0	4	3	2	2	2	1
3-Portal Inflammatory cells infiltration	0	1	4	2	1	3	3	0
4-Hepatocellular necrosis	0	2	3	2	2	2	3	0
5-Hyperplasia of biliary epithelium	0	3	2	2	0	5	2	0
6-Hemorrhage	0	4	2	1	7	0	0	0

[1]Number of rats with lesions per total examined (7 rats).
[2]Severity of lesions was graded by estimating the percentage area affected in the entire section.

(34-37) as the liver is the primary site for microsomal activation of this drug (38). The prime cause may be reactive oxygen species (ROS) generation including hydrogen peroxide and superoxide anion during CPA metabolism which in role exhaust anti-oxidant mechanisms of liver (3, 39). This action may be exaggerated by CPA reactive metabolite (Acrolin) which can react with hepatic GSH causing its depletion (40) which collectively increase susceptibility of lipid peroxidation and hepatic tissues injury (41).

The previous mechanisms of CPA-induced hepatotoxicity may explain the significant increase in serum hepatic enzymes activity (AST, ALT and GGT) of CPA-treated animals which in a concomitant with decreased activity of CAT and SOD enzymes in addition to depletion of GSH in hepatic tissues of the same group may represent a sufficient indicators for the occurrence of oxidative injury of hepatocytes consistently with several previously recorded findings (20, 36, 42). Several studies have reported that GL extract is a good free radical scavenger (13, 43, 44), so treatment with GL rendered some protection against CPA-hepatotoxicity which was evidenced by a significant decrease in serum activity of AST, ALT and GGT which accompanied by a significant increase in hepatic tissues content of GSH and activity of antioxidant enzymes (CAT and SOD) in CPA+GL-treated group when compared to the CPA-treated group. Hepatoprotective effect of GL against oxidative damage was previously detected and proved (18). Albumin is one of plasma proteins which is synthesized by liver so, determination of its levels is considered one of best indicators to evaluate hepatic function efficacy (45) and this may demonstrate its decrease in both of CPA and CPA+GL-treated groups which may be attributed to hepatotoxic

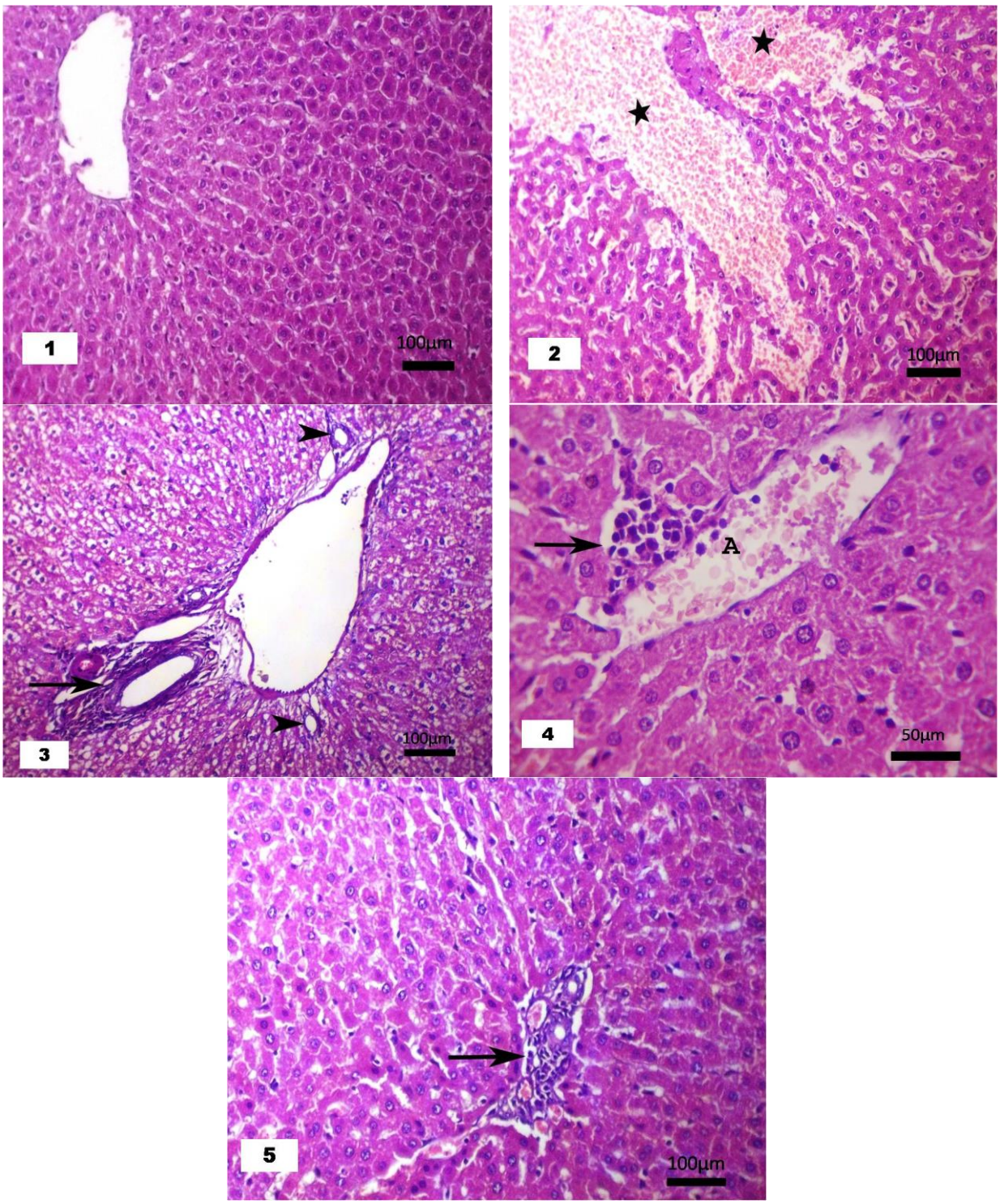

Figure 1: (1) Control group, a section in the liver of a rat showing normal hepatocytes (H&E, X200). **(2)** CPA-treated group, a section in the liver of a rat showing focal hemorrhage (stars, H&E, X200). **(3)** CPA-treated group, a section in the liver of a rat showing thickening of portal area with mononuclear cells infiltrate and fibrosis (arrow) and hyperplasia of biliary epithelium (arrow heads) besides hydropic degeneration or vacuolation of most of hepatocytes (H&E, X200). **(4)** CPA+GL-treated group, a section in the liver of a rat showing congested blood vessel (A) and necrosis hepatocytes with inflammatory cells infiltrate (arrow, H&E, X400), **(5)** CPA+GL-treated group, a section in the liver of a rat showing mononuclear inflammatory cells in the portal area (arrow, H&E, X200)

impact of CPA, while hyperglobulinemia of hepatocellular disorders, appearing as an inflammatory response to liver injury due to the probable increase in production alpha and beta globulins by liver and/or immune- globulins by plasma cells (45,46). The previous explanation may clarify the reasons for hypoalbuminemia and hyperglobulinemia which significantly decreased A/G ratio of CPA-treated animals which could be attributed to CPA hepatotoxic effects. The treatment with GL significantly ameliorated these changes in proteinogram and clearly lessens the decrease in A/G ratio caused by CPA treatment, thereby GL may exhibit a considerable suppressive effect on hepatic inflammatory reactions caused by CPA, this ameliorative effect of GL on hepatic inflammatory state was concluded formerly (18).

4-hydroxynonenal is the major lipid peroxidation product which is produced in the tissues as a result of cellular exposure to oxidative stress (47). It has a suppressive effect on cytochrome C oxidase enzyme activity (48,49) which is the key enzyme of aerobic cell respiration that contributes to the formation of ATP (essential energy molecules for cell livability and viability), so deficiency of this enzyme represent a risk for the living cells (50). The proved oxidative stress caused by CPA administration may demonstrate the increment in hepatic tissues level of 4-hydroxynonenalwhich in turn may be the cause for the decrement in these tissues activity of cytochrome C oxidase enzyme. Another explanation for inhibition of hepatic cytochrome C oxidase enzyme in CPA-treated rats may be the great susceptibility of its mRNA as well as protein to oxidative damage (51).

The later changes were alleviated by the dosage of powerful antioxidant GL extract to the rats of the CPA+GL group. Hence, the antioxidant activity of GL was reflected on the effects of its supplementation as a sole treatment to the rats of GL-treated group, as it successfully boosted the hepatic antioxidant biomarkers (GSH, CAT, SOD), which in role, decreased hepatocytes destruction and leakage of hepatic enzymes to the blood and finally decreased hepatic level of 4-HNE and enhanced the activity of cytochrome C oxidase enzyme as compared to control group.

In this study, histopathological examination of liver, affirmed that CPA can cause damage to the liver which was evidenced by presence of several different hepatic lesions, which might be caused by cellular damaging potential of CPA metabolites in relation to generation of ROS in agreement with the current studies on CPA-induced hepatotoxicity (38,42,52). These histopathological changes correlated strongly with the altered serum enzymes activity and hepatic antioxidants depletion of CPA-treated group. Semiquantiative evaluation of hepatic lesion confirmed the biochemical results which illuminated that the treatment with GL extract effectively ameliorated the abnormal hepatic histopathological lesions caused by CPA, and this may be attributed to its powerful antioxidant ability in prevention of ROS membrane damage (12,16,18).

Conclusion

Our results reported the role of 4-hydroxynonenal production and inhibition of cytochrome C oxidase enzyme in CPA-induced hepatic damage. In addition, it clarified and supported that the treatment with GL extract can reduce oxidative hepato-cyto damage caused by treatment with cyclophosphamide due to its powerful antioxidant effect, so it can be used during treatment with this anti-cancer drug to minimize its deleterious effects on the liver.

Conflict of interest

The authors declare that she has no conflict of interest.

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Original Research Article

THE MODULATORY EFFECT OF DIETARY BETAINE ON INTESTINAL ABSORPTIVE CAPACITY, LIPOGENESIS AND EXPRESSION OF LIPID METABOLISM- AND GROWTH-RELATED GENES IN NILE TILAPIA FED ON SOYBEAN MEAL-BASED DIET

Taha Ismail[1], El-Desoky Nassef[1], El-Sayed Hegazi[1], Abdel-Nasser Bakr[1], Eman M. Moustafa[2]*, Walied Abdo[3], Zizy I. Elbialy[4]

[1]Department of Nutrition and Clinical Nutrition, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt. [2]Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Kafrelsheikh University. [3]Department of Pathology, Faculty of Veterinary Medicine, Kafrelsheikh University. [4]Department of Fish Processing and Biotechnology, Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University

*Corresponding author, E-mail: emantarek2002@yahoo.com

Abstract: The current experiment was randomly designed in a 2×2 factorial design. Two dietary protein sources were utilized; fish meal and soybean meal; with betaine incorporation to both of them. This study aimed to determine the impact of incorporation of betaine into soybean meal-based (SBM) diets through its effects on growth performance, intestinal healthiness and expression of some lipid metabolism- and growth-related genes. Fish (19.84±0.20 g) were stocked in 12 aquaria and allotted into triplicate four groups (10 fish per aquarium). Four test diets were formulated to contain fish meal (FM) as a positive control, FM with betaine (FM + Betaine), SBM diet and SBM with betaine (SBM + Betaine), respectively. After 60 days, dietary betaine improved the growth performance of fish fed FM or SBM as revealed by higher final body weight, body weight gain and average daily gain and lower feed conversion ratio. Monitoring the whole-body composition revealed that addition of betaine to diet relatively augmented flesh protein content and reduced its fat content. In addition, betaine incorporation in diets significantly (P<0.05) increased the intestinal villi length especially in the jujenal portion as well as the numbers goblet cells. Furthermore, betaine had a downregulating effect on expression of lipid metabolism-related genes, fatty acid synthetase (*Fas*) and lipoprotein lipase (*Lpl*) and upregulating effect on insulin growth like factor-1 (*Igf-1*) gene in liver. It could be concluded that dietary supplementation of betaine incorporation to soybean-based diets enable nutritionists to substitute FM in fish diet. Also, betaine could improve growth performance, carcass quality (through increasing protein and decreasing lipid in fish) and enhance intestinal functions capability.

Key words: Betaine; growth performance; insulin like growth hormone factor; lipid metabolism related genes; Nile tilapia; soybean meal

Introduction

Fish serve as a vital source of human dietary protein worldwide, particularly for African

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people (1-3). Natural fisheries are progressively exhausted and the aquaculture end up basic means to compensate the need in this fundamental wellspring of dietary protein (4). Along these lines, to repay the insufficiency in animal protein resulted from the expanded interest for fish as human sustenance, fish farming is quickly extending everywhere throughout the world (5). Aquaculture contributes the greater part of the overall fish production all over the world. In Egypt, the aquaculture industry gives about 77% of the all national fish production providing arround one million ton annually (6-8). Progressive enhancement of this industry must be correlated with corresponding improvement of fish feed quality representing 70 % of the total culturing costs (7, 9).

Nile tilapia (*Oreochromis niloticus*) represents the most prevalent freshwater fishes in Egypt, widely cultured because of its high growth rate, cheap price, palatability, ability to withstand stress and diseases, ability to spawn easily and the minimal requirements in regard to management and energy inputs (10). Heightening of aquaculture production frameworks exposes fish to various ecological stressors like; poor water quality, over-crowdness, improper handling and transport which may contrarily influence their growth and health, as well as limiting the outcome of aquaculture systems (11).

Nutrition assumes as a basic job in the growth, advancement and prosperity upkeep of fish (12). Years ago, fish meal was used as the basic protein source in tilapia diets (13). Due to the extending cost and unreliable supply of this ingredient, numerous attempts have been admitted to upgrade the growth performance and decrease the production costs of cultured tilapia through replacement of fish meal with another protein sources either from terrestrial animals or plants. This was done by utilization of probiotics or prebiotics as safe dietary supplements which have neither residue in the cultured fish nor harmful impacts for fish consumers (14-15). However, increasing amount of the fish meal alternatives in the diet may result in decreasing the diet palatability to fish; decreasing feed intake and subsequently decreasing fish growth rate (16).

Betaine (trimethylglycine) is synthetized from choline oxidation (17), present in most organisms and is fundamentally extracted from sugar beet processing (18). Interestingly, betaine was recorded to improve growth performance, fish health status, feed digestibility as well as flesh quality and immune status of fish species (19, 20). It was used as a feed attractant and appetizer through stimulating the olfactory bulb, increasing feed intake and minimizing the feed wastage and water pollution (21). In addition, betaine can play a role in the osmoregulation through increasing both water retention of perturbed cells and cytoplasmic osmotic pressure via raising the temperature and ionic tolerance of critical enzymes and cellular membranes that results in protection of intracellular enzymes against osmotically or temperature induced inactivation (22, 23). Moreover, betaine acts as a methyl donor so it plays a great vital role in energy metabolism and synthesis of methionine, carnitine, phosphatidyl choline, creatine and protein (24, 25). Betaine plays a fundamental role in lipid metabolism suggesting that it is a lipotropic factor having a hepatoprotective function (26-28). Dietary betaine additionally has the ability to reduce body lipid deposition (29, 30).

To the best of our knowledge, there is little published data on the supplemental effects of betaine on growth performance, intestinal absorptive capacity, intestinal morphometry, lipid metabolism and growth-related genes in Nile tilapia (*O. niloticus*). Thus, the current study was conducted to role out the effect of incorporation of betaine to soybean-based diets and to assess its effects on growth performance, intestinal absorptive capacity, and expression of lipid metabolism- and growth-related genes in Nile tilapia.

Material and methods

The feeding trials were carried out at the Nutrition and Clinical Nutrition Department, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt, for 10 weeks during 2018. Animal ethics committee, Faculty of Veterinary

Medicine, Kafrelsheikh University, Egypt; affirmed and approved the protocol and conducting of the study.

Preparation of the experimental diets

Two basal diets were formulated to be isonitrogenous and isocaloric in terms of crude protein (30%) and digestible energy (3.05 kcal g^{-1}). Each diet was based on either fish meal (FM) or soybean meal (SBM) as the main source of dietary protein. Then each diet was supplemented with 2 g betaine per kg diet (FM, FM+betaine, SBM and SBM+betaine) (Table 1) as suggested by Luo *et al.*, (31). Betaine was purchased from Biochem Ltd. Company, Egypt and incorporated to the diets after firstly mixed with dicalcium phosphate, limestone, salts and premix until the mixture became homogenous and finally added to the other finely ground concentrate with stirring. Proximate chemical analysis of diets was performed according to Association of Official Analytical Chemists methods as shown in (Table 2).

Experimental design

The experiment was randomly designed in a 2×2 factorial design. Two dietary protein sources were used with betaine incorporation to both of them. Thus, a total of four treatments run in triplicate were allotted into12 glass aquaria (60×40×30 cm) provided with a constant aeration.

One hundred and twenty healthy Nile tilapia (*O. niloticus*) (initial body weight, 19.84 ± 0.20 g) were obtained from a private farm in Kafrelsheikh governorate, Egypt. Prior to the experiment, fish were acclimatized to basal diet for 2 weeks. At the beginning of the experiment, fish (n= 10) were stocked into each glass aquaria, and each diet was assigned to triplicate aquaria. Fish were fed till apparent satiation twice daily (at 9 a.m. and 5 p.m.) for 60 days and weighted every ten days in order to determine the growth and healthy status. Water parameters were monitored twice a week using water analysis device (Lamotte device, USA**)**. Temperature ranged from (24-26°C), 6 mg L^{-1} dissolved oxygen, 6.5-7 pH, ammonia adjusted to the normal permissible limits (< 0.1 mg total ammonia) and 14 h of light and 10 h of dark photoperiod with 40 % daily water change.

Proximate chemical analysis

At the end of the feeding trial, fish were starved for 24 h, harvested and weighed individually then euthanized through over anesthetization using MS222 as a local anesthetic drug. 4 fish from each group were stored at (-20 ºC) for proximate chemical analysis of fish tissue. Moisture content was carried out by drying the samples in a hot air oven at 60 ºC for 48 h. Ash content was determined by samples incineration at 550 ºC for 6 hrs. Crude protein (N×6.25) was assessed using the Kjeldahl method after acid digestion. Fat and fiber were determined using ANKOM Technology Method.

Morphometrical examination

Snippets of the different intestinal portions were sampled from another four fish and immediately fixed in a Bouin solution for histological observations. Fixed samples were subjected to dehydration through ascending grades of alcohol solution series (70 to 100%). After dehydration process, tissues were deparafinized in xylene, embedded in paraffin, sectioned (5 µm) and stained with hematoxylin and eosin (H&E). The heights of intestinal villi in all parts were measured using ImageJ version 1.36 (National Institutes of Health). Mean villus height for each section was based on an average of 10 villus heights/section. Goblet cells No. was determined according to the equation: goblet cell number = goblet cell number in 8 fields of x 200 magnification power / total area examined per each fish, and was expressed as (No. of goblet cells/mm^2)

Quantitative real-time PCR (qRT-PCR)

Liver samples were collected, quickly frozen in liquid nitrogen then kept at −80°C for gene expression analysis, then total RNA was extracted from 50 mg of liver tissue (n=3/group) using TRIzol reagent (easy-RED™, iNtRON Biotechnology). RNA quality was verified using formol gel electrophoresis examination. The cDNA was synthesized from each RNA sample (2 µg) using SensiFAST™ cDNA syn-

thesis kit (Bioline, United Kingdom). As presented in Table (3), gene specific primers of fatty acid synthase (*Fas*), lipoprotein lipase (*Lpl*), and insulin like growth factor-1 (*Igf-1*), were used to determine the expression in liver tissue and β actin was selected as a reference gene for normalization of gene expression. Real time PCR (qPCR) was carried out using SYBR® Premix and Stratagene MX3000 qPCR system. The reaction mix consisted of a 2 µl cDNA sample, 12.5 µl SYBR® Premix Ex TaqTM, 0.25 µl PCR forward/reverse primers (10 µM), and 10 µl nuclease-free water. The thermo-cycling conditions for the reaction were as follows: 95 ºC for 5 min, followed by 40 cycles consisting of 95 ºC for 10 s, annealing for 15 s at primer specific annealing temperature. Dissociation curve analyses were performed beginning at 65°C and ending at 95°C, with incremental increases of 0.5°C every 5s to validate the specificity of the PCR products. All samples were run in duplicate. Relative gene expression levels were evaluated using $2^{-\Delta\Delta ct}$ as previously described (32).

Calculations and statistical analysis

The following variables were calculated:

Total body weight gain (TWG)(g/fish) =final body weight (W2) – initial body weight (W1)**.**

Average daily gain (ADG) = (W2 – W1) / t (experiment duration = 60 days)

Feed intake (FI, g $fish^{-1}$ 60 $days^{-1}$) = (offered feed – feed refusal recovered and dried)/number of fish

Feed conversion ratio (FCR) = feed intake (g)/weight gain (g).

The results were presented as mean ± standard error (SE) of three replicates. All results were analyzed by one-way analysis of variance (ANOVA). When ANOVA identified differences between groups, multiple comparisons among means were made with Duncan's new multiple range test. Differences were considered significant at (*P*<0.05**)**. All statistical analyses were performed using the SPSS 24.

Results

Growth performance

As shown in table (4**)**, addition of betaine to diets based on soybean meal protein significantly (*P*<0.05**)** increased the feed intake. Fish fed on FM+betaine showed the highest FBW and BWG followed by that fed on SBM+betaine. Also, supplementation of betaine significantly (*P*<0.05**)** increased FBW and BWG in fish fed SBM+ betaine as compared to fish fed SBM only. FCR was markedly decreased in fish fed on FM+ betaine or SBM+ betaine when compared to the other groups. Also, FI significantly differed according to the source of dietary protein either from FM or SBM-based diet.

Whole body chemical composition

Analysis of whole-body composition presented in table (5) showed that crude protein content was significantly (*P*<0.05**)** increased by addition of betaine either with FM or SBM diets. FM+ betaine showed relatively higher CP content than other experimental treatments. Fat content was significantly (*P*<0.05**)** decreased by addition of betaine to fish diets. There were no significant differences in moisture, ash, soluble carbohydrate and ADF content among all dietary treatments.

Assessment of intestinal absorptive capacity

Duodenal, jujenal and ileal villi morphometry were showed in (Table 6) and illustrated in (Fig. 1). Betaine significantly increased the intestinal absorptive capacity of fish supplemented with FM diet (*P*<0.05**)**, while, SBM markedly decreased intestinal villi parameters in comparison with fish supplemented with FM diet. Interestingly, supplementation of betaine improved the jujenal villi length in FM and SBM groups. Also, the number of goblet cells per area along the intestinal mucosal lining was increased in fish fed FM diets than fish fed with SBM as a protein source. Incorporation of betaine with SBM demonstrated marked restore of goblet cells like FM diet.

Expression of Fas, Lpl and Igf-I genes in liver tissue

Relative mRNA expression of *Fas, Lpl, Igf-1* genes in liver of Nile tilapia fed on different protein sources with and without addition of betaine was shown in Figure 3. Hepatic *Fas* and *Lpl* mRNA levels showed upregulation in fish fed on the diets deprived from betaine (*P*<0.05**)**. Interestingly, addition of dietary betaine to either fish meal or soybean-based diets markedly decreased *Fas* and *Lpl* mRNA levels (*P*<0.05**)**. Fish fed on FM+ betaine had significantly (*P*<0.05**)** higher hepatic *Fas* mRNA level as compared to fish fed SBM+ betaine. *Igf-I* mRNA level in the liver of Nile tilapia fed on dietary betaine with SBM was significantly (*P*<0.05**)** upregulated as compared to fish fed SBM only. Fish fed diet with FM+ betaine showed significantly (*P*<0.05**)** higher hepatic *Igf-1* expression than other groups.

Table 1: Diets formulation and experimental design

Item (%)	FM	FM+ betaine	SBM	SBM+ betaine
FM	35.5	35.5	—	—
SBM	—	—	56.5	56.5
Wheat bran	3.8	3.7	6.7	6.6
Rice polishing	16	16	—	—
Corn grains	25	25	25	25
Wheat middling	18.9	18.8	4.96	4.86
Soya oil	—	—	1.2	1.2
Fish oil	—	—	1.8	1.8
Nacl	—	—	0.33	0.33
Minerals[1]	0.05	0.05	0.05	0.05
Vitamins[2]	0.05	0.05	0.05	0.05
Vitamin C	0.1	0.1	0.1	0.1
Dicalcium phosphate	—	—	2.4	2.4
Limestone	—	—	—	—
Lysine hydrochloride	—	—	—	—
DL methionine	—	—	0.31	0.31
Antimycotoxin	0.1	0.1	0.1	0.1
CMC	0.5	0.5	0.5	0.5
Betaine	—	0.2	—	0.2

[1]Minerals (Egypt pharma company) each 5 kgs of this product provide the following: 5000 mg Copper; 5 mg..Cobalt; 5000 mg..Iodine; 100 mg..Selenium; 30000 mg Iron; 40000 mg magnesium; 10000 mg manganese; 150000 mg Zinc; Calcium carbonate ad to 1000 gm.

[2]Vitamins (Egypt pharma company) each 5 kilograms of this product provide the following: 5000000 IU Vit.A; 1000000 IU Vit. D3; 50000 mg Vit. E; 10000 mg K3; 20000 mg B1; 20000 mg B2; 20000 mg B6; 20 mg B12; 100000 mg Niacin; 5000 mg Folic; 100 mg Biotin; 50000 mg Pantothenic Acid; Calcium carbonate ad to 1000 gm.

Table 2: Proximate chemical analysis of the basal diets

	FM	SBM
DE (Kcal/kg)	3045	3059
CP %	29.9	29.92
Fat %	6.25	3.6
ADF %	6.57	7.17
Ca %	1.9	0.7
Total P %	1.35	1.02
Available p %	0.71	0.46
Na %	0.15	0.15
Lysine %	1.98	1.67
Methionine %	0.75	0.73
Threonine %	1.18	0.99
Tryptophane %	0.31	0.24

ADF: Acid detergent fiber DE: digestible energy SBM: Soybean meal
CMC: Carboxy-methyl cellulose FM: Fish meal.

Table 3: Primer sequences used for qPCR analysis

Gene	Primer sequence (5'-3')	Annealing Temperature	References
Fas	F: TGAAACTGAAGCCTTGTGTGCC	60ºc	Tian *et al.,* (33)
	R: TCCCTGTGAGCGGAGGTGATTA		
Lpl	F: TGCTAATGTGATTGTGGTGGAC	59ºc	Tian *et al.,* (33)
	R: GCTGATTTTGTGGTTGGTAAGG		
Igf-I	F: TCCTGTAGCCACACCCTCTC	60ºc	Costa *et al.,* (34)
	R: ACAGCTTTGGAAGCAGCACT		
β-actin	F: CCACACAGTGCCCATCTACGA	63ºc	Qiang *et al.,* (35)
	R: CCACGCTCTGTCAGGATCTTCA		

Fas: fatty acid synethase *Lpl*: lipoprotein lipase *Igf-1*: insulin like growth factor-1.

Table 4: Effect of dietary betaine on growth parameters of Nile tilapia fed on FM and soybean.

	IW (g)	FBW (g)	BWG (g)	ADG (g)	FI (g)	FCR
FM	19.95±0.21	50.07± 0.42^{b}	30.11±0.27^{b}	0.50±0.00^{b}	48.68±0.52^{a}	1.62± 0.03^{b}
FM+ betaine	19.62±0.13	54.61± 0.33^{a}	34.99± 0.20^{a}	0.58±0.00^{a}	50.24±0.25^{a}	1.44± 0.00^{c}
SBM	19.90±0.19	43.17± 0.54^{c}	23.27±0.73^{c}	0.39±0.01^{c}	42.67±0.77^{b}	1.84± 0.03^{a}
SBM+ betaine	20.00±0.08	51.16±0.30^{b}	31.16±0.34^{b}	0.52±0.01^{b}	49.29±0.55^{a}	1.59± 0.01^{b}

IW: Initial weight FBW: Final body weight BWG: Body weight gain
ADG: Average daily gain FI: Feed Intake FCR: Feed Conversion Ratio.
**Values are means ± standard error (n = 10). Means with different small letter in the same column differ significantly ($P< 0.05$).

Table 5: Effect of dietary betaine on whole-body composition of Nile tilapia fed on FM and soybean

	Moisture%	CP%	Fat%	Ash%	Soluble carbohydrates%	ADF%
FM	57.83±0.30	18.78±0.17^{cb}	15.80±0.15^{a}	2.76±0.17	3.49±0.54	1.35 ± 0.17
FM+ betaine	57.92±0.29	19.85±0.15^{a}	14.82±0.36^{ab}	2.79±0.3	3.37±0.41	1.25 ± 0.10
SBM	58.2±0.31	18.52±0.4^{b}	15.33±0.21^{b}	2.79±0.31	3.61±1.07	1.48 ± 0.10
SBM+ betaine	57.62±0.28	19.35±0.34^{ab}	14.59±0.18^{b}	2.87±0.43	4.53±0.71	1.04 ± 0.08

CP: crude protein ADF: acid detergent fiber.
**Values are means ± standard error (n=4). Means with different small letter in the same column differ significantly ($P< 0.05$).

Table 6: Effect of dietary betaine on of intestinal villi length of Nile tilapia fed on plant and animal protein sources

Treatments	Villi Length (μm)		
	Duodenum	Jujenum	Ileum
FM	290.69±18.16	530.35±30.93^{bc}	355.98±30.05^{a}
FM+ betaine	351.20±32.03	684.92±26.96^{a}	414.96±37.16^{a}
SBM	278.73±23.06	465.48±24.04^{c}	220.44±19.76^{b}
SBM+ betaine	290.18±31.43	605.62±43.47^{ab}	255.62±30.66^{b}

Values are means±standard error (n = 4). Means with different small letter in the same column differ significantly ($P< 0.05$).

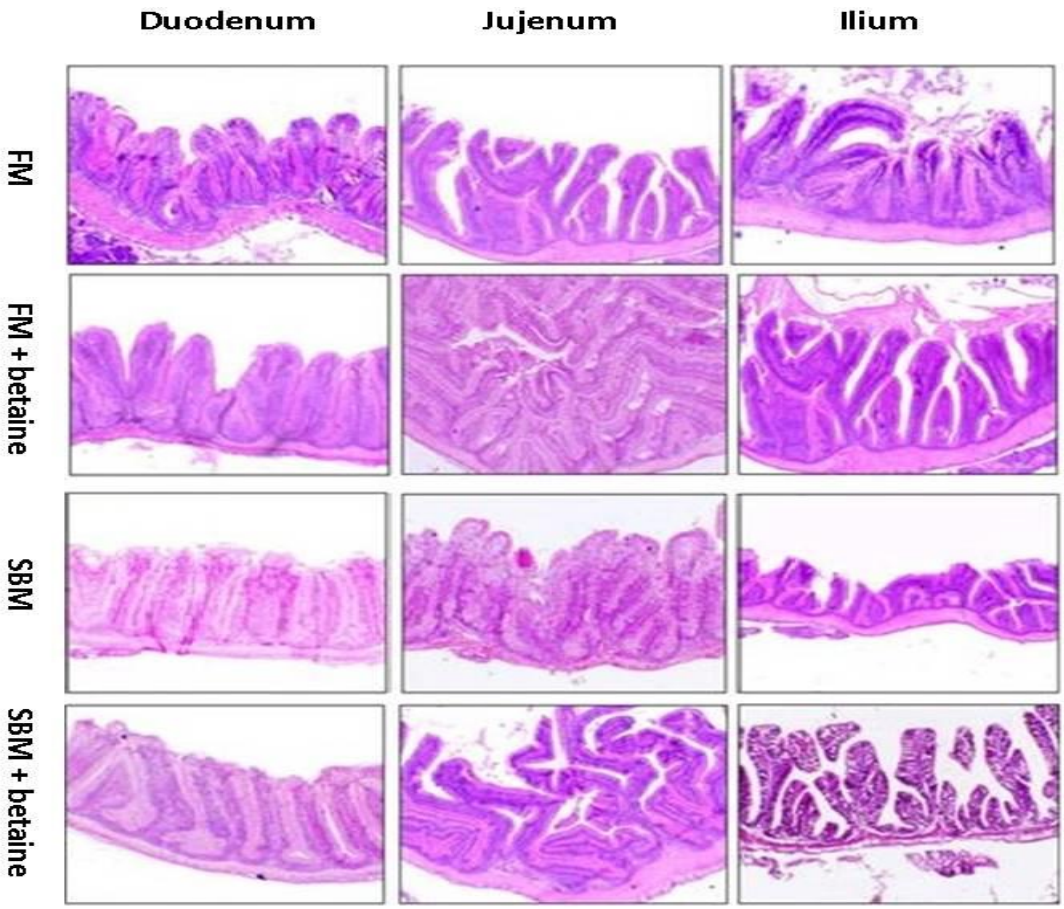

Figure 1: Haematoxylin & eosin stained photomicrograph, assessed the effect of dietary betaine on the duodenal, jujenal and ileal villi length (µm) of Nile tilapia fed on soybean meal and fish meal for 60 days

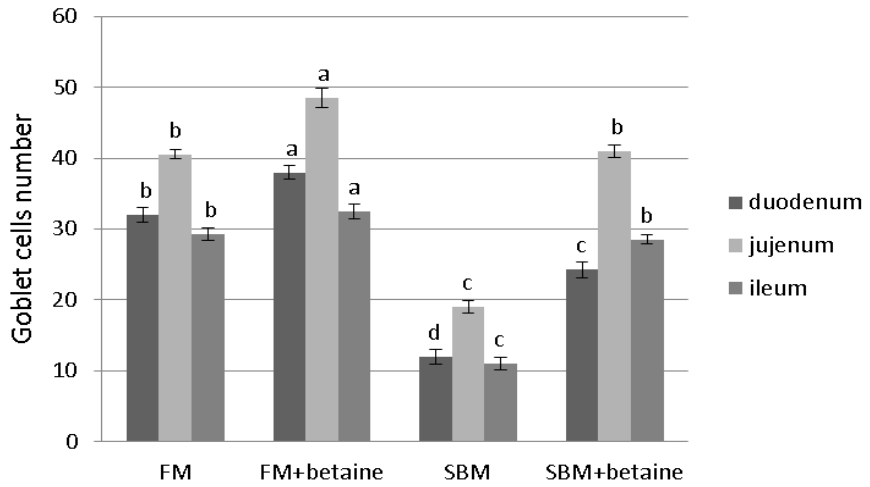

Figure 2: Effect of dietary betaine on intestinal goblet cells number of Nile tilapia fed on soybean meal and fish meal. Values are means±standard error (n = 4). Means with different small letters on columns for each organ differ significantly ($P< 0.05$)

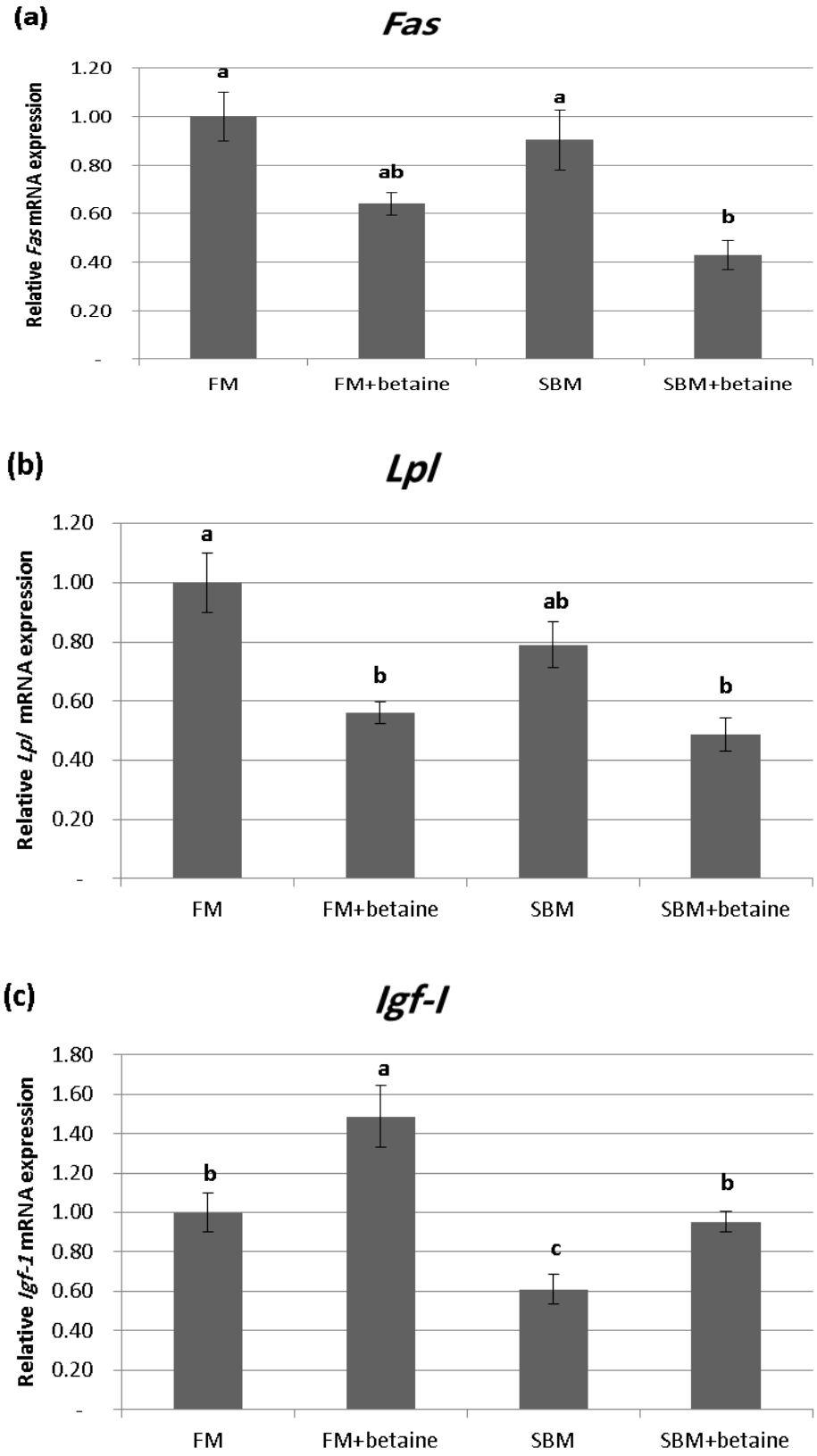

Figure 3: Effect of dietary betaine and different protein sources on mRNA level in liver **(a)** *Fas*: fatty acid synthetase; **(b)** *Lpl*: lipoprotein lipase; **(c)** *Igf-1*: insulin like growth factor-1 in Nile tilapia fed for 60 days. Values are expressed as the mean ± SE (n = 3). Data with different letters on columns statistically differ ($P< 0.05$)

Discussion

Growth is the fundamental key of energy loss and/or gains in the aquatic environment; which can be measured mainly by determination of the weight gain. Because of the expanding cost and untrustworthy supply of fish meal, various trials have been applied to redesign the growth performance and decline the production expenses of refined tilapia through substitution of fish meal with another plant protein sources. This was performed by usage of probiotics or prebiotics as protected dietary enhancements which have neither buildup in the refined fish nor unsafe effects for the fish customers (14, 15).

In the current study, fish fed FM showed higher BWG and FBW than SBM-based diets. The result is coincided with Fontainhas-Fernandes *et al.,* (36) who reported that dietary FM was the main protein source showing the highest FBW and Khan *et al.* (37) who reported that inclusion of dietary FM up to 35% showed marked increase in the growth rate. This may be attributed to high protein quality of FM and its palatability. Betaine incorporation to SBM based diet improved feed intake relatively similar to FM. Similarly, betaine was used as a feeding stimulant in red sea bream (38), dover sole (39), European eel (40), Tilapia (31), juvenile grouper (41), and Chinese soft-shelled Turtle (42). This may indicate that betaine improved palatability of the diets which might be through stimulation of cephalic index induced by betaine smell and taste. Conversely, Hughes (43) suggested that betaine not affect the feed intake in Chinook Salmon Fry.

In addition, betaine incorporation in SBM-based diets not only increased FI but also growth performance compared to FM diet levels. Similar results were also reported in Indian major carp (25), rainbow trout (44), pike perch (45), and in tilapia fed 0.5% betaine (31). Kasper *et al.* (46) found that dietary betaine can spare the entire choline requirement in Nile tilapia in contrast with Vieira *et al.* (47). Regarding to the feed conversion ratio (FCR) which is considered as one of the most economic indicators, addition of betaine to FM or SBM-based diets decreased FCR. Diet based on SBM+ betaine achieved the same effect FM on feed efficiency. This result is similar to that reported by (25) who used 0.25% of betaine in carp fish diet, (48) who used 0.5% betaine in juvenile fresh water prawn fish and (26) who got the best FCR in channel cat fish (26). Decreased FCR might be attributed to the improvement of growth performance (31). Recently, Zou *et al.* (49) reported that feeding Nile tilapia with 0.6% betaine in diet containing high level of plant protein significantly lowered FCR. The beneficial effect of betaine on growth performance may be attributed to its feeding stimulant effect together with increased intestinal amylase and protease activities (49, 50). The positive effect of betaine on growth performance might be also due to alternation in the metabolism which needs further examination. However, the improvement of FCR in this study was due to the rapid growth rate which saves maintenance requirements.

Interestingly, betaine supplementation to SBM-based diets increased the body protein and decreased the fat content. This may be attributed to betaine role as a methyl donner, sparing methionine amino acid needed for protein synthesis. In addition, betaine plays a key role in synthesis of phosphatidyl choline, carnitine and creatine. Moreover, it was suggested that betaine role as a methyl donner enhances lipid metabolism, stimulating the hepatoprotective function resulting in prevention of the fatty liver (26). Furthermore, the incorporation of betaine at 0.4% in *Allogynogenetic gibel* carp diet resulted in lowering in the hepatopancreas, muscle and the whole body lipid content (51). Additionally, Wang *et al.* (27) reported that supplementation of dietary betaine decreased lipogenesis and increased fatty acids oxidation. The above evidence suggests that betaine incorporation into Nile tilapia diets provides the priority for protein synthesis which consumes smaller energy amounts than fat synthesis.

Assessment of intestinal healthiness including absorptive capacity and digestive

functions through the height of intestinal villi, thickness of muscular layer and the number of goblet cells is an important morphometry indicator in case of evaluating different feed strategies in aquatic animals (52, 53). In the current study, betaine incorporation at 0.2% increased the intestinal villi length and number of goblet cells. Improving the intestinal absorptive and protective functions might be due to increased surface area available for absorption and/or due to secreted mucous from numerous goblet cells which has bactericidal effect through covering receptors of pathogens and keeping the integrity of intestinal epithelium (54, 55).

Lipid metabolism variations induced by betaine incorporation was monitored by changing in mRNA level of *Fas* which affects the synthesis of body fat and thereby affects the body fat accumulation (56). *Fas* and *Lpl* are considered key factors in lipid metabolism and fat deposition in the body (57). Current results revealed decreased *Fas* mRNA level associated with betaine incorporation in diet. Similar results were reported by Kim *et al.* (58) who revealed that protection of liver against steatosis could be occurred through supplementation of dietary betaine which decreased *Fas* and ACC (acetyl-CoA carboxylase) through affecting Forkhead Box O1 (FoxO1) binding peroxisome proliferator activated receptor gamma (PPARγ). Current results are similar to that reported in *Allogynogenetic gibel* carp (51). Additionally, hepatic *Lpl* mRNA level markedly decreased with supplementation of dietary betaine. Oku *et al.* (59) reported that the expression of *Lpl* was tissue specific therefore, the effect of dietary betaine on *Lpl* expression needs further examination for Nile tilapia different organs other than liver.

Igf-1 considers the major anabolic factor needed for growth (60). *Igf-1* mRNA expression in the current study showed upregulation with addition of dietary betaine and down regulation when fish fed SBM-based diets without betaine which could subsequently reduce growth performance. Similar results were reported by Men *et al.* (61) who found that when fish fed on plant protein based diets at high level (60%) showed lower hepatic *Igf-1* than that fed 30% in Japanese seabass.

Conclusion

From the obtained results, it could be concluded that betaine incorporation with 0.2% level to SBM-based diet improve palatability, growth performance, intestinal absorptive capacity, body protein on expense of lipid body accumulation, hepatic *Igf-1* gene expression and reduce the lipogenesis through markedly decrease *Fas* and *Lpl* mRNA level in liver.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

THE EFFECTS OF GROUP VERSUS SINGLE HOUSING ON HOME CAGE BEHAVIOUR IN TWO STRAINS OF LABORATORY MICE

Ahmed F. Abou-Elnaga[1], Ibrahim F. Rehan[2]*, Ralph RJ. Thompson[3], Usama A. Abou-Ismail[1], Motamed E. Mahmoud[4], Radi A. Mohamed[5], Hesham H. Mohammed[6], Ahmed A. Sabek[7], Mohamed Z. Elhussiny[8].

[1]Department of Husbandry and Development of Animal Wealth, Faculty of Veterinary Medicine, Mansoura University, P.O. Box 35516, Egypt, [2]Department of Animal Behaviour and Husbandry (management, genetics and breeding), Faculty of Veterinary Medicine, South Valley University, 83523, Egypt, [3]University of Edinburgh, UK, [4]Department of Animal Behaviour and Husbandry (management, genetics and breeding), Faculty of Veterinary Medicine, Sohag University, 82524, Egypt, [5]Department of Aquaculture (Animal and Fish Behaviour and Welfare), Faculty of Aquatic Sciences and Fisheries, University of Kafrelsheikh, P.O. Box 33511, Egypt, [6]Department of Veterinary Public Health, Faculty of Veterinary Medicine, Zagazig University, 44511, Egypt, [7]Department of Veterinary Hygiene and Management, Faculty of Veterinary Medicine, Benha University, 13736, Egypt, [8]Department of Animal & Poultry Behaviour and Management, Faculty of Veterinary Medicine, Aswan University, 81528, Egypt

*Corresponding author, E-mail: ibrahim.rehan@vet.svu.edu.eg

Abstract: Studying the behavioural patterns of animals in their house may help to understand their needs, but there has been a little investigation of home-cage behaviours in commonly used inbred strains of mice such as C57BL/6 and DBA/2. Therefore, understanding behavioural patterns in these mice is important for neuroscience research. For the first time, this experiment was carried out to investigate the long-term effects of housing conditions (single vs. group) on home cage behaviour of C57BL/6 and DBA/2 mice, in order to reveal differences between C57BL/6 and DBA/2 in home-cage behaviours and in response to single-housing. Sixty-four mice (32 mice/strain) were housed either singly (SH) (n= 32) or in four groups, each group contained 8 animals. Home cage behavioural patterns were recorded weekly using ethogram-based instantaneous sampling for 5 consecutive weeks. Regardless of strain, single housed (SH) mice displayed higher levels of grooming and bedding directed-behaviours and were more frequently seen in-the-crawl ball, and had lower levels of feeding behaviour compared to their group housed (GH) conspecifics. There were significant strain differences in anxiety-related behaviours with the DBA/2 strain demonstrating higher levels of sleep, feeding and grooming behaviour and frequent presence in-the-crawl ball, and lower levels of exploration, locomotion and bedding-directed behaviour compared to the C57BL/6 strain. The results therefore suggest that different housing systems influence home cage behaviours of laboratory mice with the mice of the DBA/2 strain appearing more anxious. These findings may also have great implications for researchers to decide the most appropriate phenotype to use in measuring neural response–relevant behaviours in novel animal/human models.

Key words: anxiety; behaviour; C57BL/6; DBA/2; ethogram; home-cage

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Introduction

The housing conditions of laboratory rodents have been demonstrated to induce changes in their behaviour, physiology and pathology (1) which in turn could impact the reproducibility and validity of experimental results. An important aspect of the housing conditions is the social environment (e.g. single versus group housing). Improving the housing conditions of laboratory rodents may enhance welfare in these animals by promoting their ability to cope with the environment, and may also improve the accuracy of experimental data by providing a more valid animal model for research, and may therefore reduce the number of animals used.

The hypothesis that alterations in home-cage behaviour can upset neural circuit function has attracted much consideration of the recent years both in the context of disease detection and more commonly to quantify food consumption and activity parameters (2). Moreover, great effort was directed towards the 'mouse phenome' relating characterization of common inbred strains to various behavioural tests (3). Significant differences have been found between inbred mice strains in spontaneously emitted activity, particularly in the open-field test (4). In addition, large genetic differences in movement as a response to stimulant administration (5) could provide evidence of genetic associates with many behaviours, such as sleep (6). Studies carried out on inbred strains of mice have revealed consistent differences in behaviour due to the biological functions of their genotype (7). In addition, many different inbred genetic lines showed differences in behaviour which pose challenges when seeking functional explanations for specific neural mechanisms (8). Therefore, there is interest in assigning particular behavioural phenotypes as characteristic features to easily differentiate each mouse strain and further to develop easy criteria to assess their behavioural competences (9).

C57BL/6 and DBA/2 are two of the commonly used inbred strains of laboratory mice. Numerous behavioural differences have been reported between them (10); furthermore, environmental manipulations have induced remarkable differences in epigenetic effects between strains (11). Studying home-cage behavioural differences between strains can yield several advantages for behavioural phenotypic purposes e.g. reliable measurement of stable behavioural circadian rhythms that are highly responsive to environmental signals, such as light and human interference (12).

Individual housing is generally not recommended for social laboratory rodents such as mice as it compromises their welfare, but there remains some confusion surrounding the impact of individual housing on mice. Many authors illustrated that single housing, compared to group housing of mice does not amplify urinary corticosterone level (13-16), whereas others agreed that individual housing for social animals like mice is a stressful condition (17). Measures of physiological stress; however, do not necessarily correlate with subjective welfare of the animal. Together with the conflicting research on stress this highlights the need for a further investigation onto the impact of individual housing on mice, and how this might differ between strains, especially on the consequences for their behaviour. In the present study, the home-cage behaviour of single and group housed C57BL/6 and DBA/2 mice was investigated using ethogram-based behavioural observation.

Material and methods

General animal housing

Sixty-four adult male mice (32 C57BL/6J and 32 DBA/2), purchased from the Animal House, Theodor Bilharz Research Institute, Giza, Egypt, were used in this experiment. The mice were 10 weeks of age on arrival and had an average body weight about 28 g. After transportation, all animals were given 2 weeks to habituate to their new environment and to adjust to the new lighting regime and were therefore 12 weeks of age at the start of the experiment. All cages were supplied with sawdust (as bedding material) and a crawl-ball (115 mm, with 3 × 58 mm holes, polycarbonate ball, Lillico, UK) as an enrichment object. Cages were inspected daily and were cleaned once a week,

during which mice were removed and re-housed in clean cages with new bedding material. Mice had *ad-libitum* access to pelleted food (Mice chow®, Oil and Soap Manufacturing Company, El-Gharbia, Egypt) and tap water, were maintained under conditions of controlled temperature (22±2 °C) and humidity (60%) and were checked daily. The room was maintained under a 12:12 h light/dark cycle. Lights were turned off at 19:00 h and on at 07:00 h with a continuous dim red light allowing observation in the dark phase.

The group housed mice were marked on their tails by using a permanent marker pen to allow individual identification and these marks were refreshed every week throughout the experimental period. Likewise, the single housed mice were handled every week to avoid the behavioural changes between the two groups.

All experimental procedures were performed in accordance with the agreement for the Animal Care and Use and Approval of Ethics Committee for Animal Experimentation of Mansoura University, Egypt and care was taken to comply with the 3R concept.

Experimental treatments

A total of 64 mice representing two strains (32 mice of DBA/2J strain and 32 mice of C57BL/6J strain) were arbitrarily assigned to one of the following two experimental treatments:

1) "Single housing" (SH): 16 mice of DBA/2J strain and 16 mice of C57BL/6J strain (n= 32) were housed singly in Plexiglas cages (27.5 cm length × 16.5 cm width × 21.5 cm height).

2) "Group housing" (GH): 16 mice of DBA/2J strain and 16 mice of C57BL/6J strain (n= 8) were housed in groups of four in Plexiglas cages (27.5 cm length × 16.5 cm width × 21.5 cm height).

All animals were introduced to their particular experimental treatments at 12 weeks of age and were naïve to the housing conditions. They were kept under the same housing condition until they were 17 weeks old; the age at which data collection was stopped.

Behavioural assessment

In order to allow mice to habituate to the presence of the observer, the observer entered the experimental room 10 min before the observation started (18). Behaviour of the mice in each cage was recorded in the usual housing location during the dark phase when mice are most active. Observations were made in real time using an instantaneous scan sampling method with 10s intervals. Each sample interval was prompted by an audio cue via headphones, and the behaviour was recorded into a check sheet. Observation sessions, were carried out consecutively on the same day, and lasted 640 s (100s per mouse, yielding 10 scans per mouse for each of 64 mice). Observation was carried out for each cage once per week for five consecutive weeks. This meant a total of 50 scans were recorded per mouse over the entire experimental period. A complete ethogram of mice's behaviours is shown in Table 1.

Data analyses

Data was organized and summarized and tested for normality, linearity and homogeneity of variance. Then the average percentage of scans spent performing each behavioural pattern for each individual mouse was calculated by dividing the number of scans for each behavioural variable by the total number of scans and the resultant value was multiplied by 100. Two-way ANOVA was used to test for differences in behavioural patterns between the two strains of mice (DBA/2 and C57BL/6J) within the two experimental treatments (group and single housing) and result were expressed as Means ± standard error (M ± SEM) as independent variables followed by Bonferroni-PostHoc tests to compare each strain to its housing type using SPSS V.20. In cases of the significant interactions (interaction between two variables), e.g. housing system*strain, general linear model was used to compare means of behavioural patterns with different housing system as a factor and strain as a dependent variable.

Results

Main effect of experimental treatment

The output of the two-way ANOVA-test showed that there was an effect of housing conditions on the home cage behaviour of mice with the mice in the SH treatment displaying higher levels of enrichment-directed behaviours ($F_{3,60}$= 27.59, P= 0.001) (Fig. 1) than those in the GH, and those of C57BL/6J displayed higher levels of bedding-directed behaviours ($F_{3,60}$= 5.647, P= 0.035) (Fig. 3) compared to their conspecifics in GH treatment. On the other hand, GH mice showed higher levels of ingestive behaviour compared to SH mice ($F_{3,60}$= 10.851, P= 0.01) (Fig. 4).

Main effect of strain

Several behaviour patterns recorded in this study showed an effect of mouse strain: Enrichment-directed behaviour and sleep were higher in DBA/2J mice in both housing conditions compared to C57BL/6J ($F_{3,60}$= 30.654, P= 0.001) (Fig. 1) ($F_{3,60}$= 44.075.94, P= 0.001) (Fig. 5) respectively, in contrast, exploration ($F_{3,60}$= 54.327, P< 0.001) (Fig. 6) and locomotion ($F_{3,60}$= 37.623, P< 0.001) (Fig. 7) were higher in C57BL/6J .

*Housing*strain interaction effects*

SH mice of the DBA/2J strain displayed higher levels of grooming ($F_{3,60}$= 9.204, P= 0.01) (Fig. 2). Whereas bedding-directed behaviours in SH treatment ($F_{3,60}$= 9.661, P< 0.009) (Fig. 3) were more frequently performed by C57BL/6J mice than those of DBA/2J. Furthermore, feeding behaviour were highly significant in GH treatment in DBA/2J mice ($F_{3,60}$= 11.083, P= 0.006) (Fig. 4).

Table 1: Ethogram for behavioural elements recorded

Behavioural category	Description
Feeding behaviour	Rears up to gnaw at food pellets through the bars of the food hopper.
Grooming	Lick its fur, groom with the forepaws, or scratch with any limb.
Sleep	Lying un alert with both eyes closed, curled up on its side, with its face tucked into its body and motionless.
Exploratory behaviour (investigation of food and environment)	Investigate food; investigate a non-food object in the environment (Sniffing cage wall, cage top and cage floor, (air-in) sniffing air inside the cage).
Bedding-directed behaviours	Digging, sniffing bedding, bedding manipulation (pushing bedding material forwards or backwards with nose, forepaws or hind legs) and burrowing.
Locomotion	Movement in the cage.
In-the-crawl ball	Mice were encapsulated by the crawl ball in which the whole body of the animal- with or without its tail- was inside the ball.

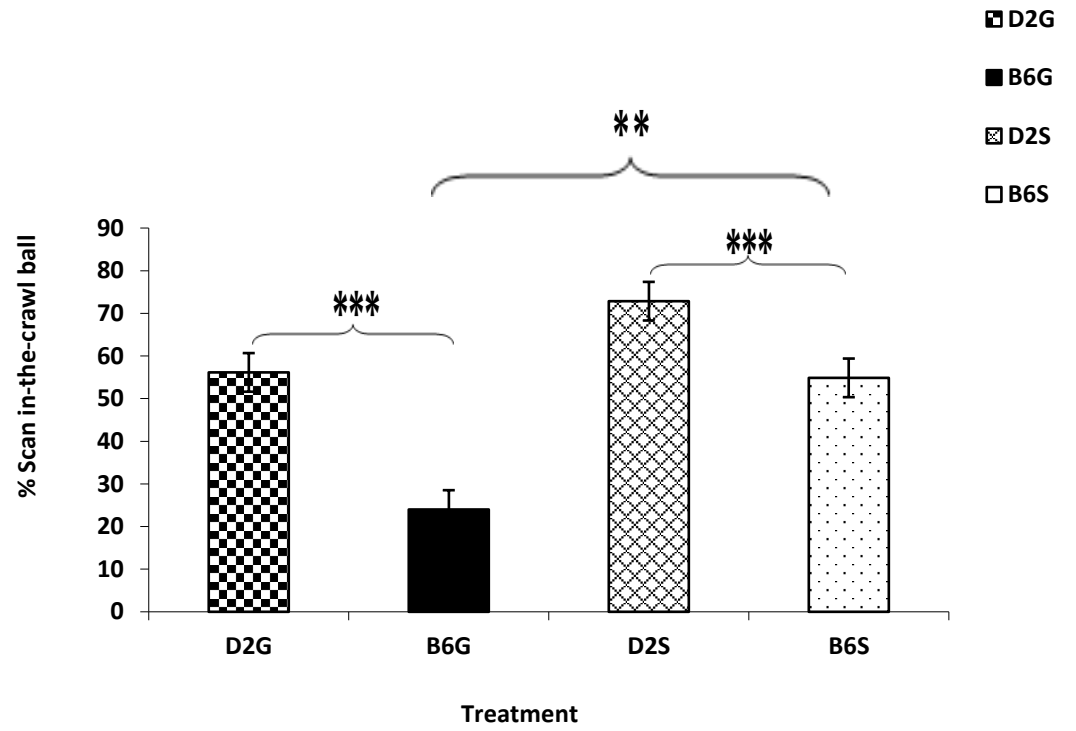

Figure 1: Average % of scans in the crawl-ball by the mice of the two inbred strains in the two experimental treatments. Data represent mean ± SEM, *** P<0.001, ** P<0.01. D2G: GH-DBA/2; B6G: GH-C57BL/6J; D2S: SH-DBA/2; B6S: SH- C57BL/6J

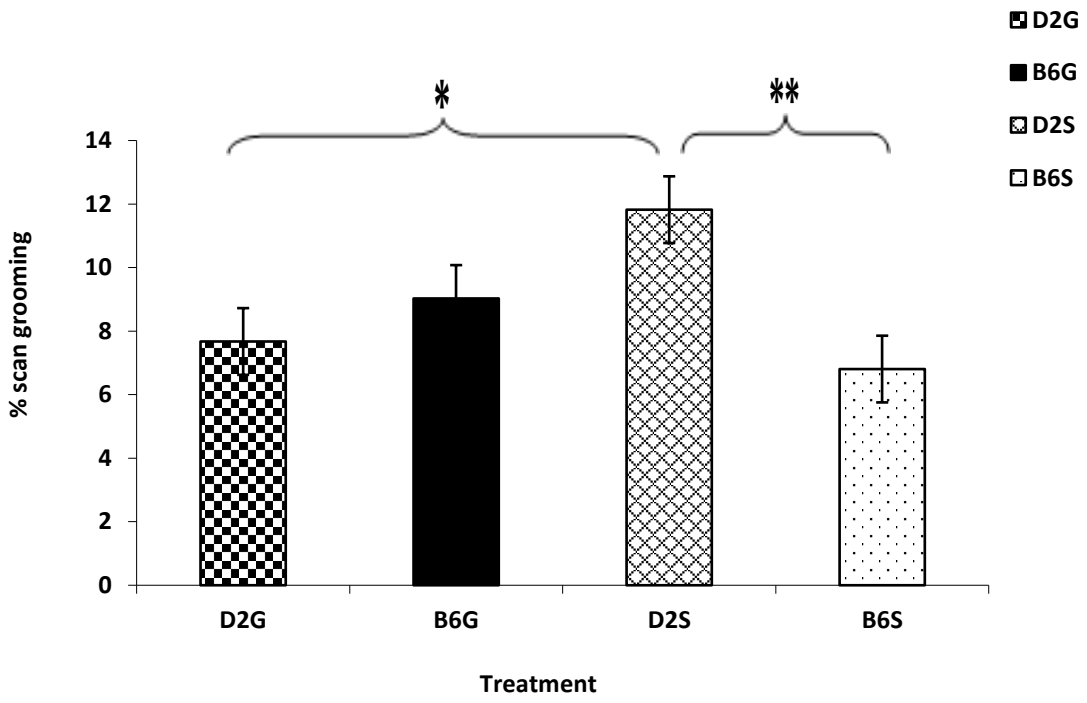

Figure 2: Average % of scans grooming by the mice of the two inbred strains in the two experimental treatments. Data represent mean ± SEM, ** P<0.01, * P<0.05. D2G: GH-DBA/2; B6G: GH-C57BL/6J; D2S: SH-DBA/2; B6S: SH- C57BL/6J

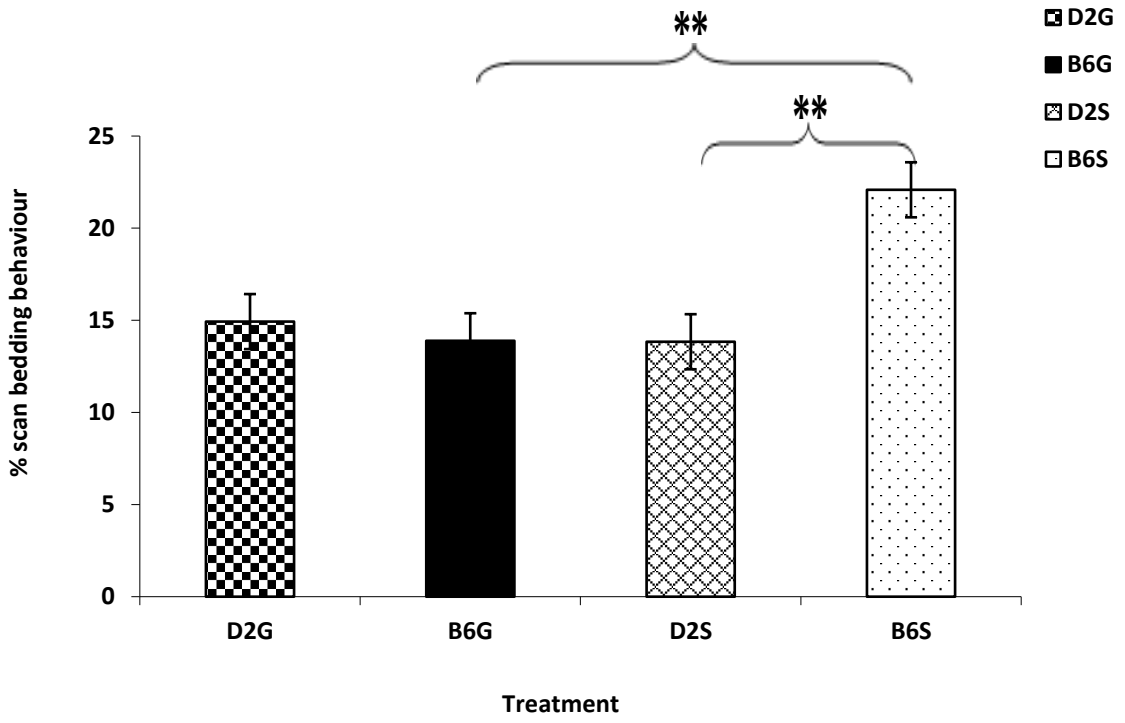

Figure 3: Average % of scans in bedding-directed behaviour by the mice of the two inbred strains in the two experimental treatments. Data represent mean ± SEM, ** P<0.01. D2G: GH-DBA/2; B6G: GH-C57BL/6J; D2S: SH-DBA/2; B6S: SH- C57BL/6J

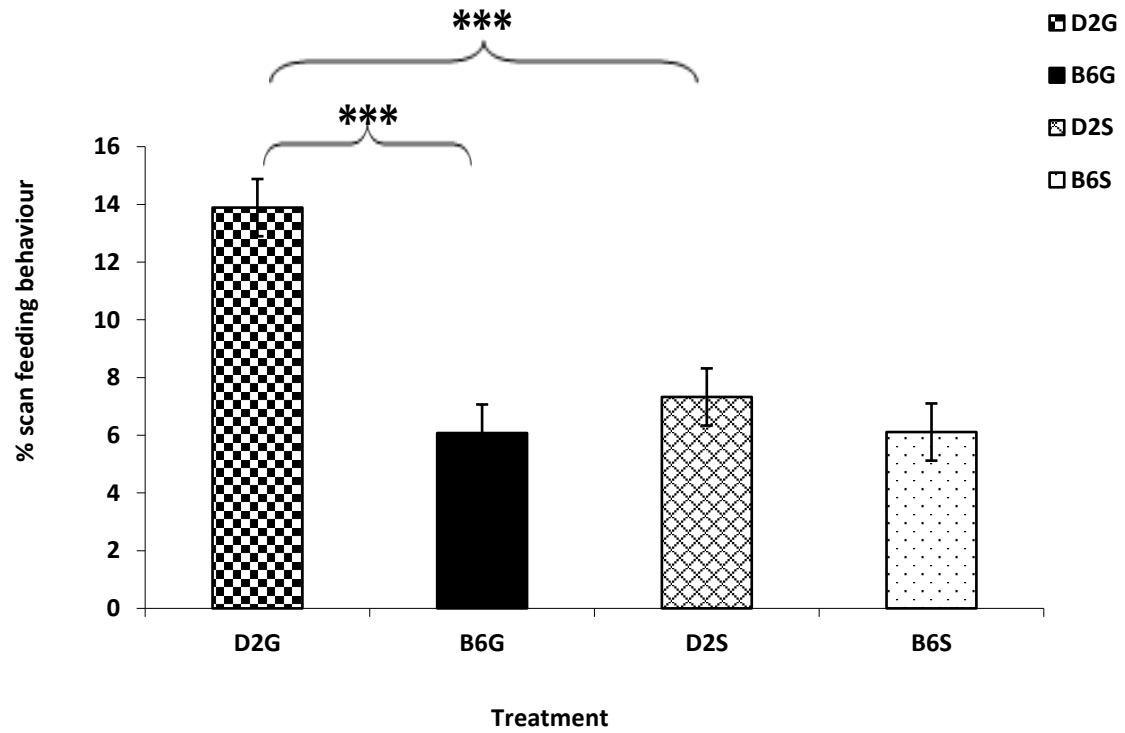

Figure 4: Average % of scans in feeding behaviour by the mice of the two inbred strains in the two experimental treatments. Data represent mean ± SEM, *** P<0.001. D2G: GH-DBA/2; B6G: GH-C57BL/6J; D2S: SH-DBA/2; B6S: SH- C57BL/6J

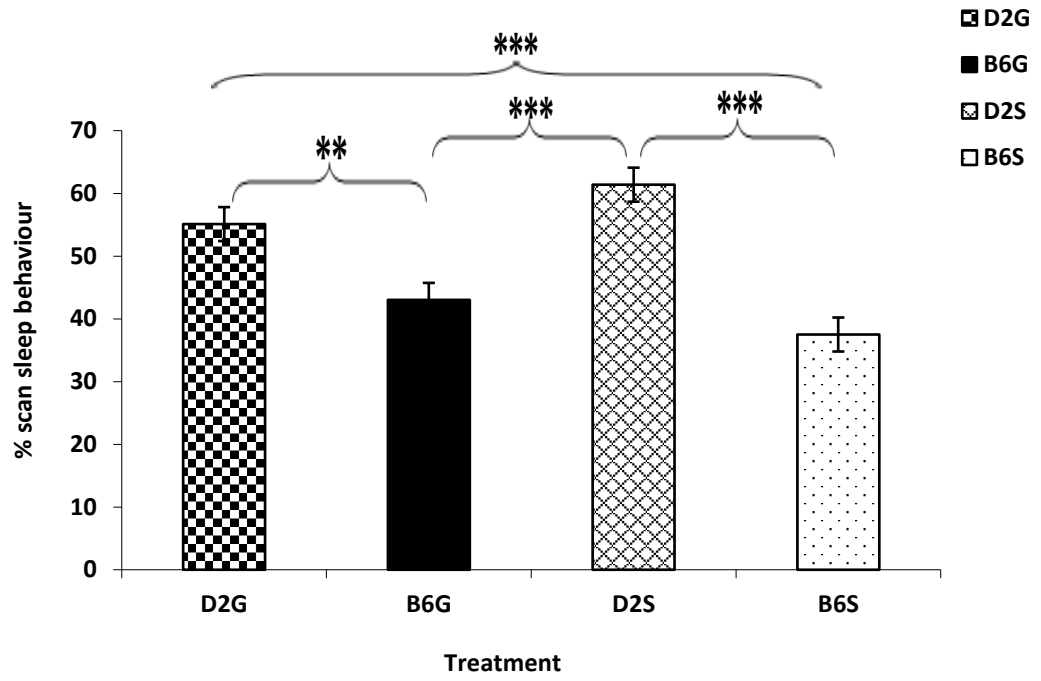

Figure 5: Average % of scans sleeping by the mice of the two inbred strains in the two experimental treatments. Data represent mean ± SEM, ** P<0.01, *** P<0.001. D2G: GH- DBA/2; B6G: GH-C57BL/6J; D2S: SH-DBA/2; B6S: SH- C57BL/6J

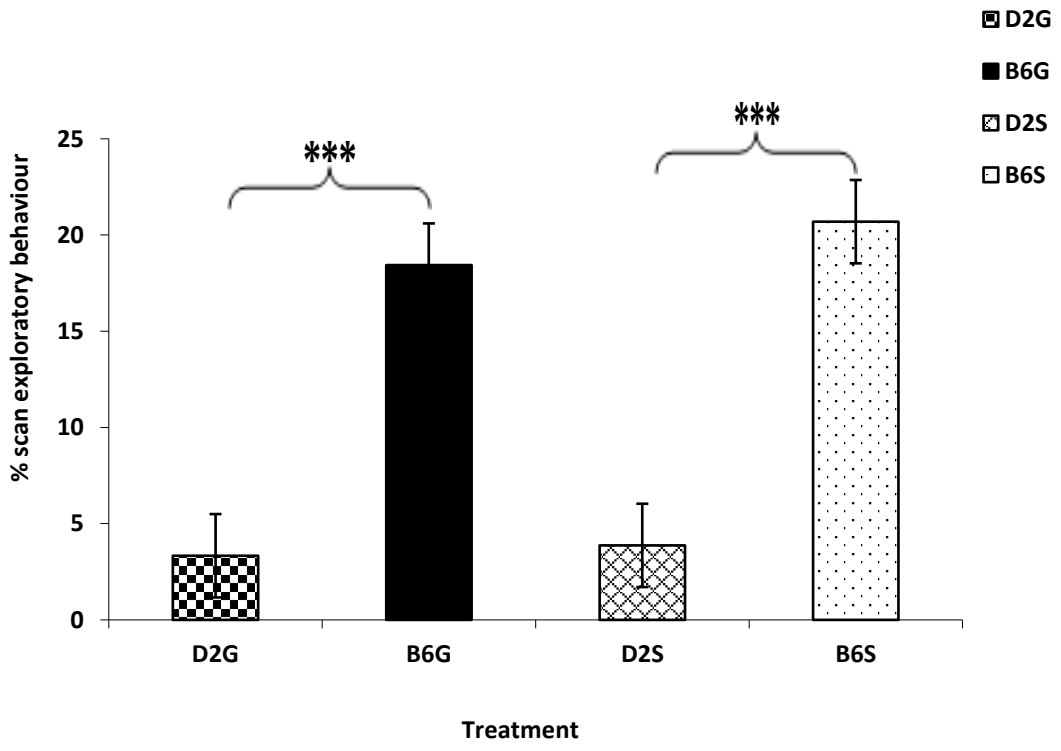

Figure 6: Average % of scans in exploratory behaviour by the mice of the two inbred strains in the two experimental treatments. Data represent mean ± SEM, *** P<0.001. D2G: GH-DBA/2; B6G: GH-C57BL/6J; D2S: SH-DBA/2; B6S: SH- C57BL/6J

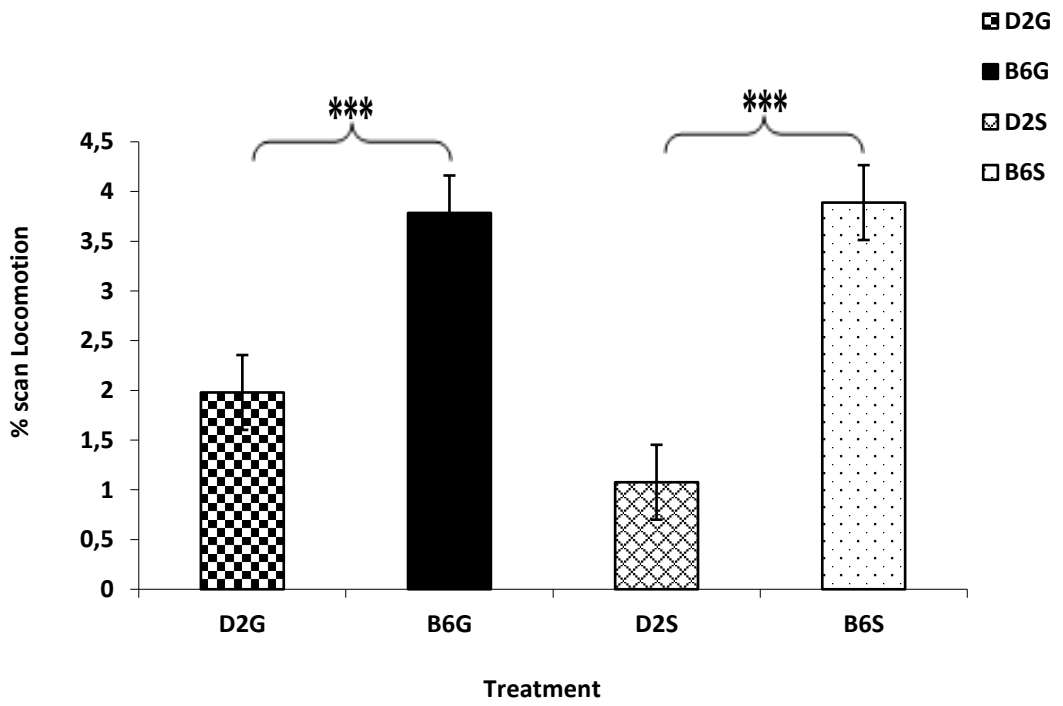

Figure 7: Average % of scans in locomotion by the mice of the two inbred strains in the two experimental treatments. Data represent mean ± SEM, *** P<0.001. D2G: GH- DBA/2; B6G: GH-C57BL/6J; D2S: SH-DBA/2; B6S: SH- C57BL/6J

Discussion

The aim of this experiment was to examine whether home cage behaviours vary between laboratory mice experiencing different housing conditions between two of the commonly used strains of mice, namely DBA/2J and C57BL/6J. The results of this study show clear differences between mice in different social environments where SH increased the levels of some behaviours including presence in the crawl-ball, grooming and bedding-directed behaviour whereas GH enhanced other behaviours such as feeding behaviour. The strain of the mice also had a significant effect on their home cage behaviours with individuals of the DBA/2J strain were more frequently seen sleeping, feeding, grooming, and in-the-crawl ball, and less frequently explorative, locomotive and directing behaviours towards bedding materials compared to mice of the C57BL/6J strain.

Although home cage behaviours play an integral role in monitoring stress responses and anxiety, there have been remarkably few studies comparing these behaviours particularly between the C57BL/6J and DBA/2 strains of laboratory mice. There has been some confusion surrounding the differences in behaviour between the strains. Frequent observations in many behavioural tests such as the novelty-induced locomotor activity test demonstrated that C57BL/6 was a highly active strain and that the DBA/2 was an intermediate one (19). Likewise, other reports showed that C57BL/6 was significantly more active (20) than DBA/2 strain. Other studies, however, could not establish a significant difference in activity between the two strains (21), while some found that DBA/2 strain was significantly more active than the C57BL/6 strain (22).

The results presented here showed a strain difference in the time period of sleep behaviour, with individuals of the DBA/2J strain sleeping

more than those of C57BL/6J strain in both housing conditions (GH and SH). This result supports that of (23) who found that DBA/2J mice were more anxious than C57BL/6J. Previous studies showed that mice with elevated levels of anxiety slept more and showed longer sleep latencies than those with low levels of anxiety (24). Differences in the levels of exploratory behaviour reported between strains of mice in the current experiment can also be explained in the light of anxiety levels of the two strains. Lower levels of exploratory behaviour expressed by individuals of DBA/2J could be due to the fact that they are more anxious than C57BL/6J. It has been previously shown that exploratory behaviour is influenced by levels of anxiety in which high level of anxiety suppresses exploration, and anxiety is associated with behavioural transition from ongoing behaviours such as transition from exploration to flight (escape) or other defensive behaviours (25-27).

Studies have compared locomotor activities between these strains of mice; however, these studies often compared their performance in different locomotor tests and the conclusions were inconsistent. For instance, C57BL/6J was shown as a higher-activity mouse strain than DBA/2J (28) whereas; no significant activity difference between the two strains (21). It was also found that DBA/2J mice were more active than C57BL/6J (22). Our findings compared their performance in their original home cages and showed that C57BL/6J mice performed more locomotor activity than DBA/2. Such results agree with those who illustrated that background strain differences and genetic mutations can affect locomotion (29). Furthermore, our results can fit with other findings which associated excessive motor activities with C57BL/6J mice (19). These results indicate that there are significant differences in activity and exploration between strains of mice. Therefore, differences between inbred mice should be considered when interpreting results from studies using inbred mouse strains (30).

Our data also showed a strain effect and treatment effect for being in-the-crawl ball. DBA/2J mice were seen in the crawl ball more frequently than C57BL/6J and this can be explained in reference to their high level of anxiety. DBA/2 mice were more prone to hide inside the crawl ball due to their high level of anxiety and fear response. The crawl ball can encapsulate small rodents like mice therefore providing them with shelter and retreat. This is consistent with the performance of these strains in the elevated plus maze where DBA/2J was shown to spend more time in the closed arms of the maze than C57BL/6J (31). On the other hand, SH mice of the two strains were more frequently seen in-the-crawl ball compared to GH counterparts. This could be due to SH mice treated crawl ball as the only surrogate physical contact in their environment that can receive their attention at the expense of other behaviour, whereas GH mice directed much of their behaviour towards conspecific animals in the cage.

Our results also found greater feeding behaviour in DBA/2J mice compared to C57BL/6J in GH mice. It was reported that food ingestion of individually housed male DBA/2J mice was greater than that of male C57BL/6J by 5-10% (32, 33). Similarly, it was found that food intake of DBA/2 mice was 50% higher than in C57BL/6J (34). The same author did not explain the difference between their results in 2005 and 2007 but given that DBA/2 mice have higher basal metabolic rate by 30% and a core temperature 0.7°C higher than C57BL/6J (35), small differences in ambient temperature could explain the strain difference.

The reason for high levels of feeding performed by DBA/2J mice in GH conditions compared to SH could be their high levels of energy expenditure and movement activity. SH DBA/2J mice were found to sleep more and to move less than their conspecifics in GH situation therefore consuming less food. It could also be due to high levels of social interaction (both aggressive and non-aggressive) in GH situation. On the other hand, low level of food intake in SH condition could be due to stress experienced by these animals. Chronic stress has been shown to have an anorexic affect (reduces food and water intake) (36) in laboratory rodents.

There was also a strain-housing interaction effect on the level of grooming behaviour with DBA/2J self-groomed more than C57BL/6J in single housing situations. The higher level of grooming expressed by DBA/2J mice could be due to the high levels of sleep in these animals. Self-grooming was reported as the most time-consuming activity of the laboratory rodent awake time and second most time-consuming activity in laboratory rodents after sleep and was reported to be concentrated around sleeping time. It takes place after sleeping, but also occurs when the animal prepares for sleep (37). But it could also be due to the high levels of anxiety in these animals (38, 39) as grooming could be acting as an alternative satisfier or "tension- reducer". Similarly, it was illustrated that grooming is extensively identified as a behavioural marker of stress in rodents (40), suggesting the possibility that more grooming in DBA/2J mice especially in single rather than group housed situation may be due to the high level of anxiety in this strain. The reason for the finding that SH mice groomed more than GH mice could be the stress of single housing. It has been illustrated that mice are social animals thus individual housing is a kind of stressful condition for them (41).

The results of the current experiment also revealed that SH mice of the C57BL/6J strain exhibited higher levels of bedding-directed behaviours compared to those of the GH. The increase in the level of bedding-directed behaviour by the SH mice could be explained as an attempt to escape. Single housing of laboratory rodents in laboratory cages has been shown to be stressful and to increase specific forms of behaviours termed as 'escape-related' (42). Whereas the increased level of bedding-directed behaviours by the C57BL/6J than DBA/2J mice could reflect their high level of activity and exploration.

Conclusion

Our findings clearly highlight the importance of social factors and strain in modulating the behaviour of inbred mice and call for cautious interpretations of behavioural changes incurred by these factors. Moreover, understanding strain differences in behaviours of DBA/2J and C57BL/6J mice may help us in finding better animal models of specific neurobehavioural responses in terms of genotype-dependent sensitivity of animals to stress and its relation to their behaviours. For example, our data on higher locomotion in C57BL/6J mice suggest that C57BL/6J mice may be a better choice than DBA/2J in order to study the effects of genetic mutations and drug administration likely to affect such behaviour.

Contributions

Ahmed F. Abou-Elnaga and Ibrahim F. Rehan designed the survey protocol, supervised data collection procedures and drafted the final version of the manuscript; Ralph RJ Thompson, Usama A. Abou-Ismail, Motamed E. Mahmoud, Radi A. Mohamed and Hesham H, Mohammed analysed the data and shared in experimental protocol; Ahmed A. Sabek and Mohamed Z. Elhussiny have finalized the experimental design and revised the manuscript. All authors contributed to, editing, and approving the final manuscript as submitted.

Competing financial interests

The authors declare that they have no competing financial interests and non-financial interests.

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Original Research Article

AMELIORATING THE TOXIC EFFECTS OF CYPERMETHRIN BY SESAME OIL IN MALE RABBITS

Sahar M. El-Sheshtawy[1]*, Ghada I. A. El-Gobary[2], Nashwa A. Omar[3], Nessrin A. Shawky[3]

[1]Toxicology, Animal Health Research Institute, Tanta Lab, Egypt, [2]Biochemistry, Animal Health Research Institute, Tanta Lab, [3]Pharmacology, Animal Health Research Institute, Tanta and Zagazig Lab, Egypt.

*Corresponding author, E-mail: saharmelsheshtawy@gmail.com

Abstract: This study intended to evaluate the beneficial protective effects of sesame oil (SO) on cypermethrin (CYP)-induced sub chronic toxicity in rabbits. Male rabbits were divided into 4 groups; I: control; II: Cypermethrin (CYP, 48 mg/kg body weight, bw); III: CYP (48 mg/kg bw)+SO (1 ml/kg bw); and IV: SO (1 ml/kg bw). All treatments were orally administered three times per week for 9 weeks. CYP induced toxic effects on liver and kidney as revealed by a significant increase in serum liver enzyme activities with a significant decrease in total protein and albumin in addition to a significant increase in creatinine and uric acid levels. The effect of CYP on male fertility was demonstrated by a significant decrease in testis and epididymis weights, epididymis sperm counts, sperm motility with a significant increase in sperm abnormalities. Besides, a significant decrease in testosterone levels along with a significant increase in prolactin levels. CYP induced oxidative stress distinguished by a significant increase in malondialdehyde (MDA) levels in addition to a significant decrease in reduced glutathione (GSH) contents and superoxide dismutase (SOD) activity. Also, CYP produced a significant increase in sperm DNA damage. Co-treatment of SO with CYP improved all alterations induced by CYP. In conclusion SO has beneficial protective effects as it tends to dampen CYP sub chronic toxicity in male rabbit.

Key words: sesame oil; cypermethrin; oxidative stress; male fertility; rabbit

Introduction

Cypermethrin (CYP) is synthetic pyrethroid widely used in agriculture. Due to the lipophilic nature of pyrethroid insecticides, they conglomerate in biological membranes (1), resulting in stimulation of free radical formation leading to oxidative stress in mammals (2). CYP is absorbed via the gastrointestinal and respiratory tracts and confers discriminatory distribution into lipid-rich internal tissues (3). CYP initially thought to be unharmed for household implementation, however later a number of reports displayed its reproductive poisoning in mammalian and non-mammalian laboratory and wildlife animal species (4). The reproductive toxicity of CYP is a major concern because spermatogenesis may be vulnerable to chronic exposure to chemicals at very low dose. Due to existence of CYP as lipid soluble and of

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small size, it passes through cell membrane and may damage DNA (5, 6). It was found that CYP raises the chromosomal deviation, and micronuclei in human peripheral lymphocytes (7) and has adverse effects on male adult rats reproduction by decreasing testicular weight, epididymis sperm counts, and motility (8, 9).

Sesame (*Sesame indicum*) has phenolic compounds, non-protein amino acids, alkaloids, cacogenics glycosides, polyunsaturated fats and lipids, mucilage, phospholipids, thiazole, disulphide, ketones, aldehyde, vitamins as B1, B2, C and E and trace elements such as calcium, magnesium,iron, copper, zinc and phosphorus (10, 11). Sesame contains abundant lignans like lipid-soluble lignans (sesamin and sesamolin), sterol and water-soluble lignan glycosides (sesaminol triglucoside and sesaminol diglycoside) with varied antioxidant attributes which have the capability to improve fertility potency of male reproductive system. (12). Sesame seed could improve the adverse effects of reactive oxygen species on testicular parameters (13, 14). Sesame has protective role on the male reproductive tract due to binding to the estrogen receptors (ER) and modification of androgen receptor (AR) activity in the testis (15, 16).

Therefore, this study was performed to evaluate the toxic effects of CYP on liver and kidney function, male reproductive toxicity in male rabbits and its amelioration by sesame oil.

Materials and methods

Materials

Cypermethrin (10% E.C, C22H19Cl2NO3, molar mass 416.30 g/mol, synthetic pyrethroid. α-cyano-3-phenoxybenzyl (1RS) - cis, trans- 3-(2,2-diclorovinyl)-2, 2-dimethyl-cyclopropane carboxylate, 10% purity) was purchased from Cenavisa Laboratorios Cemi Pendra Estela s/n 43205 Reus (Spain). Sesame oil was obtained from Cap. pharm.

Experimental animals

Twenty eight sexually-mature, healthy White New Zealand rabbits 6-8 months old, weighting about 2.000–2.100 kg with clinically normal genitalia and apparently healthy were obtained from private farm and were kept in stainless steel wire mesh cages under sanitary hygienic conditions. The room temperature was kept at 25-27°C with 45-70% humidity and 12-h light .The rabbits were fed on a standard pelleted ration according to (17, 18) with chemical composition of Crude protein % 18, Digestible energy Kcal/kg of diet 2600, Crude fiber % 10-12, Calcium % 1.2, Phosphorus % 0.8 Lysine % 0.75, Methionine and % 0.65cysteine. The drinking water was available *ad libitum* throughout the study and being acclimatized for two weeks prior to the experiment.

Experimental design

Rabbits were allocated into 4 equal groups (7/group). All treatments were applied orally three times per week for 9 weeks to cover all the spermatogenesis period.The rabbits in the first group were considered as control and not given any treatment. The second group received CYP [48 mg/kg bw (19)].The third group was given CYP (48 mg/kg bw) + SO [1 ml/kg bw (20)]. The fourth group was administered SO (1 ml/kg bw). Body weight was recorded in the non-fed state at the beginning of study (initial weight) and at time before slaughter (final weight). Weight earning (final body weight (g)- primary body weight (g) was calculated.

Blood samples

Blood samples were collected at end of experimental period (9 weeks). Blood samples were gathered from the marginal ear vein using vacuum tubes, left to coagulate and then centrifuged at 3000 rpm for 10 min. The sera were then stored frozen at −20°C until the biochemical analysis.

Biochemical assays

Serum levels of liver damage enzymes [aspartate aminotransferase (AST), alanine amino transferase (ALT), and alkaline phosphates (ALP)], kidney function parameters (Urea, uric acid and creatinine), albumin, total protein, calcium, sodium, potassium and magnesium, superoxide dismutase (SOD), reduced glutathione (GSH), and Malondialdehyde (MDA) were determined using commercially available kits and as previously described (19, 20). Serum total testosterone and prolactin levels were assessed

by Enzyme Linked Immunosorbent Assay (ELISA) as depicted by (21).

Seminal examination

After blood sampling, rabbits were slaughtered and seminal fluid were collected from the epididymis for seminal examination as previously described (21, 22).

Comet assay

Testes were examined for DNA damage using comet assay (single-cell gel electrophoresis) as previously described (23).

Statistical analysis

One-way analysis of variance using GraphPad Prism 7 (GraphPad Software, Inc, La Jolla, CA) was used to determine the difference between the groups. Comparison of means was carried out with Tukey's honestly significant difference test. Data were presented as mean ± standard error of mean (SEM) and significance was declared at $P < 0.05$.

Results and discussion

Body weight

Table (1) showed a notable decrease in the body weight of CYP- treated groups. Co-treatment of SO and CYP resulted in a significant increase in the body weight when compared to CYP-treated group. These results were in agreement with (24, 25) in rats and in rabbits (26).

The decrease in body weight of CYP treated group may be resulted from the effect of insecticide pyrethroid on gastrointestinal tract which subsequently leads to decrease in appetite and absorption of nutrients from gut (27).This decrease related to the liver injury (28). Also CYP intake may has a combined action of cholinergic (decrease feed intake and diarrhea) and oxidative stress and/or due to increase in breakdown of lipids and proteins as a direct effect of CYP pesticide exposure (29). (30) noted changes in epithelial cells and mucosal layer of digestive tract, resulting in reduced appetite and growth rate. Sesame oil rich in several antioxidants and chemo-preventive agents such as tocopherol, sesamolin, sesaminol and sesamin as well as mono-unsaturated and polyunsaturated fatty acids, rich in vitamin A, B and E as well as iron, calcium, magnesium and copper (31). All these compounds may be the cause which lead to a relatively positive effects and decrease the negative effect of CYP.

Biochemical investigation

The serum levels of ALT, AST and ALP in CYP intoxicated group were significantly increased in comparison with the control group (Table 2). In consistence, previous studies also showed similar increased in liver enzyme activities in CYP intoxication (32). Exposure to pesticide causes disruption of hepatic cell and leakage of enzymes from hepatocytes to blood (33). Increases of serum liver enzymes usually indicate liver damage (34). We also found that administration of SO with CYP significantly decreased these enzyme. SO is hepato-protective, anti-inflammatory, and can decrease lipid peroxidation by decreasing free radicals and increasing antioxidant properties (35).

Administration of CYP significantly increased urea, creatinine and uric acid (Table 2). Co-supplementation of SO with CYP showed a significant decrease in urea, creatinine and uric acid in comparison with CYP -intoxicated group. These results were in agreement with those reported by other studies (36). An elevation of blood creatinine level is associated with kidney damage which agree with results reports by (37). Sesame oil has an ameliorative effect as it decrease the negative effect of CYP. This may be due to its anti-inflammatory antioxidant properties (35). CYP intoxication significantly decreased total protein and albumin (Table 2). Co-administration of SO with CYP restored CYP effects towards normal levels. These results agree with (38, 39).

CYP intoxication significantly decreased calcium and sodium and increased potassium and magnesium levels in serum (Table 2). Co-supplementation of SO with CYP showed a significant elevation in calcium and sodium levels with significant decline in potassium and magnesium levels when compared to CYP-treated group. This result was parallel to that of (40). Increased potassium level could be due to CYP

effect on potassium channel leading to an alteration in the activation potential (41). The benefits effects of SO could be due to its profusion of antioxidant which interfere with renin-angiotensin system (42). Sesame oil induced significant increase in serum magnesium level l and this disagreed with the results of (43).

CYP intoxication significantly decreased testosterone and significantly elevated prolactin (Table 2). SO decreased this negative effect of CYP. Similar effect was reported by (44) who found that exposure to CYP significantly decreased testosterone synthesis. Decrease in testosterone revealed extra testicular target by pesticides and also suggests central effect for pesticides on hypothalamus pituitary axis (45). The Luteinizing hormone (LH) induced leydig cells to produce testosterone and so, decrease in LH inhibit testosterone production. Moreover, low testosterone, follicular stimulating hormone (FSH) and LH levels inhibit effective spermatogenesis and evolution of seminiferous tubules, thus causing infertility (46).

CYP inducted a significant elevation in serum levels of MDA in addition to a significant decline in reduced glutathione (GSH) contents and superoxide dismutase (SOD) activity as compared to the control group (Table 3). Co-treatment of SO with CYP mitigated the adverse effects of CYP toward control levels. (47) found that CYP inducted significant elevations in serum MDA, SOD and significant decline in serum GSH levels. This oxidative damage was normalized by co-administration of SO with CYP, where SO provided observed protection against oxidative stress induced by CYP and this agreed with (48).

Sperm count and abnormalities

There were marked sperm deformities in head and tail of CYP treated group as compared to the control group (Table 4). This result agreed with (9, 49). This may be due to inhibition of LH and FSH levels (9, 50). Degenerated stereo cilia and focal mononuclear cellular infiltration without any spermatozoa in epididymis were noticed following CYP treatment in rabbits (51). Co-treatment of SO mitigated the adverse effects of CYP toward control levels. This result agreed with (52) and may be attributed to the antioxidant properties of SO.

Weight of testes and epididymis

CYP induced a significant decrease in testicular and epididymis index weight when compared to the control (Table 4). The same results was recorded by (8). SO induced significant increase in testicular weight which may be due to increase in testosterone level, which can be lead to increased discrimination for leydig cells which stimulated by the increase in LH and thus increase testicular weight. Also, there was significant increase in epididymis weight which may be due to increase in number of protein receptors specific to testosterone which leads to increase epididymis weight (53).

Table 1: Initial and final body weight of male rabbits exposed to Cypermethrin three times/week for 9 weeks and the protective effect of Sesame oil (n=7)

	Control	CYP	CYP + SO	SO	P value
Initial body weight (gm.)	2070±97.46	2065±84.45	2060±54.77	2080±103.68	0.958
Final body weight(gm.)	3008±51.18	2100±139.82	2600±45.82	2900±15.811	0.00001*
Weight gain (gm.)	930±3.082	40±122.678	550±62.048	820±7.905	0.00001*

The result is significant at $p \leq 0.05$. CYP: Cypermethrin, SO: Sesame oil

Table 2: Serum biochemical parameters of male rabbits exposed to CYP and the protective effect of SO

	Control	SO	CYP + SO	CYP
AST (U/L)	52.5±2.32	33.75±10.05	61.5 ±0.96*	104.0±3.58**
ALT(U/L)	31.5±0.65	27.5±1.85	44.23±1.47*	81.25±1.93**
ALP(U/L)	111±5.91	112.8±2.62	117.5 ±4.94*	395.7±13.01**
TP (g/dl)	4.57± 0.11	4.65± 0.15	4.15± 0.06*	3.48± 0.193*
Alb. (g/dl)	2.85± 0.64	3.32± 0.11	2.95±.0.064*	1.97± 0.06*
Cr.(mg/dl)	0.68±0.05	0.69± 0.05	1.29± 0.21*	2.39 ± 0.186**
Urea (mg/dl)	17.0±0.82	15.5± 1.32	36.75±1.65 **	91.5± 9.31**
UA (mg/dl)	3.48± 0.25	3.6±0.11	3.7± 0.12*	4.32 ± 0.14**
Ca (mg/dl)	8.35± 0.23	9.95 ± 0.22*	8.92 ± 0.11*	7.8 ± 0.20*
Na (mmol/L)	131.0 ± 0.91	134.0 ± 1.87	128.25 ± 0.63*	114.7 ± 2.56*
K (mmol/L)	4.0 ± 0.09	4.3 ± 0.39	5.1 ± 0.07*	6.95 ± 0.2**
Mg (mg/dl)	2.0± 0.09	2.35 ± 0.06	2.23 ± 0.08	2.82 ± 0.48
Testo (ng/ml)	3.53 ± 0.28	7.67 ± 0.48*	4.25 ± 0.14	2.48 ± 0.31*
Prol (ng/ml)	10.5 ± 0.96	8.75 ± 0.75	13.0 ± 1.22	19.75 ± 1.65**

*Significant at ($P \leq 0.05$) ** Significant at ($P \leq 0.01$).Results are represented as mean ± standard error. CYP: Cypermethrin, SO: Sesame oil, AST: Aspartate transaminase, ALT: Alanine transaminase, ALP: Alkaline phosphatase, TP: total protein, Alb.: albumin, Cr: creatinine, UA: Uric acid, Ca: Calcium, Na: Sodium, Mg: magnesium, Testo: Testosterone, Prol: Prolactin.

Table 3: Lipid peroxide (MDA), super oxide dismutase (SOD) and reduced glutathione (GSH) of male rabbits exposed to CYP and the protective effect of SO

	MDA(nmol/ml)	SOD(μ/ml)	GSH (μ/ml)	P value
Control	4.48±0.486	29.5±1.516	1.096±0.145	0.00001*
CYP	7.88±0.178	20.4±1.14	1.008±0.078	0.00001*
CYP+SO	5.22±0.349	25.6±1.140	0.794±0.072	0.00001*
SO	3.10±0.200	34.0±1.581	1.300±0.700	0.00001*

*Significant at ($P \leq 0.05$). Results are represented as mean ± standard error
MDA: Malondialdehyde, SOD: Superoxide dismutase, GSH: Reduced glutathione, CYP: Cypermethrin, SO: Sesame oil

Table 4: Weight of sexual organs and epididymis sperm characters in male rabbits exposed to CYP and the protective effect of SO

	Control	Cypermethrin	Cypermethrin + Sesame oil	Sesame oil
Weight of testes(gm.)	5.09 ± 0.116*	4.138 ± 0.03*	4.304 ±0.071*	4.952 ± 0.09*
Weight of epididymis	1.80±0.100*	0.87 ±0.66*	1.118 ±0.13*	1.66 ±0.114*
Sperm concentration ($10^6 mm^3$)	34.86 ± 2.84	12.73±1.74**	22.3± 5.04*	33.6 ± 3.48
Sperm mortality%	87.6 ± 3.89	57.7±.4.14**	72.0± 3.25*	85.0 ± 4.12
Life sperm%	86.0 ± 3.22	50.7 +5.6**	64.0 ± 2.53*	80.0± 3.88
Sperm abnormality %	11.01 ± 0.28*	20.41±0.52**	15.01 ± 0.18*	9.21 ± o.23*

*Significat difference at ($P \leq 0.05$) **Significant at ($P \leq 0.01$)

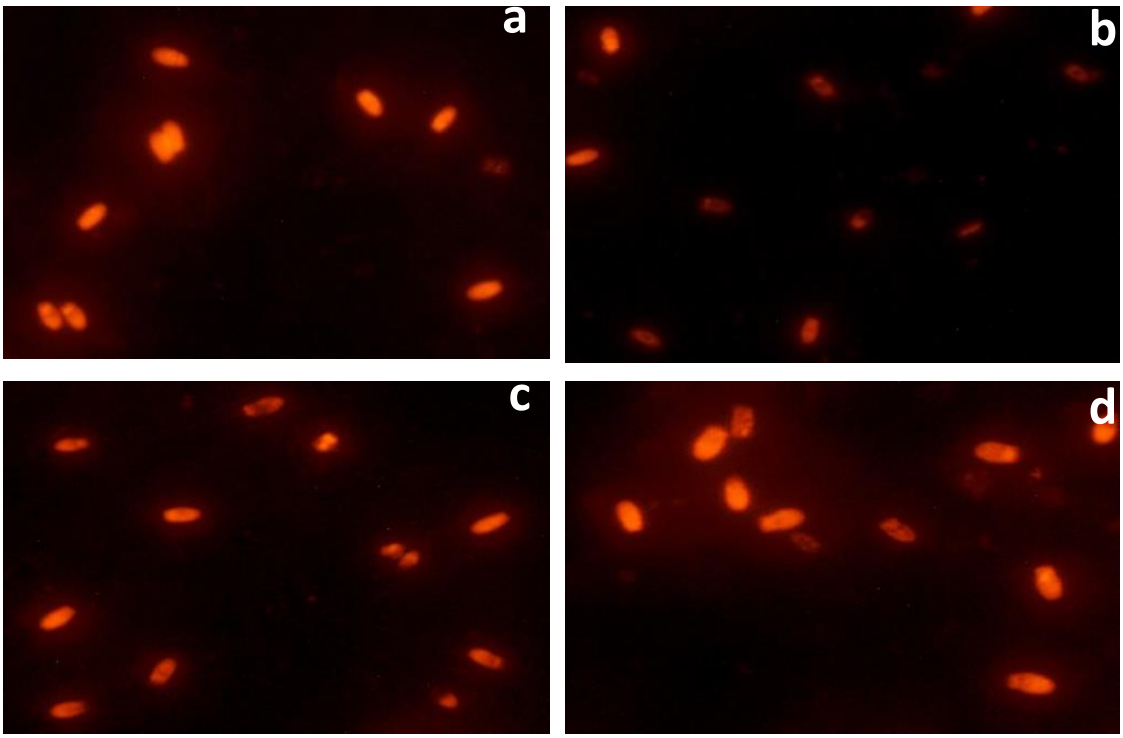

Figure 1: Comet assay results. a. Group (I) showing high incidence of genetically intact sperms. b. Group (II) displaying high incidence of genetically damaged sperms. c. Group (III) exhibiting a reduction in number of genetically degenerated sperms. d. Group (IV) revealing appearance of few genetically degenerated sperms. X 400

Comet assay

Figure 1 shows the negative effects of CYP and the defensive effects of SO on sperm DNA. The likely mechanism of pyrethroids to produce sperm DNA deterioration; may be due to their decreased molecular size and hydrophobic nature, so they cross blood testicular barrier and

reach the sperm nucleus (5). Pyrethroids have been shown to induct oxidative stress by generating free radicals which are the main cause of DNA damage (54).

Conclusion

This study confirms that SO has a beneficial protective effects as it tends to dampen CYP sub chronic toxicity in male rabbits through improvement of body weight, serum biochemical, antioxidant parameters, sperm characters as well as sperm DNA damage.

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Original Research Article

BIOREMEDIATION OF A PESTICIDE AND SELECTED HEAVY METALS IN WASTEWATER FROM VARIOUS SOURCES USING A CONSORTIUM OF MICROALGAE AND CYANOBACTERIA

Mohamed A. Abdel-Razek [1], Ahmed M. Abozeid[2], Mahmoud M. Eltholth[3, 4], Fatma A. Abouelenien[3], Sami A. El-Midany[3], Nader Y. Moustafa[5,] and Radi A. Mohamed[2]*

[1]Department of Chemistry and Toxicity of Pesticides, Faculty of Agriculture, Kafrelsheikh University, Egypt [2]Department of Aquaculture, Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University, Egypt. [3]Department of Hygiene and Preventative Medicine, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt. [4]Institute of Aquaculture, University of Stirling, Stirling, UK. [5]Department of Food Control, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt

*Corresponding author, E-mail: r.mohamed.vet@gmail.com

Abstract: The presence of organophosphate pesticides and heavy metals in water are known to be toxic to aquatic organisms. Bioremediation makes use of naturally-occurring organisms to remove pollutants from the environment. This study explored the potential of using a consortium of microalgae and cyanobacteria (*Chlorella vulgaris*, *Scenedesmus quadricuda* and *Spirulina platensis*) to remove the organophosphate pesticide malathion and the heavy metals cadmium, nickel and lead from water samples taken from varying combinations of urban wastewater and agricultural drainage water in Egypt. The fastest algal growth observed in this study was in a treatment containing the microorganismal consortium, malathion and heavy metals cultured in water samples taken from agriculture drainage and urban wastewater. Microalgae in this study were able to remove malathion from samples of wastewater with up to 99% efficacy and were able to bioaccumulate nickel at up to 95% efficacy. Moreover, microalgae demonstrated the ability to uptake lead and cadmium at up to 89% and 88% efficacy respectively. The results from this study suggest that a consortium of Chlorella vulgaris, *Scenedesmus quadricuda* and *Spirulina platensis* can be effective in remediating the pesticide malathion and the heavy metals cadmium, lead and nickel from wastewater.

Key words: bioremediation, wastewater, microorganisms, microalgae, pesticides, heavy metals

Introduction

Effluents from urban and agro-industrial wastewater are known to cause considerable contamination of rivers, lakes and seas around the world (1). Such contamination can come in the form of heavy metals, pesticides and fertilizers. One particularly toxic pesticide which is found in water sources across the globe is malathion which belongs to the organophosphate group. Malathion is a non-systemic, broad-spectrum pesticide that is used to control insects

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on vegetables, fruits and crops as well as household insects and animal parasites (2). However, malathion has been reported to have numerous deleterious effects amongst humans and animals including hepatotoxicity (3), human breast carcinoma (4), genetic disruption (5), and damage of normal hormonal activity (6). In addition to pesticides, heavy metals are also recognized as pollutants that are both commonly occurring and persistent in the aquatic environment. Heavy metals are typically introduced into water bodies via wastewater which includes industrial effluents. Of the heavy metals commonly found in water bodies, lead, cadmium and nickel are reported to be some of the most hazardous (7). In particular, through the processes of bioaccumulation and biosorption, concentrations of heavy metal ions can increase across the aquatic food chain and can be transferred to humans posing a considerable risk to health (8). Due to their toxicity and tendency to accumulate in water, pesticides and heavy metals occurring in high levels can become severely toxic to all living creatures. In fish, bioaccumulation of metals has been shown to cause either high mortality or result in many biochemical and histological changes in the surviving fish (9).

Traditional wastewater treatment processes include a combination of physical and chemical methods such as chemical precipitation, activated sludge process, carbon adsorption and microfiltration. Such techniques are used to eliminate inorganic phosphates, organic wastes, and toxins. Unfortunately, such treatment methods can be expensive to implement and moreover can be inefficient and non-environmentally friendly. In contrast, bioremediation provides an alternative method of eliminating contaminants from the environment. Most organophosphate compounds are decomposed by microorganisms in the environment as a source of carbon or phosphorus or both (10-12).

Photoautotrophic microorganisms, including green algae, are used in wastewater remediation processes due to their ability to accumulate deleterious heavy metals from aqueous effluents (13). Moreover, they have the potential to utilize organic carbon and light simultaneously as sources of energy. This provides microalgae with an essential competitive advantage over bacteria and fungi in removing organic pollutants (14). Microalgae and cyanobacteria associate with other aerobic or anaerobic microorganisms to form microbial groups that live symbiotically in a community-defined consortium. This consortium of algae and bacteria can act in a synergistic way to break down organic and inorganic pollutants much more effectively than individual microorganisms (15). For these reasons, microalgae have been used in various studies on remediation of pollutants, for both organic compounds and heavy metals. Algae are capable of the uptake and elimination of organic pollutants via both biosorption and/or metabolization (16). The major advantage of algae-mediated bioremediation is that it can achieve various goals of wastewater treatment simultaneously such as correction of pH, reduction of total dissolved solids (TDS) and removal of both chemical oxygen demand (COD) and biological oxygen demand (BOD) (17-19). Furthermore, the primary mechanism in algae-mediated remediation results in the considerable production of oxygen through photosynthesis. This natural generation of oxygen further assists in the degradation of contaminants and decreases the requirement for the utilization of mechanical aerators in traditional wastewater treatment processes (20). In addition to its extensive capacity at bioremediation, the consequent biomass created by the use of algae could be used for the formation of a wide range of value-added products. This combined method of biomass production and wastewater treatment has been termed the “integrated cultivation process”(21,22).

The aim of this study was to evaluate the growth of a combined consortium of microalgae and cyanobacteria (*C. vulgaris, S. quadricuda* and *S. platensis*) in the presence of the pesticide malathion and the heavy metals cadmium, nickel and lead, as well as their efficacy in removing these pollutants from contaminated water taken from different wastewater sources.

Material and methods

Isolation of microorganisms, enrichment and acclimatization

The algal strains *Chlorella vulgaris* and *Scenedesmus quadricuda* and the cyanobacteria *Spirulina platensis* were isolated from different water samples collected from Lake Burullus in the north of the Egyptian delta in July 2016. Subsamples of the collected water were placed on sterile agar plates that were formed by the addition of 1.5% agar to BG-11 medium. Axenic strains of microalgae were obtained through continuous sub-culturing in BG11 agar plates supplemented with ampicillin and kanamycin. Fluorescent lamps were used for illumination throughout the culture process (23). Algal strains were identified through electrochemical analysis in the Institute of Biotechnology, Universidad Nacional Autónoma de México (UNAM), Mexico (24). The cultured consortium of microorganisms was then incubated with the pesticide malathion and the heavy metals cadmium (Cd), nickel (Ni) and lead (Pb). Agricultural drainage water was collected from a known source of water emanating from agricultural lands whereas urban wastewater was collected from a known source of water emanating from urban areas in Kafr El Sheikh Governorate.

Chemicals

Malathion was obtained from the Kafr El Zayat Pesticide Company in Egypt (Malathion 98% active ingredient) and Cd, Ni and Pb were obtained in pure powder form from Sigma Aldrich® in Egypt.

Experimental design and sampling

The study used eight glass aquaria (20 cm x 40 cm x 30 cm) representing seven treatments and one control (Table 1) in three replicates. All aquaria were filled with 10 L of water from either urban wastewater alone, agricultural drainage water alone, or a combination of both, according to the treatment in question. Microorganisms were batch-cultured in the seven treatment aquaria whereas no microorganisms were added to the control. The control treatment consisted solely of pesticides and heavy metals in the test water. The study used an inoculum of microorganisms at a concentration of approximately 8 x 10^4 cell/ml. Different combinations of Cd, Pb, Ni and malathion, were then added to aquaria, according to treatment type, at concentrations of 5, 10, 10 and 25 ppm respectively. To maintain the optimum condition for the culture of microorganisms throughout the experiment, the flasks were incubated at 23 ± 1 °C in a culture room under a 24 hour photoperiod by using a 25-watt bulb in each aquarium. In addition, aquaria were supplied with continuous aeration. Samples of test water were taken at different periods throughout the duration of the experiment (0, 1st, 3rd, 7th, 14th, 21st, 28th day) for the quantification of microorganismal growth (cell count) as well as for the analysis of heavy metals and physicochemical parameters. Malathion residues were prepared for quantification by centrifuging the algal culture filtrates.

Measurement of microorganismal growth

The growth of microorganisms in the seven treatments was quantified in terms of cell count by using a T80 UV-visible spectrophotometer (OD600 PG Instruments, United Kingdom).

Analysis of malathion concentrations

For malathion analysis, 2 ml samples were taken every four days from each glass aquarium and were placed in glass tubes. These samples were then extracted twice with equal volumes of 1:1:1 hexane-acetone-dichloromethane as the extracting reagent. The mixture was homogenized for three minutes using a vortex mixer. The extracting reagent with residual malathion was filtered, dried by use of anhydrous sodium sulfate and followed by filtration through Whatman GF/B glass-fiber paper. This operation was performed sequentially, and the filtrates were mixed. The filtrate was then evaporated till dryness and resolved in HPLC-grade dichloromethane (50 μL) for analysis. Malathion residues were quantified using trace gas chromatography coupled to a Polaris Q Thermo Finnigan mass spectrophotometer (GC–MS)

using the EPA8141 method under the subsequent conditions: equity column-5; 30 m x 0.25 mm ID; 0.25 µm, temperature of oven (120 °C) for three minutes and increased at a rate of 5 °C per minute till 270 °C; temperature of injector (250 °C); injection volume 1 µl, MSD detector; scan range 45–450 amu; helium flow 30 cm/s (120 °C), transfer line (320 °C), splitless (0.3 minute), splitless liner, and double taper (25). The kinetics of the removal of malathion was conducted for a period of 54 hours.

Analysis of heavy metal concentrations

The heavy metals Ni, Pb and Cd were quantified by first gently evaporating 200 ml of water from each aquarium till dryness. The residues were then dissolved in 5 ml concentrated nitric acid (HNO_3). Subsequently, 5-10 drops of hydrogen peroxide (H_2O_2) were added in order to ensure the completion of the process of digestion. The dried residue was eluted in 1 ml HNO_3, (26) and then the concentrations of heavy metals were quantified using an atomic absorption spectrophotometer (GBC Avanta E, Victoria, Australia; Ser. No. A5616).

Physicochemical analysis of water samples

Analysis was carried out for various water quality parameters such as temperature, pH, electrical conductivity (EC) and total dissolved solids (TDS). Temperature and pH were measured using a glass electrode pocket pH and temperature meter (Digital Mini-pH Meter, Model 55, Fisher Scientific, USA) whereas TDS and EC were measured using a salinity-conductivity meter (YSI EC300, YSI Corp., Yellowstone Springs, Ohio, USA**.**

Statistical analysis

The data were tested for normality of distribution, linearity and homogeneity of variance. The final concentrations of malathion, Ni, Pb and Cd were analyzed using general linear models (SAS Statistical Analysis Software). The physicochemical parameters were analyzed using a one-way ANOVA. The level of significance was set at $P \leq 0.05$.

Results and discussion

Effect of malathion on growth of tested algal strains

Growth of the microorganismal consortium was observed across all treatments (Fig. 1). However, the highest level of growth was observed in the treatment containing both the pesticide malathion and the heavy metals cadmium, lead and nickel in the combined water from agriculture drainage and urban wastewater (treatment 'PHMAU' [pesticides and heavy metals with microorganisms in agriculture drainage water and urban wastewater]; Fig. 1). The largest increase in the number of cells throughout the experiment was observed in this treatment, reaching a maximum level of 15 x 10^6 cells/ml in day 28 of the experiment. The second highest level of microorganismal growth was observed in the treatment only containing malathion in urban wastewater ('PMU' [pesticides with microorganisms in urban wastewater]). Peak algal growth in this treatment reached 12 x 10^6 cells/ml in day 28 of the experiment. On the other hand, the lowest growth of tested algal strains during 28 days of exposure was recorded in the treatment containing just microorganisms in agricultural drainage water ('MA').

The high level of algal growth in the presence of heavy metals and pesticides observed in this study corresponds with other studies conducted elsewhere. The study by (27) found that microorganisms can indeed utilize a wide range of organic pollutants, including pesticides, as an energy source for their growth and simultaneously mineralize and degrade the compounds. Similarly, results from the study by (28) which studied the growth of *C. vulgaris* in the presence of the organophosphate pesticide dimethoate reported enhanced microalgal growth and higher protein and chlorophyll content. The stimulatory effect of malathion on the growth of microalgae reported in our study corroborates with findings in (29) 2016 This study which examined the growth response of *S. quadricuda* and *C. vulgaris* in the presence of malathion reported a considerable increase in

cell count in water containing malathion. In addition, (30) studied the impact of malathion on the growth of *Aspergiullus oryzae*. They reported accelerated growth of this microorganism when malathion was added to the culture water.

The stimulatory effect of malathion on microorganismal growth could be a result of the increase of the available phosphorus due to the breakdown by the microalgae (11). Moreover, the accelerated growth observed in the microorganisms that were cultured in the presence of malathion could be attributed to their ability to use this compound as a sole source of phosphorus in the absence of inorganic phosphate from the growth medium (31).

Effect of heavy metals on growth of the microorganismal consortium

he highest level of microorganismal growth observed in this study was in the treatment containing both the pesticide malathion and the heavy metals cadmium, lead and nickel in the combined water from agriculture drainage water and urban wastewater ('PHMAU'; Fig. 1). The second highest level of microorganismal growth observed in the treatments containing heavy metals was in the treatment consisting of just heavy metals in the sample containing only urban wastewater ('HMU'; Fig. 1). In this treatment, microorganismal growth reached a peak of 9×10^6 cells/ml at the end of experiment. The lowest level of growth was observed in the treatment containing microorganisms alone in agricultural drainage water ('MA'; Fig. 1).

The results from this study are in line with findings from a study by (32) which investigated the effects of mercury (Hg), Cd and Pb on the growth of *S. quadricuda*. The authors of this study observed enhanced algal growth in the presence of Pb and Cd ions at concentrations similar to those used in our study (5-20 ppm). However, this study also reported a deleterious effect of Hg on algal cells at any concentration. As suggested in this paper, the reason for this accelerated algal growth in the presence of Pb and Cd could be attributed to a phenomenon involving heavy metals resulting in an increase in chlorophyll content.

Breakdown of malathion by the consortium of microorganisms in wastewater

This study measured the growth potential of a consortium of microorganisms in water from different sources as well as their capability to biologically breakdown the pesticide malathion. The highest level of malathion removal by the microorganismal consortium was observed in the treatment with pesticides, heavy metals and microorganisms in the combined water from agriculture drainage and urban wastewater ('PHMAU'; 99% [24.75 mg/L] Fig. 2), followed by the treatment containing just pesticides and microorganisms in urban wastewater ('PMU'; 95% [23.5 ± 0.1 mg/L]) and the treatment containing pesticides and microorganisms in agricultural drainage water ('PMA'; 85% [21.25 mg/L]). In comparison, malathion removal in the control treatment ('PHAU') was just 15% (3.75 ± 0.1 mg/L; Table 2).

The biggest advantage in using microorganisms for the degradation of organic compounds is the synergism with bacteria. They can degrade symbiotically wherein algae provide oxygen for the bacteria via photosynthesis and in turn uptake carbon dioxide released from the heterotrophic bacteria. The considerable capacity of the consortium of microorganisms used in this study to degrade malathion may be attributed to synergistic interactions whereby the overall degradative efficiency is increased (33). For example, (34) found that *Chlorella* spp. were capable of metabolizing up to 99% of the organophosphate pesticide fenamiphos in just 4 days making *Chlorella* a potent resource for fenamiphos removal. Similarly, (35) reported that *S. quadricuda* can effectively break down the fungicides dimethomorph and pyrimethanil as well as the herbicide isoproturon. The biodegradation observed in these studies as well as the study in question could be driven by enzymes produced by these microorganisms; enzymes such as cytochrome P450 (recorded in *Chlorella* spp.) which may assist in breaking chemical bonds in the pesticide molecules (36-40).

Bioaccumulation of heavy metals by the consortium of microorganisms in wastewater

This study investigated the bioaccumulation of Cd, Ni and Pb at concentrations of 5, 10 and 10 ppm respectively in a consortium of three different microorganisms and in different types of wastewater. The highest level of Pb removal was observed in the treatment with microorganisms, heavy metals and pesticides in the combined water from agricultural drainage and urban wastewater ('PHMAU'; 89% [8.9± 0.02 mg/L], Fig. 2) followed by the treatment with only heavy metals and microorganisms in urban wastewater ('HMU'; 88% [8.8 ± 0.05 mg/L]) and the treatment with heavy metals and microorganisms in agricultural drainage water ('HMA'; 87% [8.7 ± 0.04 mg/L]). In contrast, Pb removal in the control treatment ('PHAU) was just 1% (0.1 ± 0.02 mg/L; Table 2). Moreover, the three treatments containing microorganisms, HMA, HMU and PHMAU, showed the highest levels of Ni removal, 95% (9.5± 0.07 mg/L), 93% (9.3 ± 0.06 mg/L) and 91% (9.1 ± 0.02 mg/L), respectively in comparison with the control PHAU treatment where Ni removal was just 2% (0.2 ± 0.01 mg/L). Cadmium removal was highest in the treatment PHMAU, followed by HMA and HMU with removal of 88% (4.4 ± 0.025 mg/L), 86% (4.3 ± 0.015 mg/L) and 78% (3.9 ± 0.02 mg/L) respectively in comparison with the control PHAU treatment 3% (0.15 ± 0.015 mg/L; Table 2).

In accordance with our results, (41) reported that *Chlorella* spp. were efficient in removing Cd and Ni from growth medium under laboratory conditions with a removal potential ranging from 70% to 95%. Similarly, (16) found that *C. vulgaris* was effective in removing Cd, Pb and Hg. A study by (42) demonstrated that the cyanobacteria *Spirulina platensis* can tolerate Cd at concentrations up to 100 mg/L and was able to remove Cd at a rate of ~98.04 mg/L. (43) reported a high tolerance of *Scenedesmus* spp. to Ni, Cd, copper (Cu), and zinc (Zn) at relatively low concentrations of 2-5 mg/L but higher tolerance of lead up to 30 mg/L. The biosorption of various heavy metals such as Pb, Cd, Cu, Zn by various strains of microalgae could be attributed to their capacity to carry out ion exchange. Microalgae can hold mobile metal ions (e.g., potassium, sodium, magnesium and calcium) in their structure by binding them to acid functional groups (44, 45). Therefore, ion exchange could be the primary mechanism through which microalgae uptake heavy metals (46, 47). Furthermore, algal cell walls are surrounded by a three-dimensional network of macromolecules (proteins and polysaccharides) which carries negatively charged functional groups (e.g., hydroxyl, carboxyl, phosphate or amine groups, etc.) that play a crucial role in chemical binding with metal ions and are responsible for the biosorption ability of microalgae. Due to the cationic form of major metal ions in water solution, they are adsorbed to the algal cell walls (48-51).

Physicochemical parameters of water samples

The highest pH observed in this study was in the PMA treatment (8.517 ± 0.076) whereas the lowest pH recorded (7.966 ± 0.079) was in the PHAU treatment. Overall, there was a significant increase in pH in treated water. This could be attributed to the photosynthetic activity of algae resulting in the consumption of high quantities of bicarbonates and formation of a high level of carbonates thereby raising the pH (52). The highest levels of conductivity were observed in the PMA treatment (2339 ± 264.7 µS/cm) and the lowest levels were observed in the PHAU treatment (851.0 ± 18.57 µS/cm). Conductivity increased markedly towards the end of the experiment in treatments containing microorganisms, in particular the PMA treatment (3040 µS/cm). Higher conductivity in the presence of microorganisms coincides with the results of (53). The highest levels of TDS recorded in this study were in the PMA treatment (1171 ± 133.0 ppm) and the lowest levels were recorded in the PHAU treatment (425.9 ± 9.433 ppm). In the HMU treatment, there was a slight increase in TDS (590.0 ± 23.79 ppm). Overall, there was a significant increase in TDS in treated water compared to untreated water. There were no significant differences in temperature between experimental treatments. The

reading of temperature ranged from 16.61 ± 0.81 °C in the PHAU treatment to 16.81 ± 0.805 °C in the PMA treatment (Table 3). Temperature is a critical factor as algal productivity is accelerated by increasing temperature up to an optimum temperature which varies between algal species. Optimum temperature differs with limited nutrients or light conditions and growth often decreases when algae are subjected to a sudden change of temperature (54).

Table 1: Various treatments of the different sources of wastewater representing seven treatments and one control

Treatment	
1	Pesticides with microorganisms in urban wastewater (PMU)
2	Pesticides with microorganisms in agricultural drainage water (PMA)
3	Heavy metals with microorganisms in urban wastewater (HMU)
4	Heavy metals with microorganisms in agricultural drainage water (HMA)
5	Pesticides and heavy metals with microorganisms in agricultural drainage water and urban wastewater (PHMAU)
6	Microorganisms in urban wastewater (MU)
7	Microorganisms in agricultural drainage water (MA)
8	Pesticides and heavy metals in agricultural drainage water and urban wastewater (PHAU) (Control)*

* Initial concentrations for experimental water used: Cd, Ni and Pb were found to be 0.036, 0.2and 0.025 ng/mL respectively and 0.0012 ng/mL malathion

Table 2: Removal (%) of malathion and heavy metals using a consortium of microorganisms (the microalgae *Chlorella vulgaris*, *Scenedesmus quadricuda* and cyanobacteria *Spirulina platensis*) tested in wastewater obtained from different sources with concentrations of 25 ppm of the pesticide malathion and the heavy metals cadmium (Cd), nickel (Ni) and lead (Pb) at concentrations of 5 ppm of Cd, 10 ppm of Ni and 10 ppm of Pb after 28 days of treatment. The data are means ± standard error of three replicates

	Malathion		***Cd***		***Ni***		***Pb***	
Treatment	***mg/L***	***Removal (%)***	***mg/L***	***Removal (%)***	***mg/L***	***Removal (%)***	***mg/L***	***Removal (%)***
PMU	1.25 ± 0.1	95 ± 0.4						
PMA	3.75 ± 0.75	85 ± 0.3						
HMU			1.1 ± 0.02	78 ± 0.4	0.7 ± 0.06	93 ± 0.6	1.2 ± 0.05	88 ± 0.5
HMA			0.7 ± 0.015	86 ± 0.3	0.5 ± 0.07	95 ± 0.7	1.3 ± 0.04	87 ± 0.4
PHMAU	0.25 ± 0.125	99 ± 0.5	0.6 ± 0.025	88 ± 0.5	0.9 ± 0.02	91 ± 0.2	1.1 ± 0.02	89 ± 0.2
PHAU	21.25 ± 0.1	15 ± 0.4	4.85 ± 0.015	3 ± 0.3	9.8 ± 0.01	2 ± 0.1	9.9 ± 0.02	1 ± 0.2

PMU; Pesticides with microorganisms in urban wastewater, PMA; Pesticides with microorganisms in agricultural drainage water, HMU; Heavy metals with microorganisms in urban wastewater, HMA; Heavy metals with microorganisms in agricultural drainage water, PHMAU; Pesticides and heavy metals with microorganisms in agricultural drainage water and urban wastewater, PHAU; Pesticides and heavy metals in agricultural drainage water and urban wastewater

Table 3: Analysis of physicochemical parameters of the various sources of wastewater throughout the duration of the experiment

Treatment	Sample time								
	Parameter	Unit	Zero	Day1	Day3	Day7	Day14	Day21	Day28	Mean ±SEM
	pH	-	8.42	8.3	8.21	8.27	8.84	8.56	8.46	8.437±0.081
	Temperature	°C	18.7	18.8	18	17.7	16.7	14.3	13.4	16.80±0.812
PMU	EC	µS	992	1008	1123	1380	3030	2640	2340	1788±324.5
	TDS	ppm	496	504	560	690	1520	1330	1180	897.1±163.8
	pH	-	8.43	8.39	8.34	8.31	8.83	8.72	8.60	8.517±0.076
	Temperature	°C	18.7	18.8	18	17.7	16.7	14.4	13.4	16.81±0.805
PMA	EC	µS	1653	1672	1728	2120	2990	3170	3040	2339±264.7
	TDS	ppm	826	836	864	1060	1500	1590	1520	1171±133.0
	pH	-	8.45	8.46	8.51	8.44	8.54	8.55	8.46	8.487±0.017
	Temperature	°C	18.6	18.6	17.9	17.5	16.7	14.2	13.3	16.69±0.803
HMU	EC	µS	1076	1080	1096	1128	1177	1296	1404	1180±47.36
	TDS	ppm	538	540	548	564	589	648	703	590.0±23.79
	pH	-	8.5	8.48	8.5	8.45	8.52	8.54	8.45	8.491±0.013
	Temperature	°C	18.5	18.6	17.9	17.5	16.6	14.2	13.3	16.66±0.798
HMA	EC	µS	1651	1663	1684	1728	1770	1887	1986	1767±47.57
	TDS	ppm	825	832	843	864	885	944	990	883.3±23.45
	pH	-	8.37	8.42	8.5	8.11	8.40	8.58	8.49	8.410±0.057
	Temperature	°C	18.5	18.6	17.9	17.5	16.6	14.3	13.3	16.67±0.791
PHMAU	EC	µS	1001	1004	1017	1144	1789	1980	1898	1405±173.5
	TDS	ppm	501	502	509	572	896	990	949	702.7±86.76
	pH	-	8.37	8.31	8.23	8.12	8.28	8.26	8.18	8.250±0.031
	Temperature	°C	18.5	18.6	17.9	17.5	16.6	14.3	13.3	16.67±0.791
MU	EC	µS	1031	1052	1125	1247	1536	1911	2210	1445±174.4
	TDS	ppm	515	526	563	623	769	956	1110	723.1±87.81
	pH	-	8.44	8.36	8.28	8.04	8.10	8.33	8.31	8.266±0.054
	Temperature	°C	18.6	18.6	18	17.6	16.6	14.3	13.4	16.73±0.792
MA	EC	µS	997	1008	1051	1092	1236	1578	1848	1259±124.6
	TDS	ppm	498	504	525	546	618	788	925	629.1±62.39
	pH	-	7.9	8.04	8.27	7.64	7.79	8	8.12	7.966±0.079
(PHAU)	Temperature	°C	18.5	18.5	17.9	17.5	16.6	14.1	13.2	16.61±0.81
(Control)	EC	µS	806	808	819	834	857	898	935	851.0±18.57
	TDS	ppm	403	404	410	417	429	449	469	425.9±9.433

PMU; Pesticides with microorganisms in urban wastewater, PMA; Pesticides with microorganisms in agricultural drainage water, HMU; Heavy metals with microorganisms in urban wastewater, HMA; Heavy metals with microorganisms in agricultural drainage water, PHMAU; Pesticides and heavy metals with microorganisms in agricultural drainage water and urban wastewater, MU: Microorganisms in urban wastewater, MA; Microorganisms in agricultural drainage water, PHAU; Pesticides and heavy metals in agricultural drainage water and urban wastewater, EC; electrical conductivity, TDS; total dissolved solids

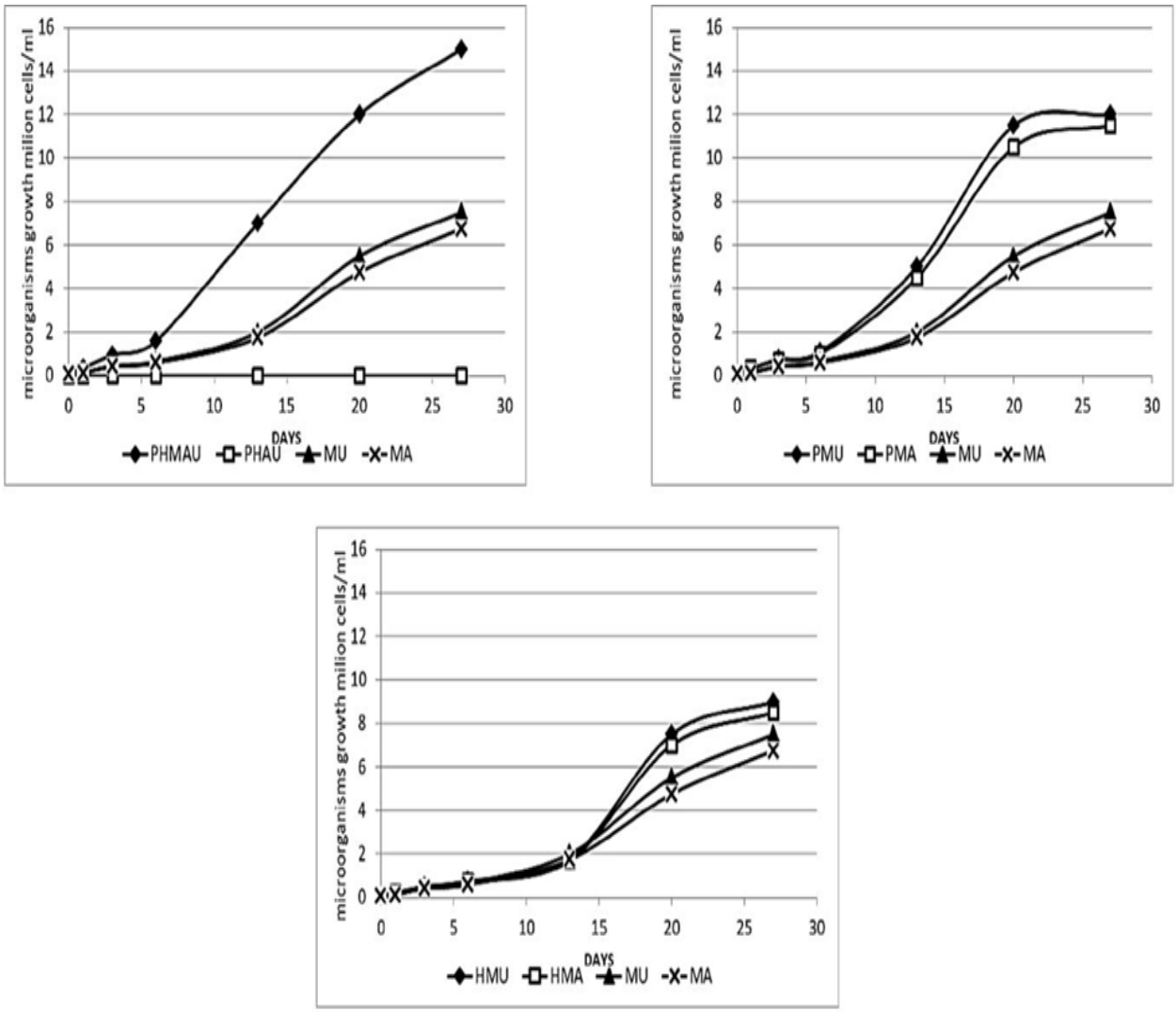

Figure 1: Growth curves of a consortium of *Chlorella vulgaris*, *Scenedesmus quadricuda* and cyanobacteria *Spirulina platensis* incubated in wastewater from different sources with heavy metals (5 ppm of cadmium, 10 ppm of nickel and 10 ppm of lead) and 25 ppm of the pesticide malathion

PMU; Pesticides with microorganisms in urban wastewater, PMA; Pesticides with microorganisms in agricultural drainage water, HMU; Heavy metals with microorganisms in urban wastewater, HMA; Heavy metals with microorganisms in agricultural drainage water, PHMAU; Pesticides and heavy metals with microorganisms in agricultural drainage water and urban wastewater, MU: Microorganisms in urban wastewater, MA; Microorganisms in agricultural drainage water, PHAU; Pesticides and heavy metals in agricultural drainage water and urban wastewater

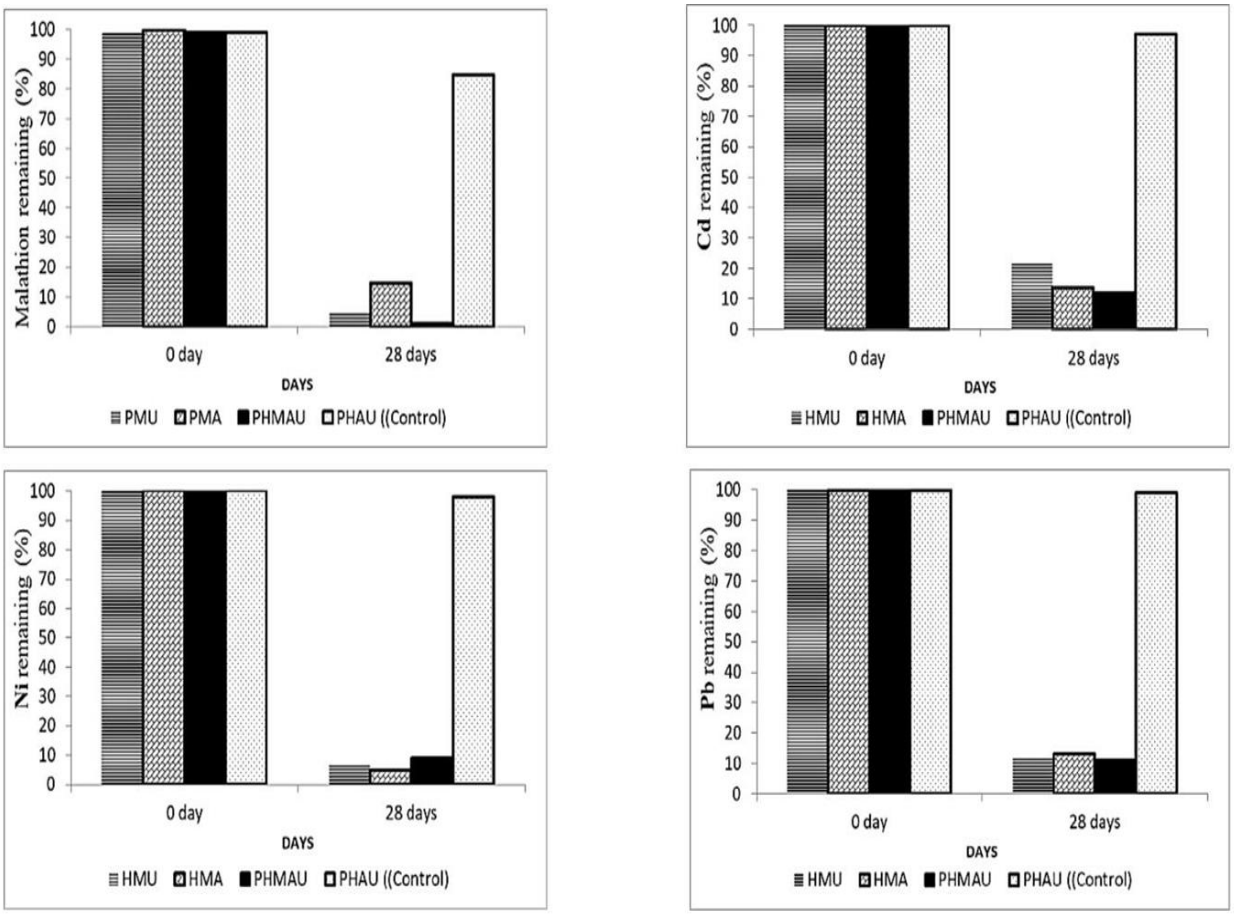

Figure 2: Residual malathion, cadmium, nickel and lead after treatment in wastewater from different sources containing a microalgal consortium (*Chlorella vulgaris*, *Scenedesmus quadricuda* and *Spirulina platensis*) for 28 days. Malathion was added at a concentration of 25 ppm in addition to 5 ppm of cadmium, 10 ppm of nickel and 10 ppm of lead

PMU; Pesticides with microorganisms in urban wastewater, PMA; Pesticides with microorganisms in agricultural drainage water, HMU; Heavy metals with microorganisms in urban wastewater, HMA; Heavy metals with microorganisms in agricultural drainage water, PHMAU; Pesticides and heavy metals with microorganisms in agricultural drainage water and urban wastewater, MU: Microorganisms in urban wastewater, MA; Microorganisms in agricultural drainage water, PHAU; Pesticides and heavy metals in agricultural drainage water and urban wastewater

Conclusion

A consortium of *Chlorella vulgaris*, *Scenedesmus quadricuda* and *Spirulina platensis* can be considered an effective method for remediation of the pesticide malathion and heavy metals in polluted wastewater. Moreover, heavy metals and pesticides can provide a source of energy for microorganisms thereby increasing the overall potential for degradation.

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Conflict of interest

The authors declare that they have no conflict interests.

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Original Research Article

GENETIC DIVERSITY AMONG TWO COMMON POPULATIONS OF *CANIS LUPUS FAMILIARIS* IN EGYPT BY USING MITOCHONDRIAL DNA HVR1 SEQUENCE

Mostafa A. Elmadawy[1]*, Seham El-Kassas[2], Safaa E. Abdo[3], Atsushi Nagai[4], Yasuo Bunai[4]

[1]Department of Forensic Medicine and Toxicology, Faculty of Veterinary Medicine, Kafrelsheikh University, 33516 Egypt, [2]Animal, Poultry and Fish Breeding and Production, Department of Animal Wealth Development, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt, [3]Genetics and Genetic Engineering, Department of Animal Wealth Development, Faculty of Veterinary Medicine, Kafrelsheikh University. [4]Department of Legal Medicine, Graduate School of Medicine, Gifu University, 1-1 Yanagido, Gifu-City, 501-1194, Japan.

*Corresponding author, E-mail: drmostox@yahoo.com, ORCID 0000-0002-7174-1370

Abstract: The current study aimed to investigate the variation of mtDNA hypervariable region 1 (*HVR1*) among Egyptian Baladi and German shepherd dogs in Egypt with respect to their phylogenetic origin. Blood samples were obtained from two dog breeds; Egyptian Baladi (n = 46) and German shepherd (n = 42) and used for genomic DNA extraction and PCR amplification of the mtDNA *HVR1* using primers H15360 and L16106. The determined haplotypes were aligned to the sequences of the first published dog mitochondrial genome (Accession No. U96639). We identified 22 different haplotypes from 34 single nucleotide polymorphisms (SNPs) including 2 insertion-deletion polymorphisms among the Egyptian Baladi dogs and 12 haplotypes from 22 SNPs among the German shepherd dogs. Four haplogroups (A, B, C, and D) were identified in the two breeds, their distribution includes 78% of Egyptian Baladi dogs and 76% of German shepherd dogs, respectively were located in the haplogroup A. While 19 % of the German shepherd and 15% of Egyptian Baladi dogs were found in the haplogroup B. 5% of the detected haplotypes of the two breeds were belonged to haplogroup C. 2% of the detected haplotypes of Egyptian Baladi dogs were classified to a haplogroup D. High haplotype and nucleotide diversities were found in the two breeds indicating a lack of genetic differentiation and a recent population growth. The later was confirmed in the Egyptian dogs with the negative values of the neutrality tests and their clustering in the same clade within the phylogenetic tree.

Key words: Egyptian Baladi dogs; German shepherd dogs; HVR1; mtDNA; genetic diversity

Introduction

Dogs (*Canis lupus familiaris)* participate in human life in plentiful ways as such, they protect human from enemies and often could act as ‘‘silent witnesses’’ in forensic casework (1).

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They also, assist with herding and guarding livestock by protecting domestic animals from predators (2). The origin and evolution of the domestic dog are depending on many factors including the place of origin (3). Historically, their domestication occurred when the primitive dogs were obtained from their wild ancestor, the gray wolves (*Canis lupus lupus*) which was further selected to form many dog breeds with specialized characteristics (3, 4). After domestication, several breeds of dogs have been recognized all over the world as a result of the selective breeding either by inbreeding from the same ancestral lines, or by mixing dogs from very different lines (5).

In Egypt, dogs are well known, popular and highly regarded. They were probably domesticated in Egypt in the Pre-Dynastic eras" and served as hunters and as companions for the Egyptians (6). There are many well-known dog breeds such as Egyptian Baladi and German shepherd dogs. However, there is a lack of accurate breed identification for these animals despite the numerous efforts to study dog phylogeny. Several reasons have been listed for this limitation and for the infrequent use of canine evidence in forensic investigations. The absences of validated microsatellite markers or short tandem repeats (STRs), low yield of canine DNA and accurate canine databases are the main causes (7). However, the isolation and analysis of canine mitochondrial DNA (mtDNA) can dramatically improve the identification of the genetic diversity among dog breeds.

The mtDNA is highly informative because of its characteristics such as small molecular weight, high variability and maternal transmission (8-10). The canine mtDNA is approximately 16,728 bp, which consists of 13 protein-coding genes, 22 tRNAs, 2 rRNAs, and polymorphic non-coding regions referred to as hypervariable regions (HVR1 and HVR2) (11). HVR1 is highly polymorphic and can be effectively used in forensic investigations because it can be successfully amplified from limited or severely degraded DNA (7, 12). Besides, HVRI is more effective than HVR2 in studying molecular evolution of animals because the HVR2 is associated with mtDNA replication and translation with limited mutations making it less polymorphic (13).

In Egypt, there is a lack of works on dog breeds diversity and phylogenetic studies among the existence native and foreign breeds and the only conducted researches include DNA polymorphism among pure-breed dogs. Moreover, genetic differences between dogs in Egypt and those in other countries are currently unknown. We therefore investigated mtDNA *HVR1* diversity among 46 Egyptian Baladi dogs and 42 German shepherd dogs in Egypt to establish a genetic database for these two dog breeds, screening for DNA polymorphisms and phylogenetic analysis searching for their origin and relationship.

Material and methods

Sampling and DNA extraction

Forty six Egyptian Baladi dogs and 42 German shepherd dogs were randomly chosen because they are the most common dog breeds in Egypt. About 1.5 ml blood was collected from the forelimb (cephalic vein), and transferred into potassium EDTA containing tubes and then preserved in ice tank till transferred to the Lab. The samples obtained from German dogs were collected from private clinics in Cairo and Mansoura cities after obtaining written consent from the owners. Samples from Baladi dogs were collected at Faculty of Veterinary Medicine, Kafrelsheikh University. Total genomic DNA was extracted from the collected samples using QIAamp DNA Blood Mini Kit (QIAGEN GmbH - Germany) according to the manufacturer instructions. The quality of the extracted DNA was tested for any fragmentations using 1% agarose gel electrophoresis stained with ethadium bromide.

PCR amplification and DNA sequencing

HVR1 region was amplified with specific primers H15360 and L16106 listed in the Table 1. The target fragment corresponded to the sequence at position 15,458–16,727 in the complete annotated nucleotide sequence of the do-

mestic dog (*Canis lupus familiaris*) mitochondrial genome (14). Total volume of PCR mixture was 25 µL contained 1 µl DNA, 0.5 µL of each primer (20 µM each), 2.5 µL of 10x PCR buffer, 0.5 µl Taq polymerase, 2.5 µl dNTPs mix and 17.5 µl milliQ water. PCR amplification was carried out under the conditions previously described by (15). After amplification, The PCR products were purified using ethanol precipitation method depending on the previous method described by Ausubel et al., (16). Sequencing of all samples, in both directions using cycle sequencing ready reaction kit (Applied Biosystems, Foster City,CA) was then done in a BigDye™ Terminator Ver.1.1 using specific primers for sequencing (Table 1**)**.

Statistical and phylogenetic analysis

All the obtained sequences of HVR1 were edited and aligned with the dog mtDNA genome I. D: U96639 (14) as a reference using MEGA software version X (17). Each sequence was first trimmed to approximately 653-bp (from15459–16112 bp of the reference dog mtDNA genome (U96639). Ugene software version 1.26 (18) was used to determine the haplotypes. The sequence of each HVR1 mtDNA haplotype in this sequenced region was deposited in National Center for Biotechnology Information (NCBI) GenBank database under accession numbers (MK050465 to MK050498). Power of discrimination (PD) of the dog mtDNA HVR1 haplotypes were calculated according to the Tamura and Nei model of evolution (19). The statistical quantities for the DNA sequences, including number of haplotypes, nucleotide diversity and Fu and Li's D and F test were performed by using DnaSP 5.10.1(20). Maximum likelihood phylogenetic trees were constructed among the Egyptian Baladi dog and German shepherd dogs and the dog mtDNA genome (U96639) using MEGA software version X (17). Also, the genetic relationship of the identified haplotypes was graphically presented by median-joining network using the network program NETWORK version 5.0.1.0 (21).

Results

Genetic diversity and differentiation

A 653-bp sequence spanning the *HVR1* region was successfully amplified from forty six Egyptian Baladi and forty two German shepherd dogs. The nucleotide sequences of this *HVR1* segment were aligned and clustered into 22 unique haplotypes, represented by 46.83% in Egyptian Baladi dogs (EGYBD1-EGYBD22) while only 12 haplotypes, represented by 26.19% were determined in the case of German shepherd dogs (GYSD01-GYSD12) (Table 2 and 3). Each sequence of these haplotypes covered from 15459–16112 bp of the reference dog mtDNA genome (U96639) (14). The nucleotide sequences of all haplotypes of the two dog breeds were deposited into the GenBank database under accession numbers listed in (Table 2 and 3). The frequencies of these haplotypes among the Egyptian Baladi and German shepherd dogs were calculated and mentioned in Table 2 and 3. The most frequent haplotype among the Egyptian Baladi dogs was EGYBD1 which was detected in 10 individuals from the 46 dogs with a frequency of 0.217 followed by EGYBD18 and EGYBD22 with a frequency 0.109 (Table 2). Among the German shepherd dog, the GYSD11 was the most frequent with a frequency 0.262 followed by GYSD12 and GYSD04 with frequencies 0.190 and 0.143, respectively (Table3).

The analysis of the 653-bp of Egyptian Baladi dog *HVR1* (Table 4) by DNAsp software (20) resulted in the presence of 33 polymorphic sites with 34 single nucleotide polymorphisms (SNPs) including 2 insertion-deletion polymorphisms in the 46 *HVR1* sequences. These polymorphic sites contained 13 singleton variable sites with two variants (the presence of a different nucleotide in only one sequence) at these positions 2, 17, 99, 134, 172, 178, 252, 292, 356, 390, 409, 501, and 581. While there were no singleton variable sites with three variants detected. Besides, they contained 19 parsimony informative sites with two variants (the presence of different nucleotide repeated at least in 2 sequences) at 50, 68, 137, 153, 154, 162, 167,

169, 174, 185, 192, 194, 342, 357, 454, 497, 545, 567, and 625. In addition, one parsimony informative site with three variants was found at position 181. Moreover, eight transitional pairs (si) were reported through the 653-bp sequences without transversional pairs (sv) and the transitional transversional ratio (R = si/sv) was 31.1. Likewise, the analysis of the German shepherd dog sequences (Table 4) resulted in presence of 21 polymorphic sites with 22 SNPs. Among them, six singleton variable sites at position 1, 3, 4, 11, 13, and 15 were noticed. Total numbers of 14 two variants parsimony informative sites were found at position 2, 5, 6, 7, 8, 10, 12, 14, 16, 17, 18, 19, 20, and 21. On the other hand, one three variants parsimony site was noted at 9. The transitional transversional ratio was 24.00 due to presence of 7 transitional pairs without transversional nucleotides were reported. Moreover, the power of discrimination was calculated to be 0.908318 for the Egyptian dogs and 0.851474 for the German shepherd one, respectively.

The nucleotide (π_n) and haplotype (H) diversity coefficients were calculated for the Egyptian Baladi and German shepherd dogs (Table 5). The German shepherd dogs showed higher haplotype diversity compared to the Egyptian ones hence 1 ± 0.034 compared to 0.987 ± 0.018 were reported, respectively. The two dog breeds showed, to some extent, a similar nucleotide diversity which was 0.01299 in the case of Egyptian Baladi dogs and 0.01158 for the German shepherd. The average number of pairwise differences (π) between haplotypes within the dog populations varied from 8.472 in the case of Egyptian Baladi dogs to 7.561 for the German shepherd dogs. Moreover, the Fu and Li's D and Fu and Li's F tests indicated that the Egyptian Baladi dogs showed the lowest values; -0.55934 and -0.58080 compared to 0.38302 and 0.37188 for the German shepherd dogs, respectively.

Phylogenetic analysis

The maximum likelihood analysis, based on kimura-2 parameter model (+G+I) with 1000 bootstraps replicates of the 22 haplotypes of Egyptian Baladi dogs and the 12 haplotypes of German shepherd dogs included in the current study resulted in the phylogenetic tree shown in Fig. 1. Two main clades were observed A, and B. Within the clade A and B, two main subclades; A1 and A2 and B1 and B2 were noticed, respectively. These subclades were further divided into lower subclades. Through the tree, there were no demarcations between the Egyptian Baladi and German shepherd haplotypes. Most of the Egyptian haplotypes were located in the clade A. The EGYBD07 haplotype was located in a separate subclade (A2). These clades were supported 1 and 97 bootstraps. Likewise, the ML analysis of the 22 Egyptian Baladi dogs' haplotypes, the 12 ones of the German shepherd dogs and the reference dog mtDNA genome (U96639) (14) was done using MEGA software version X (17), the resulted phylogenetic tree was shown in Fig. 2. The evolutionary analysis by Maximum Likelihood method showed a tree with the highest log likelihood (-23500.98). Two main clades, A, and B were observed which further subdivided into many subclades. These subclades were also, split into lower subclades. Through the tree, reference dog mtDNA genome (U96639) was shown to be the most probable ancestor. Most of the haplotypes were located in the clade A. The EGYBD07 haplotype was located in a separate subclade (A2). These clades were supported 16 and 95 bootstraps.

For the genetic linkage of the detected haplotypes with the reference dog mtDNA genome (U96639), the genetic network (Fig. 3) was drawn using the NETWORK software version 5.0.1.0. The GYSD12 and EGYBD22 haplotypes (node H_1) were directly connected to U96639 ancestor (node H_1). While, GYSD10 and EGYBD15 (Node H_3), GYSD09 (node H_4), GYSD08 and EGYBD08 (node H_5), GYSD05 (node H_8), GYSD02 (node H_11) and EGYBD02 (node H_27) could be linked to the ancestral node (H_1). Also, it was shown that the most frequent haplotypes (EGYBD01 for the Egyptian Baladi dogs and GYSD11 for German shepherd dogs) were unlinked to the U96639 ancestor.

Four haplogroups (A, B, C, and D) were identified in the studied two dog breeds (Fig. 4,

A and B). Their distribution in the two breeds was 78% of Egyptian Baladi dogs and 76% of German shepherd dogs, respectively were located in the haplogroup A. Besides, 19 % of German shepherd and 15 % of Egyptian Baladi dogs were found in the haplogroup B. While, the two dog breeds shared the same percentage (5 %) for haplogroup C. Haplogroup D was noticed in 2 % of the Egyptian Baladi dogs only.

Table1: List of primers used for PCR amplification and sequencing of *HVR1* in dogs

Primer name	Primer sequence (5'-3')	Purpose	Reference
H15360	ATTACCTTGGTCTTGTAAACC	PCR amplification & sequencing	(7, 15)
L16106	AAACTATATGTCCTGAAACC	PCR amplification & sequencing	(7, 15)
H15422	CTCTTGCTCCACCATCAGC	Sequencing	(15)
H15840	TACTCCAATCCTACTAATTC	Sequencing	(15)
L16102	AACTATATGTCCTGAAACCATTG	Sequencing	(15)

Table 2: Sequence variations, GenBank accession No. and frequencies of the 22 haplotypes detected in 46 Egyptian Baladi dogs

	15464	**15475**	**15508**	**15526**	**15557**	**15592**	**15595**	**15611**	**15612**	**15620**	**15625**	**15627**	**15630**	**15632**	**15636**	**15639**	**15643**	**15650**	**15652**	**15710**	**15750**	**15800**	**15814**	**15815**	**15848**	**15867**	**15912**	**15938**	**15955**	**15959**	**16003**	**16025**	**16039**	**16083**	**GenBank Ac. No.**		
Haplotype	.	T	C	C	T	T	C	T	T	T	T	A	C	C	T	T	A	T	G	C	C	T	C	T	G	A	C	G	C	C	A	T	T	A	U96639	No.	F
EGYBD1	.	.	.	.	.	.	.	.	.	.	.	G	.	T	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	C	.	.	MK050465	10	0.2173913
EGYBD2	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	C	.	.	MK050466	1	0.0217391
EGYBD3	.	.	.	.	.	.	.	.	.	.	C	G	.	T	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	C	.	.	MK050467	1	0.0217391
EGYBD4	.	.	.	.T	.	.	T	.	C	.	C	.	.	T	.	G	G	.	A	.	.	C	T	C	.	.	T	.	T	.	G	.	.	G	MK050468	3	0.0652174
EGYBD5	.	.	.	.T	.	.	T	.	C	.	.	.	.	T	.	G	G	.	A	T	.	C	T	C	.	.	T	.	T	.	G	.	.	G	MK050469	2	0.0434783
EGYBD6	.1C	.	.	T	.	.	.	.	C	.	.	.	.	T	.	G	G	.	A	.	.	C	T	C	.	.	T	.	T	.	G	.	.	G	MK050470	1	0.0217391
EGYBD7	.	.	.	.	.	C	.	.	.	.	C	.	T	T	C	A	.	.	.	.	.	C	T	C	A	.	T	.	.	T	G	.	C	G	MK050471	1	0.0217391
EGYBD8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	MK050472	2	0.0434783
EGYBD9	.	.	.	.	.	.	.	.	.	C	.	G	.	.	.	A	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	MK050473	2	0.0434783
EGYBD10	.	.	.	.	.	.	.	.	.	.	.	G	.	T	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	C	.	.	MK050474	1	0.0217391
EGYBD11	.	.	T	T	.	.	.	C	.	.	.	.	.	.	.	G	.	C	.	.	.	C	T	.	.	.	T	.	T	.	G	.	.	.	MK050475	1	0.0217391
EGYBD12	.	.	T	T	.	.	.	C	.	.	.	.	.	.	.	G	.	C	.	.	.	C	T	.	.	.	T	D	T	.	G	.	.	.	MK050476	1	0.0217391
EGYBD13	.	.	.	.	.	.	.	.	.	.	.	G	.	T	.	A	.	.	.	.	.	.	T	.	.	G	.	.	.	.	.	C	.	.	MK050477	1	0.0217391
EGYBD14	.	.	.	.	.	.	.	.	.	.	.	G	.	T	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	G	MK050478	1	0.0217391
EGYBD15	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	C	.	.	MK050479	1	0.0217391
EGYBD16	.	.	.	.	.	.	.	.	.	C	.	G	.	.	.	A	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	MK050480	3	0.0652174
EGYBD17	.	.	.	.	.	.	.	.	.	.	.	G	.	T	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	C	.	G	MK050481	1	0.0217391
EGYBD18	.	.	.	.	.	.	.	.	.	.	.	G	.	T	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	MK050482	5	0.1086957
EGYBD19	.	C	.	.	.	.	.	.	.	.	.	G	.	T	.	A	.	.	.	.	T	.	T	.	.	.	.	.	.	.	.	C	.	.	MK050483	1	0.0217391
EGYBD20	.	.	.	T	.	.	T	.	C	.	.	.	.	T	.	G	G	.	A	.	.	C	T	C	.	.	T	.	T	.	G	.	.	G	MK050484	1	0.0217391
EGYBD21	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	G	MK050485	1	0.0217391
EGYBD22	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	MK050486	5	0.1086957

Table 3: Sequence variations, GenBank accession No. and frequencies of the 12 haplotypes detected in 42 German shepherd dogs

	15508	**15526**	**15553**	**15595**	**15612**	**15620**	**15627**	**15632**	**15636**	**15639**	**15643**	**15650**	**15652**	**15665**	**15800**	**15814**	**15815**	**15912**	**15955**	**16003**	**16025**	**16083**	**GenBank Ac. No.**		
Haplotype	C	C	A	C	T	T	A	C	T	T	A	T	G	T	T	C	T	C	C	A	T	A	U96639	No	F
GYSD01	.	T	.	.	C	.	.	T	.	G	G	.	A	.	C	T	C	T	T	G	.	G	MK050487	1	0.0238095
GYSD02	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	T	.	.	.	.	.	.	MK050488	1	0.0238095
GYSD03	.	.	.	.	.	C	G	.	.	A	.	.	.	.	.	T	.	.	T	.	.	G	MK050489	2	0.047619
GYSD04	.	T	.	T	C	.	.	T	.	G	G	.	A	.	C	T	C	T	T	G	.	G	MK050490	6	0.1428571
GYSD05	.	.	G	.	.	.	.	.	.	A	.	.	.	.	.	T	.	.	.	.	.	.	MK050491	2	0.047619
GYSD06	.	T	.	.	C	.	.	T	.	G	G	.	.	.	C	T	C	T	T	G	.	G	MK050492	1	0.0238095
GYSD07	T	T	.	.	C	.	.	.	.	G	.	C	.	.	C	T	.	T	T	G	.	.	MK050493	2	0.047619
GYSD08	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	MK050494	3	0.0714286
GYSD09	.	.	.	.	.	.	.	.	.	A	.	.	.	C	.	T	.	.	.	.	C	.	MK050495	1	0.0238095
GYSD10	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	T	.	.	.	.	C	.	MK050496	4	0.0952381
GYSD11	.	.	.	.	.	C	G	.	.	A	.	.	.	.	.	T	.	.	T	.	.	.	MK050497	11	0.2619048
GYSD12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	MK050498	8	0.1904762

Table 4: Haplotype diversity (H), nucleotide (πn) diversity, mean number of pair-wise differences (π) between haplotypes within populations, polymorphic sites (p.s.), number of haplotypes (Hn), Fu and Li's D and F tests, and Tajima's D in two dog breeds

Breed	H ± SD	(π_n)	π	p.s.	Fu and Li's D	Fu and Li's F	Tajima's D
Egyptian Baladi	0.987 ± 0.018	0.01299	8.472	33	-0.55934 (P > 0.10)	-0.58080 (P > 0.10)	-0.35475 (P > 0.10)
German Shepherd	1 ± 0.034	0.01158	7.561	21	0.38302 (P > 0.10)	0.37188 (P > 0.10)	0.16720 (P > 0.10)

Table 5: Haplotype diversity and power of discrimination

Parameter	Egyptian Baladi dogs	German Shepherd dogs
Total number of animals	46	42
No. of haplotypes	22	12
Power of discrimination	0.908318	0.851474
SNPs	34	22
Total number of singleton mutations	13	6
Number of parsimony informative sites	20	15
Transitional Pairs (si)	8	7
Transversional Pairs (sv)	0	0
R = si/sv	31.1	24.0

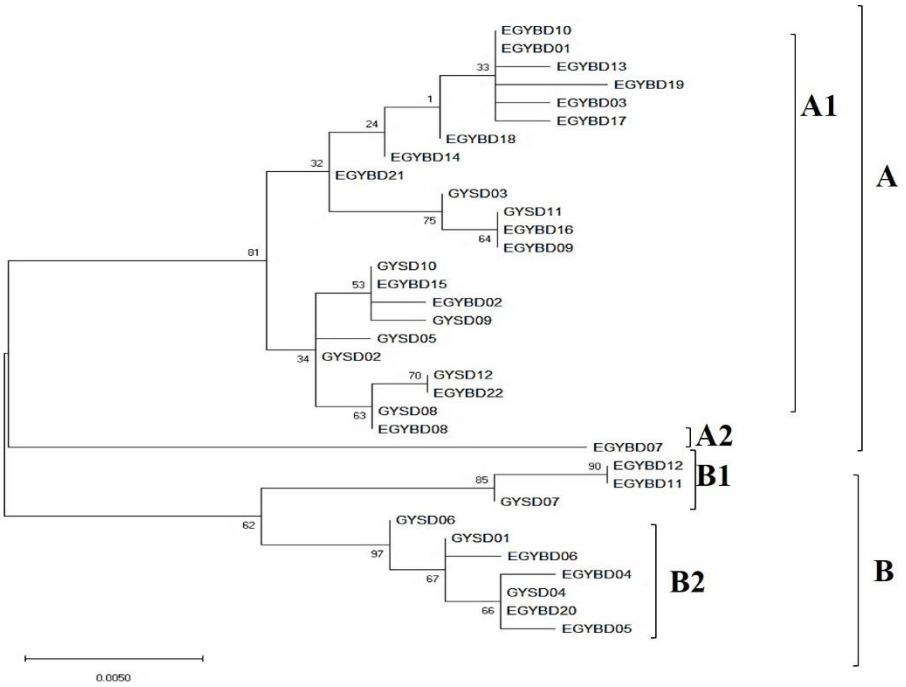

Figure 1: The second Maximum likelihood tree based on kimura-2 parameter method (+G+I) of 34 haplotypes of the two dog breeds included in this study. The number of bootstrap replications=1000. The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model. The tree with the highest log likelihood (-23500.98) is shown

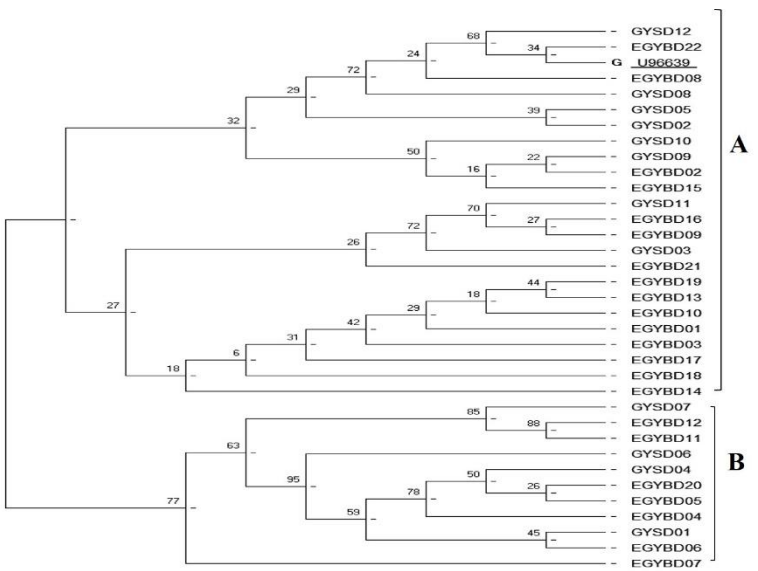

Figure 2: The second Maximum likelihood tree based on kimura-2 parameter method (+G+I) of 34 haplotypes of the two dog breeds and U96639 included in this study. The number of bootstrap replications=1000. U96639 is the first published dog mitochondrial genome. G refers to the most common ancestral line

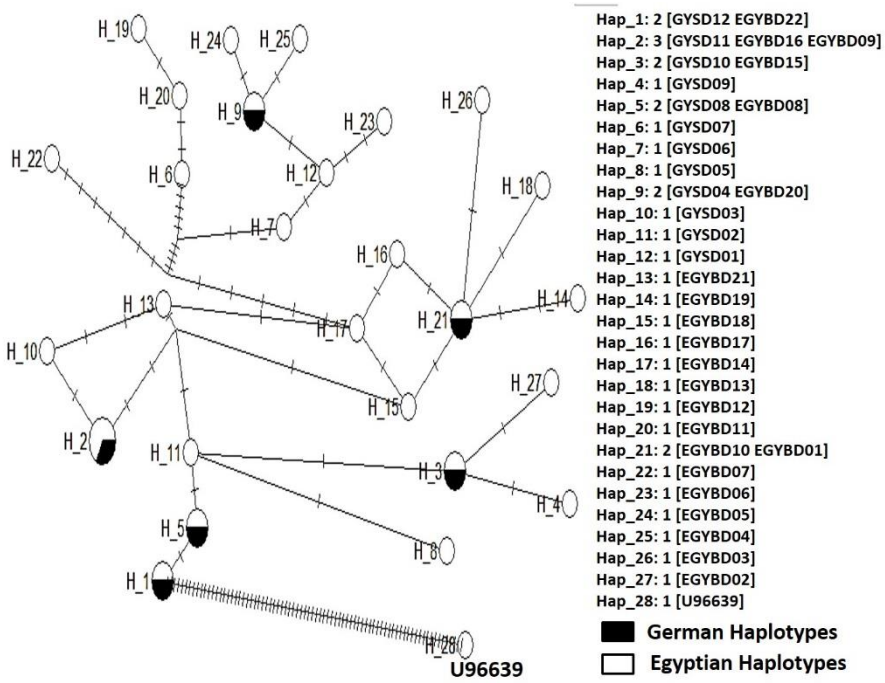

Figure 3: Median-joining network (Ɛ = 0) depicting Genetic relationships among dog breed mtDNA *HVR1* haplotypes from this study using Network v5.0.1.0. Circled areas are proportional to the corresponding haplotype frequency

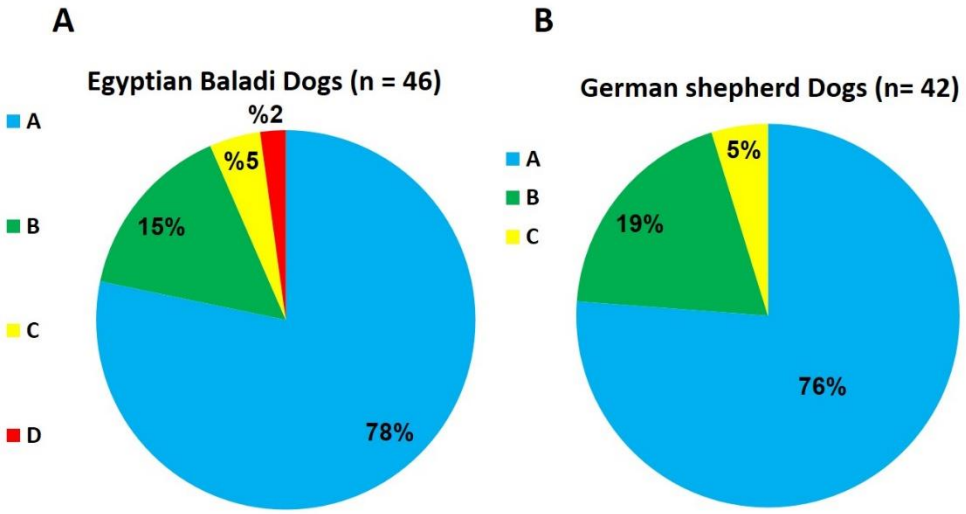

Figure 4: Mitochondrial DNA haplogroup distributions in the most common Egyptian dog breeds

Discussion

German shepherd and Egyptian Baladi are widely distributed and the most common dog breeds in Egypt. These dogs are kept mainly for guarding and have a crucial role in the forensic cases (2). However, up to date, there is missing information on the genetic diversity between these two breeds. Also, there are no effective controlled breeding programs for these breeds. So, for many purposes, such as forensic cases, it is highly recommended to differentiate between these two breeds.

The canine mtDNA, especially *HVR1*, is highly polymorphic that can be used effectively as a DNA marker for the breed identification (22). However, little is known regarding using *HVR1* in dog breeds identification in Egypt. As the haplotype diversities are informative tools about the history of animals, and for the breed identification. So, the high diversities reported in the current study probably explain the lack of differentiation between the Egyptian Baladi and German shepherd dogs in Egypt (23). These results agreed with the previous studied (11, 24) which reported high haplotype diversities although using different dog breeds and nucleotide numbers. Moreover, the Egyptian Baladi dogs had high haplotype diversity almost, as high as that noticed in the case of German shepherd dogs. This might imply that Egyptian dogs were developed from several breeds over their breeding history. In addition, the high genetic variability among the two studied breeds probably indicates that the dog populations in Egypt have been undergoing a rapid expansion in recent history (25). Plus, an increased effective population size, and a reduced genetic drift might be inferred from the identified high genetic diversity (26). Likewise, the reported high nucleotide diversities for the two breeds might elucidate high genetic differentiation and presence of large differences between haplotypes (27). The rate of genetic diversity is determined effective population size, and rate of mutation. Where, the larger the population size, the higher the genetic diversity (28). So, it is perhaps occurred due to a relatively large long-term effective population size rather than any severe bottleneck during dog evolution (29). Also, the detected higher haplotype and nucleotide diversities probably indicate that the studied populations recently divergent from each other (30).

Additionally, the detected high haplotype and nucleotide diversities could be a signature of a rapid population expansion (31). This was confirmed by the genetic distant from the most frequent haplotypes and the ancestral haplotype (U96639) (25). Another evidence of the rapid population expansion was concluded from calculating the Tajima's D test and Fu's Fs tests that are usually used to find out the population expansion. For the Egyptian Baladi dogs, the Tajima's D test and Fu's Fs values were non-significantly negative. The overall negative values of these neutrality tests perhaps designated an excess of the rare mutations in populations, which might suggest a recent population expansion as well as an evidence of a selective sweep (32). While for the German shepherd dogs, the positive non-significant values of Tajima's D test and Fu's Fs probably implied low levels of both low and high frequency polymorphisms and a balancing selection (32). These non-significant results observed in case of Egyptian and German dogs indicated non-significant variations and the absence of a clear population structure (33).

The phylogenetic analysis of the different haplotypes found in the two dog breeds under study and the reference dog mtDNA genome (U96639), was performed. The analysis revealed the presence of two main clades subdivided to many subclades. Also, most of the Egyptian and German haplotypes as well as the reference dog mtDNA genome (U96639) were clustered in the same clade indicating absence of clear demarcations and a strong relationship between the haplotype sequences of the present study and the reference dog mtDNA genome (U96639). This might indicate that two studied breeds are descended from the same ancestral line, recently originated, and mutations that account for their differences have yet to be reported and become fixed (23). Additionally, similarities among the Egyptian Baladi and German shepherd dogs were revealed from the

haplogroup distribution in which the major haplogroup (A) was identified in the two breeds.

Conclusion

In conclusion, this study aimed to use the mtDNA *HVR1* to differentiate between the most common dog breeds in Egypt; German shepherd and Egyptian Baladi. The calculated high genetic diversity indicates a lake of differentiation between the two breeds. The most frequent haplotypes of the two breeds was belonged to haplogroup A followed by haplogroup B and C. The negative neutrality tests imply recent population growth of the Egyptian Baladi dogs. The results of this study would be helpful in advancement of Egyptian forensics and animal genetic studies.

Disclosure statement

No conflicts of interest, financial, or otherwise, are declared by the authors.

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Original Research Article

IMPACT OF BIOMOS AND AGRIMOS DIETARY SUPPLEMENTATION ON GROWTH PERFORMANCE, FEED UTILIZATION AND IMMUNOLOGICAL PARAMETERS OF NILE TILAPIA (*Oreochromis niloticus*) FINGERLINGS

Fawzy I. Magouz [1], Mahmoud F. Salem[2], Eman M. Moustafa[3*], Salwa A. Elkhamy[1]

[1]Department of Animal Production, Faculty of Agriculture, Kafrelsheikh University, Postal code: 33516, Egypt, [2]Sakha, Aquaculture Research Unit, Central Lab for Aquaculture Research, Abbasa, Sharkia- ARC, Egypt, [3]Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Kafrelsheikh University

*Corresponding author, E-mail: emantarek2002@yahoo.com

Abstract: This study aimed to evaluate the potential benefits of *Biomos*® and *Agrimos*® as prebiotics in Nile tilapia diets. Seven experimental treatments were formulated from 30% protein basal diet to contain *Biomos*® and *Agrimos*® at levels of 0.1, 0.2 and 0.3 % for each, in addition to the control diet without any additives. Three hundreds and fifteen fingerlings of Nile Tilapia *(O. niloticus)* with average initial weight (7 ± 0.5 g), were randomly allocated into 7 treated groups allotted into 21 glass aquaria (three replicates of 15 fish / each treatment). Each aquarium measured 60× 35× 40 cm^2. The fish were fed at 3% fish biomass along the experiment which lasted for 15 weeks. The results revealed significant improvements in growth and all feed utilization parameters in the prebiotic supplemented groups. The diets containing *Biomos*® (0.1%) and *Agrimos*® (0.2%) revealed the highest growth and protein utilization parameters values. Experimental fish carcass composition was relatively affected by the different dietary treatments. The hematological, biochemical and immunological parameters of the experimented groups indicated significant increase in *Biomos*® and *Agrimos*® treated groups. The achieved results demonstrated that *Biomos*® and *Agrimos*® at levels of 0.1% and 0.2%, respectively could be used in Nile tilapia diets without negative effects on growth, feed utilization, blood and immunological parameters. Hence, Biomos® and Agrimos® could be added to commercial diets to improve tilapia fingerlings immune response.

Key words: agrimos; biomos; growth; immunity; *Oreochromis niloticus*

Introduction

Aquaculture in Egypt is the main source of fish production. It represents 77% of the absolute fish production of which 85% were delivered by the developed pond-based aquaculture around the Nile Delta lakes (1). Tilapia is the most generally developed species in these ponds which speaks to over 65% of the all out aquaculture production (2) because of the expanded dimension of intensification and cultured regions (3). Heightening of aquaculture production frameworks exposes fish to various ecological stressors like; poor water quality,

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over-crowdness, improper handling and transport which may contrarily influence their growth and health, as well as limiting the outcome of aquaculture systems (4-6).

Nutrition assumes an essential job in the growth, development and wellbeing upkeep of fish (7). Few years ago, fish meal was utilized as the primary protein source in tilapia diets. Because of the expanding cost and insecure supply of this ingredient, many attempts have been attempted to enhance the growth performance and decline the production expenses of cultivated tilapia. This was done through utilizing probiotics or prebiotics as safe supplements which have neither residues in the farmed fish nor harmful effects for the consumers (8-10).

Prebiotics are non-digestible dietary carbohydrates which get away from the assimilation in the upper gastrointestinal tract. Prebiotics advantageously influence the host by specifically invigorating the growth and/or activating the metabolism of health-promoting bacteria in the gastrointestinal tract (11, 12). Likewise, they modify the gut bacterial composition through changing the kind of substrate gave to the current gut microbiota (13, 14).

Mannan oligosaccharide (MOS) is a yeast cell wall derived feed ingredient. It works locally in the gut enhancing assimilation and gut wellbeing in animals through diminishing the colonization of undesirable bacteria. Furthermore, MOS functions as a prebiotic, favoring development of helpful bacteria in the gut (15, 16). AGRIMOS® is a particular mix of MOS and ß-glucans separated from the yeast cell walls of *Saccharomyces cerevisiae;* that are especially critical to help the non-specific immune system of the animal (17). Although few investigations revealed the dietary prerequisites of biomos and Agrimos to maintain growth, while, immunological responses still not very much archived particularly on the fish resistance level (17).

The current study was directed to evaluate the potential benefits of Biomos® and Agrimos® dietary supplementation on growth performance, feed utilization, body chemical composition, internal organs indices, hematological parameters, immunological response and economical efficiency of Nile Tilapia (*O. niloticus*) fingerlings under Egyptian conditions.

Material and methods

This study was conducted in fish Aquaculture Research Unit in Kafr El-Sheikh Governorate. All handlings of fish were directed according to the guidelines for animal care and use for scientific purposes built up by the Ethics Committee of the Faculty of Agriculture, Kafrelsheikh University, Egypt (Approval Date: 18-03-2018).

Diet preparation

A basal diet was formulated from commercial ingredients including fish meal, soybean meal, yellow corn, wheat bran, vitamins, minerals mix and fish oil. The dry ingredients were grounded utilizing a feed processor into little size particles. Seven diets were formulated from the basal diets by adding the prebiotics at different concentrations. Contents and chemical composition of each diet were exhibited in Table (1; A & B).

The ingredients were weighed and blended by a mixture blender for 20 minutes. A constant Biomos and Agrimos levels was added for all diets except control diets. After homogenous blending, every hundred gram diet was gradually added to the blend as indicated by (18). The diets were cooked on water exaporator for 20 minutes. The diets were pelleted through grain machine and the pellets were dried at room temperature for 24 h before utilized. The pellets were gathered and spared in plastic bags and stored in a refrigerator at 4^0C through the experimental period to dodge nutrients deterioration.

The utilized feed additive, Biomos® and Agrimos®, were commercial natural enhancers blend; (Bio-Mos®; Alltech, Inc., Nicholasville, KY, USA), (Agrimos®; LALLEMAND ANIMAL NUTIION, FRANCE).

Experimental design

The experiment was performed using 315 Nile Tilapia (*O. niloticus*) fingerlings (weighing on average 7 ± 0.5 g). They were collected from a private fish farm in Al Reyad, tolompate

7, Kafr El-Sheikh Governorate. All collected fish were kept in a fiberglass tank, for three weeks for accommodation; where fish were fed a commercial diet (containing 30% dietary protein level (CP). After the accommodation period, the fingerlings were randomly divided into 7 groups of 45 fingerlings / each group allotted into three replicates of 15 fingerlings / each replicate). Fingerlings were put in glass aquariums of 60 × 35 ×40 cm in size contained 70 L of water, (15 fish/ aquarium) and were equipped with effective aeration system. The seventh Groups 1 (control group) were fed a commercial diet, Group 2to 4 were fed diets supplied with 1, 2, 3 kg/ ton) of Biomos. While, groups 5 to 7 were fed diets supplemented with 1, 2, 3 kg/ ton of Agrimos.

Fish were fed the experimental diets for 12 weeks at a rate of 3% of the all-out stocking biomass/aquarium. Diets were applied twice a day (at 8:00 am & 14:00 pm). Fish were weighed at fortnightly intervals along the experimental period and the feed amounts were corrected by the change in live body weight. Fish excreta and feeding wastes were expelled by siphoning and about half of water in every aquarium was day by day replaced by dechlorinated new water.

Determination of fish growth parameters

The fish were totally weighed (15 fish/each replicate) using an electronic balance**.**

Total weight gain (TWG), average daily gain (ADG), specific growth rate (SGR), survival rate (SR %), feed conversion ratio (FCR), and protein efficiency ratio (PER), were calculated according to following equations:

Total weight gain (TWG) (g) = last body weight – beginning body weight (19).

Average Daily Gain (g/fish/day) = TWG (g)/trial period (d).

Specific growth rate (SGR % / day) = [Ln last body weight- Ln introductory body weight] × 100/trial period (d) (20).

SR= Total number of fish at the end of the experiment × 100/ absolute number of fish at the start of the experiment.

FCR= feed consumption (g)/Live weight gain. (21).

PER=Live weight gain (g)/protein intake (g). (21).

Determination of diet proximate analysis

Dry matter, crude protein, ether extract, crude fiber and ash contents of the experimental diets and the whole body of fish at the end of the experiment were performed according to AOAC (22).

Hematological investigations

Toward the finish of the experiment, twelve fishes from every group (4 fishes /every replicate) were randomly sampled and weighed. Anti-coagulated blood samples were taken from the caudal vein for blood analysis and differential leukocyte count. Due to the small fish size, blood samples collected from 3-4 fish were pooled according to Urbinate & Carneiro (23).

Red blood cells count (RBCs$\times 10^6$/mm) and white blood cells count (WBCs$\times 10^3$/mm) were determined according to the method described by Stoskopf, (24). Hemoglobin concentration (Hb g/dl) was estimated according to the method of Zinkl (25). Packed cell volume (PCV %) was estimated by the micro-haematocrite method described by Decie & Lewis (26).

Determination of internal organs indices

Toward the finish of the experiment, four fishes from every treatment were slaughtered and the abdominal cavity was directly opened to evacuate liver, kidney, spleen and gonads then weighed separately. Liver index (HSI), kidney index (KSI), spleen index (SSI) and gonads index (GSI) were calculated as follows:

Hepato somatic index (HSI %) = 100 × [liver weight (g) / body weight (g)] (27).

Kidney somatic index (KSI %) =100× [kidneys weight (g) /body weight (g)] (28).

n somatic index (SSI %) = 100× [spleen weight (g) / body weight(g)] (29).

Gonado somatic index (GSI %) =100× [gonads weight (g) / body weight (g)] (30).

Immunological parameters

Phagocytic activity (PA) and index (PI) were determined according to Kawahara et al., (31).

The nitro blue tetrazolium assay was used to investigate the respiratory burst activity as previously described (32). The lysozyme activity was examined by the technique described by Demers and Bayne (33) depending on the ability of lysozyme to lyses Gram positive lysozyme delicate bacterium; *Micrococcus lysodeikticus*.

Biochemical parameters

Total antioxidant capacity (TAC) of liver tissue was performed by the technique described by Prieto et al., (34). Catalase (CAT) activity was performed utilizing spectrophotometric assurance of hydrogen peroxide (H_2O_2) which framed stable complex with ammonium molybdate (35).

Statistical analysis

The obtained data were statistically analyzed utilizing general direct models technique adjusted by SPSS (36) for users guide, with a restricted ANOVA. Means were statistically compared for the significance ($P < 0.05$) using Duncan's multiple range test (37).

Results

In the current study, the physiological responses of *O. niloticus* fingerlings to Biomos® and Agrimos® were researched through assurance of fish development and hematological parameters. It was observed that there was a huge increase in absolute weight gain (TWG) and average daily gain (ADG) in all prebiotics treated groups except in T7 group compared with control group (T1). The most elevated qualities were noticed in the T2 and T6 groups. However, the specific growth rate (SGR) was fundamentally expanded in T2 group only. Survival rate percent (SR%) was significantly increased in T2, T3, T5, T6 groups with most noteworthy qualities reported in the case of T2 & T5 groups as shown in table (2: A).

The impacts of the two utilized prebiotics on feed intake, food conversion rate (FCR) and protein efficiency ratio (PER) were summarized in table (2: B). It was noticed that the best food conversion rate and protein efficiency ratio values were in T2 and T6 groups.

Chemical composition of the experimental fish body, average dry matter (DM), crude protein (CP), ether extract (EE), ash and nitrogen free extract (NFE) were determined and summarized in table 3. There was a critical difference in the DM in case of T2, T3, T6 and T7 groups with most astounding increment found in the T3 group. While, CP% was fundamentally expanded in T2, T5 followed by T3 group contrasted with other treated groups. On the other hand, ether extract was altogether diminished in all treated groups contrasted with control one. The most astounding estimation of ash content was recorded in T3 and T5 groups and the least incentive in T7 group.

RBCs, and WBCs count, Hb, and PCV demonstrated noteworthy increases in all prebiotics supplemented groups contrasted with the control one (Table 4). The highest level of RBCs count was recorded in T3 and T6 groups. The results of serum total proteins, albumin and globulin, showed non-significant increases in all probiotic treated groups contrasted with the control group except T4 group which expanded essentially. The T4 group demonstrated a huge decline in AST level. No noteworthy changes were observed in ALT in all groups.

Regarding immunological parameters, phagocytic activity and index, respiratory burst activity, lysozyme, total antioxidant and catalase activities were altogether expanded and increased in all groups with most extreme dimensions on account of group 2 and group 6, respectively (Table 5).

Discussion

In aquaculture, probiotics can be admitted either as feed added substances or as added substances to the water (38, 39). The shape and span of prebiotic and probiotic administration can impact their viability on fish health (8). The dietary supplementation of pre- and probiotics has been archived as a superior strategy of guaranteeing the effectiveness of the probiotic bacterial colonization in the fish gastrointestinal tract (7, 14, 40).

Aqua feeds industry are focusing mainly on getting double advantages of both upgraded development and resistant reaction of vast majority

Table 1A: Composition of the experimental diets offered for each group

Ingredients	Diet1 control	Diet2	Diet3	Diet4	Diet5	Diet6	Diet7
Fish meal (72% CP)	10	10	10	10	10	10	10
Yellow corn	22	22	22	22	22	22	22
Soybean meal (45% CP)	42	42	42	42	42	42	42
Wheat bran	20	20	20	20	20	20	20
Fish Oil	5	5	5	5	5	5	5
Vit&Min	1	1	1	1	1	1	1
Biomos	-	0.1	0.2	0.3	-	-	-
Agrimos	-	-	-	-	0.1	0.2	0.3

Table 1B: Chemical analysis of the experimental diets (% on DM basis).

Ingredients	Diet 1 control	Diet 2 (0.1% Biomos)	Diet 3 (0.2% Biomos)	Diet 4 (0.3% Biomos)	Diet 5 (0.1% Agrimos)	Diet 6 (0.2% Agrimos)	Diet 7 (0.3% Agrimos)
Dry matter	92.78	92.38	92.22	92.07	92.56	92.60	92.66
Crude protein	31.08	31.08	31.08	31.08	31.08	31.08	31.08
Ether extract	6.32	5.62	4.71	4.94	6.71	6.02	6.10
CF	1.10	2.21	1.92	1.93	2.13	2.09	2.21
Ash	6.73	6.65	6.45	9.37	6.49	6.61	6.14
NFE	54.77	54.44	55.84	52.68	53.59	54.2	54.47
Gross energy(GE) (Kcal/100g)[1]	458.265	445.622	448.879	437.775	459.625	453.861	454.254
Digestible energy(DE) (Kcal/100g) [2]	321.82	318.4	321.32	307.37	327.94	319.51	317.83
P/E ratio[3]	67.82	72.39	71.35	72.61	71.21	69.60	67.38

[1] Gross energy (Kcal/100g), based on 5.6Kcal/g protein, 9.44 Kcal/g lipid, 4.1 Kcal/g carbohydrate.

[2] Digestible energy (Kcak/100g), based on 5.0Kcal/g protein 9.0Kcal/g lipid, 2.0Kcal/g carbohydrate. According to (Wee & shu, 1989).

[3] P/E (protein to energy ratio)= mg crude protein/Kcal of gross energy.

Table 2A: Effect of Used prebiotics on growth parameters and survival rate of Nile tilapia

Treatment	I.W.	F.W.	T.W.G (g/fish)	A.D.G (g/fish/day)	S.G.R (%/day)	S.R.%
T1	7.02±.02	24.93±0.06^{b}	18.10±0.20^{d}	0.16±0.03^{c}	1.3±0.03^{b}	95.5±2.2^{c}
T2	6.97±.02	29.46±0.78^{a}	23.50±0.36^{a}	0.27±0.01^{a}	1.8±0.16^{a}	100±0.00^{a}
T3	7.00±.00	27.36±0.08^{ab}	20.86±0.84^{ab}	0.21±0.01^{ab}	1.5±0.03^{ab}	97.7±2.2^{b}
T4	7.00±.00	26.96±1.00^{ab}	20.13±0.98^{b}	0.18±0.04^{b}	1.43±0.03^{b}	95.5±4.4^{c}
T5	7.32±.32	27.00±1.01^{ab}	20.86±1.2^{ab}	0.21±0.01^{ab}	1.6±0.18^{ab}	100±0.00^{a}
T6	7.00±.00	28.86±0.57^{a}	21.86±0.59^{ab}	0.23±0.01^{ab}	1.7±0.15^{ab}	97.7±2.2^{b}
T7	7.01±.01	25.06±0.86^{b}	18.26±0.84^{cd}	0.16±0.03^{c}	1.40±0.05^{b}	95.5±2.2^{c}

T1=Diet 1 (control group), T2= Diet 2 (0.1% Biomos), T3= Diet 3 (0.2% Biomos), T4=Diet 4 (0.3% Biomos), T5= Diet 5 (0.1% Agrimos), T6= Diet 6 (0.2% Agrimos), T 7= Diet 7 (0.3% Agrimos), I.W. = Initial Weight, F.W. = Final Weight, T.W.G = Total weight gain, A.D.G = Average daily gain, S.G.R = Specific growth rate, S.R.% = Survival Rat

Table 2B: Feed intake, feed conversion rate and protein efficiency ratio of Nile tilapia in response to prebiotics supplementation

Treatment	FI	FCR	PER
T1	42.16±.16^{c}	2.33±.03^{a}	1.26±.08^{b}
T2	48.90±1.50^{a}	1.97±.08^{b}	1.53±.03^{a}
T3	47.43±.51^{a}	2.10±.17^{ab}	1.43±.06^{ab}
T4	46.33±.83^{ab}	2.22±.13^{a}	1.30±.15^{b}
T5	45.33±1.51^{abc}	2.13±.08^{ab}	1.46±.03^{ab}
T6	46.16±1.63^{ab}	1.99±.04^{b}	1.53±.03^{a}
T7	42.70±1.40^{bc}	2.20±.26^{a}	1.33±.08^{b}

T1=Diet 1 (control group), T2= Diet 2 (0.1% Biomos), T3= Diet 3 (0.2% Biomos), T4=Diet 4 (0.3% Biomos), T5= Diet 5 (0.1% Agrimos), T6= Diet 6 (0.2% Agrimos), T 7= Diet 7 (0.3% Agrimos), FI: Feed Intake, FCR: Feed conversion rate, PER: Protein efficiency ratio

Table 3: Composition Analysis of fish body fed graded levels of Biomos and Agrimos

Treatment	DM	CP	EE	ASH	GE Kcal/100g
T1	24.52±0.01^{c}	58.92±0.01^{c}	17.54±0.01^{a}	17.84±0.02^{b}	518.89
T2	24.34±0.02^{d}	64.32±0.01^{a}	13.33±0.01^{b}	18.42±0.01^{a}	502.140
T3	25.16±0.01^{a}	61.52±0.01^{b}	13.57±0.01^{c}	19.16±0.01^{a}	496.187
T4	24.52±0.01^{c}	57.52±0.01^{c}	13.33±0.02^{c}	18.42±0.01^{a}	491.940
T5	24.55±0.01^{c}	64.32±0.01^{a}	15.07±0.01^{b}	18.71±0.00^{a}	510.242
T6	24.65±0.00^{b}	58.66±0.01^{c}	17.17±0.01^{a}	18.17±0.00^{a}	515.180
T7	24.10±0.00^{e}	59.72±0.01^{b}	15.33±0.01^{b}	17.74±0.00^{b}	508.708

T1= Diet 1 (control group), T2= Diet 2 (0.1% Biomos), T3 = Diet 3 (0.2% Biomos), T4= Diet 4 (0.3% Biomos), T5= Diet 5 (0.1% Agrimos), T6= Diet 6 (0.2% Agrimos), T 7= Diet 7 (0.3% Agrimos). DM= Averege dry matter, CP= crude protein, EE= ether extract, GE= gross energy

Table 4: Effect of Biomos and Agrimos on haematological parameters and serum biochemical analysis in *Oreochromis niloticus*

	T1	T2	T3	T4	T5	T6	T7
RBCs (x10/mm^3)	2.8±.02^{c}	2.9±.02^{bc}	3.9±.05^{a}	3.2±.12^{abc}	3.3±.07^{abc}	3.8±.09^{a}	3.5±.53^{ab}
Hb (g/100ml)	8.1±.11^{a}	9.2±.70^{a}	10.57±.67^{a}	10.51±1.04^{a}	9.5±.26^{a}	8.3±.21^{a}	10.9±1.6^{a}
PCV (%)	24±0.00^{b}	26±2.0^{ab}	30.5±2.5^{a}	30±1.00^{ab}	27.5±.50^{a}^{b}	26.5±.50^{a}^{b}	32±3.00^{a}
WBCs (x10^3/mm^3)	31.9±5.01^{a}	37.7±7.9^{a}	41.3±10.1^{a}	38.7±.7^{a}	44.4±4.4^{a}	32.2±1.5^{a}	36.3±7.3^{a}
Total protein (g/dl)	4.8±.10^{b}	5.2±.20^{ab}	5.1±.24^{ab}	5.9±.11^{a}	5.6±.24^{ab}	5.7±.39^{ab}	5.8±.38^{ab}
Albumin (g/dl)	2.9±.08^{a}	3.06±.06^{a}	3.02±.21^{a}	3.4±.08^{a}	3.3±.05^{a}	3.4±.22^{a}	3.2±.19^{a}
Globulin (g/dl)	1.9±.02^{b}	2.14±.14^{a}^{b}	2.11±.03^{ab}	2.49±.03^{a}	2.3±.18^{ab}	2.41±.17^{a}^{b}	2.41±.19^{a}^{b}
ALT (U/I)	5.10±.28^{a}	5.11±.02^{a}	5.11±.30^{a}	4.5±.32^{a}	5.3±.10^{a}	4.8±.04^{a}	5.11±.19^{a}
AST (U/I)	77±.93^{a}	76.6±.4^{a}	75.1±4.01^{a}^{b}	69.8±.04^{b}	78±.90^{a}	72.8±.57^{a}^{b}	62.8±.40^{c}

T1=Diet 1 (control group), T2= Diet 2 (0.1% Biomos), T3= Diet 3 (0.2% Biomos), T4=Diet 4 (0.3% Biomos), T5= Diet 5 (0.1% Agrimos), T6= Diet 6 (0.2% Agrimos), T 7= Diet 7 (0.3% Agrimos). RBCs = Red Blood Cells, HB = Haemoglobin, PCV = Packed Cell Volume, WBCs = White Blood Cells, ALT = Alanine aminotransferase, AST = Aspartate amninotransferase

Table (5): Effect of prebiotics supplementation on immunity and biochemical parameters

Treatment	Phagocytic activity	phagocytic index	Respiratory burst activity	lysozyme activity	Total antioxidant activity	Catalase activity
T1	16.47±1.02^{c}	4.27±0.08^{a}	2.41±0.03^{Da}	0.04±0.001^{Da}	0.38±0.002^{Fa}	2.05±0.15^{Ea}
T2	42.55±1.66^{ab}	19.33±0.41^{ab}	8.72±0.022^{Aa}	0.83±0.002^{Aa}	1.95±0.006^{Aa}	7.78±0.08^{Aa}
T3	31.35±1.73^{a}	13.26±0.27^{ab}	5.37±0.021^{Ba}	0.34±0.003^{Ba}	1.21±0.02^{3Ca}	3.56±0.05^{Ca}
T4	29.63±3.11^{bb}	11.47±0.74^{a}	4.68±0.044^{Cb}	0.39±0.003^{Cb}	1.54±0.002^{Ab}	5.31±0.08^{Ab}
T5	28.27±1.64^{abc}	11.37±0.44^{a}	4.23±0.032^{Ba}	0.025±0.005^{Ba}	1.11±0.002^{Da}	2.14±0.17^{Db}
T6	39.93±1.27^{ab}	16.25±0.36^{b}	7.29±0.027^{Aa}	0.76±0.003^{Aa}	1.52±0.002^{Ba}	5.13±0.13^{Ba}
T7	25.87±3.47^{bc}	9.62±0.89^{a}	3.85±0.052^{Cb}	0.31±0.004^{Cb}	1.12±0.002^{Bb}	4.33±0.02^{Bb}

T1=Diet 1 (control group), T2= Diet 2 (0.1% Biomos), T3= Diet 3 (0.2% Biomos), T4=Diet 4 (0.3% Biomos), T5= Diet 5 (0.1% Agrimos), T6= Diet 6 (0.2% Agrimos), T 7= Diet 7 (0.3% Agrimos)

Aqua feeds industry are focusing mainly on getting double advantages of both upgraded development and resistant reaction of vast majority of farmed fish species, as well as preventive medicinal services by means of numerous healthful procedures to guarantee its manageability in the aquaculture system (41). The nutritional status is the main key that affects the immune status of cultured fish species so as to finally get a superior assurance (42).

In the current study, the increased growth parameters among prebiotic treated groups may be attributed to improved feed utilization in fish. The results are similar to those reported by some authors (43-47); but in contrast to those reported by Genc et al., (48).

The improvement of food conversion rate, the best was recorded in T2 and T6 groups, may be due to the effect of used prebiotics in the current study which led to decreased amount of feed necessary for producing one unit of fish leading consequently to production cost reduction. The results are in agreement with some authors (45, 46, 49). The dietary MOS could altogether build the intestinal microvilli length or potentially thickness prompting expanded nutrient absorptive capacity (43, 46, 49).

The results of chemical composition of the experimental fish body, average dry matter, crude protein, ether extract, ash and nitrogen free extract, are in a partial consent to Orban et al., (50), where they recorded that body composition was strongly affected by their feed composition. In the current study, there were no critical contrasts recorded in body composition among groups; however, an expansion was noticed in protein level of carcass prawn with expanding the Agrimos® incorporation level in the diet. These outcomes are like those announced by numerous authors (45, 48, 51, 52).

The elevated number of WBCs may be attributed to the improved defense response as a result of Biomos and/or Agrimos feed supplementation. The obtained result is similar to those reported by some authors (53-55). However, it is in contrast to other authors (56, 57). The variety in the results of haematological parameters might be due to the probiotic type and dose, fish physiological status, species, size, age, ecological conditions and dietary routine (58). The increased level of total proteins in all prebiotic treated groups may be attributed to improved body defense and stronger innate response of fish as a result of prebiotic supplementation (53, 59, 60). The results were in agreement to some authors (57, 61); however, in contrast to Andrews *et al.* (53). Fluctuations in hematological and serum biochemical factors may be species-related and rely upon the

incorporation rates of MOS, diet ingredients and/or the raising time frame period (62).

This study demonstrated that the immune response of *O. niloticus* fingerlings was fundamentally influenced and expanded in all groups with the maximum level in group 2 and 6. Lysozyme is a standout amongst the most fundamental safe reactions of fish. It is originating from neutrophils and macrophages emitted into blood and mucus to apply bacteriolytic impacts (63) helping organisms to oppose bacterial, viral and parasitic diseases (64). The present outcomes demonstrated that the dimensions of lysozyme action were fundamentally modified due to Biomos and Agrimos supplementation. The highest serum lysozyme activity was observed in fish encouraged eating diet with 0.1% Biomos diet and 0.2% Agrimos, respectively. The expanded lysozymal action might be due to the immune-stimulatory effects of dietary Biomos and Agrimos (42).

Respiratory burst activity, a key for innate immunity, could be estimated utilizing nitroblue tetrazolium (NBT) (65, 66). It shows the oxidative capability of reactive oxygen species (ROS) like; hydrogen peroxide, superoxide anions, and hydroxyl radicals (67), delivered by initiated phagocytic cells and in charge of killing or degrading engulfed materials, including microbes (67). In the present study, group 2 (0.1% Biomos) and group 6 (0.2% Agrimos) demonstrated a huge upgrade of NBT toward the finish of the investigation. It implies that both Biomos and Agrimos assumes a critical job in activating antioxidant defense systems including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (68). The expanded bactericidal, lysozyme, and antioxidant activities in the present investigation might be attributed to the resistant stimulatory impacts of dietary Biomos and Agrimos.

Conclusion

It could be concluded that prebiotics supplementation of Biomos® and Agrimos® are highly beneficial in *O. niloticus* fingerlings diets resulting in an increased nutrient utilization and improving growth rate, hematological, biochemical parameters, immunological responses and survival rate. From the obtained results, it is preferable to use Biomos® and Agrimos® as feed additives at levels of 0.1% and 0.2%, respectively, with commercial feeds to improve tilapia fingerlings immune response.

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Original Research Article

DIGESTIVE ENZYMES, IMMUNITY AND OXIDATIVE STATUS OF NILE TILAPIA (*Oreochromis niloticus*) REARED IN INTENSIVE CONDITIONS

Mahmoud A.O. Dawood[1]*, Mustafa Shukry[2], Mohamed Mamdouh Zayed[3], Amira Alaa El-Dein Omar[4], Amr I. Zaineldin[5], Mohammed F. El Basuini[6]

[1]Department of Animal Production, Faculty of Agriculture, Kafrelsheikh University, 33516, Egypt, [2]Department of Physiology, Faculty of Veterinary Medicine, Kafrelsheikh University. [3]Department of Aquaculture, Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University. [4]Department of Fish Diseases and Management, Faculty of Veterinary Medicine Kafrelsheikh University. [5]Animal Health Research Institute (AHRI-DOKI), Egypt, [6]Department of Animal Production, Faculty of Agriculture, Tanta University, 31527, Egypt

*Corresponding author, E-mail: mahmouddawood55@gmail.com

Abstract: High stocking density is significantly disturbing the growth and productivity of aquatic animals. Digestive enzymes, immunity and oxidative status of Nile tilapia were investigated in case of culturing in several densities. Fish (14.3±0.03 g) were stocked in 12 aquaria (60 L) at four densities of 10 (SD10), 20 (SD20), 30 (SD30) and 40 (SD40) fish per aquarium for 30 days. Fish growth, feed efficiency ratio, digestive enzyme activity and dissolved water oxygen significantly (P<0.05) decreased, while the total ammonia increased with increasing stocking density. Immunoglobulin and NBT levels decreased significantly (P<0.05) in SD40 set compared to SD20 set without no differences with the other two groups. Lysozyme activity reported the highest significant (P<0.05) values in SD10 and SD20 groups over the high stocking density group (SD40) without no difference with SD30 group. Bactericidal, phagocytic activities and phagocytic index reported significantly (P<0.05) lower values in fish reared in SD30 and SD40 groups than fish reared in SD10 and SD20 groups. Peroxidase activity also showed significantly (P<0.05) low values in SD40 and SD30 groups with the weakest activity in SD40 group. Total serum protein lowered relatively in SD30 and SD40 groups without no differences with the other groups. Furthermore, fish reared at high stocking densities resulted in significantly (P<0.05) decreased superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) activities as well as increased malonaldehyde (MDA) activity in blood of tilapia suggesting suppressed antioxidant response. In conclusion, intensive conditions depressed the growth, digestive enzyme activity, immunity and oxidative status of Nile tilapia.

Key words: digestive enzyme activity; growth; immunity; nile tilapia; oxidative status; stocking density

Introduction

Intensive aquaculture conditions can affect the water quality negatively; thus, can markedly threaten the fish health and productivity. It has

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been found that increased stocking led to high ammonia accumulation, hydromineral balance and mortality in different fish species including, tambaqui, winter flounder, Nile tilapia and Japanese flounder (1-4).

The low growth performance is concretely affected by the intensive conditions due to decreased feed intake. The feed utilization is related to the activity of microbiota and digestive enzyme activity in the gastrointestinal tract (GIT), which increases feed digestibility and utilization and ultimately improves the growth and health status of fish (5). Protease, lipase and amylase are the major digestive enzymes, which play the main roles in feed digestion and absorption. If the activity of these enzymes increases, overall body metabolism may increase (6). The activities of digestive enzyme were depressed in Nile tilapia and Japanese flounder when reared in intensive conditions (3,4). Additionally, oxidative stress and immunosuppression are the direct features occurring during the intensive conditions, which either caused by the physiological stress (7) or water quality deterioration, such as a decrease in dissolved oxygen and an increase in ammonia levels (8). Due to intensive rearing conditions, the fish respiration rate increases leading to hydromineral imbalance (9, 10). Although not studied, high stocking densities may suppress immune responses of the fish, because physiological stress (11) and low water quality (12) are sources of immunosuppression. As a result, fish become more susceptible to infectious diseases.

Nile tilapia (*Oreochromis niloticus*) is one of the most important cultured fresh water species in the world. Tilapia is usually farmed in Egypt using intensive culture system which often caused stressful circumstances and depressingly disturb their growth and wellbeing (13). Low growth performance was observed in tilapia reared in intensive conditions as reported by Liu et al. (3) and Wu et al. (14). For Nile tilapia, there are enough information about the growth performance and feed utilization of fish reared under intensive conditions. However, there is little data on the effect of stocking density on the digestive enzyme activity, immunity and oxidative status of Nile tilapia. Moreover, available information on the relationship between physiological changes with the growth of Nile tilapia stocked at different densities was limited. Therefore, this study aims to investigate the effect of intensive conditions on growth performance, digestive enzyme activity, immunity and oxidative status of Nile tilapia.

Material and methods

Fish, diet and experimental protocol

Tilapia fingerlings were obtained from a private farm located in Kafrelsheikh, Egypt, and transported to Animal Production Department, Faculty of Agriculture, Kafrelsheikh University, Egypt. After 2 weeks acclimation, 300 tilapia (14.3±0.03 g) were put into 12 glass aquaria (60 L) and distributed in four stocking densities; at 10 (SD10), 20 (SD20), 30 (SD30) and 40 (SD40) fish per aquarium. Each aquarium was provided with an air stone for aeration. Feeding rate was fixed at 2 to 3 % of body weight per day with two feeding times 8:00 and 15:30 hr for 30 days. Fish fed diets prepared as described by Dawood et al., (13). The nutritional profile for each diet was confirmed by AOAC (15). The leftover feed was siphoned out after 3 h and 50 % of water was replaced daily with fresh, dechlorinated water of similar temperature. Lighting in the culture unit was set at 12:12 light: dark cycle throughout the study.

Water quality parameters were monitored regularly throughout the experimental period. Water temperature, pH and dissolved oxygen (DO) were measured using thermometer, portable digital pH meter (Martini Instruments Model 201/digital) and Waterproof Portable Dissolved Oxygen (model Hanna waterproof IP67). Total ammonia-nitrogen was measured calorimetrically.

Sampling schedule

All fish were fasted for 24 hr prior to final sampling. Fish were individually measured for final body-weight. Then, the intestine sampled for digestive enzymes analysis from 9 fish per experimental group. Fish were randomly caught and euthanized by “diluted tricaine methanesulfonate (MS-222; 400 ppm ratio; Sigma-Aldrich, Egypt)”. Intestine aseptically

taken, washed with PBS (pH 7.5; 1 g per 10 mL), homogenized and centrifuged for 5 min at 8000 rpm. The supernatant was then kept at 4°C.

The total protein content was measured using diluted homogenates following the method of Lowry et al. (16) using bovine serum albumin as a standard. Protease activity was evaluated according to Anson (17) using Folin phenol reagent, and amylase activity was measured according to Jiang (18) and Worthington (19) using iodine solution to reveal non-hydrolysed starch. Protease and amylase activities were both expressed as "specific activity" (units per mg of protein).

Specific activity of lipase was assessed based on the protocol described by Borlongan (20) and Jin (21) with olive oil as a substrate. Fatty acid, derived from enzymatic hydrolysis of triglyceride on stabile emulsion of olive oil, was titrated with NaOH. One unit of specific activity of lipase was determined as the volume of NaOH 0.05 N needed to neutralize fatty acid released after 6 hours-long incubation with substrate. Lipase activity was expressed as "units per gram" of intestine content.

Blood collection and immunological assays

Blood was collected from the caudal vein of 9 anaesthetized fish per group and quickly put into 1 ml EDTA coated vials for whole blood and non-coated vials for serum collection. The blood samples were left for 30 minutes till blood clotting then serum separation by centrifugation at 3000 rpm for 10 minutes. Serum samples were stored at -20 °C until further analysis. Blood total serum protein was carried out by RA-50 chemistry analyzer (Bayer) using readymade chemicals (kits) supplied by Spinreact Co. Spain, following manufacturer's guidelines. Immunoglobulin M (IgM) was measured by an ELISA assay using a commercial kit (Cusabio; Wuhan, Hubei, China). The result of IgM was expressed as mg per dl.

Respiratory burst activity of the whole blood was quantified by the nitro-blue-tetrazolium (NBT) assay according to Secombes (22). The NBT reduction was measured using the microplate reader (Optica, Mikura Ltd, UK) at 630 nm.

Lysozyme activity was measured following Parry et al. (23). The result was expressed as "a reduction in absorbency of 0.001/min". Serum bactericidal activity against *Aeromonas hydrophila* was detected by following Rainger and Rowley (24). The results were recorded as survival index (SI). Values were calculated as follows: "SI = CFU at end / CFU at start x100".

The total peroxidase activity of serum was also assessed using the spectrophotometer at 540 nm as described by Quade and Roth (25) and partially modified by Sahoo et al. (26).

Phagocytic activity and phagocytic index were determined following Kawahara et al. (27). The number of phagocytized cells was counted in the phagocytic cells to calculate the phagocytic index according to the following equations: "Phagocytic activity (PA) = Macrophages containing yeast/Total number of Macrophages x100; Phagocytic index (PI) = Number of cells phagocytized/Number of phagocytic cells".

Oxidative status

Superoxide dismutase (SOD), malonaldehyde (MDA), catalase (CAT), and glutathione peroxidase (GPX) in fish serum were measured using the diagnostic reagent kits following the manufacturer's (Cusabio Biotech Co., Ltd; China) procedure.

Growth performance calculations

During the final sampling, all fish per tank were weighed separately. Growth performance and feed utilization were evaluated using weight gain (WG), specific growth rate (SGR) and feed efficiency ratio (FER). Calculations were made using the following formulae: WG (%) = (FBW– IBW) ×100/IBW; SGR (%BW/day) = 100((lnFBW -lnIBW)/T); FER = WG /FI. Where FBW = body weight final (g), IBW= body weight initial (g), T = duration of the trial in days, WG = wet weight gain (g) and FI = estimated feed intake (g).

Statistical analysis

Shapiro-Wilk and Levene tests confirmed normal distribution and variance homogeneity. All statistical differences were assessed by one-way ANOVA tests (SPSS version 22, SPSS Inc., Il, USA) with Duncan's as post-hoc test where differences in experimental groups occurred. The level of significance was accepted at $P<0.05$. All data are presented as means ± standard error (SE).

Results

Water quality values

Water physicochemical characteristics are shown in Table 1. Intensive conditions led to significant ($P<0.05$) decrease in dissolved oxygen (DO) and increase in total ammonia levels of rearing water. The lowest DO and the highest total ammonia values were detected in SD30 and SD40 groups. No significant ($P>0.05$) differences were detected among all the groups in terms of rearing water temperature and pH levels.

Growth and feed efficiency

Growth performance of fish (FBW, WG and SGR) decreased significantly in SD40 group than the other groups (Table 2). Also, feed efficiency ratio decreased significantly ($P<0.05$) in SD40 compared to SD10, while no significant ($P>0.05$) differences were reported among the other groups (Table 2). Survival rate lowered significantly ($P<0.05$) in SD30 and SD40 groups than the low stocking density group (SD10) without no differences with SD20 group.

Digestion enzymes

Amylase, lipase and protease enzymes showed significantly ($P<0.05$) higher activities in fish reared in low stocking densities (SD10 and SD20 groups) over the high stocking density (SD40) without no significant difference ($P>0.05$) with SD30 group (Fig. 1).

Immune response

High stocking density led to significant immunosuppression in tilapia as declared by the blood immunity in Figure 2. Immunoglobulin and NBT levels decreased significantly ($P<0.05$) in SD40 group compared to SD20 group without no differences with the other two groups. Lysozyme activity reported the highest significant ($P<0.05$) values in SD10 and SD20 groups over the high stocking density group (SD40) without no difference with SD30 group. Bactericidal, phagocytic activities and phagocytic index reported significantly ($P<0.05$) lower values in fish reared in intensive conditions (SD30 and SD40 groups) than fish reared in low stocking density (SD10 and SD20 groups). Peroxidase showed significantly ($P<0.05$) lower value in SD40 and SD30 groups compared to the other groups with the lowest level in SD40 group. Total serum protein lowered relatively in SD30 and SD40 groups without no differences with the other groups.

Oxidative status

Oxidative and antioxidative enzymes activities are shown in Figure 3. SOD and CAT decreased significantly ($P<0.05$) in SD30 and SD40 groups compared to SD10 and SD20 groups. Similarly, GPX exhibited significantly ($P<0.05$) lower activity in SD30 and SD40 groups than SD10 and SD20 groups with the highest level in SD10 group. However, MDA increased significantly ($P<0.05$) in fish reared in intensive conditions (SD30 and SD40 groups) over fish reared in low stocking density (SD10 and SD20 groups).

Discussion

Aquaculture is based on the culture of fish in an optimal environmental and culture conditions. High stocking density is among the rearing strategies of aquatic animals. In this system the water components are utilized efficiently in order to get higher fish production per unit of rearing water. Nevertheless, over stocking density can be a risky stress which suppress the growth, survival, immune response and oxidative status (28, 29).

Table 1: Water quality parameters of Nile tilapia reared under different stocking densities

Item	Test group			
	SD10	SD20	SD30	SD40
Temperature (ºC)	27.07±0.15	27.1±0.24	27.2±0.2	27.4±0.13
pH	7.2±0.06	7.23±0.12	7.17±0.09	7.23±0.07
Dissolved oxygen (mg L^{-1})	$5.43±0.09^{c}$	$4.83±0.15^{b}$	$4.4±0.15^{a}$	$4.2±0.06^{a}$
Total ammonia (mg L^{-1})	$0.57±0.03^{a}$	$1.22±0.2^{b}$	$1.71±0.09^{c}$	$1.86±0.2^{c}$

*Values expressed as means ± SE (n = 3). Different superscript letters indicate significant differences for each pair-wise comparison between treatments

Table 2: Growth and feed efficiency of Nile tilapia reared under different stocking densities

Item	Test group			
	SD10	SD20	SD30	SD40
IBW	14.43±0.23	14.2±0.17	14.23±0.34	14.37±0.33
FBW	$31.18±3^{b}$	$34.6±2.67^{b}$	$32.83±2.25^{b}$	$28.28±2.31^{a}$
WG (%)	$115.06±20.78^{b}$	$138.61±18.4^{b}$	$126.42±15.53^{b}$	$95.01±16^{a}$
SGR	$1.26±0.15^{b}$	$1.44±0.13^{b}$	$1.35±0.12^{b}$	$1.1±0.14^{a}$
FER	$0.85±0.03^{b}$	$0.79±0.07^{ab}$	$0.77±0.06^{ab}$	$0.73±0.02^{a}$
Survival	$100±0^{b}$	$96.67±1.67^{ab}$	$92.22±1.11^{a}$	$92.5±1.44^{a}$

*Values expressed as means ± SE (n = 3). Different superscript letters indicate significant differences for each pairwise comparison between treatments

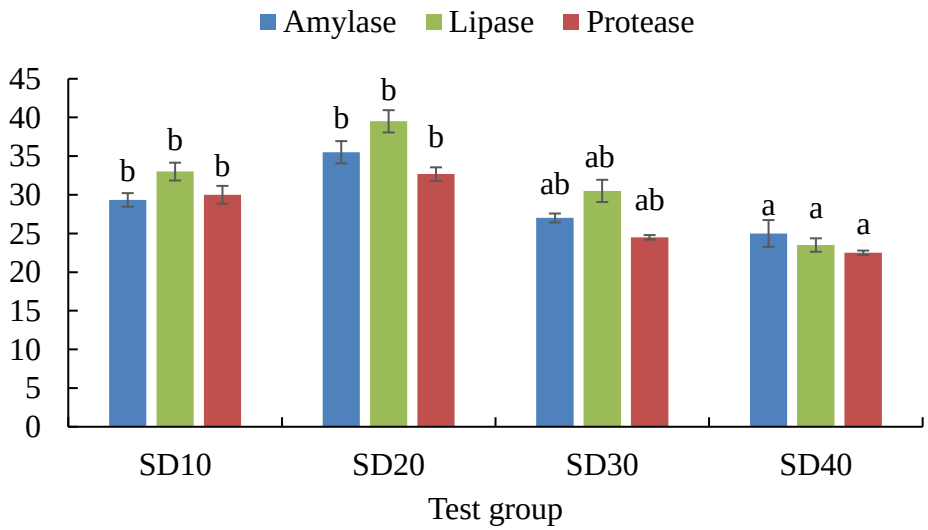

Figure 1: Digestive enzymes activities of Nile tilapia reared under different stocking densities. Values are expressed as mean ± SE from triplicate groups. Bars with an asterisk are significantly different from those of control group (P<0.05)

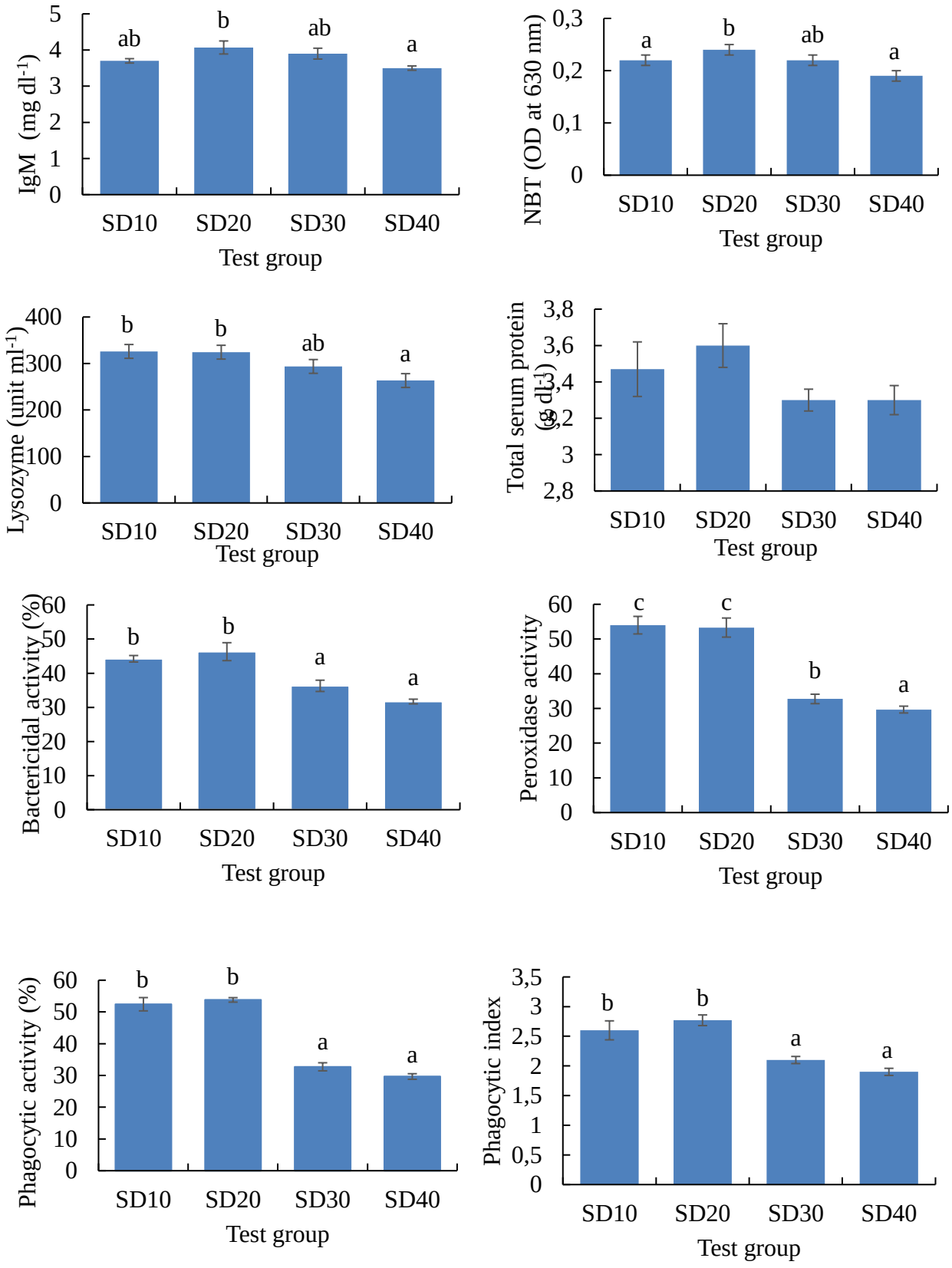

Figure 2: Activity of blood immune responses in Nile tilapia reared under different stocking densities. Values are expressed as mean ± SE from triplicate groups. Bars with an asterisk are significantly different from those of control group (P<0.05)

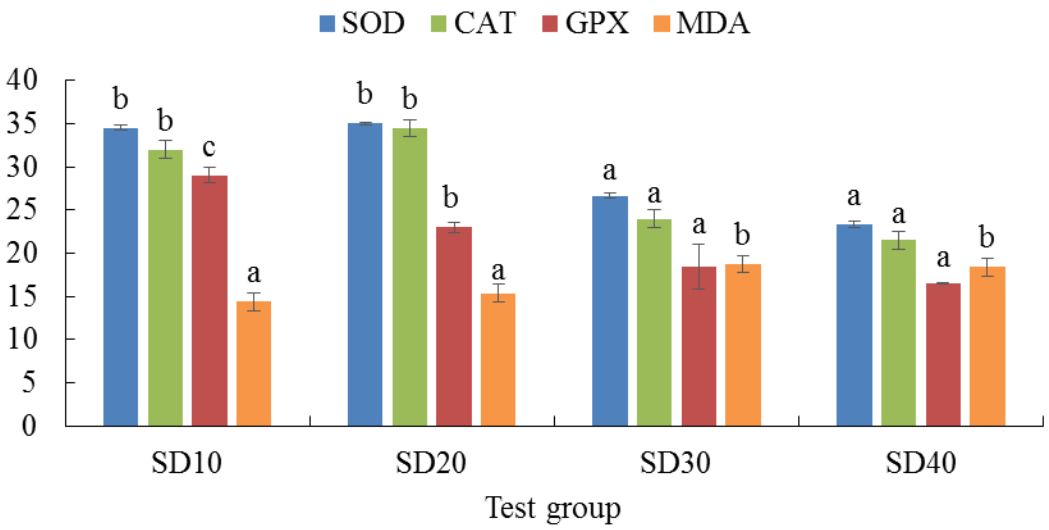

Figure 3: Oxidative status [SOD (IU L^{-1}), CAT (IU L^{-1}), GPX (IU L^{-1}) and MDA (nmol ml^{-1})] of Nile tilapia reared under different stocking densities. Values are expressed as mean ± SE from triplicate groups. Bars with an asterisk are significantly different from those of control group (P<0.05)

The current study illustrated that the growth performance of tilapia reared in intensive conditions (SD40) was slower than the other densities indicating that growth was influenced by the unreasonable stocking density. Parallel results also were obtained in tilapia and other fish species (14, 28, 30). Wendelaar (31) concluded that the low growth of fish reared in intensive conditions is due to the high level of required energy level to deal with stress which resulted in low available energy for fish growth. Survival rate is a significant parameter to express the welfare and health status of fish (32). In agreement with our study, fish reared in intensive conditions showed depressed survival rates (14, 30, 33).

In this study, compared with the low stocking density (SD10), fish reared in intensive conditions (SD30 and SD40) showed lower feed efficiency ratio (FER). Low FER is related to energy metabolism rate (30, 32). Fish reared in intensive conditions are usually exposed to severe-stressor circumstances that increase the required energy (9, 29). The extra energy requirements were probably met by mobilizing body resources, resulting in lower growth and feed utilization (34).

The decreased growth and feed efficiency of Nile tilapia in this study might be related to decreased activity of digestive enzymes. Major digestive enzymes produced by fish are protease, lipase and amylase to play its role in feed digestion and utilization. If these enzymes increase the overall body metabolism may increase (3,6). It is possible that intensive rearing conditions can weaken the digestion and utilization process of feed by affecting the activity of digestive enzymes. Further microbiome and proteomic studies are required to reveal the effect of intensive aquaculture conditions on the intestinal digestive enzymes and microbes.

Water quality is one of the main factors which affect the growth and feed utilization of fish (32, 35). The present study showed that high stocking density causes higher stress in Nile tilapia, leading to higher oxygen consumption and an increased ammonia level. There are many studies on the effects of stocking density on water quality with almost similar results. Similarly, increased stocking density led to lower DO and higher CO_2 levels (1, 14, 33). Randall and Tsui (36) reported that fish under stressful conditions excrete more ammonia and the present results suggest that the fish in high stocking densities experienced more severe stressed status. This is supported by the results of immune response and oxidative status.

It is well known that stress causes immunosuppression in fish (3). High stocking stress clearly declined soluble immune components in Nile tilapia with similar results in Senegalese sole and Rainbow trout (28). It has been proposed that, stressful conditions resulted in high produced corticosteroid levels which significantly inhibits the production of cytokines and immune responses (11). In this study, fish reared at high stocking densities exhibited suppressed immune responses "e.g. NBT, lysozyme activity, IgM, bactericidal activity and phagocytosis". Immunoglobulins are "heterodimeric glycoproteins that play a vital role in recognizing natural antigens and exist in the skin, gill and gut mucus, bile as well as systemically found in the plasma of fish" (37). Low serum IgM was noticed in tilapia of SD40 group. The respiratory burst activity (NBT) is a reliable parameter used to detect oxidative radical production which reflect the immunity of cultured fish to show its ability to resist the infectious diseases and environmental stressors (22, 38).

The lysozyme activity can breakdown the polysaccharide walls of pathogenic bacteria and offers stronger innate immune defense in fish against stressors (32). The lysozyme activity depends on the leucocyte counts which produce lysozymes that catalyse the glycosidic bonds of pathogenic bacterial cell walls resulted in enhanced complement system and phagocytosis (39). In agreement with our results, high stocking density reduced lysozyme and peroxidase activities, suggesting some degree of immunosuppression in Solea senegalensis, Gilthead seabream, Rainbow trout and Nile tilapia reared in intensive conditions (29, 40, 41, 42).

Phagocytosis is one of the significant cellular immune system components in fish (43). Its role is to guarantees that fish can avoid pathogen attacks efficiently by recognize the pathogens and to bound their spread and progress (44). Our study demonstrated decreased bactericidal and phagocytosis, suggesting weakening immune response and tolerance against high stocking density. These results suggested that the resistance to the pathogenic bacteria could be weakened because of the intensive conditions.

The reactive oxygen species (ROS) is produced by animal cells in the presence of several antioxidant defense mechanisms. The oxidative stress normally happens when the production and removal of ROS is unbalanced, since the oxidative damage of cultured species is directly related to the quality of rearing environment (45). Among the antioxidant enzymatic defenses SOD, GPX and CAT enzymes (46, 47). In this study, fish reared in intensive conditions resulted in decreased SOD, CAT and GPX activities as well as increased MDA activity in blood of tilapia suggesting suppressed antioxidant response. The depression was a response to the continuous stresses of stocking density and might reflect the limited abilities for antioxidant systems in tilapia to wholly remove these harmful SOD, finally leading to oxidative damage (28, 40, 48). Usually, the antioxidant system is activated to control the ROS which resulted in oxidative stress (49). High level of lipid peroxidation is a result of excessive ROS production which ends by the production of MDA. High MDA levels finally leading to oxidative damage to DNA, protein and cytoplasm (50). The obtained results revealed increased MDA in fish at high stocking level indicated cell damage. Earlier reports also revealed decreased antioxidant enzyme (SOD, CAT and GPX) (40, 48) and increased oxidative enzyme (MDA) levels in fish reared in intensive conditions (28).

Conclusion

It can be concluded that, the intensive rearing conditions can impair the welfare of tilapia fingerlings and depress the growth, digestive enzyme activity, immune response and oxidative status.

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Original Research Article

UTILIZATION OF SUGAR BEET BY PRODUCTS IN FEEDING GROWING RAHMANI LAMBS

Mohamed Ali[1], Nabil Eweedah[1], Hamed Gaafar[2]*, Metwally Abd El-Salam[2]

[1]Department of Animal Production, Faculty of Agriculture, Kafrelsheikh University, Egypt. [2]Animal Production Research Institute (APRI), Agricultural Research Center, Ministry of Agriculture, Egypt.

*Corresponding author, E-mail: hamedgaafar@gmail.com

Abstract: Twenty female Rahmani lambs with an average initial body weight 31.01±0.68 kg were randomly divided into four similar groups (5 in each group). To receive one of the tested rations, Ration1 containing 50% concentrate feed mixture 1 plus 50% berseem hay as a control ration. In rations 2 and 4, 50% of berseem hay was replaced by dry sugar beet tops. While, in rations 3 and 4, 50% energy ingredients (yellow corn grains, wheat bran and rice bran) were replaced by dry sugar beet pulp in concentrate feed mixture 2. Ration 3 had significantly (P<0.05) the highest digestibility coefficient of all nutrients followed by rations 4 and 2, while, while ration 1 had the lowest values. Digestible crude protein had nearly the same trend, but total digestible nutrients and digestible energy were significantly higher in ration 3 compared with the other rations. A logarithmic scale of acidity or basicity of rumen liquor were nearly similar for all the different groups. Dry sugar beet pulp in rations 3 and 4 showed significantly the higher concentrations of total volatile fatty acids and ammonia nitrogen compared to the other rations. Dry sugar beet pulp increased dry matter, total digestible nutrients, digestible crude protein and digestible energy, but tended to decrease with dry sugar beet tops compared with control group. Growth rate and feed conversion were significantly higher (P<0.05) for lambs fed ration 3 compared with the other rations. Lambs fed ration 3 recorded significantly the highest net revenue and economic efficiency.

Key words: growing lambs; dry sugar beet pulp; dry sugar beet tops; growth performance

Introduction

In Egypt, there is a shortage of available fed for ruminant. So that a growing attention is being focused on the use of crop by-products such as sugar beet by-products. Using of these by-products will decrease the fed cost as well as limitation the environmental pollution.

In 2017 a large area was (about 545000 feddans) cultivated with sugar beet in Egypt, which produced about 650000 tons of dried sugar beet pulp (DSBP) as a by-product of' sugar beet manufacture. It also produced about 6.8 million tons of fresh sugar beet tops (FSBT) as an agricultural by-product (3).

Sugar beet pulp may be used as a partial or whole replacement of energy sources in mixed rations of ruminants (21, 17). Also, dried sugar beet tops (DSBT) was used as good roughage in feeding ruminants with appreciable reduction

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in feeding cost without any health troubles (6, 22, 4 and 12).

The objectives of this study were to investigate the effect of feeding female Rahmani lambs on ration containing DSBP as partial replacement 50% of energy sources (Yellow corn grains, wheat bran and rice bran) and/or DSBT as partial replacement 50% of berseem hay (BH) on feed intake, digestibility, rumen fermentation activity, some bloods parameters, growth performance, fed conversion and economic efficiency.

Material and methods

The present study was carried out during summer 2017 at private farm, El-Hamol, Kafr El-Sheikh Governorate, Egypt in cooperation with the Animal Production Department, Faculty of Agriculture, Kafrelsheikh University, Egypt.

Sugar beet tops (SBT) were collected after harvesting sugar beet crop and spread on ground, then turning it from time to time (before sunrise and after sun down) till drying (the drying period was about 7 days) then material was collected and stored. While DSBP was obtained from the Dakahlia Company of Sugar, Dakahlia Governorate as a by-product of sugar beet industry.

Twenty female Rahmani lambs weighted 31.01±0.68 kg were used in feeding trial that lasted 90 days. Animal were divided into four similar groups (5 lambs each). Animals were randomly assigned in to four experimental ratios. R1 containing 50% concentrate feed mixture (CFM1) plus 50% berseem hay BH (control ration). In R2 and R4 50% of BH was replaced by DSBT, while, in R3 and R4 50% of energy sources (yellow corn grains, wheat bran and rice bran) in CFM1 were replaced by DSBP in CFM2. Crude protein in CFM2 was adjusted by adding 0.6 urea (46% N) to compensate the lower protein content in DSBP. Each 1 ton of CFM1 contained 110 kg soybean meal, 300 kg yellow corn grains, 300 kg wheat bran, 250 kg rice bran, 10 kg common salt, 15 kg limestone, 7.5 kg premix, 5 kg sodium bicarbonate and 2.5 kg yeast culture. Whereas, CFM2: 50% from sources of energy (yellow corn, wheat bran and rice bran) were replaced by DSBP with addition 4.2 kg urea over the 1 ton to adjust protein content.

Animals were weighed before morning feeding on 2 consecutive days at the beginning and at the end of the experimental and once biweekly during the experimental period. At the end of the feeding trial, three lambs from each group were used in three digestion trials to evaluate the previous rations by using acid insoluble ash (AIA) technique (29). Samples of feed ingredients, ration and feces were taken for chemical analysis according (1). Digestible energy was calculated as follows: DE= TDN× 0.04409 (24).

At the last day of the digestibility trail, rumen liquor samples were taken 3 hr after the morning feeding by a rubber stomach tube. The samples were filtered through a double layer of cheese cloth. The rumen pH value was determined directly by HANNA pH meter (HI-8424 Sophisticated micro-processor, pH meter). The rumen samples were transferred into covered plastic tubes with two drops of formalin for stopping microbial activity and kept in a deep freezer until analysis. Total volatile fatty acids (TVFA's) concentration was estimated by using steam distillation method (30). While Ammonia-N (NH_3-N) concentration was determined using magnesium oxide (Mgo) as described by (1)

Blood samples taken at the same time of rumen liquor collection from the jugular vein in clean tubes and then were centrifuged at 2500 rpm for 20 minutes to separate blood serum using serological pipettes. Samples of whole blood were collected using EDTA as anticoagulant and immediately directed to hematological determination. Different items of the blood picture were carried out, the counts of red blood cells (RBC's) and white blood cells (WBC's) (20), hemoglobin (Hb) (9), total protein (TP) (8), albumin (AL) (14) and GOT and GPT (26). The data were analyzed using IBM SPSS Statistics general linear models procedure (15).

Results and discussion

Results in Table 1, revealed that a variation in chemical composition among the different

feed ingredients. The CF content was higher, whereas the contents of CP and NFE were lower in DSBP compared to the average of other energy sources (yellow corn grains, wheat and rice bran). It reflects the differences in CF and NFE contents between CFM1 and CFM2, while CP content was adjusted in CFM2 by adding urea to become nearly the same for both concentrate feed mixtures. Also, CP content was nearly similar for both BH and DSBT. While, CF content was higher, but NFE and ash contents were lower in BH compared with DSBT. The calculated composition of experimental rations showed that CP content was nearly similar for different rations. Moreover, CF content increased with DSBP (R3), but it decreased with DSBT (R2). However, NFE content had opposite trend, which decreased with DSBP (R3 and R4) and increased with DSBT (R2). The EE content decreased with DSBP R3 and R4. Ash content increased with DSBT (R2). Similar results found that introducing DSBP in rations of buffaloes and sheep resulted in an increase in CF content (10, 25). Also, replacing BH by DSBT in rabbit's ration decreased CF content and increased NFE and ash contents (13).

The digestibility coefficients and nutritive values of experimental rations are shown in Table (2). The digestibility coefficient off all nutrients in R3 (containing DSBP) had the highest significantly values ($P<0.05$) followed by R4 (containing DSBP + DSBT) and R2 (containing DSBT) while, the R1 (control) had the lowest values. The DCP% had the same trend nearly but, the TDN and DE values were significantly higher in R3 compared with the other rations. The improvements of digestibility and nutritive values with DSBP might be due to their higher fiber content (Table 1), which reduce the outflow rate of feed in the rumen as well as urea nitrogen supplemented with DSBP may be stimulate the rumen microorganisms. Although, the digestibility coefficients of all nutrients improved with DSBT compared to BH. These results agreed with those obtained by (23) indicated that using DSBP at level 25 and 50% in growing Angora goats rations increased the digestibility of DM, OM, CP and CF as well as nutritive value as TDN than control ration. On the other hand, (18) feeding Rahmani rams on complete pelleted rations with replacing 50 and 100% of BH by DSBT significantly increased the digestibility of DM, OM and NFE but, the digestibility of CP and EE and the values of TDN and DCP were decreased significantly higher. (4) concluded that DSBT could be used successfully as a replacer to 50% from BH in ration of growing lambs without any detritus effects on productive and reproductive performance.

Rumen liquor parameters presented in Table (3) showed that pH values were nearly similar for the different groups. The pH values of all rations were between 6.47 and 6.60 which were with the normal range (27). No significant differences in rumen pH values were detected while, the concentration of total VFA's and NH_3-N significantly increased between Rahmani rams consuming rations containing different levels of DSBP (0, 25 and 50%) as replacement of concentrate mixture, (5). Rations 3 and 4 contained DSBP showed significantly ($P<0.05$) higher concentrations of TVFA's and NH_3-N while, the lower TVFA's concentration was in R1 and NH_3-N concentration was in R2. Adding urea with concentrate feed mixture containing DSBP (CFM2) led to increase ruminal NH_3-N concentration, which simulate rumen microorganisms utilizing resulting in more fermentation of soluble carbohydrates and structure fiber producing VFA`s. (23) indicated that the total VFA's concentration significantly increased as the proportion of DSBP increased in the Angora goats ration. (11) didn't find any significant differences in the ruminal pH between Rahmani rams fed different rations containing 100% BH as control or rations contained 50 and 100% of BH were replaced by DSBT.

Results of blood hematology in Table (3) revealed significant differences ($P<0.05$) in the counts of white and red blood cells among the different groups, which R4 had the highest values followed by R3 and R2, while R1 had the lowest values. However, hemoglobin concentration and hematocrit percentage were nearly similar for different groups. The values of blood

hematology obtained in this study were within the normal range for sheep being 5-14.5 x 10^3/µl for WBC's, 8.2-12.3 x 10^6/µl for RBC's, 9-14 g/dl for hemoglobin and 25-38% for hematocrit (28).

Serum parameters in Table (3) showed that the concentrations of total protein and globulin were significantly (P<0.05) higher in R3 and R4 followed by R2, while R1 had the lower values. On the other side, the concentrations of glucose and total lipids as well as the activity of GOT and GPT were significantly (P<0.05) higher in R1 followed by R2, while both R3 and R4 had the lower values. These results revealed that DSBP was more effective in whole blood and serum parameters than that DSBT. While, (4) found that no significant differences on blood parameters when lambs fed ration contained DSBT as replacement of BH at rate 50 and 100 % compared with control group (BH). (25) declared that incorporation SBP in sheep ration decreased plasma concentrations of triglyceride, cholesterol, urea and uric acid compared to control ration (CFM). Finally, all serum blood parameters values were within the normal range.

Table 1: Chemical composition of ingredients, concentrate feed mixtures and experimental rations

Item	DM %	Composition of DM %					
		OM	CP	CF	EE	NFE	Ash
Ingredients							
Soybean meal	92.46	89.70	44.04	5.38	2.09	38.19	10.30
Sources of energy							
Yellow corn grains	91.42	98.68	9.49	1.74	4.23	83.22	1.32
Wheat bran	90.49	95.18	13.89	9.04	3.76	68.49	4.82
Rice bran	90.41	87.87	13.97	9.52	15.29	49.09	12.13
Average	90.77	93.91	12.45	6.77	7.76	66.93	6.09
Dry sugar beet pulp	89.28	95.91	9.70	24.98	2.18	59.05	4.09
Berseem hay	89.45	89.70	13.45	27.64	2.45	46.16	10.30
Dry sugar beet tops	87.65	79.18	13.78	12.74	2.28	50.38	20.82
Concentrate feed mixtures							
CFM1	91.21	90.22	15.48	6.22	6.45	62.07	9.78
CFM2	90.60	90.96	15.49	13.97	4.25	57.25	9.04
Experimental rations							
R1 (control)	90.33	89.96	14.46	16.93	4.45	54.12	10.04
R2	89.43	84.70	14.63	9.48	4.37	56.22	15.30
R3	90.03	90.33	14.42	20.80	3.35	51.76	9.67
R4	89.58	87.70	14.53	17.08	3.31	52.78	12.30

Table 2: Digestibility coefficients and nutritive values of experimental rations

Item	R1	R2	R3	R4	SEM
Digestibility coefficients, %					
DM	65.25^{c}	66.42^{bc}	68.13^{a}	67.70^{ab}	0.38
OM	66.30^{c}	67.03^{b}	69.28^{a}	67.50^{b}	0.35
CP	66.53^{c}	67.03^{bc}	69.09^{a}	67.45^{b}	0.31
CF	61.48^{c}	63.60^{b}	66.30^{a}	64.25^{b}	0.55
EE	76.50^{c}	78.90^{bc}	82.73^{a}	80.03^{b}	0.74
NFE	64.05^{c}	67.48^{b}	69.28^{a}	67.65^{b}	0.58
Nutritive values, %					
TDN	62.35^{b}	61.45^{b}	65.85^{a}	62.44^{b}	0.56
DCP	9.62^{c}	9.82^{b}	9.96^{a}	9.80^{b}	0.04
DE, Mcal/kg DM*	2.75^{b}	2.71^{b}	2.90^{a}	2.75^{b}	0.02

a, b, c: Means in the row with different superscripts differ significantly (P<0.05)

Table 3: Rumen liquor, whole blood and serum parameters of growing lambs in experimental groups

Item	R1	R2	R3	R4	SEM
Rumen liquor					
pH value	6.47	6.50	6.60	6.55	0.03
TVFA's (meq/100 ml)	12.11^{b}	13.19^{ab}	14.82^{a}	14.46^{a}	0.38
NH_3-N (mg/ 100 ml)	16.32^{ab}	15.28^{b}	19.50^{a}	18.60^{ab}	0.72
Whole blood					
WBC's (x $10^3/\mu l$)	8.12^{b}	8.57^{ab}	9.05^{ab}	9.50^{a}	0.23
RBC's (x $10^6/\mu l$)	9.74^{b}	10.39^{ab}	10.68^{ab}	11.10^{a}	0.24
Hb (g/dl)	9.67	9.97	10.57	10.97	0.27
HTC (%)	26.77	26.83	27.20	27.37	0.37
Blood serum					
Total protein	7.13^{b}	7.60^{ab}	8.07^{a}	8.13^{a}	0.15
Albumin	3.83	3.88	3.97	4.00	0.05
Globulin	3.30^{b}	3.72^{ab}	4.10^{a}	4.17^{a}	0.15
Glucose	51.00^{a}	48.67^{a}	45.33^{b}	45.33^{b}	0.82
Total lipids	153.00^{a}	149.00^{a}	135.33^{b}	133.00^{b}	3.11
GOT	46.78^{a}	44.67^{ab}	43.56^{b}	41.89^{b}	0.65
GPT	16.67^{a}	15.67^{ab}	14.83^{b}	14.50^{b}	0.32

a, b: Means in the row with different superscripts differ significantly (P<0.05)

Table 4: Average daily feed intake, live body weight, total and daily weight gain of growing lambs in experimental groups

Item	R1	R2	R3	R4	SEM
Average daily feed intake:					
As fed basis, kg/head/day:					
CFM1	0.718	0.696	-	-	
CFM2	-	-	0.740	0.725	
Berseem hay	0.718	0.348	0.725	0.362	
Dry sugar beet tops	-	0.348	-	0.362	
Total	1.436	1.392	1.480	1.449	
As dry matter basis, kg/head/day:					
DM	1.297	1.245	1.332	1.298	
TDN	0.809	0.765	0.877	0.810	
DCP	0.125	0.122	0.133	0.127	
DE, cal/head/day*	3.56	3.37	3.86	3.57	
Live body weight, total and daily weight gain:					
Duration (day)	90	90	90	90	
Initial weight (kg)	31.38	30.58	30.83	31.24	0.68
Final weight (kg)	43.16^{ab}	42.37^{b}	44.06^{a}	43.23^{ab}	0.98
Total weight gain (kg)	11.78^{b}	11.79^{b}	13.23^{a}	11.99^{b}	0.47
Average daily gain (g)	130.89^{b}	131.00^{b}	147.00^{a}	133.22^{b}	5.23
Relative growth rate, %	37.54^{b}	38.55^{b}	42.91^{a}	38.38^{b}	0.97

a, b: Means in the row with different superscripts differ significantly (P<0.05)

Feed intake as dry matter, TDN, DCP and DE was nearly similar for all groups (Table 4). (4) and (2) showed that no significant differences (P>0.05) in total dry matter and crude protein intake by sheep fed rations containing DSBP.

Live body weight, total and daily weight gain of growing lambs in different experimental groups are shown in Table (4). While, final live body weight, total and average daily body weight gain were significantly (P<0.05) higher for lambs fed R3 contained DSBP compared with the other groups. The relative growth rate of lambs fed R1, R2, R3 and R4 were 37.54, 38.55, 42.91 and 38.38%, respectively. These results showed that DSBP achieved the higher growth rate of growing lambs, which confirmed with higher digestibility and nutritive values (Table 2) and rumen fermentation activity (Table 3). These results agreed with those obtained by (16) who found that increasing average daily gain of lambs fed diet containing 25 or 50% DSBP as a replacer of CFM. (12) showed that average daily gain of lambs was significantly higher with replacing yellow corn by DSBP. (18) didn't find any significant differences in average daily gain of growing lambs fed rations containing DSBT at rate 0, 50 and 100% in complete pelted rations as replacement of BH. (11) reported that no significant differences for the average daily gain of crossbred ewe lambs fed rations containing DSBT as a replacement of BH (0, 50 and 100% DSBT).

Feed conversion of growing lambs fed the experimental rations presented in Table (5). Incorporation of dry sugar beet pulp and tops in rations of growing lambs significantly (P<0.05) improved feed conversion. Lambs in control group fed R1 showed the highest amounts of DM, TDN, DCP and DE required per one kg live weight gain, whereas those fed R3 containing DSBP had the lowest values of DM and DCP and those fed R2 containing DSBT had the lowest TDN and DE values. The improvement of feed conversion with DSBP attributed to the improvement of ADG (Table, 5).

Table 5: Feed conversion and economic efficiency of growing lambs in experimental groups.

Item	R1	R2	R3	R4	SEM
Feed conversion:					
DM, kg/kg gain	9.91^{a}	9.50^{ab}	9.06^{b}	9.74^{ab}	0.15
TDN, kg/kg gain	6.13^{a}	5.84^{b}	5.97^{ab}	6.08^{ab}	0.06
DCP, kg/kg gain	0.955^{a}	0.931^{ab}	0.905^{b}	0.953^{a}	0.03
DE, Mcal/kg gain	27.20^{a}	25.73^{b}	26.26^{ab}	26.80^{ab}	0.23
Economic efficiency:					
Feed cost (LE/day)	4.00^{a}	3.03^{c}	3.40^{b}	3.31^{b}	0.07
Feed cost LE/ kg gain	30.56^{a}	23.23^{b}	23.13^{b}	24.85^{b}	1.26
Output of weight gain (LE/day)	7.20^{b}	7.21^{b}	8.09^{a}	7.33^{b}	0.29
Net revenue (LE/day)	3.20^{c}	4.18^{ab}	4.69^{a}	4.02^{b}	0.29
Net revenue improvement %	0.00^{c}	30.63^{ab}	46.56^{a}	25.63^{b}	0.65
Economic efficiency	1.80^{c}	2.38^{a}	2.38^{a}	2.21^{b}	0.07

a, b, c: Means in the row with different superscripts differ significantly (P<0.05)

Moreover, feed intake decreased with feed DSBT (Table, 4). These results are illustrated with those obtained by (19) who found that efficiency of feed utilization was markedly improved in cows fed beet pulp as compared with those given corn. (16) reported improvement in feed efficiency of lams by feeding in rations containing DSBP at rate 25 or 50% as replacing of CM. (11) showed that feed efficiency (kg feed DM/kg gain) was nearly similar when fed crossbred ewe lambs on rations containing DSBT as a replacement of HB (0, 50 and 100% DSBT) and were no significant differences for all groups.

Results of economic efficiency in Table (5) showed that feeding rations contained DSBP and DSPT significantly (P<0.05) reduced average daily feed cost compared to control group and the lowest value was with DSPT in R2. In addition, feed cost per one kg weight gain was significantly (P<0.05) higher with control group compared to the other groups containing DSBP and/or DSPT. This may be due to the lower price of DSBP than those of corn gain, wheat and rice bran as well as lower price of DSBT than that of BH. Meantime, R3 contained DSBP had significantly (P<0.05) the higher output of daily weight gain compared with the other groups, which attributed to higher ADG of lambs fed R3 (Table, 5). Moreover, lambs fed R3 recorded significantly (P<0.05) the highest net revenue followed by R2 and R4, while R1 had the lowest values. Furthermore, lambs fed R2 and R3 had significantly (P<0.05) the highest economic efficiency followed by R4, while R1 had the lowest value. These results agreed with those obtained by (7) who found that feed cost per kg liveweight gain decreased with increasing amounts of DSBP in the rations. (25) reported that replacing CFM with SBP supplemented with 10% soybean meal (SBM) in sheep ration led to decrease total daily feeding cost and Feed cost LE/ kg gain and improved daily profit by 38.07% compared to control ration. (4) and (11) reported that feed cost as LE/kg gain decreased significantly with improved the economic efficiency for crossbred ewe lambs on rations containing DSBT as a replacement of HB (50 and 100% DSBT) compared with control

Conclusion

From these results it could be concluded that replacing 50% of energy sources (yellow corn, wheat bran and rice bran) by dried sugar beet pulp and replacing berseem hay by dried sugar beet tops in rations of growing lambs improved their performance concerning digestibility, rumen fermentation activity, blood parameters, growth rate, feed conversion and economic efficiency.

Conflict of interest

Authors declare that no conflict of interest.

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Original Research Article

GROWTH AND REPRODUCTION PERFORMANCE OF JAPANESE QUAILS (*Coturnix coturnix japonica*) UNDER VARIOUS ENVIRONMENTS OF LIGHT COLORS

Hassan E. Elkomy, Ayman E. Taha*, Heba A. Basha, Magda I. Abo-Samaha, Mohamed M. Sharaf

Department of Animal Husbandry and Animal wealth Development, Faculty of Veterinary Medicine, Alexandria University, Egypt

*Corresponding author, E-mail: ayman_soma2007@yahoo.com

Abstract: Light colors are important environmental factors affecting growth and reproductive performance of Japanese Quails. In the current study, a total number of 1060 of Japanese quail chicks were used to assess the effect of white, red, and green light color on body weight, age of sexual maturity, laying parameters as well as responses of reproductive organs. Results revealed that Japanese quails reared under red color light had higher ($P<0.05$) body weight at five weeks of age, relative growth rate (RGR), the first 42 days of egg production, relative ovaries and testicle weights, sperm motility and fertility and hatchability percentages (243.28 g, 184.50 %, 79.76 %, 4.33 %, 4.05 %, 90.50 %, 83.47 % and 83.47 %; respectively) than those raised under green and white light colors. Also, quails subjected to red color light during growth reached sexual maturity earlier (39.34 d) than birds subjected to white and green light colors (44.87 and 48.45 d). We conclude that using red light color during the period of growth and laying in Japanese quails improves growth and reproduction performances.

Key words: Japanese quails; light colors; growth; reproduction performances

Introduction

Light is an environmental factor affecting quail production, light stimuli vary by intensity, wavelength, duration and color, all of which aggregate to influence the physiological, production, reproduction as well as behavioral parameters of the birds (1). Poultry have retinal photoreceptors that are responsible for vision, while the non-visual photoreceptors (extra-retinal) are responsible for detecting photoperiod and adapting their physiology to the environment (2). Light stimulates directly the GnRH pathway that increases the amount of gonadotropins, which enhancing the ovarian development. Simultaneously, the increase in day length decreases the amount of melatonin and GnRH, and removing the inhibition on the stimulatory axis. Increased photoperiod enhances the sexual maturity of chicks due to induction of gonadotropin and sex steroids secretion with subsequent increment in production (3).

Interestingly, light color and its wavelength represent important factors that judge the performance of birds. It has been concluded that the red light diminishes cannibalism and feather pecking; blue light has a calming effect; however, orange-red and blue green color light have

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enhancing chicken reproduction and growth (4), respectively. There were variations among the performance parameters of broiler chicks reared under various light intensities (5). However, no improvement was reported in broiler chicks exposed to various intensities of light, although green light color enhanced growth performance of such chicks as compared to orange, red and yellow light (6). However, Kim et al. (7) found that birds performed better when reared under red light without affecting feed conversion ratio (FCR).

Hatchability of quail eggs collected from birds exposed to green bulbs was higher than those subjected to other light treatments (8, 9)**.** Meanwhile, quails reared under red and incandescent lights had higher body weight than those reared under blue and green lights (8)**.** The increased photoperiod to enhance the earlier sexual maturity, which may be altered according to the spectral output, Japanese quails exposed to red light reached to sexual maturity two weeks earlier than those exposed to blue or green light (8)**.** Hens reared under red light laid eggs significantly earlier than those under incandescent light and blue light. The mean age at first egg of chicken pullets in red light was earlier by two to eleven days compared to pullets in all other light treatments (6)**.** Similarly, laying hens maintained under red and white lights had early sexual maturity than hens maintained under green light (10). Moreover, light color affects egg numbers of laying hens (7, 10, and 11). This study aimed to investigate effects of light color on growth and reproduction parameters of Japanese quails.

Material and methods

Experimental design and birds management

This study was performed using 1060 one day old Japanese quail chicks using rearing pens at the Department of Animal Husbandry and Animal Wealth Development, Faculty of Veterinary Medicine, Alexandria University, Egypt. Chicks were randomly distributed into three light treatments (white light n=426, red light n=320 and green light n=315). Pens were cleaned, disinfected and fumigated using formaldehyde gas. The chicks were brooded at a temperature of 35˚C using automatic gas heaters as a source of heat and illumination. Temperature was gradually reduced 3˚C weekly till reach room temperature 24 ˚C at the fifth week of rearing. Birds were placed in environmentally controlled lightproof rooms separated from each other by a wooden chip board. At the fifth week, Japanese quails were moved to wire cages and grouped to sire families. Each family consisted of one male and three females (1:3 sex ratios) according to Karousa et al. (12). Cages were separated by lightproof plastic sheets to prevent transmittance of light. Additionally, all windows were blacked out by light proof plastic sheets.

Birds were fed add-libitum commercial starter diets containing 22% crude protein and 3100 kcal/kg of metabolizable energy meeting (13) requirements. While at the laying period, laying diet had calcium content of 3.5%; the birds also had free access to water. Animals were exposed to 24 h lighting during the brooding and growing periods. At laying period, the duration of light was adjusted by using a timer to 16 hours light and 8 hours darkness (16 L: 8 D). All light sources were equalized to 9W. The following light treatment regimens were used. Treatment 1 (White light): A total number of 426 Japanese quail chicks were reared in separate pens under white LED light (W) with wavelength 380 to 770 nm from hatching till five weeks; the birds were then moved to laying batteries containing 20 sire families illuminated with the same light. Treatment 2 (Red light): 320 Japanese quail chicks were reared in a separate pen illuminated with red LED bulbs (R) with wavelength 618 to 635 nm. At the fifth week, Japanese quails were moved to separate battery containing 28 sire families illuminated with same light. Treatment 2 (Green light): 314 Japanese quail chicks were reared in a pen illuminated green LED bulbs (G) with wavelength 515 to 535 nm. At the fifth week, Japanese quails were moved into laying batteries (14 sire families illuminated with same light). Bird eggs were collected from each treatment (white, red and green) daily and stored for 7 days then disinfected and incubated in previously cleaned and fumigated incubators by spraying TH4® (2

ml/liter water) to assess the effect of continuous use of different light colors on hatching performance (hatchability and fertility percentages) of Japanese quail eggs.

Hatching performance

Fertility % = (Number of fertile eggs/ Total number of eggs set) × 100

Hatchability of the total eggs% = (Number of hatched chicks/ Total number of eggs set) × 100

Hatchability of fertile eggs % = (Number of hatched chicks/ Number of fertile eggs set) × 100

Growth and reproductive performance

Birds were weighed at hatch and every week till five weeks of age to the nearest gram using digital scale with accuracy 0.001g (WANT®), and then the differences between two successive weights were calculated to estimate the relative growth rate according to Brody (14).

Relative growth rate = (W2 – W1)/ 0.5 (W1+W2) × 100, Where: W1= is the weight at any week and W2= is the weight at next week. Age of sexual maturity (age at the first egg) was expressed in days from hatching day till the day of the first egg of the quail hens. Egg production was recorded for each sire family of quails for the first 42 days after complete sexual maturity (15) with the estimation of hen day egg production (HDEP) for the same period.

HDEP%= (Total of eggs produced/ Number of live hens) × 100

Male and female reproduction organs

A random sample of three males and three females per treatment were selected at 98 days of age and weighed then slaughtered and dissected for removal of testes and ovaries. The right and left testes were immediately weighed to calculate the gonadosomatic index (GI = testes weight /body weight x 100).

Semen was also collected from vas deferens immediately after slaughtering of males. Sperm motility was then evaluated by two trained evaluators in samples placed on glass slides (37 °C) at 400x magnifications. Sperm motility was expressed as the percentage of mobile cells. The ovary was immediately weighed and relative weight to live body weight (ovary weight / body weight ×100) was calculated.

Statistical analysis

Data of body weight, weight gain and relative growth rate were analyzed by general linear model (GLM) procedure using **SAS** (16) according to the following model: $X_{ijk}= \mu + T_i + S_j + D_{i*j} + e_{ijk}$; μ= population mean, T_i= treatment effect (dark, green and red light), S_j= sex effect (M= male and F= female), D_{i*j} = the interaction between treatment and sex, e_{ijk}= random errors,

Data of hatchability, fertility, egg quality parameters, egg production, age at sexual maturity and reproductive organ parameters were analyzed by analysis of variance (ANOVA) using SAS (16) and the significance between different treatment groups were assessed using Duncan's test. Prior to analysis, percentages of embryonic mortality were arcsine transformed to corresponding value according to Snedecor and Cochran (17).

Reults and discussion

Effect of light color on Growth

The effects of different light colors on body weight of Japanese quails from hatch day till five weeks of age were showed in Table 1. The overall hatch weight was not significantly different between treatment groups. Similarly, this parameter did not differ between the sexes in each group. During the first week, the overall body weight in red light treated birds (55.25 g) was significantly higher than green and white light groups (49.92 and 30.62 g, respectively). Birds reared under red light also had significantly higher overall body weight at the 2nd, 3rd, 4th and 5th week of age (101.61, 160.26, 202.38 and 243.28 g, respectively) than those reared under green and white lights. Females reared under red and white colors recorded significantly higher body weight than males at the 2nd, 3rd, 4th and 5th week of age. In contrast, gender effect was not apparent for green light until 5th week of age.

Table 1: LS Means ± SE of body weight for Japanese quails subjected to different treatments of light color

Light Group	Sex	Body weight (g)					
		Hatch	W1	W2	W3	W4	W5
White	F	9.35±0.06^{a}	31.25±1.27^{a}	75.62±0.61^{a}	120.00±0.89^{a}	179.41±1.14^{a}	221.85±1.32^{a}
	M	9.29±0.09^{a}	29.98±1.86^{a}	73.38±0.90^{b}	114.48±1.30^{b}	170.64±1.67^{b}	206.41±1.93^{b}
Overall		9.32±0.05^{A}	30.62±1.12^{C}	74.50±0.54^{C}	117.24±0.79^{C}	175.02±1.01^{B}	214.13±1.17^{C}
Green	F	9.77±0.11^{a}	50.04±2.30^{a}	92.23±1.11^{a}	154.28±1.61^{a}	206.45±2.07^{a}	246.18±2.39^{a}
	M	9.63±0.10^{a}	49.81±2.19^{a}	91.23±1.06^{a}	150.80±1.53^{a}	201.18±1.97^{a}	222.89±2.28^{b}
Overall		9.70±0.08^{A}	49.92±1.59^{B}	91.73±0.77^{B}	152.54±1.11^{B}	203.82±1.43^{A}	234.54±1.65^{B}
Red	F	9.48±0.12^{a}	57.06±2.47^{a}	104.46±1.19^{a}	164.78±1.73^{a}	208.43±2.22^{a}	263.19±2.56^{a}
	M	9.53±0.12^{a}	53.44±2.54^{a}	98.76±1.23^{b}	155.75±1.78^{b}	196.33±2.29^{b}	223.39±2.64^{b}
Overall		9.50±0.08^{A}	55.25±1.77^{A}	101.61±0.85^{A}	160.26±1.24^{A}	202.38±1.60^{A}	243.28±1.89^{A}

Overall means carrying different capital letters within the same column are significantly different (P < 0.05)
Means carrying different small letters within group within same column are significantly different (P < 0.05)
F= Female, M= Male, W= Week, LS Mean = least square mean

Table 2: LS Means ± SE of relative growth rates for Japanese quails subjected to different treatments of light color

Light Group	Sex	Relative growth rate (%)					
		R1	R2	R3	R4	R5	R6
White	F	107.29±0.50^{a}	82.83±0.73^{a}	45.49±0.46^{a}	39.70±0.35^{a}	21.18±0.41^{a}	183.70±0.12^{a}
	M	104.80±0.74^{b}	84.06±1.07^{a}	43.69±0.67^{b}	39.42±0.52^{a}	19.14±0.60^{b}	182.71±0.18^{b}
Overall		06.04±0.45^{C}	83.45±0.65^{A}	44.59±0.41^{B}	39.56±0.31^{A}	20.16±0.36^{A}	183.21±0.11^{B}
Green	F	129.13±0.91^{a}	59.17±1.31^{a}	50.44±0.83^{a}	29.04±0.64^{a}	17.70±0.74^{a}	183.24±0.21^{a}
	M	131.37±0.87^{a}	58.75±1.26^{a}	49.26±0.80^{a}	28.76±0.61^{a}	10.19±0.71^{b}	181.73±0.21^{b}
Overall		130.25±0.63^{B}	58.96±0.91^{B}	49.85±0.57^{A}	28.90±0.44^{B}	13.94±0.51^{B}	182.48±0.15^{C}
Red	F	139.65±0.98^{a}	59.00±1.42^{a}	44.89±0.90^{a}	23.41±0.69^{a}	23.85±0.80^{a}	185.72±0.23^{a}
	M	137.26±1.01^{a}	59.64±1.46^{a}	44.87±0.92^{a}	23.13±0.71^{a}	13.86±0.82^{b}	183.26±0.24^{b}
Overall		138.45±0.70^{A}	59.32±1.02^{B}	44.88±0.64^{B}	23.27±0.49^{C}	18.85±0.57^{A}	184.50±0.17^{A}

Overall means carrying different capital letters within the same column are significantly different (P < 0.05)
Means carrying different small letters within group within same column are significantly different (P < 0.05)
F= Female, M= Male, W= Week, LS Mean = least square mean

Table 3: Means ± standard errors for sexual maturity of Japanese quails subjected to different treatments of light color

Treatment/Light Color	Age at first egg (days)
White	44.87±0.36^{b}
Green	48.45±0.40^{a}
Red	39.34±0.21^{c}

Means carrying different litters within the same column are significantly different (P<0.05)

Table 4: Means ± standard errors for hen day egg production (HDEP %) of Japanese quails subjected to different treatments of light color

Weeks	Treatment		
	White	Green	Red
Wk1	70.47±4.07[a]	64.63±2.53[a]	73.13±1.63[a]
Wk2	69.52±3.23[b]	56.12±2.13[c]	81.29±1.54[a]
Wk3	77.38±2.39[a]	74.15±1.63[ab]	79.25±2.42[a]
Wk4	61.19±3.13[b]	60.89±2.70[b]	79.25±1.38[a]
Wk5	78.57±2.43[a]	66.33±2.42[b]	80.27±1.92[a]
Wk6	80.48±1.54[b]	74.83±1.69[c]	85.37±1.27[a]
Total	72.94±1.61[b]	66.16±1.44[c]	79.76±1.00[a]

Means carrying different litters within the same row are significantly different (P<0.05)

Table 5: Means ± SE for egg weight in grams, external and internal egg quality parameters of Japanese quail eggs subjected to different treatments of light color

Parameters	Light treatment		
	White	Green	Red
Egg weight (g)	14.52 ± 0.16 [a]	13.72± 0.15 [b]	13.26 ± 0.29 [b]
Egg shape index	78.07 ± 0.40 [a]	78.19 ± 0.52 [a]	78.50 ± 0.51 [a]
Egg volume	12.34 ± 0.31 [a]	11.52 ± 0.27 [b]	11.63 ± 0.53 [b]
Yolk+albumen %	78.92 ± 0.50 [b]	82.99 ± 0.29 [a]	82.23 ± 0.42 [a]
Shell %	21.08 ± 0.98 [a]	17.01 ± 0.65 [b]	17.77 ± 0.90 [b]
Yolk index	0.46 ± 0.08 [c]	0.52 ± 0.07 [a]	0.49 ± 0.09 [b]
Haugh unit	93.02±0.72 [b]	95.44±0.44 [a]	96.44±0.60 [a]

Means carrying different litters within the same row are significantly different (P<0.05)

Table 6: Means ± standard errors for relative ovaries, testes and percentage of sperm motility weight of Japanese quails subjected to different treatments of light color for 14 weeks

Parameters (%)	Light Color / Treatment		
	White	Green	Red
Relative Ovaries weight	3.43±0.41[a]	4.27±0.96[a]	4.33±0.18[a]
Relative testes weight	3.53±0.30 [a]	3.47±0.24 [a]	4.05±0.20 [a]
Sperm motility	75.00±2.89[b]	84.33±7.22[ab]	90.50±1.26[a]

Means carrying different litters within the same row are significantly different (P<0.05).

Table 7: Means ± standard errors for fertility, scientific hatchability and commercial hatchability percentages of the first generation of Japanese quail eggs subjected to different treatments of light color

Light Color / Treatment	Fertility (%)	Hatchability (%)	
		Fertile eggs	Total eggs
White	81.17±0.44 [b]	84.80±0.35 [a]	77.40±1.25 [b]
Green	85.37±0.56 [a]	86.93±0.91 [a]	80.53±0.62 [a]
Red	90.07±1.13 [a]	92.53±1.27 [a]	83.47±0.58 [a]

Means carrying different litters within the same column are significantly different (P<0.05).

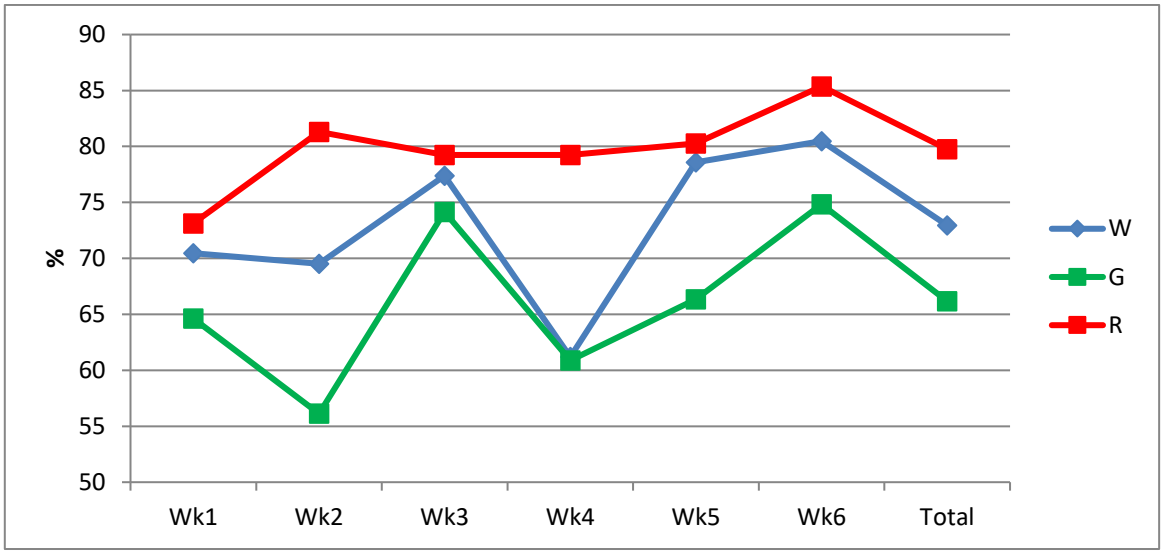

Figure 1: Means for hen day egg production (HDEP %) of Japanese quails subjected to different treatments of light color

W= Quails reared under white light from
G= Quails reared under green light from

Data on relative growth rates of Japanese quails subjected to different light colors for five weeks are presented in Table 2. Birds reared under red light grew at significantly higher rate (R6) from zero to five weeks of age (184.50%) than those reared under white and green light colors (183.21 and 182.48, respectively). Moreover, the overall growth rate of females was significantly higher than that of males in all light treatments.

These results are similar to those reported by others who found that Japanese quails reared under red lights had higher body weight than those reared under blue and green lights after five weeks (8). They speculated this was due to higher weight of reproductive organs as testes weight of male Japanese quail at five weeks of age with red light was double that of green light. Similarly, Li et al. (18) and Reddy et al. (19) reported growth-stimulating effect of red light in laying hens and documented increased weights and activity of reproductive organs (higher weight of the ovary and oviduct and more yellow yolk follicles). In contrast, Baxter et al. (10) found that hens reared under Green light had significantly higher body weight gain than birds under Red and White lights after 23 weeks of age. The sex effect on body weight of Japanese quail was also reported by (20) and (21) who found that female weights were significantly higher than those of males.

Age at sexual maturity

The onset of sexual maturity of Japanese quail subjected to different treatments of light color, (based on the age at first egg) was presented in Table 3. Females of Japanese quail reared under red light treatment reached sexual maturity significantly earlier (39.34 d) than those exposed to white light (44.87 d), while significantly late sexual maturity was recorded by females reared under green light (48.45 d). The result agreed with (22) who found that red light induced sexual maturity of broiler breeder birds 7 and 14 days earlier than green and blue light treated respectively. Also, laying hens maintained under red and white light had early sexual maturity than hens maintained under green light. Red light resulted in higher steroid and gonadotropin concentrations and higher neuropeptide mRNA expression (10). Similar results recorded in Japanese quail exposed to Red light reached sexual maturity two weeks earlier than those exposed to blue or green light (8).

Egg production

Table 4 and Figure 1 were illustrated the hen day egg production (HDEP %) of Japanese quails subjected to different light color treatments for six weeks after complete sexual maturity. There was no difference in HDEP among different light treatments during the first week of egg production. However, red light treatment birds had significantly higher HDEP percentage from the 2nd week till the 6th week of egg production, followed by white color treated group while the lowest HDEP% was recorded for green light treated group. The total HDEP percentage was significantly higher in red light treatments than white and green light treatments where the highest HDEP percentage recorded for red light (79.76%) and the lowest HDEP percentage recorded for green light (66.16%). These results are similar to those reported by Baxter et al. (10) and Hassan et al. (11) who found that red monochromatic light improved egg production of the laying hens more than blue or green treatments whereas the white light had an intermediate effect. Moreover, Yang et al. (22) reported that highest cumulative egg production values of laying hens occurred in the red-light treated birds. The same results found in Japanese quails brooded and kept for the 16-week production period under red or white light laid significantly more eggs than those brooded and kept under green or blue light (8).

The present study revealed that long-wavelength light (red and white) expedited the age of sexual maturation (ASM) and increased level of egg production in birds, while short wavelength light (green) delayed sexual maturation and reduced egg production. These observations are similar to those of Mobarkey et al. (23), who found that long wavelengths (red light) contain more energy so they are able to penetrate through the skull and brain tissue and stimulate the hypothalamus to produce gonadotropin releasing hormone. As such, hypothalamic photoreceptors might be stimulated by lower wavelength but high intensity of blue/green light (24). As well, it was suggested that stimulation of retinal photoreceptors by green light might be able to inhibit reproduction (25); the possible mechanisms for such inhibition still unclear; however, contribution of serotonin is suggested due to its synthesis in hypothalamus and retina (23).

Egg quality

Some external and internal egg quality parameters of Japanese quails subjected to different treatments of light color were presented in Table 5. Egg weights of white light treated hens were heavier ($P<0.05$) than those treated with green and red light colors (14.52, 13.72 and 13.26 g, respectively). The same results were reported by Er et al. (26), who found that egg weight was significantly higher in white and green light than Red one. In addition, Hassan et al. (11) found higher egg weight under green light than that under red light. However, other reports found that light color did not affect the average egg weight during the laying period (8, 27). Non-significant differences for egg shape index among different light colors. However, there were significant differences in egg length and width. Also, non-significant difference of the egg shape index between light treatments was recorded (22). On the other hand, white treated hens showed the highest egg volume (12.34 cm^3) and shell % (21.08%). The results agreed with those reported by Pyrzak et al. (28), who found shell quality and percent of the shell did not affect by red, green and blue light treatments of the White Leghorns laying hens.

Hens treated with green and red light colors produce eggs with higher albumen plus yolk, yolk index and Haugh unit (HU) than hens exposed to white light color. The result agreed with others who found non-significant difference in HU between red, green and blue light (11, 29), but disagreed with Yang et al. (22), who found that HU of the eggs in the white-light treated group was significantly higher than red, green and yellow light. Moreover, non-significant differences for yolk and albumin percentage among different light colors were recorded (30).

Male and female reproductive organs

Relative ovary weight of female Japanese quails subjected to different light colors for 14 weeks were presented in Table 6. Japanese quail females reared under red and green light recorded higher non-significant (P>0.05) relative ovary weights (4.33 and 4.27%, respectively) than those reared under white light color (3.43%). Moreover, Japanese quail males reared under red light had higher non-significant relative testicle weights than those reared under green and white light colors, in addition sperm motility of males subjected to red color were significantly higher (P<0.05) than those subjected to white color but not significant with those exposed to green light color. These results agreed with Kim et al. (7), who found non-significant difference in ovary weight at 20 weeks of age among the different light treatments. Also, Carson et al. (31) and Pyrzak et al. (32) found non-significant difference in the rate of gonadal maturation in pullets illuminated with blue, green, or red monochromatic light incandescent light. On the other hand, Hassan et al. (11) found that laying chickens exposed to red LED light presented heavier ovaries compared with those exposed to green and blue LED lights. Moreover, Woodard et al. (8) found that testes weight of male Japanese quail at five weeks of age with red light was double that of green light. While, Retes et al. (33) found that white and red LED bulbs caused higher weights of testes and higher sperm motility in the quails at 35 days of age.

Hatching parameters

Fertility and hatchability percentages of Japanese quail eggs reared under different light colors were presented in Table 7. Quails reared under red and green light colors had higher (P<0.05) fertility and hatchability of the total eggs than those reared under white light colors, on the other hand, non-significant differences for hatchability of fertile eggs were recorded between different treated groups. Yang et al. (22) reported better fertility percentages for the eggs produced in the green-light treated breeders, while higher hatchability was recorded for birds reared under white light color. On the other hand, Retes et al. (33) found that fertility of quail eggs produced in green light treated birds higher than white, red and blue LED treatments. While, Non-significant differences for hatchability percentages of Japanese quail eggs produced in different light colors were recorded (8).

Conflict of interest

None of the authors have any conflict of interest to declare.

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Original Research Article

INFLUENCE OF DIETARY VITAMIN C SUPPLEMENTATION ON GROWTH PERFORMANCE, BLOOD BIOCHEMICAL PARAMETERS AND TRANSCRIPT LEVELS OF HEAT SHOCK PROTEINS IN HIGH STOCKING DENSITY REARED BROILER CHICKENS

Ramadan S. Shewita[1], Karima El-Naggar[1], Walaa S. H. Abd El Naby[2]*

[1]Department of Nutrition and Veterinary Clinical Nutrition, Faculty of Veterinary Medicine, Alexandria University, Egypt, [2]Genetics and Genetic Engineering, Department of Animal Husbandry and Animal Wealth Development, Faculty of Veterinary Medicine, Alexandria University, Egypt

*Corresponding author, E-mail: walaa.slouma@yahoo.com

Abstract: This study was conducted to evaluate the effects of dietary supplementation of different levels of vitamin C (VC) in broiler chicken reared under high stocking density on the growth performance, blood biochemical parameters and the expression of heat shock protein genes. A total of 150, one day old mixed sex broiler chicks (*Cobb 500*) were randomly distributed in five equal groups. Group1 was reared in normal stocking density (10.6 birds/m^2) and fed on the basal diet (BD) without VC (control). While, chicks in other groups (2, 3, 4 and 5) reared in high stocking density (15.6 birds/m^2) and fed on BD supplemented with VC at 0, 200, 400 and 600 mg / kg diet, respectively. Birds reared in high stocking density showed a reduction of the final body weight and total feed intake, with high mortality (6.6%). Moreover, they revealed a significant up-regulation of *HSP70* mRNA and elevated *HSP90* and *HSF1* mRNA expression in heart and liver tissue. Graded dietary levels of VC provided variable protection against the hazard of high density through improved final body weight and total feed intake, decreased the mortality % and downregulated liver *HSP70* expression level. However, the best performance was observed in birds supplemented with 200 mg/kg VC (group 3).

Key words: chicks' growth performance; gene expression; stocking density; vitamin C

Introduction

Broiler production facing many forms of stressor including high ambient temperature, nutritional stress (imbalance or deficiency in the nutrient requirements) and vaccination programs stress as well as diseases. It is known that birds will perform better when grown in more space, hence, high stocking density (SD) is considered one of these stressful factors which have undesirable effects on broiler performance, livability, health and immune system response, as it reduces bird access to feed and water (1). It also leads to raising the environmental temperature dangerously where more metabolic heat will be added to the house air than was planned (2).

Different feed additives have a role in reducing stress in broiler feed are included in several studies (3-5). The ameliorative effects of ascorbic acid or vitamin C (VC) in many forms of

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stressors have been documented (6, 7). The heat shock proteins (*HSPs*) family is highly homologous chaperone proteins contributing to cellular protection, protein homeostasis and cell survival against a variety of environmental and metabolic stresses. *HSP70* is one of the most conserved and important protein which plays a deep role in enhancing tolerance to various stressors in broiler chickens (8,9). Different studies indicated that *HSP70* has an importance not only at high ambient temperatures but also in cell death mediated by free radicals and reactive oxygen species (10). Furthermore, in both physiological and stress conditions *HSP90* is a molecular chaperone involved in maturation and stabilization of a wide range of proteins to maintains cellular homeostasis and function (11). We hypothesized that VC could modulate the negative impacts of high stocking density as a stress factor in broilers farms

Therefore, this study aimed to investigate the effect of dietary VC supplementation with different concentrations on growth performance, some blood biochemical parameters, and heat shock protein genes expression in broiler chicks reared under high stocking density.

Material and methods

This experiment was approved by the local ethical committee of animal use from Faculty of Veterinary Medicine, Alexandria University.

Birds, experimental design and feeding Program

One hundred and fifty, one-day-old mixed sex *Cobb 500* broiler chicks were used in this study. The chicks were weighed at one day old and then randomly distributed into five groups (30 chicks/ group), each treatment has three replicates (10 chicks/replicate) in 15 compartments, every compartment was provided by a suitable feeder and waterer. All groups received their experimental diet for 6 weeks. The bird's compartments were bedded with fresh, clean chopped wheat straw forming a deep litter of 4 cm depth. The room temperature was adjusted on 33^{o}C in the first week of age after that decreased 3^{o}C/week until reaching 21^{o}C at the fifth week of age and the relative humidity was kept at 70 %. The chicks were vaccinated according to a normal regime (vaccination against Newcastle disease on days 7, 18 and 28 and infectious bursal disease (Gumboro) on day 12 of chicks age).

The broiler chicks were fed on the basal diet (BD) prepared from a corn-soybean meal based diet and were formulated to meet the nutrient requirements of poultry (12). Starter diet was given from 0-2 weeks of age, followed by grower diet (3-4 weeks) and finally finisher diet from 5-6 weeks of age. Chicks of group 1 (G1), were fed on BD without VC supplementation and reared in normal stocking density (10.6 birds/m^2), while the chicks of groups G2, G3, G4 and G5 distributed to be in high stocking density (15.6 birds/m^2) (13) and fed on the BD supplemented with VC (Introvit-C WS, Interchemie Co., Netherland) at levels of 0, 200, 400 and 600 mg/kg diet, respectively. The composition of experimental diet and its calculated analysis were presented in table (1). Data for final body weight (BWT) and feed intake (FI) were recorded. Feed conversion ratio (FCR) and body weight gain (BWG) were calculated.

Sample collection

At the end of the experiment, three blood samples from each replicate (n=9) were collected for analysis of some biochemical parameters, separation of the serum was done using centrifuge adjusted at 3000 rpm for 10 minutes then stored in -20°C. The serum was used for measuring serum total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), triglycerides, glucose, total protein, albumin, globulin, serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic amino-transferase (SGOT) using commercial kits (Biodiagnostic company, Egypt). The analysis was done by using a spectrophotometer according to the manufacture instructions. The liver and heart tissues from the same slaughter birds were collected and homogenized then snap frozen in liquid nitrogen immediately and stored at -80°C.

Table 1: Composition of experimental starter, grower and finisher diets (gm/kg diet) and calculated chemical analysis of the basal diet

Ingredients	Diet		
	Starter	Grower	Finisher
Yellow corn	542	558.8	606
Soybean meal (44%)	319	281	253.3
Corn gluten meal (60%)	71	81	48.1
Vegetable oil	29.8	41	54.4
Limestone [1]	15	15	15
Monocalcium phosphate	14	14	14
Common salt	3	3	3
Mineral Premix[2]	1.5	1.5	1.5
Vitamin Premix[2]	1.5	1.5	1.5
Methionine [3]	1	1	1
Lysine [4]	1	1	1
Anti Coccidial[5]	0.2	0.2	0.2
Antimold[6]	1	1	1
Calculated Analysis			
Crude protein (CP) %	23.1	22.18	19.39
Metaboliziable Energy (ME) Kcal / kg diet [7]	3053	3160.7	3252.6
Calorie / protein ratio [8]	132.16	142.5	167.7

[1]Limestone (contain 36% calcium). Monocalcium phosphate: contain 22 % Phosphorus and 16 % Calcium. [2]Mineral and Vitamin premix produced by Heropharm and composed (per 3 kg) of vitamin A 12000000 IU, vitamin D32500000 IU, vitamin E 10000 mg, vitamin K3 2000 mg, vitamin B1 1000 mg, vitamin B2 5000 mg, vitamin B6 1500 mg, vitamin B1210 mg, niacin 30000mg, biotin 50 mg, folic acid 1000 mg, pantothenic acid 10000 mg, manganese 60000 mg, zinc 50000 mg, iron30000 mg, copper 4000 mg, iodine 300 mg, selenium 100 mg and cobalt 100 mg. [3] DL-Methionine (Produced by Evonic Co and contain 99 % methionine), [4] Lysine = lysine hydrochloride (contain 98 % Lysine). [5] Kill cox, Produced by Arabian company for pharmaceutical industries, 6 Produced by EL TOBA CO. For Premixes & Feed El-Sadat city Egypt. [7] ME calculated according to NRC (1994), [8] Calorie /protein ratio = ME Kcal /CP%

Table 2: Primers used for quantitative real-time PCR

Gene and ID	Primer Sequence (5`-3`)	References
HSP70 (EU747335)	F: CCAAGAACCAAGTGGCAATGAA R: CATACTTGCGGCCGATGAGA	(15)
HSP90 (NM_206959)	F: GAGTTTGACTGACCCGAGCA R: TCCCTATGCCGGTATCCACA	(15)
HSF1 (L06098.1)	F:CAGGGAAGCAGTTGGTTCACTACACG R: CCTTGGGTTTGGGTTGCTCAGTC	(15)
GAPDH (NM_204305)	F: GGGCACGCCATCACTATCTTC R: ACCTGCATCTGCCCATTTGA	(16)

Table 3: Effect of different dietary VC supplementation on growth performance of broiler chickens

Parameters	G1	G2	G3	G4	G5
W0 (Initial wt.)	45.64±0.48	45.64±0.54	45.63±0.52	45.61±0.56	45.69±0.46
W6 (Final wt.)	2549.82±72.73	2470.45±80.91	2643.50±73.00	2575.18±64.14	2540.00±55.58
TBG (g)	2504.18±72.27	2424.82±80.39	2597.87±72.50	2529.57±63.60	2494.31±55.14
TBG, RTG2	103.27	100	107.14	104.32	102.87
TFI (g)	4747.17	4494.32	4590.43	4604.81	4507.83
TFI, RTG2	105.63	100	102.14	102.46	100.30
FCR	1.90	1.85	1.77	1.82	1.81
FCR, RTG	102.70	100	95.68	98.38	97.84
Mortality %	3.3	6.6	3.3	0	0

TBG = Total body gain, TFI= Total Feed intake, FCR=Feed conversion ratio (Feed intake/ body gain), RTG2= Relative to group 2

Table 4: Effect dietary VC supplementation on some blood biochemical parameters of broiler chickens

Parameters	G1	G2	G3	G4	G5
Total protein (g/dL)	6.12±0.02	6.14±0.14	6.04±0.10	6.14±0.08	6.12±0.14
Albumin (g/dL)	5.23±0.08	5.27±0.02	5.23±0.05	5.24±0.02	5.27±0.02
Globulin (g/dL)	0.89±0.10	0.87±0.13	0.81±0.13	0.90±0.10	0.85±0.13
Cholesterol (mg/dL)	200.6±5.01	198.27±2.33	189.37±6.08	191.00±1.60	190.83±5.40
Triglyceride (mg/Dl)	200.43±2.42^{a}	200.70±0.64^{a}	199.73±0.65^{a}	201.07±1.85^{a}	184.53±8.48^{b}
HDL (mg/dL)	54.50±0.99^{a}	55.93±0.18^{a}	51.80±1.10^{ab}	52.87±0.94^{b}	50.80±1.38^{b}
LDL (mg/dL)	106.01±6.44	107.19±2.30	97.62±5.67	106.92±2.24	103.13±5.76
Glucose (mg/dL)	204.07±0.94^{b}	212.97±1.09^{a}	209.70±1.66^{ab}	206.93±3.90^{ab}	206.53±2.07^{ab}
SGOT (U/100 mL)	39.67±4.67a^{b}	43.67±1.76^{a}	39.33±4.67^{ab}	39.67±4.63^{ab}	32.33±1.33^{b}
SGPT(U/100 mL)	88.33±2.03	90.33±±3.18	88.671.20	88.33±2.33	88.33±2.96

Means with different letters in the same row differ significantly (P<0.05).
HDL= High density lipoprotein, LDL= Low density lipoprotein, SGOT= Serum glutamic pyruvic transaminase and SGPT = Serum glutamic oxaloacetic amino-transferase

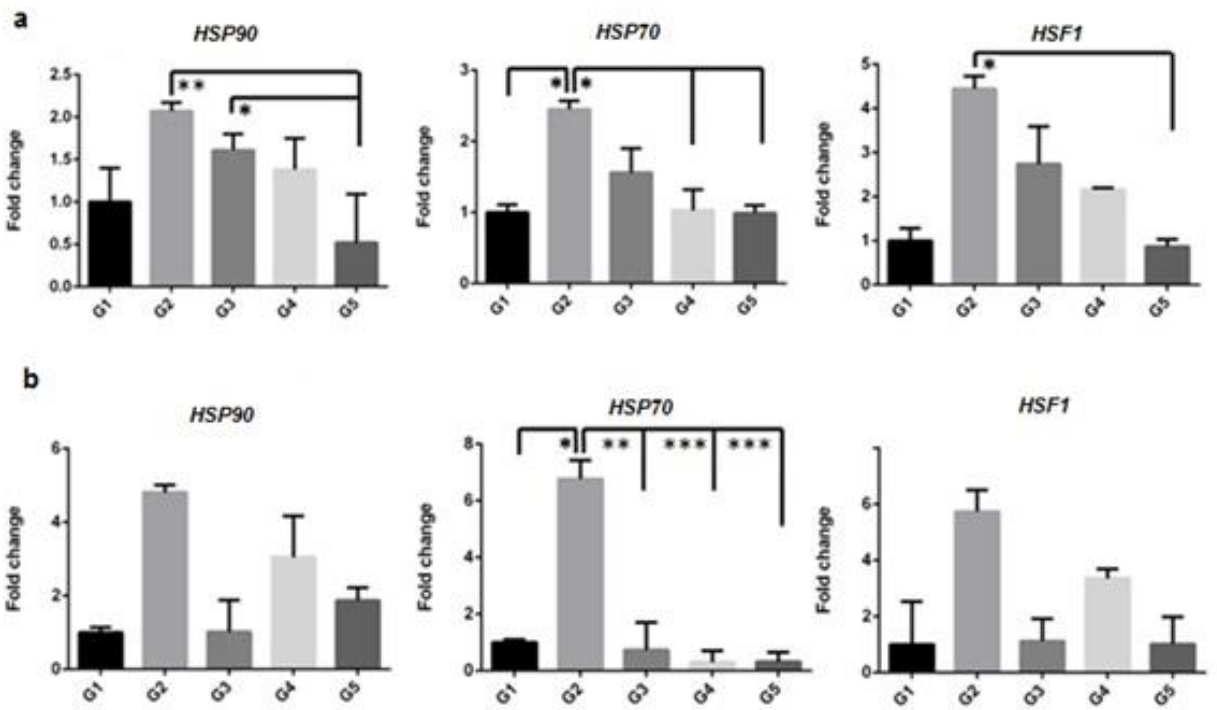

Figure 1: The mRNA expression level of heat shock proteins (*HSP90, HSP70 and HSF1*) genes in heart tissue (a) and liver (b). Asterisks on the data bars indicated when P<0.05 (*), P<0.005 (**), and P<0.0005 (***). G1 (chicks were reared in normal stocking density and fed on the basal diet), G2 (chicks were reared in high stocking density and fed on the basal diet), G3, G4, and G5 (chicks were reared in high stocking density and fed on the basal diet with VC supplementation (200, 400, 600 mg/kg diet) respectively

Total RNA isolation and cDNA synthesis

Total RNA of liver and heart tissues was isolated using the Biozol (Bioflux, Japan) according to the manufacturer instructions. The cDNA was synthesis from isolated RNA using the SensiFAST™cDNA Synthesis Kit (Bioline, United Kingdom) according to the manufacturer instructions. Briefly, 4 µl of total RNA mixed with 4 µl 5X buffer, 1 µl reverse transcriptase and 11 µl RNase\ DNase free H2O was added. The reaction was incubated at 25°C for 10 min, 42°C for 15 min (reverse transcription) and 4°C hold. The obtained synthesis cDNA was checked by glyceraldehyde 3-phosphate dehydrogenase (*GAPDH)* gene and stored at -20°C until further use.

Quantitative real-time PCR and data analysis

Quantitative Real-Time PCR (qRT-PCR) was performed for detecting the expression levels of *HSP70, HSP90* and *HSF1* genes in heart and liver tissues using specific primers for each gene (Table 2) and SensiFAST™Syber green master mix with low Rox (Bioline, United Kingdom) in the Mx3000P®System (Stratagene, USA). The amplification reaction was 20 µl consisting of 2 µl cDNA, 0.8 µl primers (50 nm), 10 µl Syber green master mix and up to 20 µl RNase\ DNase free H2O. The PCR thermal program started as 95ºC for 10 min followed by 40 cycles of 95ºC for 15 sec then 60ºC for 60 sec. The dissociation curve was carried at the end of the last cycle. The housekeeping gene (*GAPDH*) used to normalize threshold cycle value (Ct). The relative expression values were determined using comparative threshold cycle method $2^{-\Delta\Delta CT}$(14) and the results were reported as fold change differences relative to the control group (G1).

Statistical analysis

The obtained data were analyzed using the statistical analysis system (17). The significance designated at P<0.05 for the differences

among the different experimental groups for all studied parameters. The significance designated as ($P<0.05$) using one-way ANOVA between all genes in studied groups.

Results

Final BWT was improved in all groups which reared under high SD and fed diet supplemented with different levels of VC compared to G2 (Table 3). The G3 which received VC (200 mg/kg diet) showed the highest final BWT compared to G2. The G5, which received the highest level of VC (600 mg/kg) showed lower final BWT. The total body gain (TBG) of birds was improved in all studied groups supplemented with VC when compared with G2 with the highest TBG of chicks was found in G3 which subjected to high SD and supplemented with VC at 200 mg/kg diet. The lowest TBG was found in G2 that was in high SD without VC supplementation. Additionally, High SD in G2 decreased total feed intake (TFI) compared to G1. While the addition of VC in G3, G4, and G5 increased the TFI when compared with G2. The overall chick mortality percentage during the experiment was the highest in G2 (6.6 %), but it was decreased in G3 **(**3.3 %) with 200 mg/kg diet VC supplementation. Also, no mortality was observed with increased VC supplementation in G4 and G5 (400 and 600 mg/kg diet) (Table 3).

The addition of VC in G3, G4 and G5 alleviated the stress effect induced by high SD through decreasing the level of serum glucose, cholesterol, SGOT and SGPT compared with those reared in high SD without VC supplementation (Table 4).

In the present study, rearing broiler chicken in high SD leads to an alteration in the expression of *HSPs* (*HSP70* and *HSP90*), and *HSF1* in heart and liver tissue (Figure 1a and b). The expression level of *HSP70* mRNA in both heart and liver tissue revealed a significant up-regulation ($P<0.05$) in the G2 (2.35 ± 0.12 and 6.76 ± 0.66 fold) relative to the control. While *HSP70* mRNA expression in both tissues markedly lowered in chicken reared in high SD and supplemented with different concentration of VC than G2. The G2 showed an increase in expression level of *HSP90* and *HSF1* (2.07 ± 0.10 and 4.45 ± 0.28) in the heart tissue than G3, G4, and G5 supplemented with VC (200, 400 and 600 mg/kg ration, respectively). The *HSP90* expression level also showed a significant increase ($P<0.05$) in G3 than G5 (Fig. 1a). In the liver tissue, *HSP90*, and *HSF1* showed nearly similar expression pattern where they were higher in all groups especially G2 relative to the control (Fig. 1b).

Discussion

Managemental factors like SD had a significant effect on bird growth performance, health and welfare. In the present study, high SD in G2 decreased the final BWT compared with birds reared in normal SD. Similarly, Dozier et al. (18) showed that increasing the density produced some negative effects on the live performance of broilers. Addition of VC (200 mg/kg diet) to birds reared under high SD ameliorated these negative impacts on growth while failed with the higher levels of VC (600 mg / kg diet). Likewise, Elagib-Hind and Omer (19) reported that BWT was improved by the low and moderate levels (150 and 350 mg/kg) of VC compared with the higher level. Also, SabahElkheir et al. (20) observed that VC supplementation at higher doses (500 mg and 750 mg/kg) resulted in lower final weight and weight gain.

Previous studies reported that VC supplementation increased TBG (21, 22). On the other hand, others found that broiler feed intake was not affected by the VC supplementation (23, 24). The FCR was improved in all groups reared in high SD with VC supplementation (G3, G4, and G5) compared to G2, and G1. McKee and Harrison (25) also, observed an improvement in FCR of broilers as result of VC supplementation during heat stress. Decreased mortalities with VC supplementation may confirm the relationship between adding VC and its protective effect as reported by other studies (26, 27) which noticed that VC under stress and disease conditions protects the birds by improving the immune response.

Blood serum biochemical parameters are important diagnostic indicators especially under

stress conditions such as high SD. In the present study, high SD altered the activity of liver function enzymes (SGPT and SGOT), glucose, and, HDL serum concentrations (increased). The increased serum SGPT and SGOT activity in birds reared under high SD indicate that high SD might cause oxidative lesions which are in accordance with the findings of Simsek et al. (28). Dietary inclusion of VC improved these negative impacts of high SD by lowering the levels of the above-mentioned serum parameters. Similarly, Al-Darajji et al. (29) showed that plasma glucose and cholesterol concentration and plasma SGOT and SGPT activities were significantly lowered in both male and female broiler breeder reared under hot climate and supplementation with ascorbic acid. Moreover, Kucuk et al. (30) reported that supplementation with VC decreased MDA, glucose, cholesterol, and triglyceride concentrations in laying hen reared under cold stress.

The significant effect of high stocking density on *HSP70* and α1-acid glycoprotein (AGP) indicated that it was physiologically stressful condition to broiler chickens (31). Furthermore, Beloor et al. (32) reported that the expression of *HSP70* mRNA could be proper biomarkers to evaluate the stress induced by increased SD. As, *HSPs* help the stressed cells to manage the stressors, especially those affecting the protein machinery (33). Similarly, the previous study in other species (rainbow trout and sea bass) showed that *HSP70* expression level elevated in high SD (34, 35). Furthermore, Higher levels of *HSP70* gene in different tissues of birds after exposure to environmental stressors is important in the acquisition of stress tolerance (36, 37).

As other study demonstrated that supplementation of VC plays an important role in the prevention of the heat stress in poultry and improvement of their performance (38). Moreover, there is a significant ($P<0.05$) difference in *HSP70* mRNA expression in heart tissue between G2 versus G4 and G5 which fed on basal diet with 400 and 600 mg/kg ration VC supplementation. Also, a highly significant difference ($P<0.0005$) between G2 versus G4 and G5 and ($P<0.005$) between G2 versus G3 in the liver tissue were observed (Fig.1b). Similarly, Mahmoud et al. (39) demonstrated that chickens fed a diet supplemented with VC and exposed to cyclic high temperatures showed a significant decreased the expression level of *HSP70* compared with control chickens. Furthermore, Jang et al. (40) reported that the mRNA expression of *HSP70* in the liver of birds fed a diet containing VC significantly decreased compared with those birds fed basal diet under summer diurnal heat stress. In quail, *HSP70* expression in ovary and brain was decreased as the dietary VC or vitamin E supplementation increased in stressed groups (41). Hence, we suggest that VC supplementation act as an antioxidant and helps to prevent the growth of free radicals, which damage cells and subsequently reduced expression of *HSPs* in chicken reared under high SD.

As the *HSF1* is a master regulator of the heat shock genes, through activating the *HSPs* transcription by binding to heat shock element in the upstream promoter region of *HSP* genes (42). It has the ability to mediate up-regulation of *HSP70* and *HSP90* which act a critical role in survivability providing to the organisms subjected to stress (43, 44). In both heart and liver tissue in the present study, the *HSP1* expression showed more increased in G2 whish reared under high SD than G3, G4 and G5 relative to control. Its expression showed the same pattern of *HSP70* and *HSP90* expression in heart tissue and *HSP90* expression in liver tissue. Moreover, Beloor et al. (32) demonstrated that the expression levels of *HSP90* in the liver samples were higher in high density group compared with the low and standard groups but, didn't showed significant differences. The current study also showed that stress-induced due to high stocking density increases the expression of *HSP70* and *HSP90*, which play essential protective roles in maintaining the metabolic and structural integrity of the cells and organs against stress-induced injury (45-47).

Conclusions

Increasing the stocking density from 11.6 to 15.6 birds/m^2 caused stress in broiler chicks

which tended to reduce their performance, increase mortality rate, affect liver function enzymes (SGPT and SGOT) and change the expression level of heat shock protein genes. Supplementation of VC especially at 200 mg/kg chick's diet may offer a suitable nutritional strategy to overcome the disadvantageous effects of increased stocking density.

Conflicts of Interest

The authors declare no conflicts of interest

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Original Research Article

EFFECTS OF FEEDER SHAPE ON BEHAVIORAL PATTERNS, PERFORMANCE AND EGG QUALITY TRAITS OF JAPANESE QUAIL

Hesham H. Mohammed[1*], Ibrahim F. Rehan[2], Ahmed F. Abou-Elnaga[3], Radi A. Mohamed[4]

[1]Department of Veterinary Public Health, Faculty of Veterinary Medicine, Zagazig University, 44511, Egypt. [2]Department of Animal Behavior and Husbandry, Faculty of Veterinary Medicine, South Valley University, 83523, Egypt, [3]Department of Husbandry and Development of Animal Wealth, Faculty of Veterinary Medicine, Mansoura University, 35516, Egypt. [4]Department of Aquaculture (Animal and Fish Behavior and Welfare), Faculty of Aquatic Sciences and Fisheries, University of Kafrelsheikh, 33511, Egypt

*Corresponding author, E-mail: heshamvet_hosny@yahoo.com

Abstract: Offering the food to Japanese quails may help to reorganize their behavioral needs. However, limited data was reported on the effect of feeder shape on birds' behaviors, welfare, and productivity. Herein, the main objective was to assess the effects of the most available feeder shapes in the Egyptian market on the birds' behaviors. The shape of feeders were longitudinal, vertical-narrow, vertical-wide and round. All the used feeders were made from plastic, marked with a grey color and enriched with a net. A total of 180 one-day old Japanese quail (*Coturnix japonica*) were divided into four experimental groups (three replicates of each). Behavioral patterns of birds were investigated by direct observation and video recording for four days per week using scan sampling technique. The results indicated that there were feeder shape-dependent-differences in quail behaviors demonstrating the highest significant (P<0.001) levels of activity behaviors in a round feeder compared to other groups. Moreover, the highest significant (P<0.001) levels of resting behaviors within a vertical-wide feeder group. Likewise, flying behavior was recorded in the above mentioned group. However, the fear associated responses and alertness were the highest significant level (P<0.001 each) in longitudinal and vertical-wide feeders. Noticeably, the aggressive pecking was the highest significant (P<0.001) levels in birds dealt with a longitudinal feeder. Meanwhile, almost of a quail's egg quality traits demonstrated the highest significant values (P<0.001) during offering round containers for the birds. The results therefore suggested that quails might have a preference to deal more with a round feeder shape than other shapes. These findings may also have great implications to researchers, veterinarians and stakeholders to decide the most economic feeder shape of quails for greater performance and productivity.

Key words: feeder shape; welfare; egg quality; quail

Introduction

Today, the poultry industry is the most popular and enriched sources of animal protein in the form of eggs. Therefore, poultry welfare is

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essential to be deeply understood. Welfare of poultry is mainly regulated by basic principles, among which feeding plays as the most important input for intensive poultry production and has superseded effect on the financial viability of the production cycle (1). Previous and new studies have been done to maximize the feeding efficiency of chickens in areas like ingredients' selection and feed processing methods (2), the effect of feed particle size on flock performance (3), and gut development (4). However, a little data informed the impact and efficiency of different feeder shapes on birds' feeding behavior and/or productivity.

Hence, Japanese quails were inexpensive to keep and had a high immunity against common poultry diseases, we should pay attention to the tool/feeder by which offered the food to realize the birds' behavioral needs toward welfare and productivity (5). Feeding of quail has been studied in several aspects, even though few have compared the different quail feeder design in a collective study. It was reported that design way, such as size, place, geometry, spacing and/or angle of feeders can change the behavior of animals (6). Moreover, nets or partition grids covering feed trays were widely used in the poultry farms to promote a better collection of the birds around the feeders and to reduce feed competition, fighting and wastage (7). The feeder body might be in the form of a channel having a substantially C-, V- or U-shape in cross-section and comprising a passageway extending between the first and the second openings (8). Moreover, the same author stated that the body of animal feeding device may have any suitable cross-sectional shape like circular, triangular, square or oval. Likewise, the most available feeder shapes are linear, vertical and round shape in the Egyptian market. It was given by the fact that the body of the animal/bird feeding device may be designed of any size, type, shape or configuration, and it will be understood that the size of the body will be linked to the animal with which the animal feeding device is intended to be used. Therefore, the patterns of brain activation might be regulated by particular neuropeptides and/or gene expressions like immediate-early gene toward the targeted preferences (9).

Egg weight, shell weight, shell thickness, weight of albumen and yolk are the most important traits affecting egg quality under a good managemental condition (e.g., feeder shape) and fertility (10). Positive correlations among egg weight, shell weight and shell thickness have also been studied (11). However, poor egg quality results substantial economic losses to the worldwide egg industry (12). Together, it seems likely that the feeder shapes of quails may help to understand their behavioral needs towards the well-being and egg productivity. Therefore, the current study aims to check the influences of four different feeder shapes (longitudinal, vertical-narrow, vertical-wide and round) in relation to the quails' behaviour, performance and egg quality traits. Our finding may give a merit to decide the most attractive feeder shape of quail for economic purposes in quail farms.

Material and methods

This experiment was done after the approval by Ethics and Animal welfare committee of the poultry Research Unit, Faculty of Veterinary Medicine, Zagazig University, Egypt (ANWD-206).

Birds and housing

A total of 180 one-day-old Japanese quail chicks (*Coturnix japonica*), weight 7.43 ± 0.28 g purchased in one batch from Faculty of Agriculture, Zagazig University. The chicks were divided into four groups (45 quails/each) and each group was divided into three replicates (each 15 birds/0.563m^2) kept in brooder house in the same home pen. The experimental groups were categorized according to feeder's shapes (longitudinal, vertical-narrow, vertical-wide and round), as shown in Figure (1). All the used feeders were made of plastic type, marked with a red color and enriched with net to avoid the food wastage. Quail chicks were provided with 35°C ambient temperature during the 1st week of brooding and then gradually decreased by about 3.5°C/week till chicks were entirely feathered at 3-4 weeks. Each pen supplied with

10 cm thickness of sawdust (deep litter system) and hydrated lime was firstly sprinkled before spreading the well dried new litters. Available area per quail was 0.038m^2/bird. Quails were supplied with food and water as *ad-libitum* source in a synchronization program twice daily, 7 am and 5 pm, throughout the study. The basic commercial quail's starter diet through the rearing phase contained 24% crude protein and 12.45 MJ/kg metabolizable energy (13). Laying hens in all groups were fed commercial feed mixtures (Table 1), which was formulated to give the typical nutrient requirements (14) of quail contained 20% crude protein, 2.5% calcium and 11.93 MJ/kg metabolizable energy. The lighting system was 16 h of light for the period of rearing and subsequently from the six weeks of age altered to 14 h light with 10 h dark until the end of the study. The group housed quails were marked on their back by using a permanent marker pen to permit individual identification and these marks were refreshed every week throughout the experimental period.

Behavioral observation

Quails behavior was recorded by direct observation through using scan sampling technique (15), where each group was observed 3 times daily (20 min each) for 4 days weekly for the duration of the rearing period at 7 am, 12 pm and 4 pm for reporting the different behavioral patterns (see Table 2). The same individual recorded the behavioral patterns in all experimental groups through standing directly in front of each group and waiting 10 min prior the recording data to avoid any disturbance in the behaviors and to minimize the error factors (16). After scanning, the numbers of quails were counted in the observed pens to calculate the frequencies of behaviors per 1 h. These numbers were important to be used in recording the activities of quails in all treated groups (17).

Growth performance

Growth performance was recorded previously (5), where the quails were weighed on 1st day of age as one-day-old live weight and then live body weights was subsequently estimated weekly until the 4th week of age using digital balance (Sartorius 1202 MP balance, GmbH, Gottingen, Germany). Also, the average feed intake was recorded daily after calculating the feed residues. Body weight gain and feed conversion ratio were also calculated.

Egg quality traits

60 quail's eggs (from the 7th until the 10th weeks of age) were collected (15 eggs from each group). The Sartorius 1202 MP balance measured the weight (g) of whole egg, albumen, yolk and egg shell weight, while electronic digital caliper was used for calculating the whole egg length, its width and shell thickness (mm) (18-21).

Data analyses

Data were tested for distribution normality, linearity and homogeneity of variance. Data were analyzed using SAS statistical system, Package v9.2, version (22). Data were reported as means ± SEM, compared by one-way ANOVA and the Duncan's multiple range tests was used as a post hoc test. The behavioral variables did not meet the requirements of parametric tests even after transformation, therefore the equivalent non-parametric Kruskal–Wallis test was used to compare between different groups. While, the significant differences among groups were estimated by Mann-Whitney test.

Results

Effect of feeder shape on behavioral patterns

The results showed that there was an effect of feeder shapes on the behavioral patterns and performance of quails (Table 3). The highest levels of eating behavior were recorded in the group dealt with a round feeder compared to the other treatment groups. Moreover, the highest levels of activity (e.g., walking, preening, body shaking, dust pecking, leg stretch and wing stretch) were observed in the same above group (P<0.001). Furthermore, the highest level of resting period (e.g., sitting, dust-bathing and sleeping) was recorded in the group dealt with a vertical-wide feeder (P<0.001). However, the

highest fear response (e.g., stand idle and elimination) were found in the group dealt with a longitudinal feeder ($P<0.001$, $P<0.001$) and the quail vices (e.g., feather pecking, loudly sounds) were the highest recoding in the same feeder shape, with a significant level $P<0.001$ each. Meanwhile, alertness (e.g., drinking, standing) were the highest observations in the group dealt with a vertical-narrow feeder, $P\leq0.001$ each, compared to the other treatment groups.

Effect of feeder shape on bird's performance

There were significant effects of the different feeder shape on quail performance (Table 4). In particular, quail group dealt with a vertical-wide feeder in the 1st and 3rd week ($P\leq0.05$) had the highest body weight in comparison with the other groups. However, the rest of weeks had no significance differences. Moreover, there was no significant difference in feed intake, weight gain and feed conversion ratio between different feeder shapes. While the longitudinal feeder showed the highest weight gain and the lowest feed conversion ratio, and vertical-narrow feeders recorded the lowest feed intake.

Effect of feeder shape on egg quality traits

There were statistical effects of feeder shapes on the external quality (egg weight, width, length, shell weight, and shell thickness) also on the internal quality (albumen and yolk weight) of quail's eggs (see Table 5). The highest levels of egg quality traits (e.g., egg weight, egg width, shell thickness and albumen weight) represented in the group dealt with a vertical-wide feeder ($P\leq0.001$) compared to the other treatment groups. Moreover, the highest level of shell weight demonstrated in the group dealt with a round feeder ($P<0.001$) compared to the other groups. However, the high levels of egg length and yolk weight shown in the group dealt with a longitudinal feeder ($P=0.004$, $P<0.001$, respectively) compared to the other groups.

Relationship between external and internal egg quality traits

There were correlation coefficients among the external and internal egg quality traits in Japanese quail dealt with the analyzed feeder shapes (Table 6). Albumin weight was positive correlated with all external traits except shell weight, which was negatively correlated with yolk weight. Furthermore, there was negative correlation between yolk weight and shell quality (shell weight and thickness), while yolk weight correlated positively with other external egg quality.

Table 1: Ingredients of the experimental diet (kg/100kg)

Ingredients	Kg	Calculated analysis	
Yellow corn	65	Metabolized energy	11.93 MJ/kg
Soybean meal (44%)	20	Crude protein	20%
Corn gluten	5.2	Calcium	2.5%
Calcium carbohydrate & phosphate	2.1		
Soybean oil	0.2		
Premix and common salts	0.7		
Other feed additives	0.6		

Table 2: Definition of recorded behaviors

Behaviors	Definition
Eat	Head extended towards available feed resources while beak in or above the drinker appears to be manipulating or ingesting feed
Drink	Beak in contact with water in or above the drinker and appears to be drinking water
Walk	Moves forward taking one or more steps
Sitting	Head rested on something (litter or another bird) while sitting
Dust bathing	Bathing the dust with the use of wings, head, neck and legs performing vertical wing-shaking
Preen	Beak related behavior that beak touches the plumage of the bird itself
Stand	The abdomen is not touching the litter and the bird is motionless with no apparent movement of legs
Idl	Standing with motionless
Sleeping	Bird's neck is fully recumbent and the eyes permanently closed while lying
Body shaking	Raise feathers and shake body
Elemination	Dropping of fecal materials
Dust pecking	Peck floor with feet usually associated with eating behavior
Leg stretching	Extending one leg at the same side of the body
Wing stretching	Extending one wing at the same side of the body
Fly	By forcing wings displacement from one place to another
Feather pecking	Birds pecks the feather of another birds
Sound	Call or vocalization given by the bird

Table 3: Means (±SEM) of some behavioral patterns of Japanese quail using different shapes of feeders (Numbers of quails/hour)

Behavioral patterns	Shapes of feeders				*P*-Values
	Longitudinal feeder	Vertical narrow feeder	Vertical wide feeder	Round feeder	
Eat	356.58±23.98	343.50±33.89	369.67±33.86	372.17±55.53	0.36
Drink	122.17±1.27 ^{b}	134.17±1.50 ^{a}	130.50±2.17 ^{a}	105.83±1.01 ^{c}	0.000
Walk	300.00±3.78 ^{c}	318.67±3.24 ^{b}	325.83±1.96 ^{ab}	328.33±2.03 ^{a}	0.000
Sit	658.67±16.14 ^{b}	654.83±14.31 ^{b}	769.75±13.57 ^{a}	716.75±12.91 ^{b}	0.000
Dust bath	15.33±1.35 ^{b}	16.92±1.12 ^{b}	28.83±0.90 ^{a}	14.08±0.70 ^{b}	0.000
Preen	277.08±8.22 ^{b}	271.00±3.42 ^{b}	268.63±5.11 ^{b}	321.00±3.43 ^{a}	0.000
Stand	332.00±1.95^{a}	336.17±2.47 ^{a}	229.25±3.26 ^{c}	275.42±5.56 ^{b}	0.000
Idle	142.08±3.38^{a}	139.09±2.06^{a}	94.33±1.69^{c}	120.25±2.98^{b}	0.000
Sleep	353.06±8.37^{c}	356.42±3.80^{c}	444.33±2.17 ^{a}	391.83±3.25 ^{b}	0.000
Body shaking	25.58±1.93^{a}	14.66±0.56^{b}	17.58±0.76^{b}	25.66±1.26 ^{a}	0.000
Elimination	3.15±0.36^{a}	2.08±0.28^{b}	0.58±0.22^{c}	.33±0.03 ^{c}0	0.000
Dust peck	49.66±3.99^{b}	35.58±1.94^{c}	44.66±1.57^{b}	59.00±0.94^{a}	0.000
Leg stretch	47.5±2.36^{b}	51.58±1.82^{b}	46.92±1.31^{b}	60.66±2.15 ^{a}	0.000
Wing stretch	76.08±2.06^{b}	74.00±1.47^{b}	68.00±2.23^{c}	103.17±1.73 ^{a}	0.000
Fly	15.50±0.64^{c}	22.16±0,71^{b}	26.5±0.95^{a}	21.5±0.87^{b}	0.000
Feather peck	4.25±0.52	4.00±0.36	3.66±0.33	4.08±0.35	0.77
Sound	3.25±0.56	2.58±0.41	2.00±0.42	2.66±0.48	0.33

^{abc} Means within the same row having different superscripts are significantly different at $P \leq 0.05$

Table 4: Means (± SEM) of growth performance of Japanese quail using different shapes of feeders

Growth performance	Shape of feeders				*P*-Values
	Longitudinal feeder	Vertical narrow feeder	Vertical wide feeder	Round feeder	
Initial body weight (g)	6.76±0.57	7.37±0.32	7.27±0.66	8.32±0.56	0.25
Body weight in 1st week (g)	55.5±2.38^{ab}	54.41±2.08^{ab}	61.58±2.76^{a}	50.16±2.70^{b}	0.02
Body weight in 2nd week (g)	102.68±3.54	96.23±4.41	104.89±3.57	108.19±1.82	0.11
Body weight in 3rd week (g)	138.34±5.06^{ab}	135.72±4.36^{ab}	148.01±4.25^{a}	129.84±4.21^{b}	0.05
Body weight in 4th week (g)	192.20±4.93	186.06±6.92	185.98±5.19	180.11±5.49	0.52
Feed intake (g)	15.58±0.72 a	15.11±0.64 a	15.37±0.65 a	15.91±0.53 b	0.83
Weight gain (g)	185.40±5.00	178.70±6.81	178.70±5.60	171.80±5.50	0.43
Feed conversion ratio	2.38±0.15	2.39±0.13	2.44±0.17	2.62±0.13	0.60

^{ab} Means within the same row having different superscripts are significantly different at $P \leq 0.05$. g= gram

Table 5: Means (± SEM) of egg quality traits of Japanese quail using different shapes of feeders

Egg quality	Shape of feeders				*P* Values
	Longitudinal feeder	Vertical narrow feeder	Vertical wide feeder	Round feeder	
Egg weight (g)	11.48±0.18^{a}	10.59±0.23^{b}	11.61±0.15^{a}	10.36±0.16^{b}	0.000
Egg width (mm)	25.53±0.41	24.79±0.22	26.01±0.26	25.26±0.49	0.128
Egg length (mm)	32.20±0.66^{a}	30.64±0.46^{b}	32.09±0.26^{a}	30.06±0.43^{b}	0.004
Shell weight (g)	1.03±0.02^{c}	1.41±0.04^{b}	1.58±0.03^{a}	1.60±0.05^{a}	0.000
Shell thickness (mm)	0.21±0.007^{c}	0.26±0.017^{ab}	0.27±0.004^{a}	0.23±0.009^{bc}	0.001
Albumin weight (g)	6.12±0.07^{a}	5.82±0.16^{a}	6.24±0.12^{a}	5.12±0.22^{b}	0.000
Yolk weight (g)	4.33±0.16^{a}	3.42±0.15^{b}	3.78±0.09^{b}	3.64±0.12^{b}	0.000

^{abc} Means within the same row having different superscripts are significantly different at $P \leq 0.05$.
g= gram mm= millimeter

Table 6: Correlation coefficients among the external and internal egg quality traits in Japanese quails

Egg quality traits	Egg weight (g)	Egg width (mm)	Egg length (mm)	Shell weight (g)	Shell thickness (mm)
Albumin weight (g)	0.786***	0.312*	0.449***	-0.242	0.296*
Yolk weight (g)	0.610***	0.333***	0.375***	-0.362***	-0.291*

The asterisk (*) showed a significance level, $P \leq 0.05$ and the asterisk (***) showed a significance level, $P \leq 0.001$.
G= gram mm=millimeter

Figure 1: Different shape of feeders from left to right longitudinal feeder, vertical-narrow feeder, vertical wide feeder and round feeder.

Discussion

The behavioral patterns reflect a serious of the activities of endocrine and exocrine character for assessing the animal's response to its environment that consequently impairs its welfare and production (23). Herein, for the first time, we examine the influence of feeder shape on the CNS stimuli of quail hens to be targeted on their behavioral responses and production.

As a result shown in Table 3, the eating behavior had no significant difference among all groups. It means that the feeder shapes have no

clear influences on feed intake of quails. Meanwhile, the quail hens have been dealt with round shape feeders were the best group to perform several behaviors other than eating, such as, activity behaviors (e.g., walking, preening, leg/wing stretching, pecking and body shaking) and resting behaviors (e.g., sitting, sleeping and dust-bathing). All these behaviors are considered to be 'natural behaviors', and good indicators of welfare of birds (24). However, the quail hens have been dealt with longitudinal/vertical-narrow shape feeders were the groups clearly performed the fear associated responses (e.g., elimination and idle) as well as alertness behaviors (e.g., drinking and standing). By which, these behaviors are considered poor indicators of welfare of birds. Moreover, it's well-known that quails/birds can stay rested up to 70–80% of their time (25). It can be understood that the birds using a vertical-wide feeder somehow felt more comfortable by staying/resting nearby this feeder shape than others.

Changing of the management condition in quail farm might affect the performance of the birds. The result in Table 4 showed that quails dealt with a vertical-wide feeder showed significantly the best body weight, particularly in the 1st and 3rd week of age comparable to the other groups. In spite of the non-significant effects of different feeder shapes on quails' weight in the 4th week, quail dealt with a round feeder showed the best body weight and feed intake compared to the other groups. These results agreed with two other researchers who stated that some management condition can affect the growth performance of quails (26, 27), such as body weight (28) and feed consumption (29). Our results indicated that the vertical-wide and round feeder shapes can positively affect the performance of quails and it can show the good managerial conditions applied in our experiment. This preference of these feeder shapes towards the increasing the activities and performance of quails might relate to the centrally induced appetite by neuropeptide Y (30). Moreover, it is probably regulated by the high genetic expression of immediate-early gene Zenk in Pallial brain structure (9).

In this study, the average values of external quality traits of quail's eggs (e.g., egg weight, its width, its length & shell weight and its thickness) in Table 5, indicated similarities with the finding of most researchers (11, 31-35). Likewise, the average of internal quality traits of quail's eggs (e.g., albumen weight and yolk weight) indicated similarities by the finding of above researchers. It seems likely that the slight differences between the results of egg quality traits of this research and the results of the other researchers might have resulted from the genetic structure, health condition, flock age, use of different content diet in feeding, and the differences in the care and management condition of the quails (e.g., feeder shape). We reached the point that offering feeder shapes might change the behavioral patterns of quails and remain an important question whether feeder shape can affect the egg quality traits. In this study (Table 6), statistically significant correlation (M±SEM) was obtained among the average of egg weight, its width, shell weight, and shell thickness, especially in the group dealt with a vertical-wide feeder compared to the other treatment groups. Therefore, the egg weight had an indirect relation with the shell quality of the egg. It has been stated by most of the researchers reported that the shell thickness had a direct relation to egg weight (36, 37), also had a positive correlation to the shell weight (36, 38). It's well-known that shell ratio in the total egg had an opposite relation to the egg weight. It comes from the fact that the increase depending on the egg weight on the shell weight and the shell thickness was less than the increase of other components that formed the egg (35). However, by offering a vertical-wide shape and a round shape feeder to quail hens, the shell weight (1.58±0.03, 1.60±0.05, respectively) and shell thickness (0.27±0.004, 0.23±0.009, respectively) have not been clearly affected by the egg weight. Therefore, the rounded feeders have the best economic value among the other groups. It means that the egg shell quality would be evaluated by using the egg weight values due to the positive relation determined between the egg weight and the shell thickness, and the shell weight. In this study, statistically a significant

negative correlation was present between the albumen and yolk weight of the egg particularly in the group dealt with a vertical-wide shape feeder (6.24±0.12, 3.78±0.09, respectively) and the other group dealt with a round shape feeder (5.12±0.22, 3.64±0.12, respectively). It was reported that the housing system can affect egg quality and concentration of cholesterol in egg yolk in hens (39), Thusly, increasing the yolk weight in the whole egg is not preferable for human health and marketing. In this study, the group dealt with a longitudinal feeder had the significant highest yolk weight (4.33±0.16) than the other groups. However, the improvement of the albumen weight indicated the dense albumen quality to perfectly estimate the internal egg quality traits (35). In our study, almost all internal quality traits of the egg were significantly influenced based on the influence occurred in the egg weight with respect to the external quality traits. However, the yolk weight and egg length were negatively correlated to the albumen weight. This case was found to be in conformity with the findings of some researchers (40). The results were in contrast with the results mentioned that there were positive significant differences among egg weight and egg length (41).

Our finding has evidence that the quails had a more prefer to deal with rounded feeder than longitudinal and vertical ones indicating good behaviors and welfare. It has great implications for researchers, veterinarians and stockholders to decide the most economic feeder shape of quails for the maximum performance and productivity.

Conclusion

The different feeder shapes influenced the quails' behavior, welfare and productivity. Herein, the birds have demonstrated significantly the highest behavioral patterns when offering round shape feeder. However, the significant lowest activities have been detected when offering longitudinal/vertical shape feeder. It seems likely that the birds were familiarities to the round shape feeder. The quails' performance and egg quality traits had significant effects due to changes in shapes of feeder types. Therefore, for maintaining a successful and profitable quail farming business, the round feeder container as a recommended.

Contributions

Hesham H. Mohammed and Ibrahim F. Rehan are mutually contributed to this study, designed the survey protocol, supervised data collection procedures and drafted the final version of the manuscript; Ahmed F. Abou-Elnaga and Radi A. Mohamed analyzed the data and shared in experimental protocol. All authors have finalized the experimental design and revised the manuscript and then contributed to, edited, and approved the final manuscript as submitted.

Competing financial interests

The authors declare that they have no competing financial interests and non-financial interests.

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Original Research Article

EFFECT OF BEDDING MATERIALS ON DUCK'S WELFARE AND GROWTH PERFORMANCE

Hesham H. Mohammed[1]*, Asmaa I. Abdelaty[1], Al-Sadik Y. Saleem[1], Mohamed I. Youssef[1], Shereen EL. Abdel-Hamid[1]

[1]Department of Veterinary Public Health, Faculty of Veterinary Medicine, Zagazig University, 44511, Egypt

*Corresponding author, E-mail: heshamvet_hosny@yahoo.com

Abstract: Ducks spend their entire life in contact with the litter material, thus the management strategies should focus on what is the best for ducks welfare and growth performance. Herein, the main object of the present study was assessing the effects of different litter materials on the ducks' welfare and growth performance. Four groups of ducks (24 duckling for each) were kept on four types of bedding materials, sawdust, plastic slatted floor, sand and without. The present study revealed several changes in duck's behaviors due to different bedding materials, where the most frequencies of maintenance behaviors (feeding, drinking, idling, preening) were significantly ($P<0.05$) higher in plastic slatted in comparing to other materials the highest significantly in plastic slatted litter and the lowest in non-bedding litter. Moreover, the ducks reared under sawdust floor revealed the highest frequency of foraging behavior (11.48 bouts/hour). While, feather pecking and aggression were the highest in non-bedding material (74.4, 5.51 bouts/hour, respectively). The hygienic conditions inside the non-bedding floor was the worst due to increase levels of ammonia and carbon dioxide (12, 0.97 Cm^3/Litter, respectively). The ducks reared in plastic slatted floor were the best in growth performance with good signs of soundness. In non-bedding floor, the environmental stress revealed in the increase the level of plasma corticosterone hormone with bad signs of soundness. This study confirms importance of bedding materials in rearing of ducks, especially under plastic slatted floor.

Key words: duck; bedding material; behavior; performance; welfare

Introduction

Poultry has an important role amongst agricultural industries in many countries. Poultry sector in Egypt is one of the major sources of animal protein supply (1). Duck production is an aspect of the poultry industry, which is very popular in many parts of the world. Ducks represent the second largest poultry production in Africa after chicken, beside pure Egyptian breeds there were some local developed strains that established for both meat and egg production (2). Duck used for meat production, which may partly compensate the increasing demand for animal protein, where duck meat is highly appreciated as it combines the characteristics of a red meat and the dietetic characteristics of poultry meat (3). From an economic standpoint, management strategies should focus on what is

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the best for poultry welfare and growth performance (4). To increase the income from ducks rearing, producers are capable of modulating the management of ducks. The well-being of poultry and stress largely influence the poultry production (5). There are many factors which can decrease the performance and increase abnormal behavior of poultry such as management and housing (6). Deep-litter floor housing is most common when raising birds used for meat production (7).In this system, better litter management is crucial for providing good litter quality and for controlling the ammonia level inside the poultry houses. Distinct mixtures of different materials have been proposed as bedding for poultry (8). Duck litter is a mixture of duck excreta, spilled feed, spontaneously fallen feathers and farm bedding material (9). Wood sawdust is the most common used bedding material, however, there were many alternative materials that may be used such as rice and wheat straw (10), soya bean straw (11) and other dry, absorbent, low-cost organic materials. Moreover, the sand is occasionally used as a bedding material (12). The ducks spend their entire life in contact with the litter material. Therefore, its quality is considered a crucial factor of poultry welfare (13), where the good litter should be characterized by good absorption property with a reasonable drying time (14), fast drought, low price and acceptable as a fertilizer. Litter quality may play an important role in the activity levels of broilers through encouraging normal behaviors that require energetic movements such as leg exercise (walking, foraging and dusting bathing) (15, 16). Moreover, behavior is a part of an animal's interaction with its environment. Poor litter quality is considered a welfare problem in modern poultry production. For that reason, the objective of this study was to assess the effect of different bedding material on duck welfare.

Material and methods

This experiment was done after the approval by Ethics and Animal welfare committee of the poultry Research Unit, Faculty of Veterinary Medicine, Zagazig University, Egypt (ANWD-206).

Experimental animals and management

A total of ninety six one day old of Moulard duckling was collected on one batch from Faculty of Agriculture, Zagazig University. The ducks were divided randomly after one week of the brooding period into four groups (each of 24 ducklings) as according to bedding materials (17) into saw dust, plastic slatted, sand and without floor. Each group was subdivided into three replicates (each 8 birds) following identifying with wing rings and kept in brooder house in the same home pen.

Each group was kept in a pen with a floor area of 3.5 m length X 3 m width X 3 m height. Each pen had provided with one incandescent lamp of 100 watts at height 2.5 m above the level of ducks. During the experimental period, ducks were provided with full light for 1st week, then decrease gradually until reach 8h /day. Newly hatched duckling should have a proper brooding temperature, where it was measured by thermometer at the level of a bird's back and maintained at about 32-34 °C in 1st week, then decreased 3-5 °C per week until it reached 19-20 °C at 4 weeks (fully feathered ducks) (18).

Ducks were provided with *ad libitum* basic commercial duck's starter diet throughout the rearing phase that contained 22% crude protein until 5 weeks old. Then, they were fed on a grower diet with 18% of crude protein until the end of experiment (19).

Behavioral observation

It was conducted in the home pen to record different behavior for 5hrs weekly from 6 am to 6 pm by focal sample technique. Observation was done by one person standing directly in front of each group and waiting 10 minutes for acclimation of ducks (6). An observation sheet, a stop watch and photographing camera were used during the observation time for recording the behavioral pattern (20, 21). After observation, the total frequencies of normal and abnormal behavior were counted and calculated, as mentioned in Table (1) in all bedding materials.

Other welfare indicator

At the end of the study, the ducks were captured and measured the physical condition (22).

The condition of eye, nostril, feather, foot and gait was scored as the welfare indicators. The other welfare score was ranged from zero to two, where zero was the best and one was the worst.

Growth performance parameters

It was recorded previously (23), where, initial body weight (IBW) of ducks was weighed at the beginning of experiment (2^{nd} weeks age) and weekly until 10^{th} weeks age, also feed residues and thus average feed intake (FI) were recorded weekly. Average body weight gain (ABWG) was calculated by subtracting body weight between two successive weeks. Relative growth rate (RGR) was calculated by ABWG/ (initial BW+final BW) *0.5. Furthermore, feed conversion ratio (FCR) was calculated (feed intake/weight gain) over a period of experiment.

Blood sampling and cortisol level

At the end of the experimental period, blood samples had been collected randomly from 15 ducks/group, at morning to overcome the circadian variation in hormone level (23). Blood samples were obtained from wing vein into heparinized tubes, centrifuged at 4000 rpm for 15 min to obtain blood plasma, which stored on -80°C for evaluating the cortisol level, as one of stress indicating hormones (24).

Air quality hygiene

Carbon dioxide and total ammonia levels in air of each group were detected (25) to determine the hygienic level inside all experimental groups.

Statistical analysis

All statistical procedures were performed using the SAS statistical system Package V9.2 (26). One-factorial analysis of variance (ANOVA) was performed. The analysis of data distribution suggested that all traits analyzed followed a normal distribution ($P>0.05$). Pearson correlations were performed to compute the relationship of the abnormal behavior and performance parameters.

Results

The results as shown in Table (2) revealed a significant differences in the most of the duck's behaviors, where the most of normal behaviors were the highest in plastic slatted and wood shaving, respectively. While, the abnormal behaviors (feather pecking and aggression) were significantly higher ($P<0.05$) in the non- bedding floor than other groups. Likewise, the growth parameters in Table (3) had notable differences among experimental groups with significant differences, where final body weight, body weight gain, growth rate were higher in plastic slatted floor than other groups. The hygienic conditions inside the non-bedding floor was the worst due to increase levels of ammonia and carbon dioxide, as shown in Table (4). The results in Table (5) showed that ducks reared in non-bedding floor bedding materials had the worst signs of health with significant increase in the level of plasma corticosterone hormone.

Table 1: Definition of recorded behaviors

Observed behavior	Definition
Eating	Number of eating bouts on the troughs
Drinking	Number of eating bouts on the drinkers
Foraging	Number of pecking and scratching on ground, floor or other parts of pen
Idling	Standing not engaged in any activity
Activity	Either walk or run
Laying	Laying or sitting on the floor
Feather preening	Clean and care about their plumage with their peak using short and repeated action while standing or sitting
Feather pecking	Number of pecks at the feathered parts
Aggression	The ducks make hostile acts toward other birds

Table 2: Impact of different bedding material on behavior (mean±SE) of ducks

Behavioral patterns	Different bedding material			
	wood shaving	Plastic slatted	Sand	No bedding
Feeding frequency /hour	10.1±0.54 [ab]	11.97±0.99 [a]	8.74±0.79 [b]	8.37±0.45 [b]
Drinking frequency/hour	14.4±4.80[b]	29.31±6.37 [a]	9±1.58[c]	5.41±.98 [c]
Foraging frequency/hour	11.48 ±0.35 [a]	2.45±0.75 [c]	4.80±0.97 [b]	1.54±0.75 [c]
Idling frequency/hour	20.54±0.71 [a]	23.22±1.57 [a]	20.45±0.81 [a]	17±1.27 [b]
laying frequency/hour	7.62±2.39	8.60±2.48	6.97±1.18	6.42±2.18
Activity frequency/hour	23.37±1.80	19.71±1.81	23.02±1.79	20.62±1.28
Feather preening frequency/hour	11.87±2.25 [a]	12.20±1.16 [a]	10.62±0.96 [ab]	8.17±0.61 [b]
Feather pecking frequency/hour	49±13.58 [b]	21.03±2.44 [c]	56.41±19.8[b]	74.40±14.82 [a]
Aggression frequency/hour	2.60±1.14 [b]	1.98±0.91 [b]	3.68±1.73 [ab]	5.51±2.04 [a]

[abc] Means in the same row with different superscripts are significantly different at ($P < 0.05$)

Table 3: The means (±SE) of growth parameters in ducks reared under different bedding materials

Growth parameters	Different bedding material			
	wood shaving	Plastic slatted	Sand	No bedding
Initial body weight (g)	449.62±18.71	435.88±15.91	438.12±20.85	438.88±14.05
Final body weight	3175.4±74.89 [b]	3260.6±65.97 [a]	3044.3±71.17 [b]	2785.5±76.03 [c]
Total body weight gain (g)	2725.75±62.94 [ab]	2824.75±57.87 [a]	2606.18±61.91 [b]	2346.62±67.12 [c]
Weekly body weight gain (g/bird/week)	340.72±7.87 [ab]	353.09±7.23 [a]	325.77±7.74 [b]	293.33±8.39 [c]
Feed intake (g/bird/week)	968.75±78.12	964.84±73.86	955.5±71.12	861.72±68.05
Relative growth rate	1.503±0.01 [a]	1.529±0.01 [a]	1.497±0.02 [a]	1.455±0.01 [b]
Feed conversion rate	2.84±0.82	2.73±0.83	2.93±0.69	2.94±1.63

[abc] Means in the same row with different superscripts are significantly different at ($P < 0.05$). g= gram

Table 4: Levels of ammonia and carbon dioxide in air of different bedding materials groups

Environmental indicators	Different bedding material			
	wood shaving	Plastic slatted	Sand	No bedding
Total ammonia (Cm^3)	7.76±.43[b]	10±1.15 [ab]	8.40±.23 [b]	12±1.15 [a]
Carbon dioxide (Cm^3)	0.05±.005 [d]	0.13±.005 [b]	0.10±.011 [c]	0.97±.005 [a]

[abcd] Means in the same row with different superscripts are significantly different at ($P < 0.05$)

Table 5: The level of corticosterone hormone (µg/dl) and mean rank of physical condition of duck under different bedding material

	Different bedding material			
	wood shaving	Plastic slatted	Sand	No bedding
Eye	1.25	1.25	1.25	1.66
Nostril	1.16	1.16	1.33	1.66
Feather cleanliness	1.04	0.87	1.29	1.91
Feather quality	1.08	1.08	1.25	1.83
Foot pad	1.21	1.21	1.45	1.54
Gait	1.21	1.21	1.21	1.70
Corticosterone level (µg/dl)	8.475±1.90[bc]	4.82±.58 [c]	13.17±.2.52 [ab]	14.55±1.16 [a]

[ab] Means in the same row with different superscripts are significantly different at ($P < 0.05$)

Discussion

The concerning on how to manage duck's dropping under the intensive system, leading to the discovery several absorbents that were generally referred to bedding material (27). Behavioral study is considered the chief indicator to assess poultry welfare and poultry's response to the surrounding environment (17). In this study, there were several changes in duck's behaviors due to different bedding materials, as mentioned before in previous studies (17, 28-31).The bedding materials had significant effects on inestive behavior including eating,

drinking and foraging, where the frequencies of eating and drinking behaviors were significantly higher in plastic slatted in comparing to other bedding materials. While foraging bouts were the highest significance in the bedding from sawdust material. These results agreed with Mohammed et al. (17), who found significant correlation between ingestive behavior and different bedding materials. While, the present study did not agree with Karousa et al. (31), who cited that litter types had no significant effect on feeding and drinking behaviors. As mentioned before in Table (2), the frequencies of ingestive behavior (eating, drinking and foraging) were the lowest significantly in no bedding materials, which confirm the importance of bedding materials to improve the ingstive behavior. The observed differences in ingestive behavior may be due to the properties of the bedding materials affected the quality of the ration and water (30).Furthermore, Toghyani et al., (32) mentioned that there were significant difference in ingestive behavior among different bedding materials. In this study, the laying frequency was the highest in plastic slatted and the lowest in non- bedding floor, but the difference did not reach the significance. This result may be attributed to the absence of comfortable media for the resting in no bedding group. These results agree with Anisuzzaman and Chowdhury (33) and disagreed with Stub and Vestergaard (34). The ducks reared in on non-bedding material was standing less significantly without any activities in comparison to other groups. This result was agreed with Mohammed et al., (17), while was disagreed with Toghyani et al., (32), who found that standing behavior was slatted but the differences did not reach the significance. Those changes in idling and laying behaviors, as comfortable behavior (5) may be due to the variations in cleanliness, odor and other characters of different litters (35). The bouts of activity (walking and running) were higher in sawdust and sand bedding than plastic slatted and non-bedding material which may be attributed to the changes in particle size of the litter (17). The difference in duck's activity in the present study was supported by Oliveira and Carvalho (36), who cited that the locomotion of birds was affected by different type of litter. The activity in plastic slatted was the lowest which may be due to the negative correlation between activity and the rate of food consumption (37). Regarding to feather preening, the ducks reared under plastic slatted floor and sawdust were more performance of feather preening with significant difference in comparing to other groups. This result may be due to the ducks more comfort on these floors. This result was agreed with Mohammed et al. (17), and disagreed with Waitt et al. (38) and Rice et al. (39), who found that the floor types hadno significance difference on preening behaviour of ducks. Sameh et al., (40) did not record any significant differences in preening behavior among different litter materials. Moreover, the abnormal behaviors representing in feather pecking and aggressive were significantly affected by the bedding, where it was the highest in non-bedding material and the lowest in plastic slatted floor. These results agreed with Mohammed et al. (17) and disagreed with Sameh et al. (40). Likewise, the bedding materials had significant effects on the most parameters of growth performance, where the plastic slatted was the highest and non-bedding group was the lowest. The improvement in growth performance in a plastic slatted litter may be attributed to the increase of all maintenance behaviors in this litter. The ducks reared in plastic slatted group revealed a significant increase in final body weight, total body weight gain, weekly body weight gain and growth rate by comparing to other experimental groups. These results were comparable to other studies, who mention that litter materials had a significant effect in growth parameters (29, 32, 41). While, other researchers did not find a significant effect of bedding materials on growth performance (17). The feed consumption and feed conversion ratio did not reveal significant differences among the experimental group. Likewise, Davis et al., (42) and Mendes at el., (43) stated that different litters did not influence growth performance. While, other previous studies mention that bedding materials has a significant effect on feed consumption (44) and

feed conversion ratio (45). The date as mentioned before in Table (4) revealed the hygienic conditions in the study, where the levels of ammonia and carbon dioxide were significantly higher in non-bedding litter in compare to others. These results may attributed to accumulation of fecal matter and increase the humidity in non-bedding litter (46). These results go hand by hand with results obtained by Tasistro et al. (47), who stated that there was a significant interaction between bedding materials and level of ammonia. Also, Almeida et al. (28) found that the use of perforated plastic flooring can improve the air quality (less CO2 and NH3 concentration) and bird cleanliness. Nevertheless, Fraley et al. (48) mentioned that there were no differences for any of the environmental data between flooring systems. Lien et al., (49) reported that the litter absorbs moisture, reduces ammonia production and finally may affect the total body weight gain. The signs of soundness in eye, nostril, feather, feet and gait were better in presence of bedding materials, especially in plastic slatted and sawdust, respectively. While the non- bedding litter was the worst in the previous signs. These results may attributed to ability of bedding materials to absorb moisture and may reflect the good managerial conditions (49). These results were comparable to Buhr et al. (50); Waitt et al. (38); Fraley et al. (48); Karcher et al. (22). Garcia et al. (30) cited that litter material had no effect on the physical condition of birds.

The level of plasma corticosterone was the highest significance in the non-bedding group, while the ducks reared in plastic slatted group revealed the lowest level of plasma cortisol. This result may be due to the ducks in plastic slatted floor had good signs of soundness. This result disagreed with that obtained by Asaniyan et al (27) and Sameh and El-Khloya (51), who noted that litter depth and litter type had no significant influence on hematological values and welfare indices.

Conclusion

In the present study, there were several changes in duck's behaviors due to different bedding materials, where the most of maintenance behaviors were the highest significantly in plastic slatted litter and the lowest in the non-bedding litter. Furthermore, growth parameters were the better in plastic slatted and sawdust floor, respectively, while the lowest growth parameters were in non-bedding floor. The hygienic conditions inside the non-bedding floor was the worst due to increased levels of ammonia and carbon dioxide. The ducks reared under non-bedding floor revealed the worst signs of soundness, while it was the best in plastic slatted floor. The plasma corticosterone is a useful indicator for acute and environmental stress, which was the highest in non-bedding litter. This study confirms importance of bedding materials in rearing of ducks, especially under plastic slatted floor.

Conflict of interests

None of the authors have any conflict of interest of declare.

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Original Research Article

PESTICIDES AND TOXIC METALS RESIDUES IN MUSCLE AND LIVER TISSUES OF SHEEP, CATTLE AND DROMEDARY CAMEL IN SAUDI ARABIA

Ahmed M.A. Meligy[1,2], Abdallah Y. Al-Taher[3], Mohamed Ismail[3], Abdelmohsen A. Al-Naeem[4], Sabry M. El-Bahr[3,5]*, Waleed R. El-Ghareeb[6,7]

[1]Central laboratory, College of Veterinary Medicine, King Faisal University, Saudi Arabia, [2]Plant Protection Research Institute, Agricultural Research Center, Egypt, [3]Department of Physiology, Biochemistry and Pharmacology, College of Veterinary Medicine, King Faisal University, Saudi Arabia, [4]Department Clinical Studies, College of Veterinary Medicine, King Faisal University, Saudi Arabia, [5]Department of Biochemistry, Faculty of Veterinary Medicine, Alexandria University, Egypt, [6]Food Control Department, Faculty of Veterinary Medicine, Zagazig University, 44519, Egypt, [7]Department of Veterinary Public Health and Animal Husbandry, College of Veterinary Medicine, King Faisal University, Saudi Arabia

*Corresponding author, E-mail: sabryelbahr@hotmail.com; selbahar@kfu.edu.sa

Abstract: Data regarding determination of pesticide residues and toxic elements in edible tissues of different animals particularly in the camels in Eastern region of Saudi Arabia are scarce. Therefore, current study aimed to evaluate levels of forty-five pesticides and heavy metals (lead (Pb); cadmium (Cd); Arsenic (As)) in muscle and liver tissue samples that collected from forty-two animals; sheep (n=21), cattle (n=11) and dromedary camels (n=10) admitted to Al-Ahsa abattoir, Eastern province, Saudi Arabia. Seven of forty-five pesticides residues were detected in tissues by gas chromatographic mass spectrometry (GC/MS). The detected pesticides were organophosphorus (diazinon, profenofos and coumaphos), pyrethroid (cypermethrin, permethrin and lambda-cyhalothrin) and acaricide (amitraz). Diazinon has been detected in both muscle and liver tissues of all examined carcasses. However, other detected residues distributed according to species. All toxic metals have been detected in both muscle and liver tissues of all tested animals. The heavy metal loads were species independent. The concentrations of Pb and As in liver tissues were comparable to that of muscle tissues of all tested animals. However, the concentrations of Cd in liver were significantly higher than that in muscles of all tested animals. All values of detected pesticide residues and heavy metals were below the maximum residue limits (MRLs). In conclusion, pesticide residues and toxic metals were detected in meat and liver tissues of sheep, cattle and dromedary camels that admitted to Al-Ahsa abattoir, Saudi Arabia. The detected pesticides residues and toxic metals were well below MRLs; thereby no risks are associated with its human consumption.

Key words: chromatography; residues; toxic elements; meat; pesticides; animals

Introduction

The use of pesticide increased steadily during 1996-2013 in Saudi Arabia (1). In 2013, the

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total usage of insecticide was 3130.5 tons of active ingredients compare to that during 2012 (2889 tons of active ingredients). The organophosphate ranked first of pesticide used during this period (1). The Date Palme still represents the most important crop cultivated in Al-Ahsa, Eastern Province, Saudi Arabia and an element contributing in economy of the beautiful oasis, Al-Ahsa. Pesticides used in Al-Ahsa to fight the red date Palme weevil (2) and other pests that attack different varieties of vegetable crops cultivated in Al-Ahsa region (1). Contamination of the environment with pesticide residues has been documented earlier (3). Based on the reports of the toxicity and detrimental effects of pesticides, their use has been restricted worldwide. Although the use of most of pesticides has been restricted, its residues in food were detected (4). Water, plants and grasses that contaminated by pesticides residues may ingested by herbivorous animals and secreted in meat and/or milk (4). Pesticides residues contamination has been reported in processed food (5, 6) and eggs (7, 8). In addition, organochlorine pesticides have been detected in edible tissues of camel, cattle and sheep carcasses slaughtered in Sharkia Province of Egypt (9), in meat of sheep slaughtered in Jaipur area of India (10) and in fish and poultry meat in India (11). The dromedary camels are one of the important sources of meat in the semiarid and arid areas of Asia and Africa (12). Camel meat characters are near to that of other red meat animal species (12). In Al-Ahsa region, camel meat is preferable along with the other meats of sheep and cattle. However, data reporting the detection of pesticides residues in edible tissues of these animals reared in Al-Ahsa region of eastern province of Saudi Arabia are scarce. The inorganic arsenic is more toxic than organic one. However, it is reported in literatures as total arsenic (13). The exposure of animal to lead increased when feed materials mixed with contaminated soil (14). Regarding lead toxicity, cattle and sheep are the most sensitive animal species. The elimination of lead is incomplete and slow. Its half-life is approximately 250 days in ewes, between 95 and 760 days in cattle (15, 16), and 2 to18 years in humans (17, 18). Like lead, animal exposed to cadmium through plants mixed with contaminated soil (19). Cadmium classified as a human carcinogen (Group 1) by International Agency of Cancer Research particularly cancer in lungs, endometrial tissue, bladder, and breast (20). Only one report (21) originated from our laboratory demonstrated the detection of metal contents in the edible tissues of camel and sheep slaughtered in Saudi Arabia. However, in this report (21), cattle meat and liver tissues were not investigated. GC-MS used for high speed, excellent separation and quantitative analysis of many volatile pesticides (22-29). The current study aimed to evaluate levels of forty-five pesticides and heavy metals (lead (Pb); cadmium (Cd); Arsenic (As)) in muscle and liver tissue samples that collected from forty-two animals; sheep (n=21), cattle (n=11) and dromedary camels (n=10) admitted to Al-Ahsa abattoir, Eastern province, Saudi Arabia.

Material and methods

Animals and samples

Specimens of muscle (n=42) and liver (n=42) tissues have been collected from 42 animals of three species namely sheep (n=21), cattle (n=11) and dromedary camels (n=10) that have been admitted to Al-Ahsa abattoir, Eastern province, Saudi Arabia (25°22′18.1″N 49°26′21.1″E) directly after slaughter. Age of animals was determined by visual examination of teeth during the ante-mortem inspection (25). The average age of the slaughtered camel and cattle was estimated to be 5 ± 2 years, respectively while for sheep was and 8 ± 3 months. All animals were apparently healthy, active, and free from any disease. Sampled tissues for pesticide and heavy metal residues analysis were stored in plastic falcon tubes and stored at –20°C until the time of extraction and measurement of 45 pesticide and heavy metal residues. The experiments and procedures were done according to the regulations and guidelines of ethical research committee, King Faisal University, Saudi Arabia. All chemicals were purchased from Merck, Darmstadt, Germany and were of HPLC grade or the highest quality available. Specifications for pesticides standards used in the study are illustrated at Table 1.

Analytical procedure for pesticide residues

The QuEChERS EN 15662 method for pesticide residue extraction was used (30) and the obtained extracts were transferred to an auto sampler vial of GC-MS for analysis of pesticide residues.Pesticides residues have been detected by GC/MS system (Shimadzu GC/MS-QP2010 Plus, Japan) equipped with a capillary column DB-5MS (30m length, 0.25 mm thickness, 0.25m diameter). The temperature of injector was 250ºC. The temperature program of oven started with 60ºC. Hold 6 min at 7ºC/min to 200 ºC, at 5 ºC/min to 280 ºC. Hold 20 min; carrier gas, helium; purity, ≥99.999%; flow rate, 1.2 ml/min; injection port temperature, 250 ºC; injection volume, 1ul; injection mode, splitless, purge on after 1.5 min; ionization voltage, 70 eV; ion source temperature, 220 ◦C; GC/MS interface temperature, 250◦C. The analyses were done at two modes, Selective Ion Monitoring (SIM mode) and full Scan mode for enhanced sensitivity and selectivity. The limits of detection (LOD) and limit of quantification (LOQ) for all pesticides are shown in Table 2.

Analysis of heavy metals (Pb, Cd and As)

The digestion of muscle and liver samples and the GFA-EX7 graphite furnace atomic absorption spectrophotometeic (Shimadzu, Koyoto, Japan) determination of Pb, Cd and As were done as described earlier (31, 32). Preparation of standard stock solutions of Cd, Pb and As and calibration procedures were the same of that discussed in our previous works (31, 32). The LOD for the three investigated heavy metals was 1 µg/kg**.** The absorbance was obtained by adjusting the hollow cathode lamps at the operation conditions shown in Table 3.

Analytical quality control

In the present study, to check the efficiency of digestion procedures and the subsequent recovery of the pesticides and metals, homogeneous mixtures of three samples of each of muscle and liver were spiked with multi-elements and multi-pesticides solutions that contain standard solutions of all pesticides and metals considered in the present study. Solutions were spiked in a manner to attain final concentrations of 3µg/g. A mixture without any pesticides and metals was used as control. All mixtures were then subjected to the digestion procedure. The resulting solutions were analyzed three times for pesticides and metal concentrations according to the same procedures as the samples to establish confidence in the accuracy and reliability of data generated. The amount of spiked pesticides and metals recovered after the digestion of the spiked samples were used to calculate percentage recovery as described earlier (33). The pesticide recovered from both tissues in a percentage over 89% as indicated at Table 2. All heavy metals recovered in a percentage ranged between (89.6-94.2 %) and (85-89.4 %) in liver and muscle tissues, respectively (Table 4).

Specificity

The specificity was confirmed based on the presence of the transition ions (quantifier and qualifier) at the correct retention times (that was defined formerly by using one sample from each type that spiked at 10ng/g to check the retention time of pesticides) corresponding to that of the precursor ion presented in Table 1. The measured peak area ratios of qualifier/quantifier were within the range defined in EU Commission Decision 2002/657/EC when compared to the standards.

Statistical analysis

The statistical analysis was performed using the SPSS 6.1.3 software package (SAS, Cary, NC, USA). All data were analyzed for variance using ANOVA test.

Results and discussion

Data summarized in Table 1 and Figure 1 indicated that, the total number of investigated pesticides standards were 45 standards. However, gas chromatographic analysis indicated the existence of only 7 types of pesticides residues (Diazinon, Lambda-Cyhalothrin-II, Profenofos, Cis-Permethrin, Cypermethrin, Amitraz, coumaphos). The aim behind the screening of large number of pesticides was to cover all existing chemical classes that includes, organophosphates, organo-chlorine, carbamates, synthetic pyrethroids, triazines, pyrimidines, triazoles, dicarboximides, bezimid-

azole, dithiocarbamates, nicotinoids, dinitrophenol, pesticides aliphatic nitrogen, pesticides morpholine, pesticides from natural product derivatives, and pesticides of substituted urea class. However, only seven of screened pesticides have been detected in muscle and liver tissues of all tested animals (Table 1). The detected pesticides residues are of listed pesticides residues monitored in Saudi agricultural soils (34). Diazinon has been detected in muscle and liver tissues of all examined carcasses. This may attributed to the extensive use of diazinon for eradication of Ticks that affected animals related to all areas of Al-Ahsa governorate as said by the habitants of that region. Other detected pesticides residues were species related (Table 5 and 6). Profenofos residues has been detected in examined tissues of sheep and camels whereas, amitraz and permethrin have been detected only in muscle and liver of cattle carcass (Table 5 and 6). Coumaphos detected only in examined tissues of sheep carcass while Lambda-Cyhalothrin residues were detected only in muscle and liver tissues of camel carcass (Tables 5 and 6). The variation of pesticides contamination among tested animals may attribute to different contaminated areas because animals who admitted to Al-Ahsa abattoir were from different areas of Al-Ahsa governorate. The area dependent pesticides contamination has been confirmed in other study in Egypt (35). The data described in Tables 5 and 6 indicated that, all values of detected pesticide residues were below the international permissible limits (14). Parallel to the current findings, earlier report (9) demonstrated that, the organochlorines residues detected in muscle and liver tissues of camel, sheep and cattle were well below the respective MRLs set by local or international organizations. Data summarized in Tables 7 indicated that, the investigated toxic metal residues have been detected in muscle and liver tissues of all examined carcasses. The heavy metal loads were species independent (Tables 7). The concentrations of Pb and As in liver tissues were comparable to that of muscle tissues of all tested animals. However, the concentration of Cd in liver was significantly higher than its concentration in muscle in all tested animals (Tables 7). This may attribute to the fact that, the liver is main organ for detoxification in the live organism (36). The current findings come in accordance with recent work (21) reported that, liver tissues had a higher loads of heavy metals (Cd, Pb and As) than that of muscle tissues in camel and sheep slaughtered in the same area of Saudi Arabia. All values of detected heavy metals were well below the international permissible limit as described in Tables 7 (4, 13, 14, 20, 21, 37). The highest detected average of Arsenic, lead and cadmium residues either in muscle or in liver tissues were 11.6 ± 2.0µg/kg, 10.5 ± 1.8µg/kg and 0.9 ± 0.1 µg/kg whereas the arsenic, lead and cadmium MRLs are 1000µg/kg, 500µg/kg and 1000µg/kg, respectively. This indicated that, the contaminated tissues with all toxic elements are negligible and safe for human consumption. The detected values of Arsenic, lead and cadmium residues were much lower than that reported in liver and kidney bovine tissues (27.42-30.55 µg/kg; 231-226.96 µg/kg; 207.5-197 µg/kg; (38), respectively. The wide range between detected elements in the current study compared with earlier report (38) may attribute to different detection limits and sensitivity. Eelier detection limits (38) were 100µg/kg for Cd, 150µg/kg for Pb and 30µg/kg for As. However, in the current study the detection limits were 1µg/kg for the three investigated elements. The detected values of Cd and As in liver of camel (1.3 ± 0.1 µg/kg; 1.4 ± 0.4 µg/kg) and sheep (11.6 ± 2.0 µg/kg; 8.0 ± 2.0 µg/kg), respectively were near to that reported recently (21) in liver of camel (2.18 ± 0.71 µg/kg; 1.79 ± 0.66 µg/kg) and sheep (38.41 ± 25.15 µg/kg; 30.29 ± 22.57 µg/kg), respectively. The values of Cd and As in the muscle of camel (0.9 ± 0.1 µg/kg; 0.6 ± 0.1 µg/kg) and sheep (8.0 ± 1.1 µg/kg; 6.4 ± 1.2 µg/kg) respectively were near to that reported recently (21) in muscle of camel (0.29 ± 0.27 µg/kg; 0.44 ± 0.17 µg/kg) and sheep (12.89 ± 4.24 µg/kg; 10.05 ± 3.77 µg/kg), respectively. In the current study, the detected values of Pb in liver and muscle of camel and sheep were exactly comparable to that reported earlier in the same tissues of the same tested animals (21).

Table 1: Specifications for pesticides standards used in the study

No	Name of pesticide	Retention time	Quantifying ion	Qualifying ion1	Qualifying ion2	No	Name of pesticide	Retention time	Quantifying ion	Qualifying ion1	Qualifying ion2
1	Demeton-S-methyl	10.129	263	169	127	26	Propargite-I	31.003	135	350(7)	173(16)
2	α-HCH	11.108	219	183(98)	221(47)	27	Profenofos*	31.382	339	374 (39)	297 (37)
3	Diazinon *	15.670	304	179(192)	137(172)	28	Bifenthrin	32.39	181	166 (25)	165 (23)
4	HCB	17.567	284	282(51)	286(81)	29	Methoxychor	32.672	277	228(16)	212(4)
5	Phorate	18.271	260	121(160)	231(156)	30	Captan	32.782	264	149(104)	150(70)
6	β-HCH	19.954	219	217(78)	181(94)	31	Formothion	32.884	170	224(97)	257(63)
7	δ-HCH	20.108	219	217(80)	181(99)	32	p,p'-DDT	33.108	235	237(65)	246(7)
8	Heptachlor	20.503	272	237(40)	337(27)	33	p,p'-DDD	33.232	235	237(64)	199(12)
9	Aldrin	20.743	263	265(65)	293(40)	34	Cis-Permethrin *	33.759	183	184(14)	255(1)
10	Lambda-Cyhalothrin-II *	22.533	181	241(205)	243(152)	35	Captafol	34.565	149	150(79)	152(79)
11	α-Endosulfan	23.087	241	265(66)	339(46)	36	p.p'-DDE	36.154	318	316(80)	246(139)
12	chlorothalonil	23.995	264	266(109)	268(62)	37	O',p'-DDD	36.337	235	237(65)	165(39)
13	Methylparathion	25.056	263	233(66)	246(8)	38	Cypermethrin*	36.457	181	152(23)	180(16)
14	Endosulfan sulfate	25.313	241	265(66)	339(46)	39	Amitraz*	36.613	293	162(113)	132(104)
15	Dieldrin	27.196	263	277(82)	380(30)	40	coumaphos*	37.106	362	226(56)	364(39)
16	Endrin	27.432	263	317(30)	345(26)	41	Trans-Permethrin	37.231	183	184(14)	255(1)
17	O,p'-DDE	27.683	246	318(34)	176(26)	42	Fenvalerate	37.317	167	225(53)	419(37)
18	Dicofol	28.104	139	141(72)	250(23)	43	Deltamethrin	37.969	181	172(25)	174(25)
19	Malathion	28.418	173	158(36)	143(15)	44	Cyfluthrin	41.512	206	199(63)	226(72)
20	Fenithrothion	29.053	277	260(52)	247(60)	45	Esfenvalerate	46.085	419	225(158)	181(189)
21	Bromopropylate	29.452	341	183(34)	339(49)						
22	penconazol	30.101	248	250(33)	161(50)						
23	Pirimiphos-methyl	30.281	290	276(86)	305(74)						
24	Procymidone	30.448	283	285(70)	255(15)						
25	o,p'-DDT	30.767	235	237(65)	246(7)						

*Tissues detected pesticides

Table 2: Limit of Detection (LOD), Limit of Quantification (LOQ) and recovery percentages of pesticide measured using the ions for each pesticide molecule

No	Name of pesticide	RT	LOD (µg/g)	LOQ (µg/g)	Recovery %
1	Demeton-S-methyl	10.129	0.02	0.06	89.2
2	α-HCH	11.108	0.02	0.06	90.2
3	Diazinon *	15.670	0.006	0.017	95.0
4	HCB	17.567	0.02	0.06	92.1
5	Phorate	18.271	0.03	0.12	89.9
6	β-HCH	19.954	0.01	0.03	90.5
7	δ-HCH	20.108	0.01	0.03	90.6
8	Heptachlor	20.503	0.002	0.007	92.4
9	Aldrin	20.743	0.02	0.05	89.5
10	Lambda-Cyhalothrin-II *	22.533	0.004	0.01	91.7
11	α-Endosulfan	23.087	0.003	0.008	90.9
12	chlorothalonil	23.995	0.006	0.015	93.3
13	Methylparathion	25.056	0.004	0.012	94.5
14	Endosulfan sulfate	25.313	0.002	0.006	93.4
15	Dieldrin	27.196	0.02	0.008	93.1
16	Endrin	27.432	0.01	0.04	92.2

Table 2: Continuation

No	Name of pesticide	RT	LOD (µg/g)	LOQ (µg/g)	Recovery %
17	O,p'-DDE	27.683	0.02	0.06	90.5
18	Dicofol	28.104	0.003	0.009	90.7
19	Malathion	28.418	0.005	0.012	90.0
20	Fenithrothion	29.053	0.004	0.013	91.9
21	Bromopropylate	29.452	0.005	0.015	92.9
22	penconazol	30.101	0.004	0.012	93.0
23	Pirimiphos-methyl	30.281	0.006	0.018	90,1
24	Procymidone	30.448	0.01	0.030	90,7
25	o,p'-DDT	30.767	0.009	0.016	91.7
26	Propargite-I	31.003	0.009	0.015	91.4
27	Profenofos*	31.382	0.004	0.010	92.5
28	Bifenthrin	32.39	0.002	0.006	90.4
29	Methoxychor	32.672	0.006	0.012	93.7
30	Captan	32.782	0.01	0.03	94.0
31	Formothion	32.884	0.004	0.012	90.2
32	p,p'-DDT	33.108	0.002	0.006	92.9
33	p,p'-DDD	33.232	0.003	0.013	91.9
34	Cis-Permethrin *	33.759	0.004	0.010	93.7
35	Captafol	34.565	0.001	0.004	91,4
36	p.p'-DDE	36.154	0.004	0.011	89.8
37	O',p'-DDD	36.337	0.004	0.012	89.7
38	Cypermethrin*	36.457	0.003	0.009	93.9
39	Amitraz*	36.613	0.002	0.006	89.6
40	coumaphos*	37.106	0.002	0.006	94.0
41	Trans-Permethrin	37.231	0.003	0.009	90.9
42	Fenvalerate	37.317	0.02	0.06	90.8
43	Deltamethrin	37.969	0.002	0.007	94.3
44	Cyfluthrin	41.512	0.003	0.009	90.1
45	Esfenvalerate	46.085	0.01	0.03	90.2

*Tissues detected pesticides

Table 3: Programs of heating method of graphite furnace atomic absorption (GFAAS) for Arsenic (As), lead (Pb) and cadmium (Cd)

Steps	Temperature (°C)			Ramp(s)	Hold(s)	Argon flow rate (ml min−1)
	As	Pb	Cd			
Drying 1	150	150	150	5	20	250
Drying 2	200	200	200	5	15	250
Pyrolysis	1200	800	500	10	20	250
Atomization	2000	2000	1800	0	5	0
Clean-out	2000	2200	2200	1	3	250

Table 4: The recovery of heavy metals (As, Pb and Cd) from digested liver and muscle samples:-

Heavy metals	Added Heavy metals (µg/kg)	Recovered from liver (µg/kg)	Recovery (%)	Recovered from muscle (µg/kg)	Recovery (%)
As	5	4.47	89.6	4.39	89.4
Pb	5	4.69	94.2	4.44	88.8
Cd	5	4.66	93	4.25	85

AS: (Arsenic); Pb: Lead; Cd: Cadmium

Table 5: Pesticides residues (mg/kg) detected in muscle tissues (n=42) of sheep, cattle and dromedary camels admitted to Al-Ahsa Abattoir, Saudi Arabia

Animal Species	No. of Animals tested	No. of samples	No of Residues containing samples	No of detected pesticides residues	Types of detected pesticides residues	Concentration of detected pesticides residues	Permissible limit (13, 14)
Sheep	21	21	3	4	Diazinon	0.005	0.02
					Profenofos	0.015	0.05
					Cypermethrin	0.009	0.20
					Caumaphos	0.018	0.01
Cattle	10	10	3	4	Diazinon	0.017	0.02
					Cypermethrin	0.035	0.20
					Amitraz	0.033	0.02
					Permethrin	0.029	0.50
Camel	11	11	2	3	Diazinon	0.018	0.02
					Profenofos	0.019	0.05
					Lambda-Cyhalothrin	0.033	0.50

Table 6: Pesticides residues (mg/kg) detected in Liver tissues (n=42) ofsheep, cattle and dromedary camels admitted to Al-Ahsa Abattoir,Saudi Arabia.

Animal Species	No. of Animals tested	No. of samples	No of Residues containing samples	No of detected pesticides residues	Types of detected pesticides residues	Concentration of detected pesticides residues	Permissible limit (13, 14)
Sheep	21	21	3	4	Diazinon	0.012	0.02
					Profenofos	0.022	0.05
					Cypermethrin	0.015	0.05
					Coumaphos	0.024	0.01
Cattle	10	10	3	4	Diazinon	0.034	0.02
					Cypermethrin	0.041	0.02
					Amitraz	0.023	0.20
					Permethrin	0.014	0.05
Camel	11	11	2	3	Diazinon	0.027	0.02
					Profenofos	0.029	0.05
					Lambda-Cyhalothrin	0.053	0.50

Table 7: Heavy metals (µg/kg) detected in muscleand liver tissues (n=42) of sheep, cattle and dromedary camels admitted to Al-Ahsa Abattoir, Saudi Arabia.

Animal Species	No. of Animals tested	No. of samples	Heavy metals concentration (ppb)					
			*Pb in muscle tissues	Pb in liver tissues	†Cd in muscle tissues	Cd in liver tissues	‡As in muscle tissues	As in liver tissues
Sheep	21	21	4.98 ± 1.44^{aw}	7.8 ± 1.2^{aw}	0.6 ± 0.1^{ax}	1.4 ± 0.4^{ay}	6.4 ± 1.2^{aw}	8.0 ± 2.0^{aw}
Cattle	10	10	7.40 ± 2.00^{aw}	7.9 ± 1.5^{aw}	0.7 ± 0.2^{ax}	1.3 ± 0.2^{ay}	8.7 ± 2.0^{aw}	11.1 ± 2.5^{aw}
Camel	11	11	7.30 ± 1.20^{aw}	10.5 ± 1.8^{aw}	0.9 ± 0.1^{ax}	1.3 ± 0.1^{ay}	8.0 ± 1.1^{aw}	11.6 ± 2.0^{aw}

*Permissible limit of Pb (500µg/kg) (38).
†Permissible limit of Cd (1000µg/kg) (38).
‡Permissible limit of As (1000µg/kg) (38).
Values are expressed as Mean ± SD.
Means within the same column with different superscripts are significantly different ($P < 0.05$).
Means within the same row with different superscripts are significantly different ($P < 0.05$).

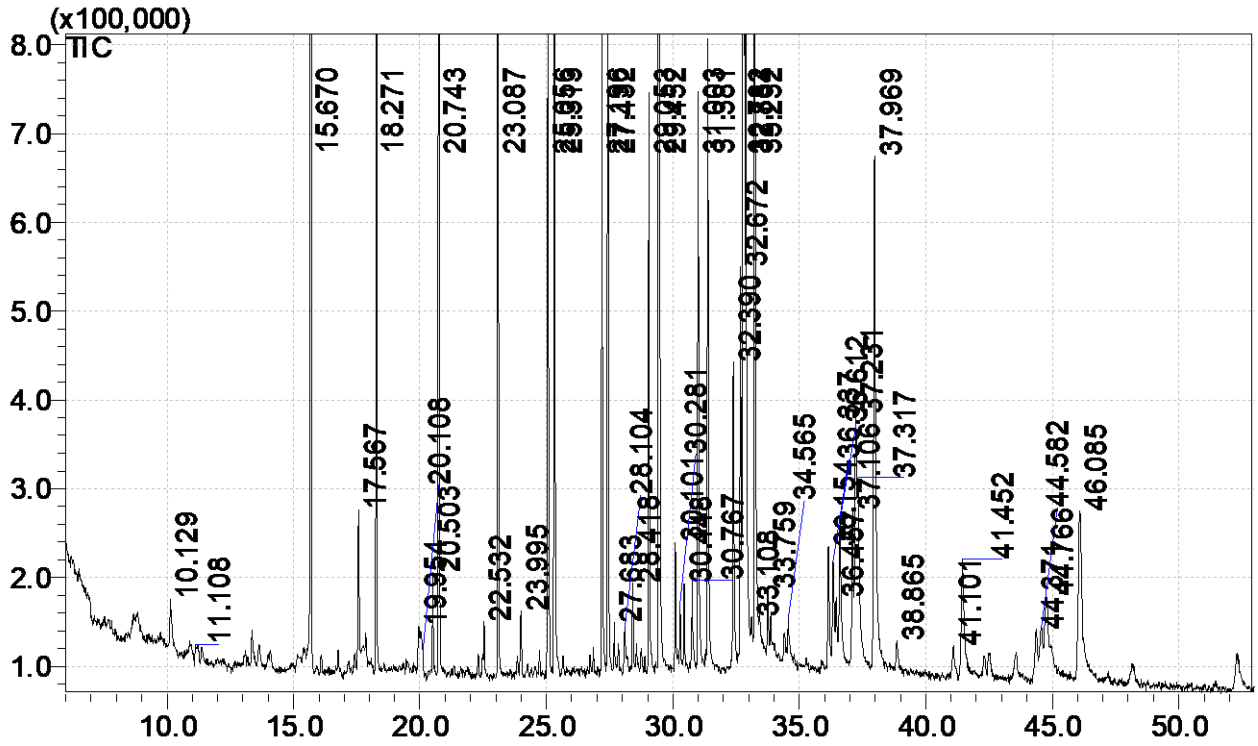

Figure 1: GC/MS chromatogram of investigated pesticides standards

Conclusion

The current study concludes the existence of pesticide residues and toxic metal residues in muscle and liver tissues of sheep, cattle and dromedary camel carcasses at Al-Ahsa governorate. Diazinon contaminated muscle and liver tissues of all examined carcasses. Other pesticide residues differed according to the areas that animals originated. The heavy metal loads were species independent. The concentrations of Pb and As in liver tissues were comparable to that of muscle tissues of all tested animals. However, the concentration of Cd in liver was significantly higher than its concentration in muscle in all tested animals. Consumption of carcasses is safe to human health because the detected pesticide and toxic metal residues were well below international permissible limit.

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Original Research Article

GREEN TEA MITIGATES STREPTOZOTOCIN-INDUCED DIABETIC MICE THROUGH ANTI-FIBROTIC ACTIVITY AND MODULATION OF PROINFLAMMATORY CYTOKINES

Hend M. Salah[1], Eman A. Moussa[1], Samaa M El. Bakr[1], Mustafa S. Atta[2]*, Foad A. Farrag[3], Ahmed S. Hafez[4]

[1]Department of Zoology, Faculty of Science, Kafrelsheikh University, 33516, Egypt, [2]Department of Physiology, Faculty of Veterinary Medicine, Kafrelsheikh University, [3]Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Kafrelsheikh University, [4]Department of Pharmacology, Faculty of Veterinary Medicine, Aswan University, Egypt

*Corresponding author, E-mail: mostafa.ataa@vet.kfs.edu.eg

Abstract: This research was planned to assess the protective effect of green tea extract (GTE) in STZ-induced diabetes in mice. Seventy-five female albino mice were used. Mice randomly allocated into five groups of 15 mice in each as follows: group 1 (control), group 2 (STZ, diabetic), group 3 (green tea + STZ), group 4 (protective group with green tea), and group 5 (green tea only). Oral administration of the green tea extract for three weeks to diabetic mice resulted in significant regaining in body weight, decreased blood glucose, cholesterol, triglyceride, free fatty acid and low-density lipoprotein cholesterol (LDL-C) levels and lowered malondialdehyde (MDA) contents and increased total protein and high-density lipoprotein cholesterol (HDL-C) as compared to untreated diabetic group. Histopathological changes were compatible with these biochemical findings. Diabetic mice pre- or co-treated with green tea also exhibited a significant downregulation in *TGFβ1* and *TNFα* genes expression. Green tea extracts had protective and antidiabetic effect in controlling blood glucose level in addition to improving lipid metabolism and body weight in diabetic mice.

Key words: diabetes; green tea; liver; TNFα; TGF-B1; mice

Introduction

Diabetes mellitus (DM) is one of the major intimidations to health in the world. It was expected to be more prevalent in 2025 (1). Hyperglycemia is associated with vascular complications and kidney diseases (2). There are several other factors that play a great role in diabetes for instance, obesity and oxidative stress which lead to more ferocious complications (3), with decreased humoral immunity (4).

DM type 1 is a consequence of the annihilation of β-cells of the pancreas (5). Deficiency of insulin primes to hyperglycemia. STZ is a structural analog of glucose (Glu) and N- acetyl glucosamine is reserved up by β-cells via the GLUT 2 transporter subsequently lead to β-cell loss via DNA destruction (6, 7). Moreover, cells that express GLUT 2 transporter like liver and kidney cells are also vulnerable to STZ. This clarifies kidney and liver complications (8, 9).

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Oxidative anxiety is an inequity among the reactive oxygen species (ROS) and antioxidant protection of the body, which play a great role in diabetic complications (10). Since many studies stated that oxidative anxiety, facilitated by diabetes-induced production of ROS, lead to progress of diabetes, it noticed that improving oxidative anxiety via antioxidants might be a successful approach for demoting diabetic complications (11, 12).

Green tea (*Camellia sinensis*) is prevalent nutraceutical antioxidant especially in Asian countries (13). Green tea has an important role in decreasing blood pressure, low-density lipoprotein cholesterol, and oxidative stress (14). Many studies proved the valuable effects of green tea on diabetic complications (15, 16) as well as it has antihyperglycemic effect in STZ diabetic animals (17). Moreover, green tea shows antioxidants and free radicals scavenger properties (18).

This study was done to evaluate the protecting pathway by which green tea alleviate streptozotocin-induced diabetes in mice.

Material and methods

Chemicals

We utilized a pharmaceutical-grade of STZ from Sigma (St. Louis, MO, USA). The STZ solution prepared by dissolving STZ in citrate buffer (0.01 M, pH 4.5) (19, 20). Chemicals consumed were of analytical grade. Total cholesterol, high-density lipoprotein (HDL)-cholesterol and triglyceride (TG) standard kits were purchased from Erba Diagnostics Mannheim Gambh, Germany.

Green tea extract

The green tea tablets, each tablet contain green tea extract 200 mg manufactured by El Obour City pharmaceutical industries. Following grinding, the obtained green tea powder dissolved in distilled water. This solution was provided to mice orally by using a stomach tube.(21)

Experimental animals

Seventy-five female albino mice, (9 – 11 weeks old, weighing about 25-30 gm was used. They obtained from Medical Technology Center, University of Alexandria, Egypt. Animals were managed according to the rules and regulations of our university committee. Female albino mice were housed in stainless steel pens of ambient temperature 23±2°C and light (12 h light/12 h dark) and free access to food and water. The mice were fed a standard diet according to (22) .

Initiation of experimental diabetes

After 2 weeks of accommodation, the mice were exposed to a 12 h fast. The mice were I/P injected with a single dose of 200 mg/kg STZ (7, 19). STZ was freshly dissolved in 0,1M cold sodium citrate buffer, pH 4.5(19, 20).after 2h from injecting we put 5% glucose and food to injected mice to avoid death(20) .we replace 5%glucose with water after 2h.The urine glucose level was checked three days after STZ injection to check the progress of diabetes. The diabetic mice were steadied for 5 days and then the experiment beginning. Mice which showed glucose levels >250 mg/dL was considered diabetic.

Experimental design

After acclimatization, mice were alienated randomly into five groups of 15 mice each: Group 1: healthy control mice received distilled water Group 2 (diabetic group): will be used as control diabetic mice. The mice were injected I/P with one dose of 200 mg/kg STZ (7, 19). In group 3 (treated group with green tea) diabetic mice were administrated green tea at a dose of 200 mg/kg bw orally by stomach tube daily for 21 days. In group4 (protective group with green tea) mice were given green tea at dose of 200 mg/kg by stomach gavage for 21 days and then diabetes was induced with a single dose of 200 mg/kg streptozotocin with continuous treatment with green tea at dose of 200 mg/kg orally for 21days. In group 5 (control green tea group) non-diabetic mice received green tea (200 mg/Kg /day)dissolved in distilled water orally by stomach tube for 21 days according to (23).

Sampling

Mice were sacrificed by cervical decapitation. Body weights of all the animals were recorded prior to the treatment and sacrifice. Weight change % = Initial weight - Final weight × 100/Initial weight

Blood samples were collected in either EDTA coated tubes for the hematological investigation or plain tubes for serum separation (24) for biochemical assays. Kidneys, liver, spleen, and pancreases after decapitation were quickly removed and washed by cold saline to remove extraneous materials. Each organ was divided into 3 specimens. The first was snap frozen with liquid N2 and kept at -80°C until use for RNA extraction. The second was fixed in 10% neutral buffered formalin for the histological examination. The third was used for transmission electron microscopy (TEM) handled using standard techniques.

Blood analysis

The serum samples for measurement of blood glucose level was determined based on glucose oxidase method (25), insulin concentrations were determined according to (26) using an insulin-ELISA kit (Morinaga Seikagaku, Tokyo, Japan). Malondialdehyde (MDA) was quantified by the method of Ohakawa et al. (27), HbA1c was appraised using DCA 2000 analyzer (Bayer, Elkhart, IN)(28, 29). Total protein concentration in serum was assayed colorimetrically using commercial kit (Diamond, Egypt) and according to Lowry et al. (1951). Serum levels of total cholesterol, triglycerides, and high-density lipoproteins cholesterol (HDL-C) were estimated by quantitative colorimetric assay (Stanbio Laboratory, Inc., Texas, USA) according to (30) and low-density lipoprotein cholesterol (LDL-C) concentration assessed according to the formula of Friedewald et al; (31). LH, FSH, and estrogen were assessed using IMMULITE chemiluminescent assay kits (DPC, Glyn Rhonwy, Llanberis, Gwynedd, UK)(32). The Horiba ABX 80 Diagnostics (ABX pentra Montpellier, France) was used for hematological examination (33).

Histopathological and transmission electron microscope examination

The histopathology was carried out according to (34) using hematoxylin and eosin staining technique. Preparation od samples for transmission electron microscope (TEM) using collagen-coated nickel grids was applied as previously described (35).

Molecular investigation

Total RNA was extracted from tissue samples using RNA extraction kit (easy-REDTM, iNtRON Biotechnology, #17063, South Korea). cDNA synthesis was performed using reverse transcription kits (Thermo Scientific, Fermentas, #EP0451) which include the following: Revert Aid H Minus Reverse Transcriptase enzyme, Oligo dT,5X Reaction Buffer, RiboLock RNase Inhibitor, and dNTP Mix. Concentration of RNA and cDNA quantify using a Nanodrop (Q5000 Uv-Vis spectrophotometer, USA).

Real-time PCR with SYBR Green (2X Maxima SYBR Green/ROX qPCR Master Mix) used to determine the expression of the target genes in the liver, with GAPDH as a housekeeping gene, according to the manufacturer protocol (Thermo Scientific, USA, # K0221). The primers used in the amplification are shown in Table 1. The web-based tool, Primer 3 (http://www-genome.wi.mit.edu/cgibin/primer/primer3_www.cgi) used to design these primers. To confirm primer sequence is unique for the template sequence; we check similarity to other known sequences with BLAST. Calculation of fold changes in gene expression was done using$2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Data was analyzed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Results subjected to Tukey's multiple comparisons post-hoc test. Values are statistically significant when $p < 0.05$.

Results

Growth weight and Biochemical analysis

Diabetic mice had a significant drop in body weight in relation to other groups.as shown in

figure 1.The diabetic mice showed a significantly increased level of serum glucose, triglycerides, cholesterol, LDL-C, VLDL-C relative to the control group and other treated groups and green tea protective groups. Serum insulin, total protein and HDL-C statistically decreased in diabetic mice as compared to the control group, treated and green tea protective groups (Table 2). Serum HbA1C pointedly increased in diabetic mice relative to the control group (Table 2). Diabetic mice showed a noteworthy increase in MDA compared to their equivalent levels in control animals. However, treated groups with green tea showed a significant diminution in the levels of MDA in relation to diabetic mice. Protective groups treated with green tea exhibited a significant reduced MDA levels as compared to diabetic mice (Table 2). LH, FSH and estrogen levels did not show any significant difference among all groups (Table 3).

Hematological analysis

RBC and WBC count, PCV, neutrophil % and platelets levels in diabetic mice decreased significantly, while MCV, MCH, and MCHC increased significantly in diabetic mice compared to the control mice. Green tea extract significantly normalized RBC and WBC count, PCV, neutrophil % and platelets in diabetic mice as well as MCV, MCH, and MCHC of diabetic mice to the control level (Table 4).

Histopathological studies

All results of histopathology were displayed in figure 2. Histopathological observation revealed that liver of the control animal showing normal hepatocytes arranged in cords around the central vein (arrowhead) whereas that of STZ-treated animal showing single cell necrosis (arrowhead) associated with active apoptosis (arrow). Green tea treated diabetic animal showing slight hepatic vacuolation (arrowhead) adding that liver of green tea group showing normal hepatocytes around the central vein (arrow) as well as liver of protective group pretreated with green tea showing normal hepatocytes arranged around the central vein (arrow). Kidney of control animal showing normal renal glomeruli (arrowhead) and tubules (arrow) while kidney (cortex) of STZ-treated animal showing marked glomerular congestion (arrowhead) and degeneration within renal tubules (arrow) while that of green tea treated diabetic animal showing normal renal glomeruli (arrowhead) and patent normal tubules (arrow). Green tea group showing normal renal glomeruli (arrowhead) and tubules (arrow). Kidney of protective group with green tea-treated animal showing renal glomeruli (arrowhead) and tubules (arrow) within the normal limits. Pancreas of control animal showing normal glandular acini (arrow) and β islets (arrowhead) while that of STZ-treated animal showing degeneration of both glandular acini (arrow) and β islets (arrowhead). Green tea treated diabetic animal showing normal glandular acini (arrow) and slight vacuolation of β-cells (arrowhead) as well as pancreas of green tea-treated animal showing normal glandular acini and β islets (arrowhead). Diabetic animal pre-and post-treated with green tea-treated animal showing normal glandular acini (arrow) and β cells (arrowhead).

Electron microscope studies

All results of transmission electron microscope were displayed in figure 3. Liver of control group showing normal hepatocytes M indicates mitochondria and N indicates nucleus. While that liver of diabetic group showing multilocular cytoplasmic fat vacuoles, decreased mitochondrial number and shrinkage of nuclear membrane. As well as, liver of green tea treated diabetic animals showing presence of small fat vacuoles, glycogen granules and multiple autophagic vacuoles. besides green tea group showing three adjacent hepatocytes separated with thin connective tissue layer and mostly normal. M indicates mitochondria, bar=500 μm., liver of protective group treated with green tea group showing moderate degree of hepatic steatosis, F indicates fat vacuoles. Pancreas (endocrine portion) of control group showing presence of large number of B secretary granules within the B cells (arrowhead), BC indicates blood capillary, pancreas of diabetic group showing exocrine pancreatic cell with noticeable decrease the number of secretory granules

and mostly immature (arrowhead) with corrugated nuclear membranes (arrow), while that Green tea treated diabetic group showing exocrine pancreatic cell revealing normal nucleus (N) and with normal network of rough endoplasmic reticulum (RER), pancreas of green tea group showing two adjacent exocrine cells with normal nuclei and surrounded with network of rough endoplasmic reticulum, in addition, pancreas (exocrine cell) of protective group pretreated with green tea showing normal nucleus surrounded with network of rough endoplasmic reticulum and revealing large number of mature secretory vesicles (arrowhead). kidney of control group showing normal lining epithelial cells of the distal renal tubules with normal mitochondria (M) with mild condensation. Adding, kidney of STZ treated group showing the lining epithelial cells of the proximal tubules with slightly condensed mitochondria and some of them showing presence of fat vacuoles (arrowhead) and with normal brush border covering (arrow). as well as, green tea treated diabetic group showing lining epithelial cells of the distal renal tubules and mostly revealing lysis of the most intracellular organelles (arrow), kidney of green tea group showing the proximal renal tubules lining epithelial cells with normal brush border and mitochondria (M), bar=500 µm., while that of protective group pretreated with green tea group showing lining epithelial cells of the distal renal tubules and mostly revealing lysis of the most intracellular organelles (arrows).

Outcome of streptozotocin, green tea on the relative expression of TGFβ1gene and TNFα gene

The results of qPCR were presented in Figure 4. A considerable upregulation in the expression of *TGFβ1* gene in liver was found following administration of Streptozotocin as compared to the control group and other treated groups. Green tea treated diabetic mice showed a noteworthy decrease *TGFβ1* gene expression as compared to the diabetic group. Furthermore, pre-treatment by green tea led to a significant decrease in *TGFβ1* expression. Green tea alone led to insignificant downregulation of *TGFβ1* gene expression as compared to the control group. *TNFα* upregulation was found following administration of Streptozotocin as matched to normal one and other treated groups. Administration of green tea with Streptozotocin resulted in a significant decrease in *TNFα* gene expression as compared to diabetic one. Furthermore, pre-treatment by green tea led to a significant reduction of *TNFα* expression. Administration of green tea alone led to momentous decreased of TNFα gene expression in relation to control group.

Table 1: Sequences of primers used in qPCR

Gene	Reverse primer (/5 ------ /3)	Forward primer (/5 ------ /3)	Size (bp)	Accession number
TGFβ1	GACGTCAAAAGA-CAGCCACTCA	GCAACATGTGGAACTC-TACCAGA	106	M13177
TNFα	CTGATGA-GAGGGAGGCCATT	GCCTCTTCTCATTCCTG CTTG	115	NM_00127860 1
GAPDH	CCTGCTTCACCACC TTCTTGA	TGTGTCCGTCGTG-GATCTGA	99	M32599

Table 2: Effect of green tea on serum glucose, HbA1C, insulin, MDA, total protein levels in STZ-induced diabetic female albino mice

	Control	STZ	STZ+Green tea	pretreated with green tea	Green tea
Glucose (mg/dl)	120.5±13 ^{c}	407±49.6 ^{a}	186±27 ^{b}	115±23 ^{c}	143±4.7 ^{bc}
HbA1C (%)	5.00±0.0 ^{c}	13.0±0.44 ^{a}	8.2±0.58 ^{b}	5.0±0.0 ^{c}	8.2±0.58 ^{b}
Insulin pg/ml	1024±0.0 ^{a}	299.0±0.0 ^{c}	606±21.7 ^{b}	1024±0.0 ^{a}	826±50.8 ^{b}
MDA nmol/ml	0.557±0.0 ^{c}	1.90±0.0 ^{a}	1.0±0.06 ^{b}	0.57±0.0 ^{c}	0.8±0.01 ^{b}
Total protein (mg/dl)	6.21±0.17 ^{a}	4.44±0.18^{c}	6.175±0.4 ^{a}	6.14±0.12 ^{a}	5.30±0.46 ^{b}

Data are expressed as mean ± SEM.Superscript of different letters in each column differ significantly (P<0.05) from each other

Table 3: Effect of green tea on LH, FSH and estrogen level levels in STZ-induced diabetic female albino mice

	Control	STZ	STZ+Green tea	pretreated with green tea	Green tea
Estrogen(pg/ml)	24.2±0.3	22.8±0.5	23.0±0.4	23.6±0.24	22.8±0.37
FSH (pg/ml)	0.18±0.0	0.17±0.0	0.15±0.00	0.18±0.0	0.17±0.0
LH (pg/ml)	0.36±0.0	0.36±0.0	0.35±0.0	0.36±0.0	0.36±0.0

Data are expressed as mean ± SEM. Superscript of different letters in each column differ significantly (P<0.05) from each other

Table 4: Effect of green tea on Haemogram in STZ-induced diabetic female albino mice

	Control	STZ	STZ+Green tea	pretreated with green tea	Green tea
RBC (10^6/ul)	8.88±0.6^{a}	7.45±0.13 ^{c}	8.31±0.18 ^{b}	8.6±0.04 ^{ab}	8.07±0.05 ^{b}
Hb (g/dl)	15.4±0.18^{a}	12.9±0.24^{c}	14.5±0.2b^{a}	14.3±0.21 ^{b}	14.5±0.2 ^{ab}
HCT %	29.48±0.3 ^{c}	37.0±0.42 ^{a}	31.2±0.33 ^{b}	31.0±0.48 ^{b}	29.2±0.20 ^{c}
MCV(fl)	40.7±0.28 ^{c}	46.0±0.28 ^{a}	43.0±0.35 ^{b}	43.1±0.23 ^{b}	40.7±0.35 ^{c}
MCH(pg)	13.2±0.09 ^{c}	17.5±0.21 ^{a}	14.1±0.13 ^{c}	13.9±0.05 ^{c}	13.65±0.1 ^{c}
MCHC (g/dl)	30.3±0.23 ^{c}	41.4±0.25 ^{a}	33.4±0.34 ^{b}	32.83±0.1 ^{b}	31.1±0.13 ^{c}
WBC (10^3/ul)	17.0±0.07 ^{a}	3.54±0.39 ^{c}	7.07±0.22 ^{b}	7.82±0.44 ^{b}	6.15±0.42 ^{c}
Lymphocyte %	65.0±0.16 ^{a}	11.94±4.1 ^{c}	45.65±4.1 ^{b}	44.59±3.3 ^{b}	61.21±0.6 ^{a}
Monocyte %	17.8±0.11 ^{a}	5.345±0.4 ^{c}	14.95±0.0 ^{b}	13.83±0.2 ^{b}	17.08±0.1 ^{a}
Granulocytes %	70.1±2.50 ^{a}	29.48±1.5 ^{c}	52.00±1.5 ^{b}	56.18±1.3 ^{b}	69.75±1.9 ^{a}
Platelets (103/ul)	637.5±54 ^{a}	349.3±17 ^{c}	422±13.9 ^{bc}	543.0±13 ^{b}	486±14.6 ^{b}

Data are expressed as mean ± SEM. Superscript of different letters in each column differ significantly (P<0.05) from each other

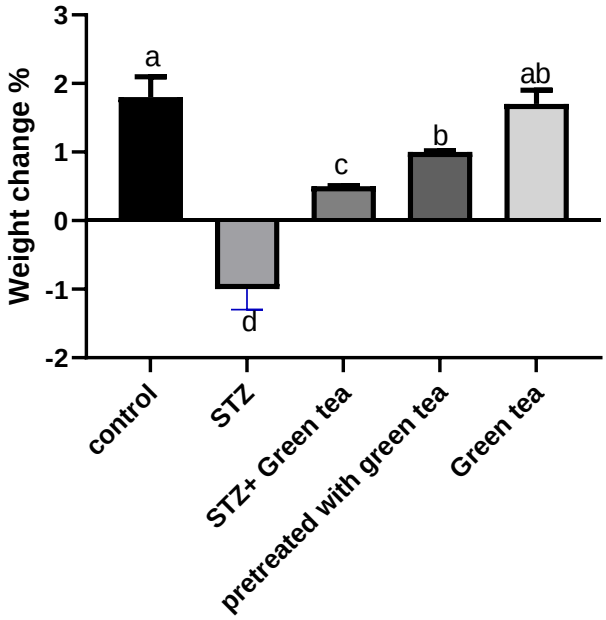

Figure 1: Effect of green tea on body weight, food intake and water intake grade in STZ-induced diabetic female albino mice. Data are expressed as mean ± SEM. Superscript of different letters in each column differ significantly (P<0.05) from each other

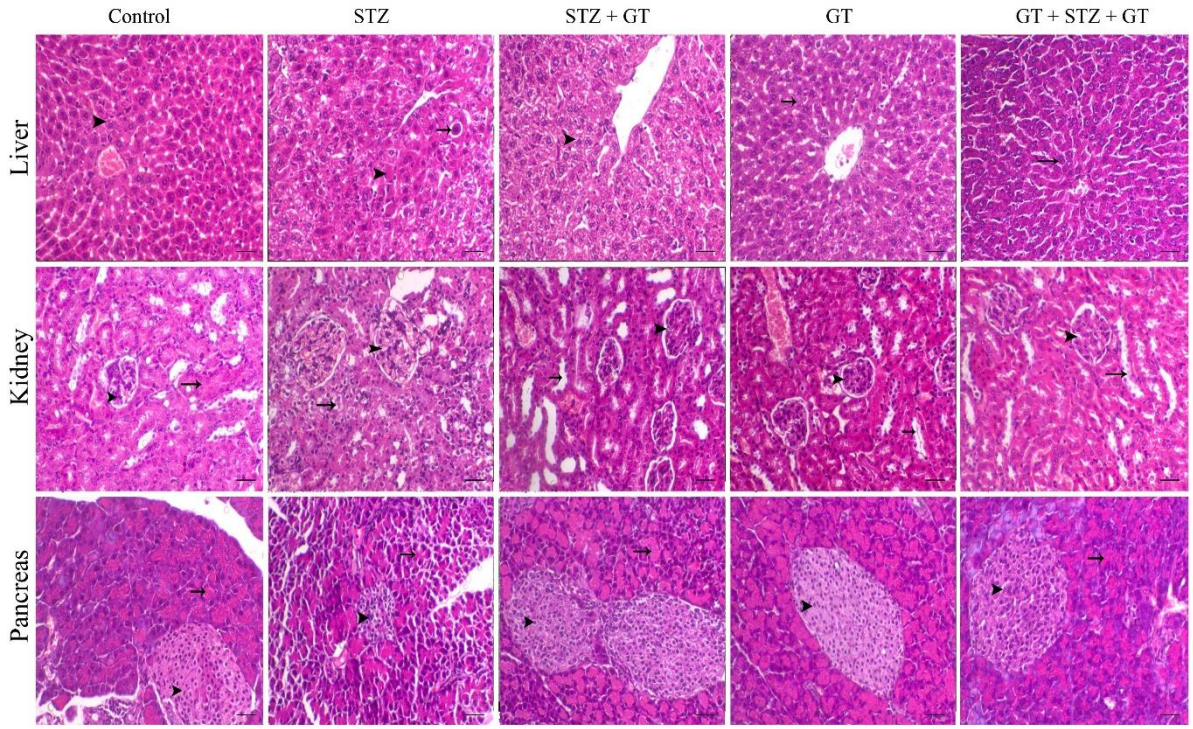

Figure 2: Histomicrograph of liver, kidney and pancreas of control group, STZ (diabetic) group, STZ+GT (green tea-treated STZ) group, GT (green tea) group and GT+STZ+GT (green tea-protective STZ) group. H&E, X200

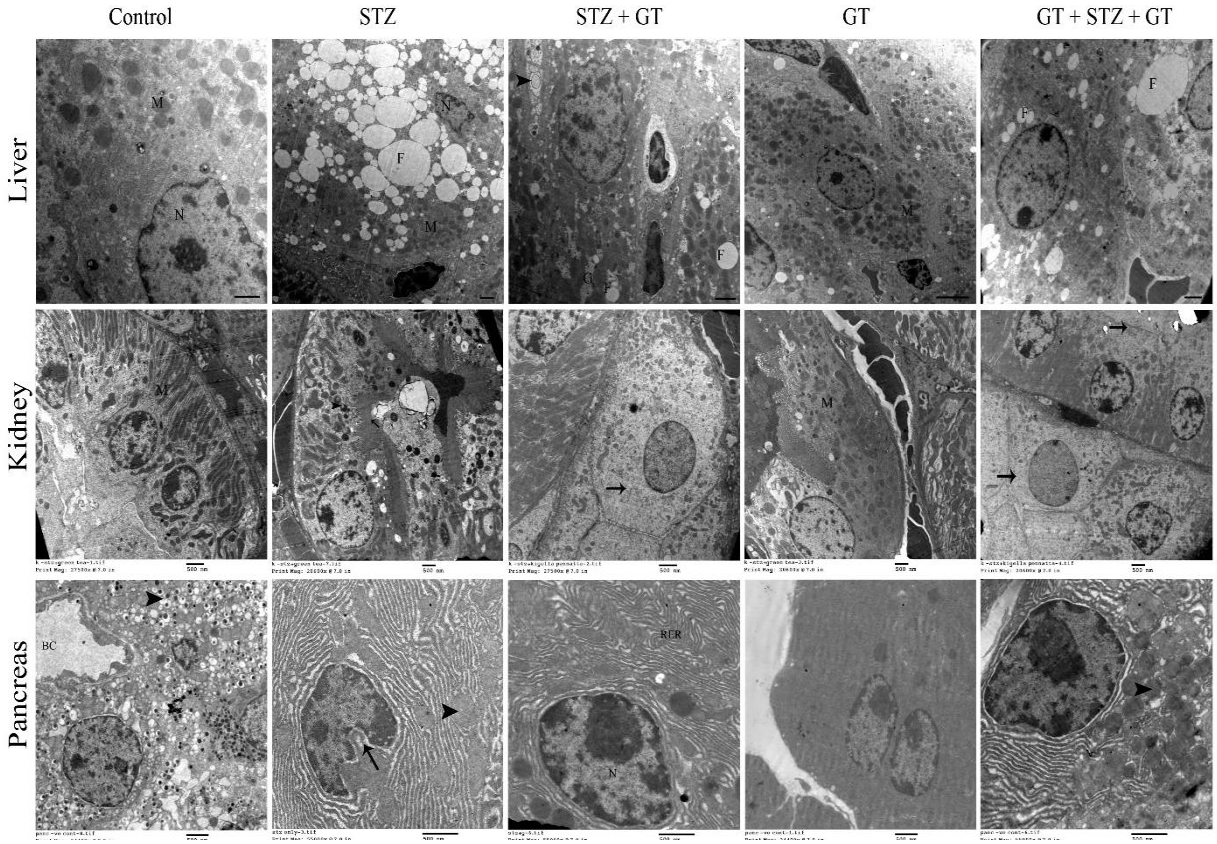

Figure 3: Electron micrograph of liver, kidney and pancreas of the control group, STZ (diabetic) group, STZ+GT (green tea-treated STZ) group, GT (green tea) group and GT+STZ+GT (green tea-protective STZ) group. Scale bar = 500 μm

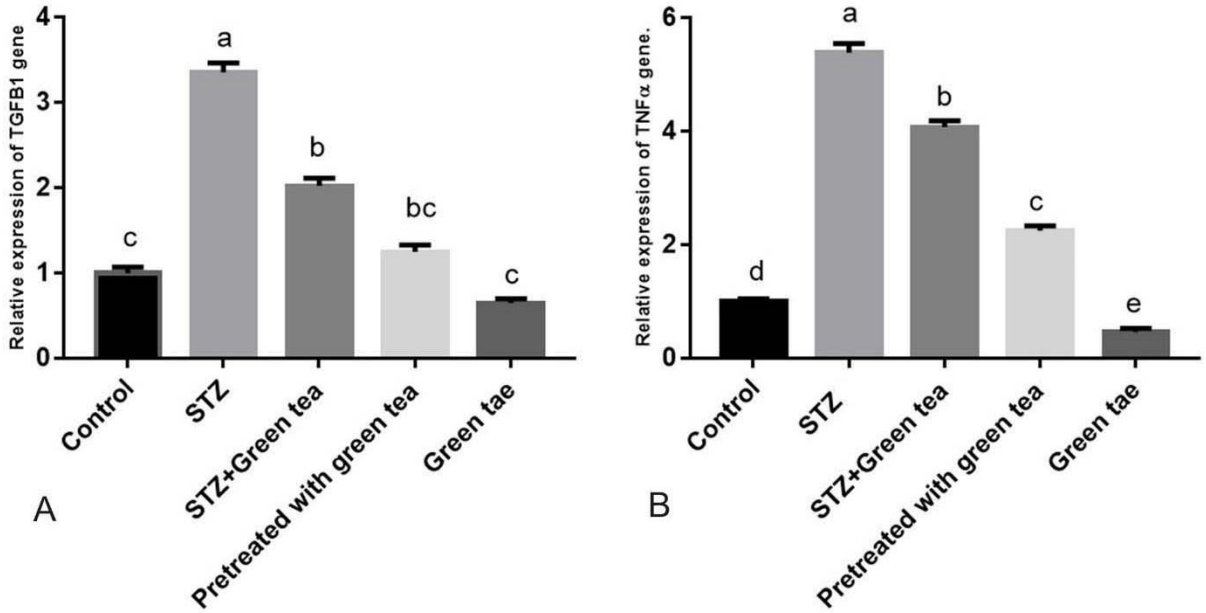

Figure 4: A. Effect of streptozotocin, green tea on the relative expression of *TGFβ1* gene. Means within the same column carrying different superscript letters are significantly different (P≤ 0.05). B. Effect of Streptozotocin, green tea on the relative expression of TNFα gene. Means within the same column carrying –different superscript letters are significantly different (P≤ 0.05)

Discussion

Diabetes is a metabolic ailment characterized by hyperglycemia. Its consequences from faults in insulin creation and this leads to disturbance in the metabolism followed by worsening of muscles which leads diabetic complications (36).

Our result revealed that diabetic mice had a significant drop in body weight as shown in figure 1. This result was consistent with (37-39) who reported momentous reduction in body weight in diabetic mice and attributed this effect to the highly muscle wasting and damage of tissue proteins.

Diabetic mice have a marked rise in the levels of serum glucose, triglycerides, cholesterol, LDL-C, VLDL-C when compared with control groups. The obtained data were parallel to that reported by (40) who found that diabetes induces substantial surges in the levels of these parameters and attributed this effect to insulin deficiency that hinder lipoprotein lipase triggering hypertriglyceridemia.

On the other hand, serum levels of insulin, total protein and HDL-C statistically decreased in STZ mice as compared to the control groups and these results agreed with (41-43) who reported similar reduction in these parameters in diabetic animals. Serum total protein in diabetic animals is obviously related to loss of structural proteins and drop in body weight which induced as a result of deficiency of insulin in diabetic mice.

Result revealed that, green tea extract in diabetic mice resulted in noteworthy regaining in body weight, decreases serum glucose in diabetic one, decrease cholesterol, triglyceride, and LDL-C levels, and improved serum total protein and HDL-C. In parallel, (44) also reported that administration of green tea extract in diabetic mice resulted in similar improvement and attributed them to the antihyperglycemic and hypolipidemic activity of green tea (45). Moreover, daily administration of green tea to diabetic mice decreased glucose levels and HbA1c levels and this result was an in consistence with (47).

In the present study, diabetic mice showed a noteworthy rise in MDA relative to control animals. However, pre and post-treatment of diabetic animals with green tea lead to a marked decrease in MDA level. These results was compatible with that obtained by (46) who reported that increase in MDA can be the result of diabetic complications.

MCV, MCH, and MCHC markedly increased in diabetic mice as compared to control mice and suggesting occurrence of anemia in diabetic mice. This result agreed with (50) who reported that anemia in diabetes was due to the increased non-enzymatic glycosylation of RBC membrane proteins, which associated with hyperglycemic oxidation of this glycosylated membrane. In contrast, green tea normalized the elevated MCV, MCH, and MCHC of diabetic mice. These results were consistent with those obtained by (50) who attributed this effect to the decreased lipid peroxide in RBC membrane which decreases hemolysis. The disrupted body defense mechanism of the diabetic animal against infections was distressed due to the neutrophil role in diabetes (48). The obtained results were similar to those obtained by (49) who reported that diabetic mice had a decreased WBC count and attributed this to Streptozotocin ability to suppress the immune system through hindrance of leukocytosis in the bone marrow. We also found a decrease in platelets levels in diabetic mice. In parallel, (51) also reported that platelet accumulation ability in diabetic mice could be due to deficiency of insulin. In contrast, green tea can return this reduced level to a level comparable to that of the control animals. These results are consistent with (52) who reported this effect to green tea ability to increase the biosynthesis of clotting factors. Similar to results obtained by (53), we found no significant change in LH, FSH and estrogen levels among the groups.

Green tea treatment led to a significant down-regulation of *TGFβ1* gene expression in liver. These results agreed with (54) who stated that green tea decreased hepatic expression of *TGFβ1* and this accountable for reduction collagen synthesis and decrease fibrosis. Administration of green tea also led to significant

downregulation of *TNFα* gene expression. In inconsistence, (55) established that green tea persuaded growth inhibition and apoptosis by decrease *TNFα* expression. TNFα expression may also have a valuable effect on diabetes since TNFα is intricate in developing diabetes.

Histopathological study and transmission electron microscope study revealed that administration of green tea can normalize the liver, kidney, pancreas and spleen that were deteriorated by STZ. In consistence, (56) reported that polyphenols (main ingredients in green tea) have anti-diabetic effects (57).

Conclusion

From the data found in this study, we can conclude that green tea has antidiabetic action though down-regulation of *TGFβ1* and *TNFα* gene expression in liver, improvement of lipid metabolism and body weight in streptozotocin - induced diabetic mice. This study verified the protective action of green tea on experimentally induced diabetic mice.

Conflict of interest

All authors declare that they have no conflict of interest.

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Original Research Article

BIOMARKERS VERSUS DUPLEX ULTRASONOGRAPHY FOR EARLY DETECTION OF ACUTE KIDNEY INJURY IN DOGS: AN EXPERIMENTAL STUDY

Mohamed A. Donia, Naglaa A. Gomaa*, Mohamed Abdelmegeid, Midhat N. Nassif

Department of Animal Medicine, Faculty of Veterinary Medicine, Kafrelsheikh University, 33516, Egypt

*Corresponding author, E-mail: naglaagomaa505@yahoo.com

Abstract: Acute kidney injury (AKI) is a common problem in dogs and is associated with high mortality. Early recognition of AKI is lifesaving and could help in clinical management. The purpose of this experimental study was to identify the accurate biomarkers for early detection of AKI in dogs. Ten healthy mongrel dogs (7 females, 3 males) were used in a crossover experimental study. Cisplatin (5 mg/kg BW) was used for induction of AKI. Serum urea and creatinine, as well as serum Sodium, Potassium, Calcium, Phosphorus, and Bicarbonate, were measured spectrophotometrically using commercial kits. However, urinary Neutrophil Gelatinase-Associated Lipocalin (NGAL) were determined using ELISA. Renal Resistive Index (RI) was calculated using Doppler ultrasound (duplex). All examinations were carried out on the same day of induction and for other 4 consecutive days. Post-mortem and histopathological examinations were carried out on two dogs. Using the repeated measure MANOVA and student T-test, the data were statistically analyzed. There were significant increases of both serum urea and creatinine beginning from the second day of infusion, while renal RI was significantly increase beginning from the third day of infusion. There are marked changes in serum electrolytes including hyperkalemia, hyperphosphatemia, hyponatremia with metabolic alkalosis. However, urinary NGAL showed a non- significant increase during the experiment. It can be concluded that renal RI can be used for early diagnosis of acute renal injury in dogs.

Key words: urinary biomarkers; renal resistive index; acute kidney injury; dogs

Introduction

Acute kidney injury is a common problem in dogs. It is costive either for diagnosis or for treatment and correlated with high mortality (1). The earlier diagnosis of such condition is regarded as lifesaving (2). There are many methods for detection of kidney injury such as estimation of glomerular filtration rate (GFR) (3). The routine technique used for detection of GFR is to measure serum creatinine concentration, but it is relatively insensitive with a delayed release in serum (4).

Novel serum and urine biomarker that can differentiate between both acute and chronic glomerular and tubular renal injury is the Neutrophil Gelatinase-Associated Lipocalin (NGAL) (5). It is a derived protein which increases in plasma and urine in acute ongoing renal injury after few hours (6). In human, NGAL

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was investigated in cases of AKI secondary to cardiac surgery, contrast-induced nephropathy and in critical illnesses. It was reported that elevated NGAL concentrations are a marker for AKI in such cases (7). Urinary NGAL is more sensitive and specific for predicting AKI and it was superior to creatinine and other markers of renal diseases (8).

One of the most advanced methods for the detection of AKI is the Doppler-based renal resistive index (RI) measurement. This is a rapid and non-invasive investigative tool that was used for early detection of AKI in the intensive care unit patients (9). Moreover, it can differentiate transient from persistent AKI in critically ill patients (10, 11). Recently, it was reported that an increase in the renal RI is an accurate and sensitive tool for early prediction of AKI in rats (12).

This study aims to compare the efficiency of selected serum biochemical parameters and the renal RI for early diagnose of AKI in dogs and to determine the most accurate, rapid, and applicable method in clinical practice.

Material and methods

Animals

Ten healthy mongrel dogs (7 females, 3 males) were used in this a cross over experimental study. Their weight ranged from 8 to 20 Kgs and their age ranged from 9 to 15 months. They considered healthy based on physical, hematological, biochemical and ultrasonographic examinations, in addition to a urine analysis. They were housed restless in separate boxes with plenty of food and water. The dogs were used as a control and treated group. In the control group, each dog was infused with normal saline at a dose rate of 5ml/kg BW. After two weeks, the same dogs were used as a treated group in which they were infused once with Cisplatin (Mylan, France) at a concentration of 5 mg/kg for induction of AKI. Physical, hematological, serum biochemical profiles, urine analysis, and ultrasonographic examinations were carried out on each dog of the treated group on the same day of induction and for other 4 consecutive days. All examinations were done after approval of the Ethics committee in Kafrelsheikh University.

Samples

Two blood samples were collected from each dog from the cephalic vein in the control and treated group. The first blood sample was about 5ml that collected in a heparinized test tube for hematological examination using Vet hematological analyzer (Mythic18, France). The second blood sample was collected in a plain test tube to obtain serum for biochemical analysis. The serum was collected by centrifugation of the clotted blood at 3000 rpm for 10 min. It was examined for blood urea nitrogen (BUN) using the colorimetric method by (BTS-302 Spectrophotometer) and (Urea Biosystem kit, Germany). Serum creatinine was estimated using the fixed time method by (BTS-302Spectrophotometer) and (Creatinine Biosystem kit, Germany). Some blood electrolytes such as Sodium, Potassium, Chloride, Calcium, Phosphorus and the Bicarbonate were measured in the serum using (Cobas C & Cobas E, France).

Urine samples were assembled by cystocentesis under ultrasonographic guide from each dog. Urine was examined macroscopically for colour and consistency. The specific gravity was measured by a standard Refractometer. The chemical examination for urine samples was accomplished using (Combi screen urine strips, Germany). Urine samples were examined microscopically for the presence of pus, cells, casts, and others. The investigation of Neutrophil Gelatinase-Associated Lipocalin (NGAL) was carried out using Sandwich enzyme-linked immunosorbent assay ELISA- kit (Canine NGAL Elisa Kit (Bioporto® Diagnostic) (13).

Ultrasonographic examination

Ultrasonographic examination was performed on unsedated dogs in both groups for detection of any changes in the tissue architecture, kidneys dimensions using 2-D mode. Renal (RI) for each kidney was measured using Doppler ultrasound (duplex), (Mindray Z5 ultrasound machine, China), with a linear transducer 5-10 MHZ.A renal interlobar or arcuate

artery was distinguished firstly by color Doppler then switched on the pulsed wave Doppler using a gate of 1.5 mm width over the selected artery. The obtained image showed the blood flow without any aliasing. Multiple pulses from one artery in three poles for each kidney right and left in each dog was recorded (14). The renal RI was measured atomically by the ultrasound machine from the selected artery.

Post mortem examination

It was performed on two dogs, one of them dead at the 5th day of the experiment and the second has been euthanized by a large dose of anesthetics and kidney specimens were picked in formalin 4% for histopathological examination.

Histopathology

Kidneys were removed, fixed in 4% buffered paraformaldehyde, embedded in paraffin with usual manner, sections were about 2.5 mm thick), and stained with periodic acid-schiff and periodic acid-methenamine silver for histologic examination under light microscope (15).

Statistical analysis

A doubly repeated measure MANOVA was used to test the significant effect of cisplatin on the parameter; urea, creatinine, urinary NGAL, and the renal RI among the examined animals at P value ≤ 0.05. This followed by univariate analysis for each parameter to examine its significant difference per day. Student T-test was used for comparison between serum electrolytes before and after the drug infusion and P value ≤ 0.05. All analyses applied using SPSS version 23.0 (IBM, New York, NY, USA).

Results

The physical examination of examined animals revealed; non-significant changes in all digital body parameters such as body temperature, heart rate respiratory rate and pulse. The mucous membranes and superficial lymph nodes were normal. Only signs of vomiting and diarrhea appeared after half hour of the drug infusion in all dogs. A degree of lethargy was clear on 5 dogs that obviously increased with time.

The physical examination of the urine samples showed changes in its colour beginning from the 3rd day of Cisplatin infusion in most dogs. The appearance of the urine changed gradually from the amber yellow colour to the turbid deep yellow then reddish bloody in two dogs. By chemical analysis, the urine samples showed traces of proteins and glucose beginning from the second day then increased gradually to three or four pluses at the end of the experiment using strips kits. The microscopical examination of urine sediments revealed a marked increase of epithelial cells, pus cells, RBCs, urate and phosphate crystals within the time of the experiment.

There were no obvious changes in the 2D ultrasonographic measurements between before and after the Cisplatin infusion. The renal dimensions (length, width, and height) were between (5.64-6.30 mm, 2.79-4 mm and 2.56-3.3 mm), respectively. On the other hand, there was a significant increase in renal RI of the interlobar arteries beginning from3rd day after infusion at P value ≤ 0.022 as in table 1 and (Fig. 1).

Concerning the blood serum biochemical analysis (Table1), revealed a significant increase in both BUN and creatinine at (P-value ≤ 0.000 and 0.002), respectively from the second day after cisplatin infusion. On the other hand, the urinary NGAL concentration showed non-significant during all the days of this study.

Regarding serum electrolyte analysis (Table 2) showed insignificant (P=0.058) decrease in serum sodium and insignificant (P =0.09) increase in the serum ionized calcium. While there was a significant increase in serum inorganic phosphorus, potassium, and bicarbonate ions at ($P\leq 0.01$, 0.01 and 0.000), respectively.

PM examination of the first dog revealed some congestion in both kidneys and intestine, while the second showed no abnormalities except slight congestion of kidneys (Fig. 2 A).

Histopathological examination showed numerous apoptotic renal epithelial cells and thickening of the glomerular membranes, periglomerular fibrosis and marked renal tubular damage (Fig. 2 B).

Table 1 Serum urea, serum creatinine, urinary NGAL and renal RI (mean and slandered deviation) after intravenous infusion of Cisplatin in dogs

	Control	Time post-infusion (day)					*P* value
		1	2	3	4	5	
Urea (mg/dl)	24.25±5.56	26±3.16	37.25±2.17**	81.25±31.19**	119.25±46**	327.5±54.65**	0.001
Creatinine (mg/dl)	0.94±0.06	0.91±0.07	1.35±0.07**	1.71±0.33**	2.72±0.28**	6.71±0.91**	0.000
Urinary NGAL (ng/ml)	0.82±0.25	2.23±1.09	1.67±0.94	2.03±1.18	2.17±0.97	2.15±1.26	0.131
Renal RI	0.62±0.03	0.62±0.02	0.64±0.03	0.68±0.03**	0.7±0.03**	0.74±0.04**	0.001

*Indicates a significant difference at different time points ($p<0.05$)
**Indicates a significant difference at different time points ($p<0.01$)

Table 2: Serum sodium, potassium, ionized calcium, phosphorus and bicarbonate (mean and slandered deviation) before and after 5th day of intravenous infusion of Cisplatin in dogs

		Before infusion	After infusion
Sodium	(mEq/l)	138 ± 2.58	126.25 ± 12.61
Potassium	(mEq/l)	4.18 ± 0.23	5.65 ± 0.9*
Ionized calcium	(mg/dl)	4.78 ± 0.25	5.28 ± 0.64
Phosphorus	(mg/dl)	4.98 ± 0.13	5.83 ± 0.59*
Bicarbonate (mmol/L)		18.48 ± 0.66	30.5 ± 4.20**

*Indicates a significant difference at $p<0.05$
**Indicates a significant difference at $p<0.01$

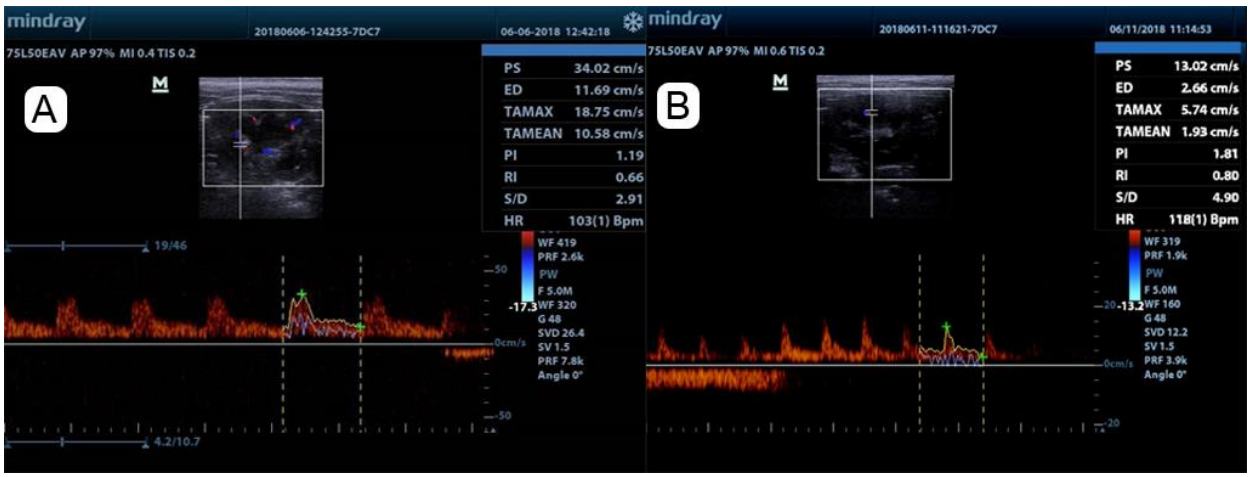

Figure 1: Pulsed wave Doppler ultrasonographic image of the interlobar artery before cisplatin infusion (A) and at the fifth day after the infusion (B)

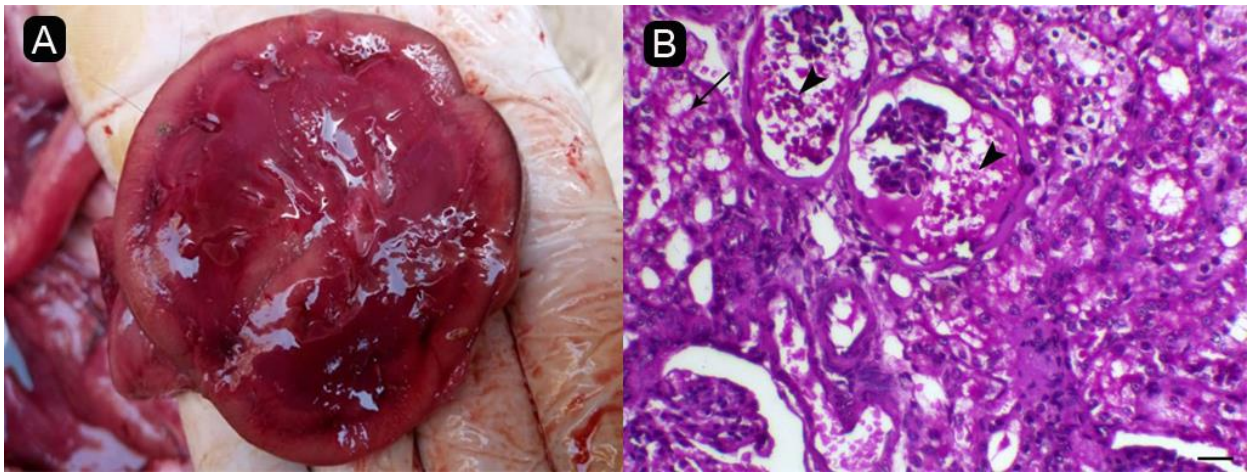

Figure 2: A sagittal section of the kidney showing congestion (A). Histopathological section of the kidney (B), shows marked necrotic changes with glomerular tufts which filled with eosinophilic materials (arrowhead) and marked degenerative and necrotic changes within the renal tubular epithelium (arrow), H&E, bar= 40 μm

Discussion

In this study, acute renal injury was induced using cisplatin 5 mg/kg BW which is a widely used chemotherapeutic agent for different malignancies. It has a marked nephrotoxic effect as it causes renal tubular cell necrosis due to the presence of platinum in its structure (16, 17). Some of the treated dogs showed vomiting and diarrhea after infusion of the drug. This could be a side effect of the cisplatin due to its irritating effect on the mucosa of gastrointestinal tract (18, 19). Days after the infusion, there was ataxia or muscle weakness appeared on the dogs and it may be contributed to electrolytes disturbance and dehydration**.**

After two days of the infusion, urine samples revealed an obvious increase of glucose and this may due to the failure of reabsorption of the glucose from the proximal convoluted tubules (20). Beginning from the 3rdday after infusion, there was a patent increase in proteins and RBCs in urine samples. This increase may be related to the decrease of glomerular filtration ate which follows the proximal tubular damage as an initial toxic effect of the cisplatin (17)**.** After two days of infusion, there was a highly significant increase in serum urea level compared with the control group. However, serum creatinine was significantly increased after three days of the infusion and this may be attributed to the impaired kidney function (15)**.** Although the estimation of urea and creatinine level in serum is routine work in the interpretation of kidney disease in the clinical practice, they have some limitations such as serum urea level may be increased in other abnormalities rather than kidney diseases such as liver dysfunction or increased dietary protein (21)**.** It may also increase in cases of gastrointestinal bleeding as a consequence of increased absorption of nitrogenous compound (22)**.** In addition, the magnitude of creatinine concentration cannot predict the origin and severity of renal dysfunction (23)**.** Moreover, its concentration is reduced in sepsis and so limits its use as a marker of kidney injury in case of bacterial infections (24).

NGAL is a neutrophil-derived protein related to lipocalin protein family (25). Its expression is upregulated by injured epithelia of renal tubules, trachea, bronchi, and also increase in the acute infections and different types of neoplasia (26, 27). NGAL is markedly upregulated in damaged proximal tubules in order to induce re-epithelization (28)**.** NGAL was reported as

an early urinary biomarker for acute renal injury in dogs (29). Besides, it can be used as a real-time indicator of progressive kidney damage (30). In this study, NGAL was insignificantly increasing after the 5th day of infusion of cisplatin. This insignificant increase of NGAL may be attributed to several causes such as the lower dose of cisplatin used in this study and the low sensitivity of the test in the detection of mild renal injury (28, 31). Therefore, the measurement of urinary NAGL is not efficient for the detection of mild kidney injury. Moreover, it increases in septic condition, so it gives a false positive result in septic cases (32).

Electrolyte disturbance usually occurs with kidney dysfunction especially those related to the renal tubules. The results of this study showed insignificant hyponatremia which may be attributed to reduced sodium conservation from damaged renal tubules (33). The significant hyperkalemia and hyperphosphatemia may have referred to the leakage of potassium and phosphate from the intracellular fluids to the extracellular fluids (43, 35). The highly significant increase of bicarbonate is unusual in such cases but, it may be due to vomition which develops an extra-renal cause of metabolic alkalosis. Alkalosis enhances calcium reabsorption in the distal nephron and this clarifies the insignificant increase in ionized calcium (36, 37).

Ultrasonography is very useful in the diagnosis of all aspects of renal diseases in clinical practice (38). In this study, each kidney was evaluated for any changes in echogenicity, length, width, height and volume by the 2 D mode. There were non-significant changes neither in the echogenicity nor in kidney dimensions when compared with the control group (39). The Doppler ultrasonography can be used for assessing the renal hemodynamic which is very useful in the diagnosis of renal arteries and veins disorders such as thrombosis (40).

The renal RI was significantly (p=0.022) increase beginning from the 3rd day of the infusion. It may have referred to vasoconstriction resulting from renin release in response to the decreased blood flow in the renal arteries secondary to renal injury (41). This result coincided with other studies on dogs with interstitial nephritis or tubular degeneration (42). In the present study, the increase of RI was parallel with the increase of serum creatinine after the third day of infusion. In another study on mice, the renal RI precedes the serum creatinine in a diagnosis of AKI after cisplatin infusion (12). However, there are some limitations for using RI in the diagnosis of all kidney diseases because it is affected by other hemodynamic disorders rather than in kidney as well as the difficulty of measuring RI without sedation of dogs which affected on its measurement (10, 12).

The histopathological picture supported the clinical and biochemical findings which indicating tubular renal injury with secondary glomerular affection. This result was similar to that described in mice kidney after using cisplatin for induction of renal failure (43).

Conclusion

The results indicate that increasing renal RI go parallel with increasing serum urea and creatinine in dogs with AKI. Although renal RI alone is not sensitive for predicting the developing of AKI, the high increase of renal RI can be used as an early signal for Acute renal injury because of its high specificity.

Conflict of interest

None of the authors have any conflict of interest to declare.

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Original Research Article

THERAPEUTIC EFFICACY OF ZINC OXIDE NANOPARTICLES IN DIABETIC RATS

Mohammed A. Kamal [1], Mohamed H. Khairy [1], Nada A. ELSadek [1], Mohamed M.A. Hussein [2]*

[1]Department of Pharmacology, Faculty of Veterinary Medicine, Zagazig University, [2]Department of Biochemistry, Faculty of Veterinary Medicine, Zagazig University, 44519, Egypt

*Corresponding author: Email: hamza_vet@yahoo.com

Abstract: This study attempted to scrutinize the potential efficacy of zinc oxide nanoparticles (ZnONPs) and the standard oral hypoglycemic drug glibenclamide in streptozotocin (STZ) -nicotinamide induced diabetic rats. Forty male Wistar rats were divided into four equal groups. Group 1 rats received saline orally and considered as a control group. Other groups were experimentally exposed to diabetes via intraperitoneal injection of STZ and group 2 was left as diabetic non-treated. Group 3 was orally administrated with ZnONPs at a low dose (1mg/kg), and group 4 orally received glibenclamide (600 µg/kg) for 30 days (duration of the experiment). Blood glucose, insulin levels, lipogram profile and pancreatic antioxidant status were improved following ZnONPs or glibenclamide administration as compared to the diabetic group. Moreover, histopathological examination revealed a better outcome in the ZnONPs and glibenclamide treated groups. Although oral treatment of ZnONPs at a low dose 1mg/kg body weight for 30 consecutive days had antidiabetic effect, this effect was less superior to glibenclamide. Therefore, further studies regarding increasing the dose of ZnONPs would be encouraging to get better anti diabetic effect.

Key words: Zinc oxide nanoparticles; antioxidant; diabetes; glibenclamide

Introduction

Diabetes mellitus (DM) is a metabolic disorder with chronic hyperglycemic and hypertriacylglycerolemic condition caused mainly due to defects in insulin secretion and/or action. The prevalence of diabetes is increasing rapidly worldwide (1). Zinc (Zn) is an essential micronutrient for pancreatic function through its effect on insulin stability and disturbance in Zn metabolism associated with diabetic complications (2). It is also a paramount player in the intracellular antioxidant machinery through its participation in the main antioxidant enzymes with free radical scavenging effect such as catalase, superoxide dismutase and metallothionein which attracted much attention in diabetes studies (3). Lower level of zinc in pancreatic tissues associated with lower insulin synthesis through β cells (4). Elevated ROS can induce oxidative damage in pancreatic tissues with subsequent increased hyperglycemia (5).

The field of nanotechnology is one of the every foremost active analysis areas in fashionable materials science and biology (6, 7). Nano-

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particles come with new properties and biomedical applications which owed to its size, distribution and morphology (8).On the other hand, the use of nanoparticles has a dark side through their generation of reactive oxygen species (ROS), resulting in oxidative damage and inflammation (9,10). Zinc oxide nanoparticles (ZnONPs) are widely used in paint, pharmaceutical industry, and cosmetic industries; furthermore, they have antimicrobial action (11). The harmful effects of ZnONPs could be detected through increasing the expression of adhesion molecules in endothelial cells, resulting in inflammation (12, 13). High dose of ZnONPs was used as a new anti diabetic agent (14) but at the same time this higher dose led to release of ROS (15, 16). However, the potential effect of ZnONPs low dose on diabetes has not been elucidated yet.

Therefore, the present study was designed to investigate the potential therapeutic efficacy of ZnONPs at low dose, relative to glibenclamide as a standard oral hypoglycemic drug, on diabetic rats with regard to lipogram profile and antioxidant status in addition to the histopathological picture of the pancreas.

Material and methods

Drugs and chemicals

Zinc oxide nanoparticles (ZnONPs) were obtained from the Faculty of Science, Department of Physics, Zagazig University in the form of dispersion. The average nanoparticle size was less than 50 nm as detected by transmission electron microscope (Fig.1). The ZnONPs distribution was detected using dynamic light scattering (DLS) technique, pH 7±0.1 for aqueous systems and density 1.7 g/ml± 0.1 at 25 ºC. The standard anti diabetic drug glibenclamide was used under a trade name of Daonil®, (Sanofi Avents Co. for Pharmaceutical Industries, Egypt, 5 mg active ingredient per tablet). Streptozotocin (STZ) was purchased from Sigma Chemicals Co., St. Louis, MO, USA.

Animals

Forty male Wistar rats, 2 months old and average body weight of 150± 20gm, were housed in metal cages at (23± 2ºC) with a light-dark (12:12 h) cycle and food and water *ad libitum*. Animals were kept two weeks before starting the experiment to be accommodated under laboratory condition. The guidelines and ethical rules of Zagazig Veterinary Medicine have been followed.

Experimental design

Animals were allocated into 4 equal groups (10 animals per group). The 1st group was considered as a control and received only saline solution. Second group was diabetic, non-treated while the 3rd and 4th groups were diabetic rats and orally received 1mg/kg body weight ZnONPs (17) and 600 mg/kg body weight glibenclamide (18) for 30 days using stomach tube.

Type 2 diabetes was induced by single intraperitoneal injection of a freshly prepared solution of streptozotocin (60 mg/kg dissolved in citrate buffer pH 4.5) then after fifteen minutes, nicotinamide (95 mg/ kg) dissolved in saline was intraperitoneally injected (19). Rats were allowed to drink 10% glucose solution over night to overcome drug-induced hypoglycemia. Rats were considered diabetic when their blood glucose reached above 250 mg/dl. The animals were considered diabetic when their blood glucose levels became above 250 mg/dl on the 3rd day after STZ injection.

Blood biochemical parameters

At the end of the experiment (after 30 days), blood samples were collected from the medial canthus of the eye in either EDTA coated tubes (for determination of blood glucose) or plain tubes (for serum biochemical analysis). Blood glucose values were recorded using commercially available kits following manufacturer's instructions (20). Serum insulin levels were evaluated using commercially available ELISA kit. Serum triglyceride, total cholesterol, High-density-Lipoprotein cholesterol (HDL-c), low density lipoprotein cholesterol (LDL-c), and very low density lipoproteins (VLDL) were determined by the methods described by (21-24).

Antioxidant status and oxidative stress assay

After euthanization, the spleen was quickly excised, rinsed with saline and tissue homogenate was prepared as previously described (25). The obtained supernatant from pancreatic homogenate was used for determination of antioxidant enzymes activities [glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD)] and lipid peroxidation (LPO) contents as previously described (26-28).

Histopathological examination of pancreas

Samples from the pancreas of all groups were collected and fixed in 10% neutral formalin for 24 h prior to routine processing in paraffin wax. Samples were cut into 5 µm sections. Slides were stained with Hematoxylin & Eosin (H&E) and examined microscopically.

For morphometric analysis of the Islets diameter, Islets were isolated by a modification of the automated method described by (29). In each animal one slide was stained and 25 images/group were taken using Am Scope 5.0 MP microscope digital camera at 400 X magnification. Islets diameters were measured by using Mitocam® plus 2.0 (Motic Images plus 2.0, china) and Islets cells were counted using Image 1.45s software (National Institute of Health USA).

Data analysis

Data were expressed as mean ± standard error (SE). Difference between groups was detected using one way ANOVA followed by Duncan's Multiple Range Test using SPSS version 21. Values at $P < 0.05$ were considered statically significant.

Results

Effect ZnONPs and glibenclamide on blood glucose and serum insulin levels

Blood glucose and serum insulin levels were significantly elevated in STZ treated animals as compared to the control animals (Table1). Both ZnONPs and glibenclamide treated animals exerted a significant decrease in blood glucose levels as compared to STZ-treated animals. However, among the treated rats, glibenclamide treatment showed better results than ZnONPs.

Effect ZnONPs and glibenclamide on lipid profile parameters

STZ treated animals exhibited a significant higher serum levels of total cholesterol (TC), triglyceride (TG), LDL-c and VLDL and a significant lower HDL-c level relative to the control group (Table 1). In contrast, ZnONPs and glibenclamide treated groups exerted a significant decrease in TC, TG, LDL-c and VLDL and a marked increase in HDL-c compared to STZ treated animals. Glibenclamide evoked a better result compared to ZnONPs treated rats.

Effect ZnONPs and glibenclamide on antioxidant/oxidative status

Antioxidant scavenging potentials for ZnONPs and glibenclamide treated animals were figured out in Table 2. The diabetic group showed a marked decrease in antioxidant enzymes (SOD, GPx, and CAT) activities and a significant increase in lipid peroxidation marker MDA. However, ZnONPs and glibenclamide treated groups exerted a significant increase in antioxidants (SOD, GPx, and CAT) activities and decrease in MDA levels as compared to diabetic animals.

Histopathological examination

Examined sections revealed normal size, population and structures of the pancreatic cells, normal Langerhans islet components in the control group (Fig., 2A). However, pancreas of diabetic rats showed degenerative changes, cytoplasmic vacuolation, apoptosis and hypo-cellularity of most of β-cells of islets of Langerhans, but alpha cells were normal in most parts (Fig., 2B). Pancreas of rats treated with glibenclamide showed normal histologic appearance with normal size, population and structures with mild congestion of islets capillaries (Fig., 2C). Pancreas of rats treated with ZnONPs showed apparently normal islet cells with preserved size and cellular population, with a few cells either apoptotic or hypertrophied (Fig. 2D).

Table 1: The Effect of ZnONPs and glibenclamide on lipid profile, blood glucose and insulin level on healthy and diabetic rats

Group	Total cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL-c (mg/dl)	LDL-c (mg/dl)	VLDL (mg/dl)	Blood glucose (mg/dl)	Insulin (µIu/ml)
Control	195.66± 2.33^{c}	120.33± 3.17^{d}	57.33± 4.48^{a}	137.64± 4.09^{d}	24.06± .64^{d}	101.8± 10.29 ^{c}	2.82± 0.067 ^{a}
Diabetic	295.28± 6.87^{a}	186.85± 2.38^{a}	26.71± 1.64^{c}	284.20± 5.06^{a}	37.37± .48^{a}	335.6± 6.15 ^{a}	0.90± 0.045 ^{d}
Diabetic treated with ZnONPs	241.66± 4.40^{b}	154.66± 2.90^{b}	34.66± 2.60^{c}	214.96± 7.04^{b}	30.93± .34^{c}	231.8± 6.15 ^{b}	1.24± 0.035 ^{c}
Diabetic treated with glibenclamide	226.25± 4.09^{b}	137.50± 1.70^{c}	47.250± 2.3^{b}	181.54± 5.68^{c}	30.93± .58^{b}	221.2± 3.70 ^{c}	1.95± 0.054 ^{b}

Means within the same column carrying different superscripts are significantly different at P<0.05

Table 2: The Effect of ZnONPs and glibenclamide on pancreatic antioxidant/oxidative stress status on healthy and diabetic rats

Group	CAT (U/gm tissue)	MDA (nmol/gm tissue)	SOD (nmol/gm tissue)	GPx (U/gm tissue)
Control	199.79± 5.681^{a}	5.623± 0.253^{c}	21.040± 1.185^{a}	116.33± 1.789^{a}
Diabetic	125.165± 3.200^{c}	22.667± 1.015^{a}	4.468± 0.322^{d}	65.200± 2.279^{d}
Diabetic, ZnONPs treated	161.48± 4.015^{b}	15.323± 1.433^{b}	9.656± 1.258^{c}	87.69± 4.614^{c}
Diabetic, glibenclamide treated	193.43± 4.768^{a}	8.837± 1.236^{c}	15.017± 0.933^{b}	101.590± 3.398^{b}

Means within the same column carrying different superscripts are significantly different at P<0.05

Table 3: Lesion scores of different changes related to islets of Langerhans among experimental groups

Lesions	Necrosis of islets cells	Apoptosis of islets cells	Degeneration of islets cells	Cytoplasmic vacuolation of islet cells	Congestion of islets capillaries	Interstitial inflammatory cells aggregations	Congestion of pancreases blood vessels	Perivascular fibrosis
Control	-	-	-	-	-	-	-	-
Diabetic, non-treated	+++	+++	+++	+++	-	-	+++	+++
Diabetic, ZnONPs treated	+	++	++	++	+++	+++	-	-
Diabetic, glibenclamide treated	-	-	-	-	+	-	-	-

(-=No, +=Mild, ++=Moderate, +++= Severe)

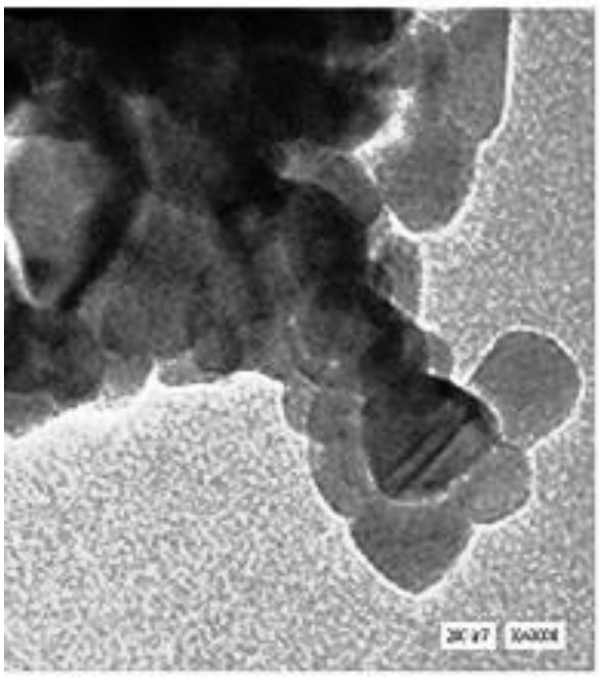

Figure 1: Transmission Electron Micrograph (TEM) image of ZnONPs. The beam of electrons transmitted through the specimen shows 50 nm

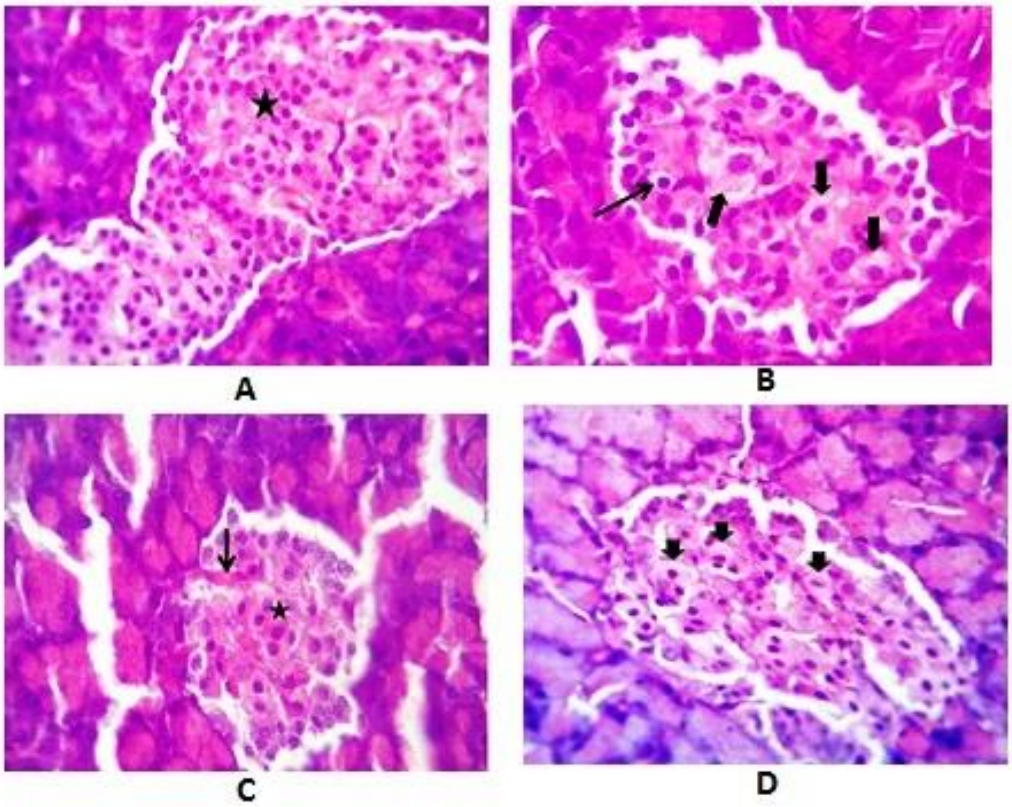

Figure 2: Photomicrograph of pancreatic tissues of experimental groups. A) Normal Langerhans islet components (star) in the control rat H&E X400. B) Diabetic rat pancreas shows hypo-cellularity of Langerhans with cytoplasmic vacuolation (thick arrows) and apoptosis (thin arrow). C) Pancreas of rat treated with glibenclamide shows normal islets structures (star) with mild congestion of islets capillaries (thin arrow). D) Pancreas of rat treated with ZnONPs shows apparently normal with preserved size and cellular population, with a few cells either apoptotic or hypertrophied (thick arrows). H&E X400

Discussion

The pathogenesis of type 2 diabetes relays on insulin resistance and chronic hyperglycemia and hyper triacylglycerolemia (30). The enhanced role of Zn in diabetes mellitus pathogenesis especially through its effect on insulin synthesis (15, 31) makes correction of zinc imbalance a matter of essence where the easily passage of oral administered nanoparticles through biological membranes makes them a promising therapeutic agent (14, 32). In the present study, we observed a significant increase in blood glucose levels in diabetic rats. This may be due to the destruction of pancreatic beta cells by STZ. This reinforces the fact that STZ induces diabetes probably through the generation of oxygen free radicals (33). The elevation of glucose in rats received STZ was due to an oxidative stress produced in the pancreas and also probably due to a single strand break in pancreatic islet DNA (34). Our results showing that oral administration of ZnONPs to STZ diabetic rats revealed a significant decrease in blood glucose levels. Rinku and Paknikar (35) results were in agreement with our results. Glibenclamide treatment showed better results when compared to ZnONPs treated group.

The significant role of insulin as a hypoglycemic hormone owed to its ability to stimulate glucose oxidation and storage of it either in the form of glycogen and triglyceride in adipose tissue (36). The inability of skeletal muscle, adipose tissue, liver and peripheral tissues to respond to insulin results in insulin resistance which is the core for type 2 diabetes pathogenesis. The pancreas is able to produce sufficient levels of insulin to maintain glucose levels beneath the diabetic threshold (37). In the present study, STZ diabetic rats showed marked depletion in serum insulin. STZ causes diabetes by the rapid depletion of β-cells; thereby bring about a reduction in insulin release. The oral administration of low dose ZnONPs or glibenclamide to STZ diabetic rats revealed a significant improvement in serum levels in insulin. This indicates ZnONPs and glibenclamide ability to improve insulin sensitivity and increased glucose utilization (15, 34). Again the best ant diabetic effect was noticed in rats treated with glibenclamide. The latter has the ability to increase pancreatic beta cells production of insulin. The long duration of glibenclamide action and its metabolites could increase its prolonged hypoglycemic risk (18).

The chronic diabetic state was also associated with dyslipidemia. Administration of STZ caused an increase in serum TC and TG (37). Similarly, we found that STZ-diabetic rats showed a marked increase in lipid profile parameters, TC, TG, LDL and VLDL, while there was a marked reduction in serum HDL-c. On a similar basis, Rinku and Paknikar, (34) found a marked elevation in serum TG following STZ administration. Oral treatment of STZ-diabetic rats with low dose of ZnONPs and glibenclamide evoked a significant decrease in serum TC, TG, LDL and VLDL but with a marked increase in serum HDL-c. Consistent with our findings, a marked reduction in lipid profile parameters following treatment with ZnONPs at 3 mg and 10 mg/kg was also reported in diabetic rats by another study (15).

Oxidative stress is defined as an imbalance between cellular production of oxidant molecule and the availability of appropriate anti-oxidants that defend against them (38). Continued oxidative stress leads to the development of chronic diseases, including diabetes mellitus, cancer, neuro-degeneration, cardio vascular and metabolic disease (39). Herein, we found that administration of STZ resulted in an observable increase in lipid peroxidation marker MDA levels and a significant decrease in the anti-oxidant activity of GPx, CAT, and SOD in pancreas as compared to the control rats. Similarly, a significant increase in hepatic and renal MDA, with a marked reduction in hepatic and renal GPx, CAT, and SOD activities were also reported in diabetic rats (20). In contrast, oral administration of ZnONPs or glibenclamide to diabetic rats showed an increase in GPx and CAT activities, with better results with glibenclamide, relative to diabetic untreated rats. Interestingly, the oral treatment with higher doses of ZnONPs (3 and 10mg) also produced a marked reduction in pancreatic GPx

and CAT activities in comparison to diabetic non treated rats (15, 40).

In the present study, STZ administration revealed degenerative changes, cytoplasmic vacuolation, apoptosis and hypo-cellularity of most of β-cells of islets of Langerhans, but alpha cells were normal in most parts. Similarly, Daisy et al., (41) found altered islet structure in rats treated with STZ at a dose of 40 and 50mg/kg body weight. Histopathological examination revealed a better outcome in the ZnONPs and glibenclamide treated groups.

Conclusion

Oral treatment of ZnONPs at a low dose 1mg/kg body weight for 30 consecutive days had anti diabetic effect. However, this effect was less superior to glibenclamide and hence further studies regarding increase the dose of ZnONPs would be encouraging.

Conflict of interest statement

None.

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Original Research Article

IDENTIFICATION OF VIRULENT NEWCASTLE DISEASE VIRUSES IN BROILERS IN KAFRELSHEIKH GOVERNORATE THROUGH THE YEAR 2017

Abd Ellwahed El-Shawadfi[1]*, Fares El-Khyat[2], AbdEl-Galil El-Gohary[2]

[1]General Organization of Veterinary Services, Ministry of Agriculture, Egypt. [2]Department of Poultry& Rabbits diseases, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt

*Corresponding author, E-mail: dr_abdelwahed1@yahoo.com

Abstract: The aim of the present study was to identify virulent Newcastle disease viruses (NDVs) circulating in broilers in Kafrelsheikh Governorate during the year 2017. Recognition of the virulent NDV strains to identify their degree of identity with the currently used vaccines against Newcastle disease in order to predict degree of protective immunity that can be offered by traditionally vaccines used against these virulent NDVs. The obtained results revealed presence of 11 samples positive for NDV as detected by inoculation into chick embryo via allantoic cavity route followed by HA and HI test. Furthermore, by using specific primers for F region to detect genotype VII, only 4 out of the positive 11 samples belonged to class II genotype VII sub genotype d were detected.

Key words: Newcastle disease virus; genotype VII; broilers; Egypt

Introduction

Newcastle disease (ND), a highly infectious viral disease targeting both wild and domestic bird species, was first detected in a poultry flock near the town Newcastle on Tyne (UK) (1). Newcastle disease virus (NDV), a member of the *Paramyxoviridae* family, is designated avian paramyxovirus serotype1. ND was first determined in Egypt by (2). According to virulence of NDV strain ND, the severity of the disease ranged o from subclinical infection to death (3). Virulent NDV strains which belong to genotype VII sub-genotype d are frequently isolated from Egyptian chicken farms causing severe economic loses especially in Kafrelsheikh Governorate (4). The pathogenesis of NDV depends on the virulence of the infecting virus and its tropism. The host to a large extent plays an important role in the virulence of NDV strains. Chickens are highly susceptible while ducks and geese may not show clinical signs when infected, even by lethal strains to chickens, in which the age, immune status of the bird, dose of the virus and route of exposure; nasal, oral, and ocular; appear to emphasize the respiratory nature of the disease (5). All these factors in addition to concurrent infection and environmental stressors go a long way to determine the course of the disease or infection (6).

Based on results obtained from phylogenetic analysis, it was postulated that gene mutations can induce conversion of the non or low virulent NDV strains into virulent or high virulent strains (7). Thus, percussions should be considered regarding the current applied diagnosis and control methods of ND, mainly due to enormous amounts of live vaccines used throughout the world (7). So, the detection of the NDV by virus isolation by inoculation in

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fertilized chicken eggs was still acting the prescribed method for the international trade and remained the method of choice for confirmatory diagnosis (8) and (9). Molecular detection of the F0 cleavage site sequence of NDV by PCR was used not only in characterization of NDV but also as a good predictor for NDV virulence (10).

Frequent occurrence of ND outbreaks in broiler flocks in Kafrelsheikh Governorate and the unknown sources of the virulent NDVs in these outbreaks were the integral factor to conduct this study. Therefore, this study aimed to recognize the new virulent NDV strains and compare the isolated sequences with those currently used in vaccines against ND. Consequently, these data could help us to achieve a perfect immune response. In the present study, we also used conventional methods and modern molecular techniques for accurate diagnosis of NDV

Materials and methods

Samples, inoculation, HA and HI

Spleen, liver, trachea, lung and cecal tonsils were collected from sick birds from 50 suspected flocks located at Kafrelsheikh governorate. Samples from each individual flock were pooled together. Pooled samples were minced and diluted with phosphate buffered saline 10%, centrifuged and used for inoculation of 10 days chick embryo via allantoic cavity route (11). Allantoic fluid was harvested after 3 days, kept at -20 until tested by HA followed by HI test using 96 well microtiter plates and reference antisera (8). The HI endpoint was determined as the highest dilution of the serum that causing complete inhibition of HA activity of 4HA unit of antigen.

Rreal time PCR (qPCR)

qPCR (F-assay)-was performed according to (12), to detect virulent NDVs dependant on the amino acid sequence around this site 112-117 of the F gene using the following oligonucleotide primers and probes.

Forward:

F+4839 TCCGGAGGATACAAGGGTCT

Reverse:

F-4939 AGCTGTTGCAACCCCAAG

Probe

F+4894 [FAM]AAGCGTTTCTGTCTCCTTCCTCCA[TAMRA]

Molecular diagnosis using RT-PCR

Before sequencing the positive isolated strains detected by qPCR, we performed reverse transcription PCR (RT-PCR) to prepare F gene for sequencing. This was conducted using the following primer sequences: NDV-M2: 5' TGGAGCCAAACCCGCACCTGCGG-3' and NDV-F2: 5'-GGA GGA TGT TGG CAG CATT-3' as previously described (13). The PCR products were visualized by 1.5 % agarose gel electrophoresis. Two ways sequencing was carried out by MacroGene Company, South Korea and the obtained sequences were annotated and analyzed by Geneious software version 4.6.4 (14).

Results

Inoculation of the 50 specimens in fertilized chick eggs resulted in 12 positive allantoic fluid samples as detected by slide hemagglutination agglutination (HA) test. The hemagglutination inhibition (HI) test revealed that 11 out of 12 allantoic fluid samples were positive for NDV (Table 1).

The qPCR (F-assay) for detection of virulent NDV revealed that only 4 (1, 5, 10, 11) out of 12 farms were positive as compared to the control FAM 18.93 with amplification plots 19.32-32.5. This indicates that these positive samples were virulent NDV isolates. While the other 8 farms (2, 3, 4, 6, 7, 8, 9, 12) were non virulent NDV isolates. (Table 2). The virulence depended on the theory of amino acid sequences in F0 at the site of 112-117 in which at least three basic amino acids at the position of residues 113 to 116 and phenylalanine (F) at 117 are present (10)

Before sequencing we isolated F gene (that encoded F-protein including the F0 cleavage site) using RT-PCR and positive samples gave PCR products with size of 109 bp similar to positive control samples (Fig.1). After sequencing, we found 2 virulent NDV isolates

NDV/Egypt/Kafrelsheikh1/2017 and NDV/Egypt/Kafrelsheikh 2/2017 strains (these sequences were deposited in GenBank databases) which both related to the genotype VII sub-genotype d. Similarly, (15) also determined genotyping of NDV isolates using the same gene.

Comparing nucleotide sequence of F-protein encoding gene of NDV/Egypt/Kafrelsheikh1/2017 and NDV/Egypt/Kafrelsheikh2/2017 isolates with some isolated virulent NDV outbreaks strains showed clustering of the two Egyptian isolates with the very virulent NDV strain NDV/Chicken/China/sdwf07/2011, NDV/Chicken/China/sdwf07/2011, EG/CK/NDV/16/Luxor.Esna/2011 and NDV/EG/CK/101/2012. Also, they were clustered with recent very virulent NDV strains NDV-F388-RLQP-CH-EG-14, NDV/Dove/Desouk/Egypt/MSS/2015 and Chicken/Israel/555/2015 (Fig. 2). All these strains belonged to genotype VII sub genotype d as agreed and confirmed by previous studies (4), (16) and (17).

Table 1: Result of NDV identification after the inoculation in fertilized chick eggs

Total samples	+ve samples to HA test	-ve samples to HA test	+ve samples to HI test	-ve samples to HI test
50	12	38	11	1
_	24%	76%	22%	2%

Table 2: Result of qPCR (F- assay) for detection of virulent NDVs

Well Name	Dye	Ct (dRn)
Pos	FAM	18.93
10	FAM	22.01
5	FAM	25.81
1	FAM	23.57
11	FAM	29.78
Neg	FAM	No Ct
8	FAM	No Ct
12	FAM	No Ct
2	FAM	No Ct
6	FAM	No Ct
3	FAM	No Ct
7	FAM	No Ct
9	FAM	No Ct
4	FAM	No Ct

Assays shown: ROX, FAM, HEX — Well types sho: Unknown

Pos (+ve) fam indicate virulent strain while **Neg (-ve)** fam indicate non virulent strain.

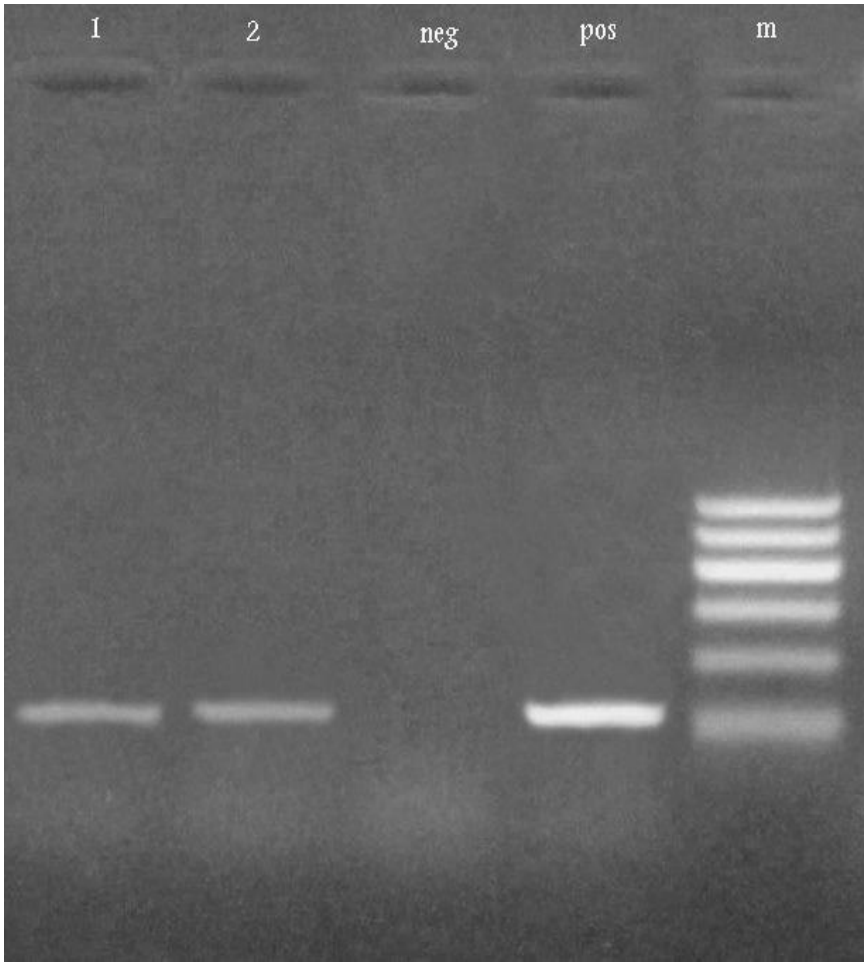

Figure 1: Ethidium bromide agarose gel electrophoresis of the RT-PCR products (109 bp). 1 and 2 were the isolated virulent NDV strains, neg: negative control, pos: positive control, M: marker (100 bp)

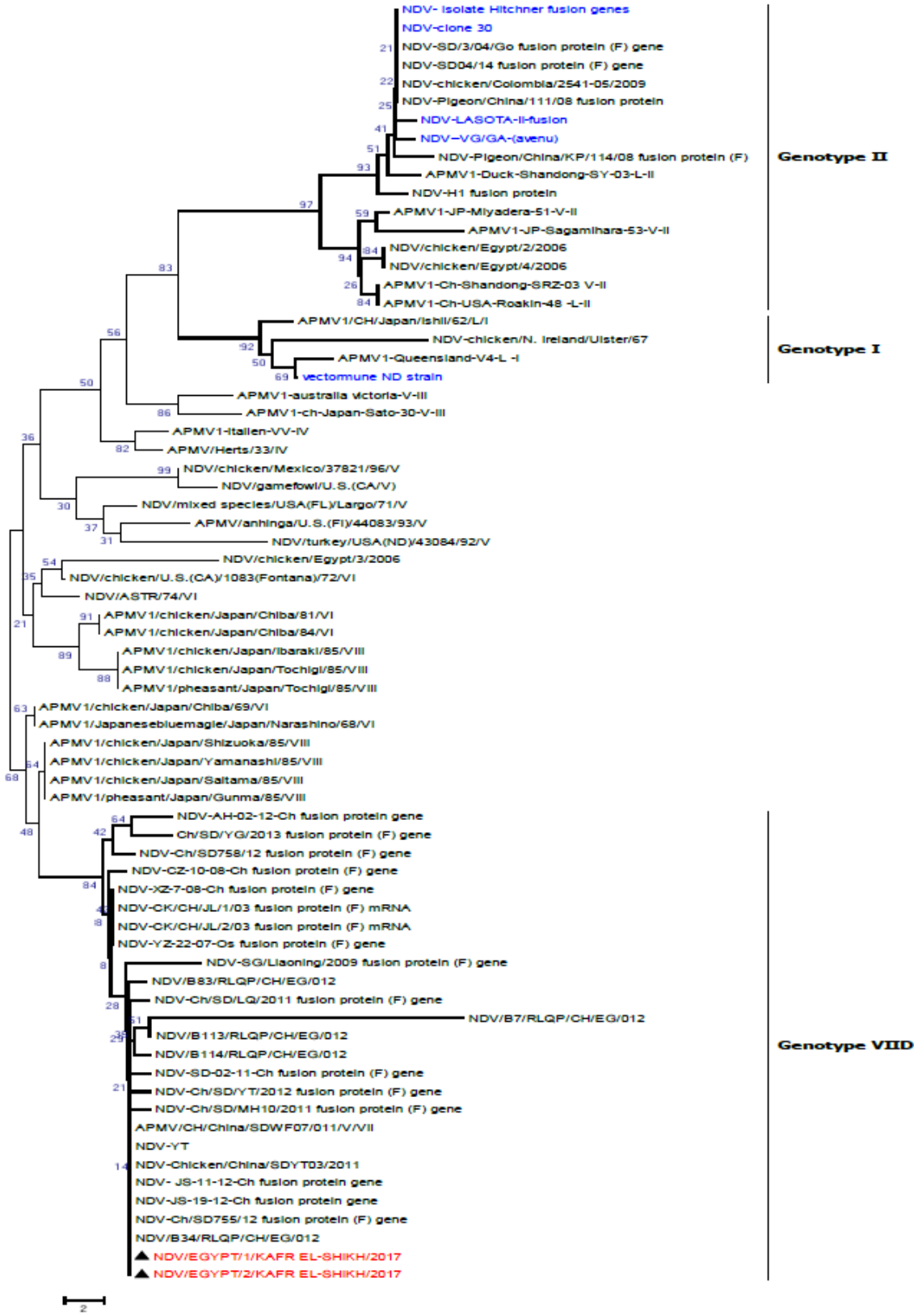

Figure 2: The phylogenetic tree of neighbor joining phylogenetic analysis based **o**n nucleotide sequence clustering of F- gene of genotype VII subtype d

Conclusion

The obtained results revealed that Egyptian chicken contained virulent NDV strains, especially class II genotype VII sub genotype d, which may be associated with severe economic losses of ND in Egypt in general and in Kafrelshiekh Governorate in particular.

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Original Research Article

MOLECULAR IDENTIFICATION OF *Aeromonas hydrophila* STRAINS RECOVERED FROM KAFRELSHEIKH FISH FARMS

Ahmed Mansour[1*], Nadia B. Mahfouz[2], Mona M. Husien[1], Mohammed A. El-Magd[3]

[1]Fish Diseases Research Department, Animal Health Research Institute, Agricultural Research Center, Dokki, Giza, Egypt, [2]Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt, [3]Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt

*Corresponding author, E-mail: drahmed_1986@hotmail.com

Abstract: The aim of this study was to recover *Aeromonas hydrophila* from Kafrelsheikh tilapia (*Oreochromis niloticus*) farms and to study its virulence. Adult fish (n = 100) exhibiting the onset of clinical signs were bacteriologically examined using aeromonas isolation base medium. The isolates were identified by PCR using *A. hydrophila*-specific 16S rRNA primers and the virulence was determined using specific primers for ten virulence genes targeting hidrolipase (*Lip*), elastase (*ahyB*), lipase (*pla/lip*), aerolysin (*aer*), cytotonic enterotoxin (*alt*), cytotoxic enterotoxin (*act*), temperature sensitive protease (*eprCAI*), serine protease (*Ahp*), haemolysin (*hlyA*), and cytotonic heat stable enterotoxin (*ast*). Molecular screening revealed presence of 35 isolates positive for *A. hydrophila*-specific 16S rRNA and 4 virulence genes (*aer*, *pla/lip*, *ast*, and *hlyA*). It also showed that the majority of the examined strains carried one or more virulence genes. These data indicate higher virulence for *A. hydrophila* in infected *O. niloticus* in Kafrelsheikh fish farms.

Key words: PCR; *A. hydrophila*; virulence genes

Introduction

Fish provide cheap healthy protein source to human especially in coast countries, such as Egypt. Therefore, it is not strange to find a fast increase in aquaculture production sector in Egypt during the last two decades. However, this huge aquaculture resulted in elevation of prevalence, pathogenicity, and drug resistance of some bacteria strains. *Aeromonas hydrophila*, a motile opportunistic aeromonad that normally lives in fish gut, is one of the most common bacteria infected a large variety of fish (including marine and fresh water fish), especially those reared in aquaculture where they subjected to many stressors (inadequate PH, extreme temperature, hypoxia, malnutrition) mainly caused by overcrowding. These stressors depress fish immunity and allow this opportunistic aeromonad to invade fish causing hemorrhagic septicemia or motile aeromonas septicemia (MAS) characterized by high morbidity and mortality thereby leading to severe economic loss to fisheries (1). The typical symptoms of this disease include tail rot, hemorrhage, ulcer and scale desquamation and exophthalmia (2).

To achieve appropriate treatment for bacterial diseases, we should first accurately identify

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not only the bacterial species but also their strains and serotypes. Unlike other animals, there some limitations regarding diagnosis of bacterial pathogens in fish due to lack of rapid, sensitive and accurate means by which fish pathogens can be detected. Although, most of bacteria can be successfully isolated following their culture on specific media, this traditional method of diagnosis failed to accurately differentiate between different strains and serotypes (2, 3). In contrast, molecular based identification can accurately identify different bacterial strains and serotypes quickly, more specifically and in a more sensitive and reliable way. Polymerase chain reaction (PCR) was successfully applied to not only detect bacterial pathogens in fish but also differentiate between different bacterial strains and serotypes (3-7). Therefore, this study was designed to screen for prevalence of *A. hydrophila* virulent strains in Kafrelsheikh governorate *O. niloticus* farms.

Materials and methods

Sampling and bacteriological examination

Fish (n = 100) showing clinical signs of skin hemorrhages were aseptically streaked on tryptic soy agar and sub-cultured until obtaining pure colonies then tested on aeromonas isolation base medium to monitor the characteristic bull eye shaped colonies. All bacterial cultures were incubated at 28°C for 24 hours. The biochemical tests were done using commercially available API20NE (Biomerieux©, France).

DNA extraction and PCR amplification

DNA extraction were done by thermolysis after culturing of bacterial strains on brain heart infusion broth and incubated at 37°C for 12 hours in shaker incubator. PCR amplification of *A. hydrophila* specific-16S rRNA gene and virulent genes was performed in thermal cycler using 25µl mixture containing 0.5 µl DNA polymerase (5 U/ml), 2.5 µl 10 X PCR buffer, 2.5 µl dNTP mixture, 1 µl of each primer (20 pmol/µl, Table 1), 5 µl genomic DNA, and 12.5 µl DNAse free water. PCR conditions were done as previously described (4-7).

Results

Bacterial culturing revealed isolation of 35 isolates of A. hydrophila from 100 fish showing typical A. hydrophila clinical signs. The isolated bacterial colonies were creamy-white, circular and convex, and 2-3 mm in diameter on TSA agar plates and resembled bull eye shape with dark green center and light periphery on aeromonas isolation base medium (Fig. 1).

Molecular characterization confirmed detection of 35 isolates positive to 16S rRNA gene with a size of 103 bp (Fig. 2). Among the screened 10 virulence genes; only 4 genes (*pla/lip*, *ast*, *aer* and *hlyA*) were found in 21 out of 35 isolates and were distributed as follow: *pla/lip*, *ast*, *aer* and *hlyA* in 5 isolates; *pla/lip*, *aer*, and *ast* in 7 isolates; *pla/lip* and *aer* in 6 isolates and *pla/lip* in 3 isolates. Fourteen strains were free from any virulence genes. The prevalence of *A. hydrophila* virulence genes among the virulent strains were distributed as follow: 100% (21/21) for *pla/lip*, 57% (12/21) for *ast*, 86% (18/21) for *aer* and 24% (5/21) for *hlyA* (Tables 2 and 3; Figs. 3-6). Depending on the frequency of the virulence genes in positive *A. hydrophila* strains, the genotypes carrying virulence genes represent (60.7%), while the genotypes free from virulence genes represent (39.4%).

Table 1: Primers used for detection of *A. hydrophila* virulence genes

Gene	Primers 5' → 3'	Size (bp)	References
16S rDNA	F: GGCCTTGCGCGATTGTATAT R: GTGGCGGATCATCTTCTCAGA	103	(7)
Hidrolipase (*Lip*)	F:AACCTGGTTCCGCTCAAGCCGTT R:TTGCTCGCCTCGGCCCAGCAGCT	65	(6)
Elastase (*ahyB*)	F: ACACGGTCAAGGAGATCAAC R: CGCTGGTGTTGGCCAGCAGG	540	
Lipase (*pla/lip*)	F: ATCTTCTCCGACTGGTTCGG R: CCGTGCCAGGACTGGGTCTT	383 - 389	
Aerolysin (*aer*)	F: CCTATGGCCTGAGCGAGAAG R: CCAGTTCCAGTCCCACCACT	431 - 1987	
Cytotonic entero-toxin (*alt*)	F: TGACCCAGTCCTGG R: GGTGATCGATCACC	442	(5)
Cytotoxic entero-toxin (*act*)	F: GAGAAGGTGACCACCAAGAACA R: AACTGACATCGGCCTTGAACTC	232	
Temperature sensi-tive protease (*eprCAI*)	F: GCTCGACGCCCAGCTCACC R: GGCTCACCGCATTGGATTCG	387	
Serine protease (*Ahp*)	F: ATTGGATCCCTGCCTA R: GCTAAGCTTGCATCCG	911	
Haemolysin (*hlyA*)	F: GGCCGGTGGCCCGAAGATACGGG R: GGCGGCGCCGGACGAGACGGGG	392	
Cytotonic heat stable enterotoxin (*ast*)	F: TCTCCATGCTTCCCTTCCACT R: GTGTAGGGATTGAAGAAGCCG	331	(4)

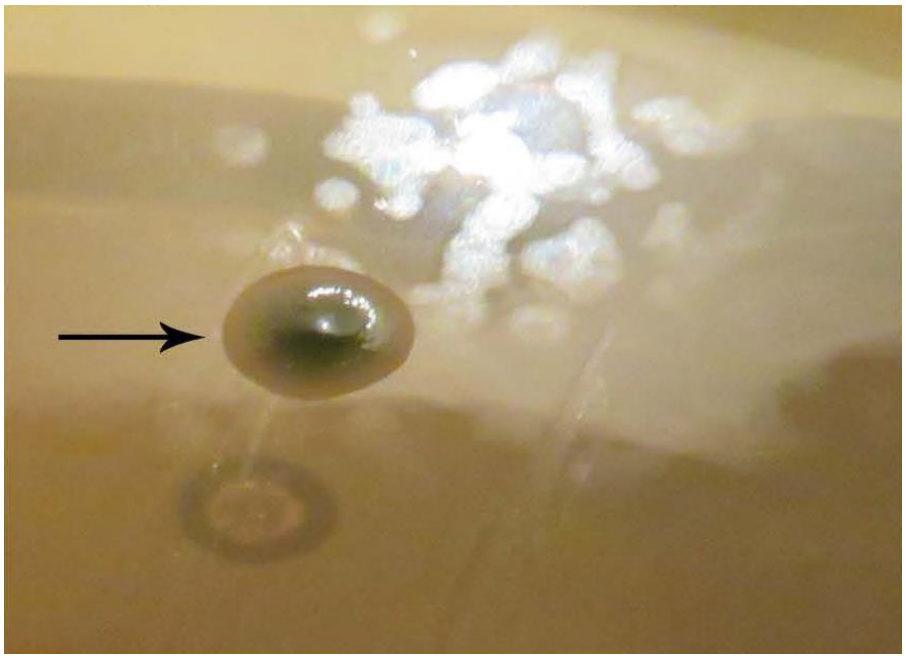

Figure 1: Characteristic bull eye shaped colony on aeromonas isolation base medium showing dark green convex center with light periphery

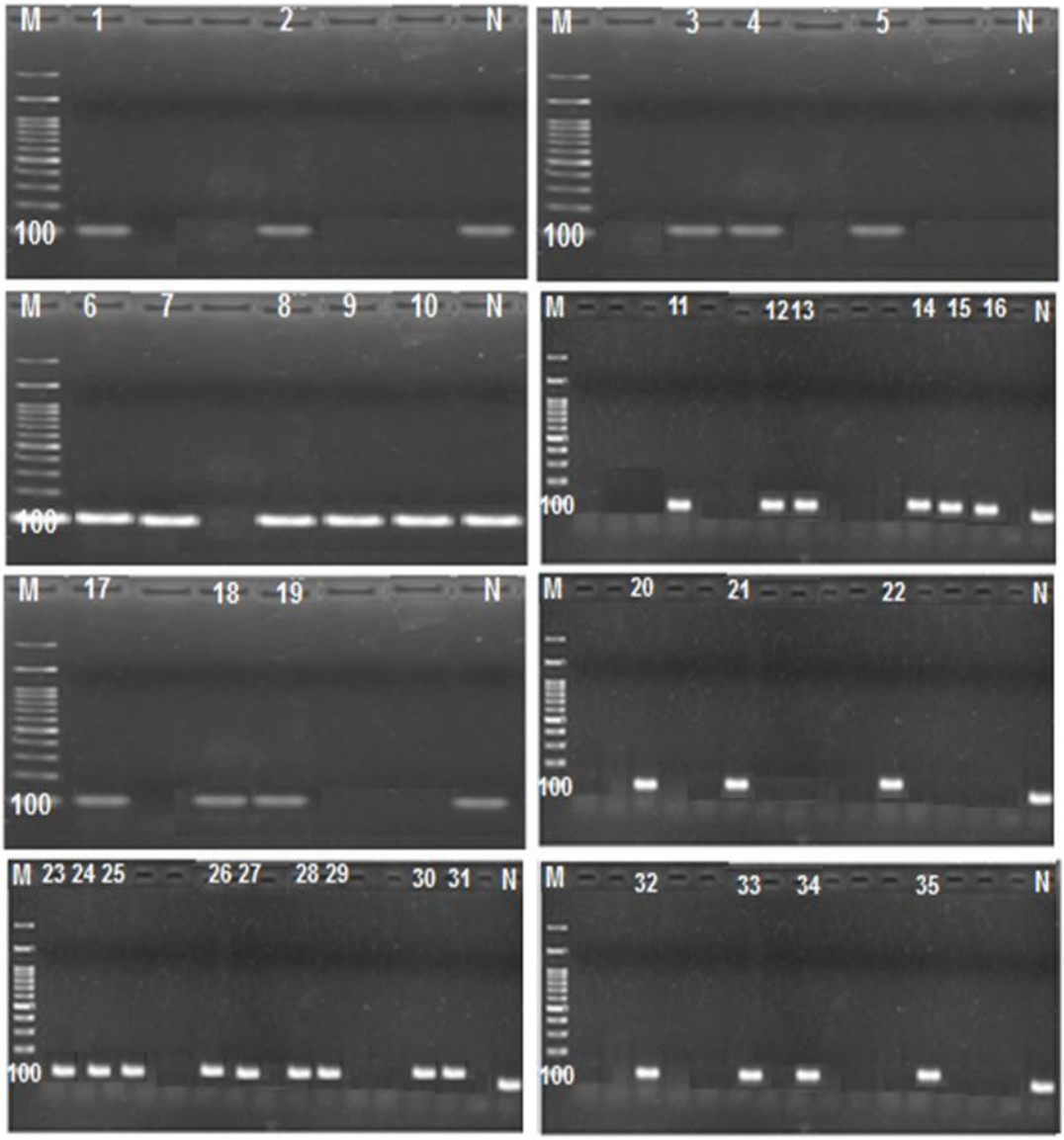

Figure 2: Electrophoretic pattern of PCR production 103 bp specific for *A. hydrophila* in 2% agarose gel stained with ethidium bromide**.** Lane M: 100 bp DNA ma Lane N: negative control **.** Lane 1- 35: positive *A. hydrophia*

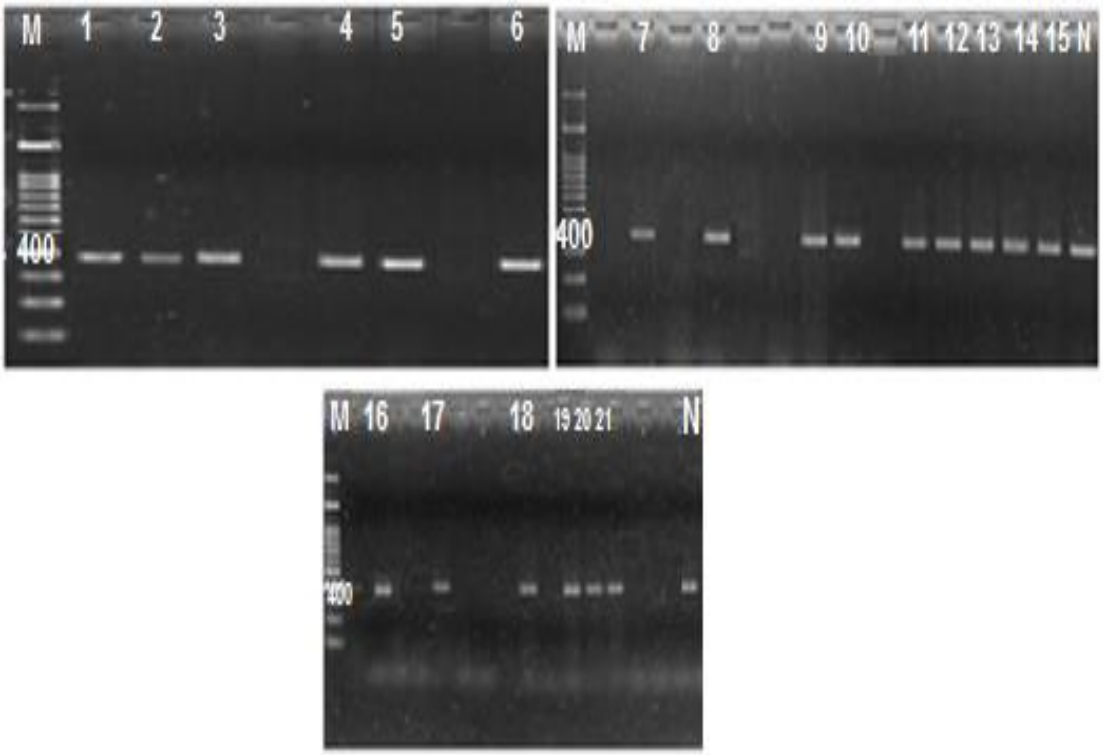

Figure 3: Ethidium bromide stained agarose gel of PCR products representing amplification of 383-389 bp amplicon of the *pla/lip* gene in *A. hydrophila***.** Lane M: 100 bp DNA marker, Lane N: negative control, Lane 1- 25: positive

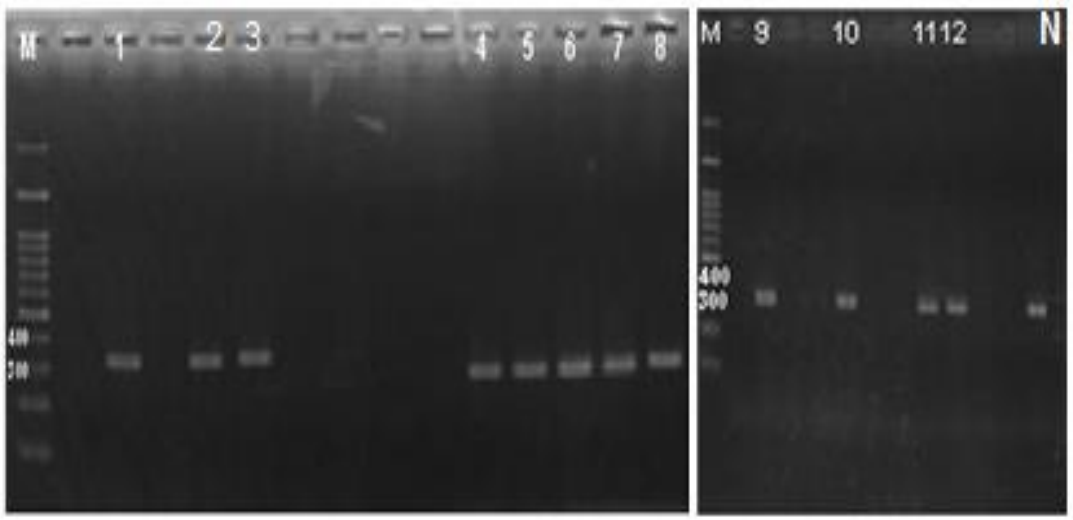

Figure 4: Ethidium bromide stained agarose gel of PCR products representing amplification of 328 bp amplicon of *ast* gene in *A. hydrophila***.** Lane M: 100 bp DNA marker , Lane N: negative control , Lane 1-14: positive

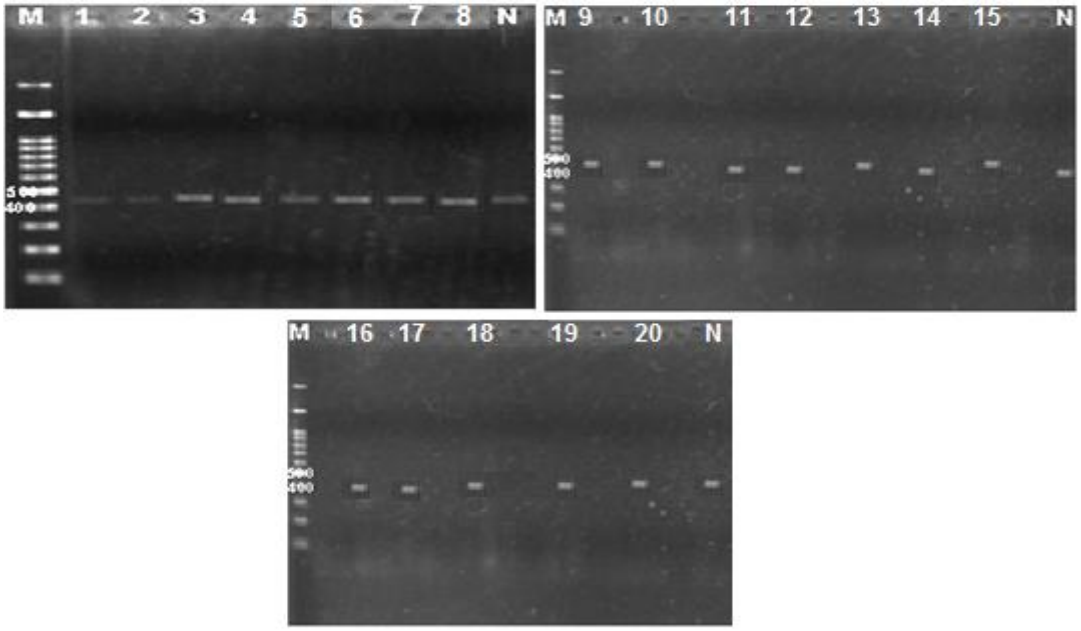

Figure 5: Ethidium bromide stained agarose gel of PCR products representing amplification of 431bp amplicon of *aer* gene in *A. hydrophila*. Lane M: 100 bp DNA marker, Lane N: negative control Lane 1- 8: positive

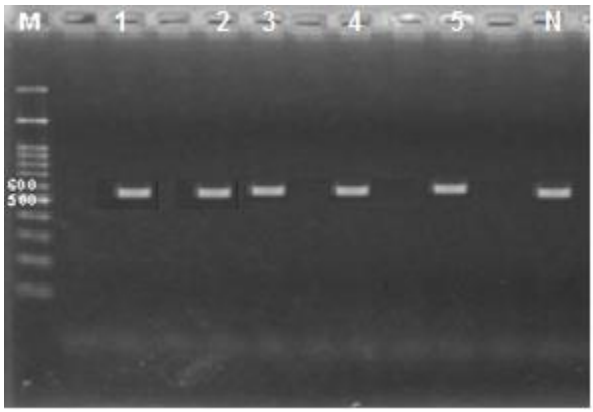

Figure 6: Ethidium bromide stained agarose gel of PCR products representing amplification of 592 bp amplicon of the haemolysin (*hlyA*) gene in *A. hydrophila* **.**Lane M: 100 bp DNA marker **.**Lane N: negative control, Lane 1- 2: positive

Table 2: List of virulence genes present in the isolated *Aeromonas hydrophila* strains

Gene	Number of isolates	Occurrence (%) in isolated strains	Occurrence (%) in virulent strains
pla/lip	21	60%	100%
ast	12	34%	57%
hlyA	5	14%	24%
aer	18	51%	86%

Table 3: Occurrence and combination of virulence genes in *A. hydrophila* strains

Gene	Number of isolates	Occurrence (%) in isolated strains	Occurrence (%) in virulent strains
pla/lip, ast, aer, hlyA	5	14%	24%
pla/lip, ast, aer	7	20%	33%
pla/lip, aer	6	17%	29%
pla/lip	3	10%	14%

Discussion

In developing coast countries, aquaculture is very important as a good source for animal protein suitable for human consumption (8, 9). This source was threatened by uncontrollable bacterial diseases especially for those caused by drug resistance bacteria and highly virulent bacteria such as *A. hydrophila* (10, 11).

PCR used in diagnosis of bacterial fish diseases, isolated from cultured fish, is a very rapid and accurate method (6, 12, 13). The molecular identification of DNA for 35 *A. hydrophila* isolates, using specific primers revealed the presence of common band at 103 bp. These results were similar to that reported by (7).

Virulence genes act as a key component in determining the potential pathogenicity of the micro-organism, acting multifunctionally and multifactorially and can be used for virulence typing of *A. hydrophila* isolates (2, 6, 14). Herein, we isolated 10 virulence genes from the positive 35 *A. hydrophila* isolates by PCR. The virulence genes in *Aeromonas hydrophila* isolates, were distributed into, fourteen isolates (40%), have no virulence genes while, twenty-one (60%) isolates have at least one or more virulence genes.

From the present work, it was evident that, the lipase (*pla/lip*) was the most frequent and important virulence gene. Lipase has the potential to change the histochemical identity of the cell membrane of the infected cells of target fish tissues thereby allowing *A. hydrophila* colonization which further induce cell necrosis (6, 15). The second more prevalent virulent gene was the aerolysin (*aer*) indicating high RBCs and cellular lysis for this bacteria (16). Presence of aerolysin in pathogenic *A. hydrophila* infections may help in diagnosis, prevention and control of the disease spreading and mortalities in aquaculture (1, 17). On the other hand, the prevalence of the cytotonic heat stable enterotoxin (*ast*) was lower than *aer* but higher than hemolysin. The *ast* has the ability to increase the vascular permeability of the gut causing detachment of the intestinal mucosa (18). Hemolysin (*hlyA*) induced lytic activities on red blood cells, causing anemia (1). Haemolytic toxins; haemolysin and aerolysin released by *A. hydrophila* may be used as a marker of pathogenicity of *A. hydrophila* (1)

Conflict of interest

The authors declare that no conflict of interest.

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Original Research Article

INFLUENCE OF BODY CONDITION SCORE ON BLOOD METABOLITES AND OXIDATIVE STRESS IN PRE- AND POST-CALVING OF FRIESIAN DAIRY COWS IN EGYPT

Mohamed E. El-Sharawy[1], Ibrahim M. Mashaly[2], Mustafa S. Atta[3], Mostafa Kotb[2], Ibrahim S. El-Shamaa[1]*

[1]Animal Production Department, Faculty of Agriculture, Kafrelsheikh University, 33516, Egypt, [2]Animal Production Research Institute, Agriculture Research Center, El-Dokki, Giza, Egypt, [3]Department of Physiology, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt

*Corresponding author, E-mail: elshamaa2008@yahoo.com

Abstract: This experiment aimed to study the relation of body condition score (BCS) with blood metabolites in dairy cow. At 8 weeks before expected parturition, 90 multiparous Friesian dairy cows were divided according to their body condition score into 3 groups: Group1: low body condition score (n=30, BCS≤2.58, thinner cows); Group2: medium body condition score (n=30, BCS≤3.12, medium BCS cows); Group3: high body condition score (n=30, BCS≤4.75, obesity cows). The cows BCS, weight and back fat thickness were recorded at -60, -21, -14, -7, 0, 7, 14 and 60 days related to calving time. Blood samples were taken at the time of BCS measurement for determination of β hydroxy butyrate, non-esterified fatty acid, haptoglobin, glucose, triglyceride, albumin, aspartate aminotransferase, cholesterol, superoxide dismutase and malonaldehyde. The results showed a significant decrease in body condition score, body weight and back fat thickness throughout the experimental period accompanied by an increase in β hydroxy butyrate, malonaldehyde and cholesterol. The correlation analysis showed that changed body condition score positively allied with cow weight (r=0.860, P<0.01), back fat thickness (r= 0.977 P<0.01), malonaldehyde (r=0.445, P<0.01) and cholesterol (r=0.342, P<0.01) and was negatively correlated with β hydroxy butyrate (r=0.416, P<0.01), haptoglobin (r=-0.232, P<0.05), non-esterified fatty acid (r=-0.457, P<0.01), albumin (r=-0.133, P>0.05) and aspartate aminotransferase (r=-0.361, P<0.01). Concisely, body condition score loss before and after calving may have significant consequences for blood metabolites, oxidative stress and body condition score profile in dairy cows.

Key words: BCS; calving; blood metabolites; Friesian cow

Introduction

The transition period extends from 21 days ante partum to 21 days postpartum and can be described as the alteration from a gestational non-lactating state to a non-gestational lactating stage (1, 2, 3). The body condition score is a reliable, simple way of estimating the nutritional status, body reserves and energy balance in dairy cows. Its evaluation is centered on the outer appearance of cows that interrelates with its body fat reserves and so, influenced by the

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energy balance (4). Lowman and Somerville (5) were the first to use a BCS Scale (4- points), even so, the scale used to measure the BCS (5-, 6-, 8-, and 10- point scales) differs between the countries. But, collectively, the low values always reflect the emaciation while the high value equate to obesity (6). Back fat thickness (BFT) related to other approaches of body condition scoring because its objective and precise. Nearby parturition, the adipose tissue begins to decompose to create fatty acids and glycerol (lipolysis) afford energy to the body. Non-esterified fatty acid (NEFA) in blood is a respectable pointer of adipose tissue mobilization (7). NEFA and β hydroxy butyrate (BHBA) are important energy metabolites that are traditionally used as indicator of negative energy balance (NEB) during transition stage (8). The determination of malonaldehyde (MDA) and activity of superoxide dismutase (SOD) during transition period as an index of oxidative stress status.

The aim of the present study was to explore the relationship between body condition score from 60 days' pre- partum until 60 days' post-partum and BCS change, BW, BFT and some metabolic blood parameters in pregnant Friesian dairy cows.

Materials and methods

The experimental protocol was approved by the Animal Care and Use Committee of Faculty of Agriculture, Kafrelsheikh University.

Animals

A total of 90 animals formed a herd of 350 Friesian dairy cows selected for experiment depend on their body weight (BW) and body condition score (BCS) 0 to 5 point scale according to the North American BCS (9), and divided into three groups (each of 30 cows) on 60^{th} days pre-partum as fairly low (group 1, ≤ 2.58 BCS,≤490 kg BW), medium (group 2, ≤ 3.12 BCS, 600 kg) and obesity (group 3, ≤4.75 BCS, ≤713 kg BW). The body condition score was performed by one person at -60, -21, -14, -7 pre-partum, at day of calving, at days 7, 14 and 60 post-partum. Animals were housed in a free stall barns, had a free access to water and fed twice daily (8 am and 4 pm) with a ration formulated according to the recommendation of NRC (10) (Tables 1 and 2).

Measurement of back fat thickness (BFT)

The investigation site is positioned in the sacral region among the caudal quarter and fifth connection line extending from the dorsal portion of the tuber ischia (pins) to the tuber coxae and is evaluated by ultrasound (4) and the data was converted to BCS.

Biochemical assessment

Blood samples were taken from the jugular vein of all cows on fixed time before morning feeding at -60, -21, -14, -7, pre- partum, at day of calving and at days 7, 14 and 60 post-partum. Serum were stored at -20 °C till essayed for NEFA, BHBA, haptoglobin (HP), and other biochemical analysis. NEFA and BHBA quantified using commercially kits (Randox laboratories Ltd, Crumlin Co., Antrim, UK), Serum Hp measured calorimetrically using a commercial kit (Phase HP kit, Tridelta Ltd., Ireland) according to the constructor's guidelines.

MDA and SOD assessed using the commercially kit (Bio-diagnostic, Egypt) according to (11). Albumin, triglycerides, glucose, cholesterol and aspartate aminotransferase assessed spectrophotometrically using autoanalyzer (MCC-3000, China) (Biomed diagnostics Germany).

Statistical analysis

Mean and standard error were calculated. The obtained data were subjected to two-way factorial analysis of variance according to the procedures out lined by Snedecor and Cochran (12). The mean value of treatments was compared according to Duncan's multiple range test (DMRT) (13). Multiple correlation coefficient analyses were used. The data was analyzed using CoStat software for windows (version 6.3).

Results

Change of BCS, BW and BFT from pre- to post- partum in dairy cows

Fluctuations of BCS in each group were presented in figure l. The obesity BCS cows

showed significantly higher BCS reduction (-1.17) 60 d pre-partum to 60 d post-partum than thinner (-0.5) and medium BCS cows (-0.67). Changes of BW and BFT were statistically significant ($P<0.05$) among the 3 groups (Fig. 2 and 3). The 3rd group showed higher BW and BFT reduction from d 60 before calving to d 60 after calving (106 kg and 1.14, respectively) compared to thinner (70 kg and 0.28, respectively) and medium groups (103 kg and 0.68, respectively). During the experimental time, highly significant positive correlations were found among reduction of BCS and both of BW ($r=0.86$, $P<0.01$) and BFT ($r=0.98$, $P<0.01$). Thus, cows with greater BCS losses had also higher BW and BFT losses (Table 3).

Metabolic parameters

Serum concentrations of BHBA, NEFA and HP in high BCS cows were significantly ($P<0.05$) higher than the other two groups before and after calving (Figs, 4-6). The differences in HP among groups were not significant. The concentrations of BHBA and NEFA increased from -7d before calving and reached the high levels on 7d post- calving and then decreased with high values as compared to those observed before parturition.

Oxidant and antioxidant status

SOD gradually increased ($P<0.05$) in the last 21 days of pregnancy (at 21 d before calving) and reached the maximum at 7d earlier calving. After parturition, SOD dropped to reach the levels recorded before calving (Fig. 7). Serum concentration of MDA was relatively steady before calving ($P>0.05$) at parturition and one week after calving (Fig. 8). The differences among groups were highly significant ($P<0.01$).

Serum biochemical parameters

The concentrations of serum glucose were gradually increased from -60 d before calving and reached the highest values at calving and then dropped sharply at 7d post-calving and back increased to the levels registered 60 d before calving (Fig. 9). Serum triglyceride was not significantly differed among groups (Fig. 10). The serum cholesterol started to increase from -60 d before calving and reached the higher values on -7d before calving and then decreased to reach the low values on 60 d post-calving (Fig. 11), while the triglyceride concentration peaked at calving and dropped 7d and 14 d post-calving and then increased 60 d post-calving. Mean serum albumin content dropped to the lowest levels at calving followed by a subsequent increase to highest levels ($P<0.05$) at 7 d post-calving then dropped to levels 60d pre-calving and followed by an increase at 60 d post-calving. The differences among groups did not significantly differed (Fig. 12). AST concentrations steady increased ($P<0.05$) from 60 d before calving to parturition and followed by a subsequent increase at one wk., 2 wk and 2 months post-calving and reached maximum level ($P<0.05$) at 60 d post- calving (Fig. 13).

Correlation study

BCS, BW and BFT were negatively ($P<0.01$) correlated with both BHBA ($r=-0.416$, $r=-0.355$, $r=-0.361$, respectively), NEFA ($r=-0.457$, $r=-0.470$ and $r=-0.448$, respectively), and AST ($r = -0.361$, $r = -0.407$ and $r = -0.341$, respectively) and were definitely ($P<0.01$) linked with cholesterol ($r=0.342$, $r=0.423$ and $r=0.323$, respectively), (Table 3).

Table 1: Chemical composition of ingredients rations during the pre- and the post- partum period

Item		Composition of DM%					
Ingredients	DM%	OM	CP	CF	EE	NFE	Ash
CFM	89.71	91.58	15.62	9.63	2.92	63.41	8.42
Corn silage	35.10	94.89	9.39	23.18	2.19	60.13	5.11
Hay	89.89	92.23	14.29	27.5	3.12	47.32	7.77
Rice straw	91.08	83.58	2.98	34.89	1.36	44.39	16.42
Rations							
Pre-partum	78.97	91.58	12.84	18.44	2.66	57.64	8.42
Close up	76.24	91.74	12.54	19.12	2.62	57.46	8.26
Post- partum	73.5	91.91	12.23	19.79	2.59	57.30	8.09

DM (dry matter); OM (organic matter); CP (crude protein); CF (crude fiber); EE (ether extract); NFE (Nitrogen free extract); Ash (Ash) and CFM (concentrate feed mixture)

Table 2: Average daily feed intake (Kg/head/day) during pre- and post-partum period

Item	Pre- Partum	Close up	Post- Partum
CFM (concentrate feed mixture)	9.5	8.5	7.6
Corn silage	9.7	12.1	14.5
Hay	3.8	3.8	3.8
Rice straw	1.9	1.9	1.9

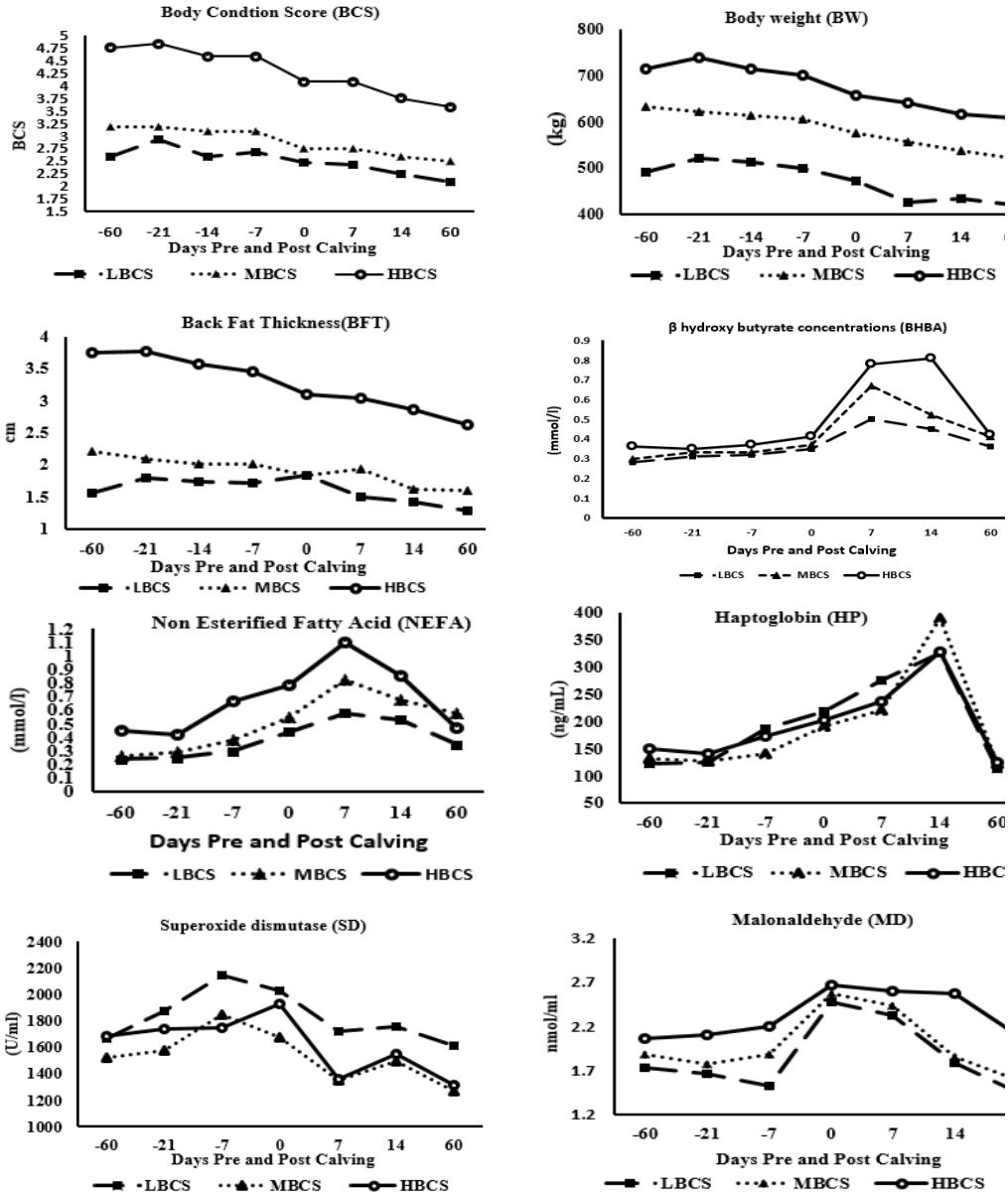
Table 3: Correlation coefficient between body condition score, body weight and back fat thickness with metabolic blood parameters and oxidative stress

Items	BCS	BW	BFT
Body Condition Score	1		
Body Weight	0.860(**)	1	
Back Fat Thickness	0.977(**)	0.884(**)	1
β hydroxy butyrate	-0.416(**)	-0.355(**)	-0.361(**)
Non-esterified fatty acid	-0.457(**)	-0.470(**)	-0.448(**)
Haptoglobin	-0.232(*)	-0.215(*)	-0.184
Superoxide dismutase	0.123	-0.019	0.054
Malonaldehyde	0.445(**)	0.383(**)	0.465(**)
Blood Serum Glucose	0.133	0.208	0.146
Blood Serum Triglyceride	0.040	0.017	-0.009
Blood Serum Albumin	-0.133	-0.145	-0.137
Aspartate aminotransferase	-0.361(**)	-0.407(**)	-0.341(**)
Blood Serum Cholesterol	0.342(**)	0.423(**)	0.323(**)

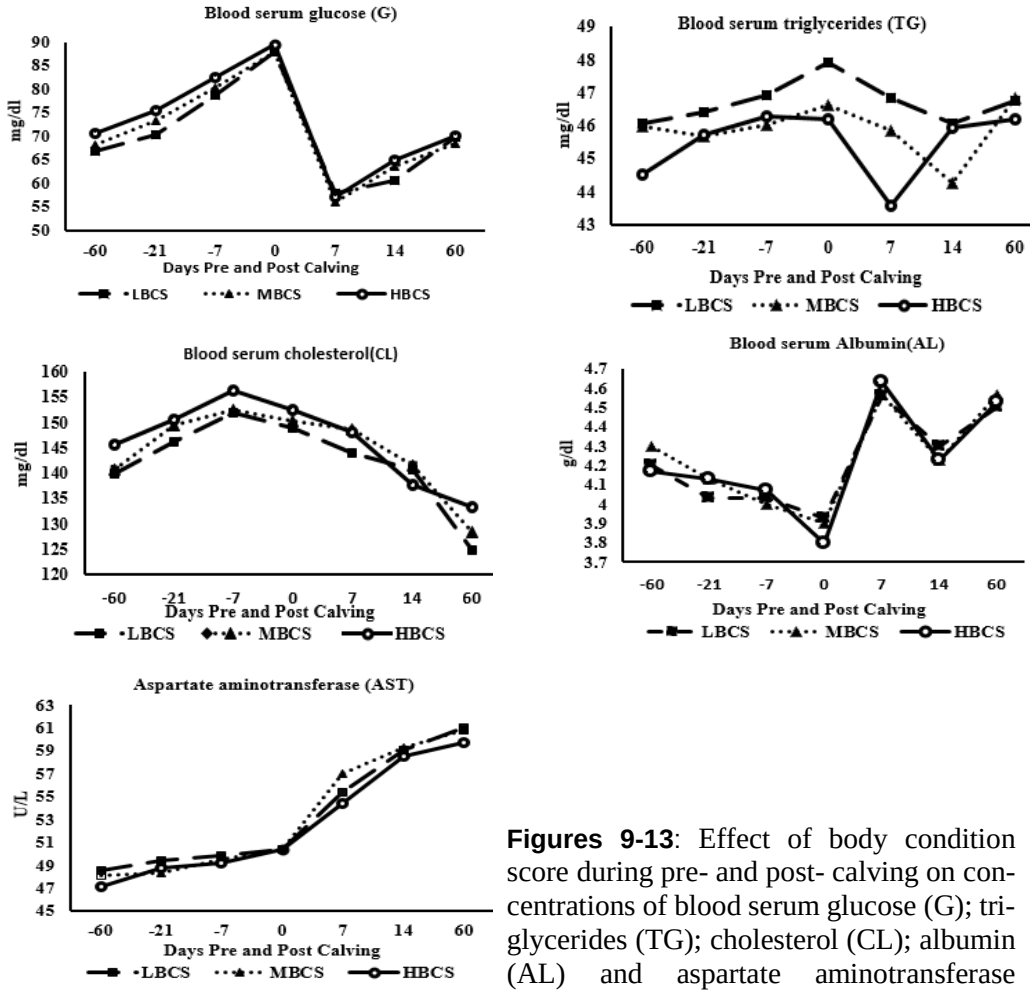
BCS (body condition score), BW (body weight), BFT (back fat thickness)

**Correlation is significant at the 0.01 level (1-tailed)

* Correlation is significant at the 0.05 level (1-tailed)



Figures 1-8: Effect of body condition score during pre- and post- calving on body condition score changes (BCS); body weight changes (BW); back fat thickness changes (BFT); β hydroxy butyrate concentrations (BHBA); non-esterified fatty acid (NEFA); haptoglobin (HP); superoxide dismutase concentrations (SOD) and malonaldehyde concentrations (MD), (Means ± SEM)



Figures 9-13: Effect of body condition score during pre- and post- calving on concentrations of blood serum glucose (G); triglycerides (TG); cholesterol (CL); albumin (AL) and aspartate aminotransferase (AST), (Means ± SEM)

Discussion

Cows with high body condition score showed higher ($P<0.05$) BCS loss pre and post-calving period than medium and thinner BCS cows. Greater loss of BCS in high BCS cows was expected and has been reported previously by Treacher et al. (14), who concluded that transition cows with great BCS waste more body weight and body condition than thinner one. In the present study, cows with high BCS had the greatest decline in BW and BFT when compared with the other groups, which is an indication of mobilizing greater body fat reserves than medium and thinner BCS cows. These results are in agreement with previous reports (6, 15, 16).

In the present study, cows with high BCS had a sharp increase in plasma NEFA and BHBA concentration, which reached the highest values at 1 week after calving, while the lowest levels were found in LBCS cows. The highest plasma NEFA and BHBA concentrations for cows with high BCS were in accordance with other studies (17) and could be explained by the high fat mobilization.

Results of the present study indicated that serum glucose increased in all groups reaching the peak at parturition then a sharp decrease 7 d post-calving, which may be attributed to cortisol and estrogen (17) or inherent hypoinsulinemia which reduce glucose uptake by tissue sensitive to insulin (18). On the other hand, García et al. (19) found that low glucose level was during calving and explained these results due to sever NEB suffered by the animals in this group that delivered high milk during the experimental time. Moreover, others found that glu-

cose concentration remained stable and increase slightly at calving reflecting an increase in gluconeogenesis in response to calving stress (20, 21). Cheng et al. (22) reported that cows in negative EB have low rates of glucose and high levels of BHBA.

In the present study, triglycerides were relatively stable at range from 43.6 to 46.9 mg/dl and no significant differences were noticed among groups. This finding is consistent with previous report by García et al. (19) and González et al. (23) who attributed this to the excess of fatty acids mobilized to liver to be as energy resource. The reasons for such case are triglycerides accumulation in the liver and triglycerides taking up by the mammary gland for milk fat synthesis and secretion (24). Reduced cholesterol concentrations starting from the time at calving and post-calving weeks were found in all groups, which could be associated with reduced DM intake (25, 26). Conversely, Mouffok et al. (20) found that cholesterol was significantly elevated in post-partum which indicates good energy nutrition (27). Total cholesterol was significantly lower on week around parturition with the lowest value one week prior to calving (28, 29). Alternatively, García et al. (19) described that cholesterol level had a gradual upsurge as the lactation advanced.

Reduced albumin concentration reaching the lowest values at the time of parturition in all groups and increase to the highest values one-week post calving. Our results are in disagreement with findings of Soca et al. (26), who found that albumin concentrations decreased after calving in low and moderate BCS cows and this may be associated with increasing of NEFA. Our results were in agreement with finding of Gheise et al. (16), who noted that the highest albumin levels at 1 d after calving. Albumin is synthesized via the liver and it's the core source of plasma thiol groups which are considered as an element of extracellular antioxidant defense against oxidative stress (30).

In the present study, the activity of aspartate amino transferase (AST) was not significantly changed by BCS on the calving day in all experimental groups and increased gradually at 7, 14 and 60 d post-calving. No significant different was found among groups pre and post-calving. The higher level of AST in dairy cattle are allied with the fatty liver syndrome, lower dry matter consumption and ketosis marks (32).

Haptoglobin (HP) concentration in the present study was significantly higher at calving day and post-calving days than before parturition. The present finding was in agreement with the finding of others (31, 32), who found that the highest value of HP was recorded in cows 3wk postpartum. This finding supported the previous results of Montagner et el. (33) who found the trend for higher HP concentrations in cows' pre-partum and the enhanced concentration in the post-partum were consistent with impaired hepatic function.

Highest MDA concentration was recorded on the day of calving in all treated groups, then continuously decreased after calving on 7, 14 and 60 d post-calving that was in agreement with the finding of others (34). Castillo et al. (35) reported that MDA is the last product of lipid peroxidation, therefore changes of MDA concentrations can be used as a biomarker of oxidative stress. SOD enzyme is the major antioxidant defense competent in protecting the cells against increased ROS (36). The increase of SOD around calving (-7 d to +7 d pre and post- calving) because of a possible homeostatic control (37).

Conclusion

This study showed that lost body condition in 8 wks before calving had an adverse metabolic status, with increased serum concentrations of NEFA, BHBA, MDA and AST post calving. In addition, high BCS cows (obesity, G3) had highest BHBA profiles that suggested they had a higher risk of subclinical ketosis, in addition, highest serum concentrations of NEFA in all cows after calving, indicated the highest mobilization of body reserves after calving.

Conflict of interest

The authors state no conflicts of interest.

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Original Research Article

EFFECTS OF DIET'S ACIDIFYING ADDITIVES ON THE INTESTINAL HISTOMORPHOLOGY IN DUCKS

Ahmed Abdelfattah-Hassan*, Hanaa M. El-Ghazali

Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Zagazig University, 44519, Egypt

*Corresponding author, E-mail: aabdelfattah@vet.zu.edu.eg

Abstract: This study aimed at evaluating the effect of acidifying additives as formic and propionic acids on weight gain and on small intestinal macroscopic, microscopic and SEM morphology in ducks. A total of 30 Mulard ducklings were used in this study and they were randomly assigned to control (given standard commercial duck diets with no acidifiers added), 1% acidifiers added to the standard diet or 2% acidifiers added to the standard diet. After rearing the ducks for 52 days, their weight was recorded and then the length of the different parts of the small intestine was measured. Then, samples were taken for histological and scanning electron microscopical (SEM) examination of the small intestine. The present results found that addition of acidifying mixture to the diet significantly improved duck's weight ($P<0.05$), also there were significant changes in length, histology and SEM characteristics of the small intestine between different duck groups. The observed effects were dose dependent, i.e. with more organic acids in the diet, the more the changes were noticeable. Our results indicate that, the addition of organic acids to duck's diet increased small intestine's length and increased the length of intestinal villi, possibly resulting in more surface area for digestion and absorption of the diet, and, therefore, greatly improved the final body weight of the study ducks.

Key words: acidified diets; organic acids; histology; intestinal villi; SEM

Introduction

Poultry meat is a good source of proteins and other valuable nutrients for human consumption, and it is considered an essential supply to meet the growing human population. The unsupervised use of antibiotics in poultry industry is still a serious problem and it poses great risks to human health; when improperly used as growth promotors. Antibiotics use as growth promotors is illegal now in Europe, since the European Union has banned their use in 2006.

Other alternatives are currently still being studied in poultry industry and one modern feeding strategy is changing the pH of the diet through addition of acidifying substances; as organic acids. These acids will slightly change the pH of the intestine, and also has direct antibacterial effects (1–3). So, it will reduce the intestinal bacterial load, improve production and eliminate the need for antibiotics use as growth promotors. Several commercial mixtures of organic acids and inorganic acids are currently in

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the market in the European Union as better replacements to growth promoting antibiotics.

Being acids, organic acids improve the digestibility of dietary proteins and amino acids in the stomach, by enhancing the digestion of proteins (4). In addition, they improve the utilization of feed and result in better feed conversion and growth rates. Moreover, an additional advantage of organic acids is their use in the preservation of broiler feed stuff (5). Therefore, the addition of organic acids to the feed ingredients preserves its properties for longer time, result in better growth in reared poultry and increase the profitability for poultry producers.

In fact, the addition of acidifying substances was described previously in poultry; to control infections such as campylobacter or to improve the weight gain of reared poultry. Several acids were used, such as, lactic acid in the drinking water (1), organic acids in drinking water (6), formic acid in feed (7) along with other additives reviewed in (8). Their use resulted in improved weight gain in poultry, because these organic acids enhance diet's protein and energy use and reduce microbial competition for these nutrients in bird's intestine (reviewed in (3)). Moreover, the addition of these acidifying substances was not harmful and did not cause damage in the intestinal epithelium, on the contrary it increased chicken-flock's biosecurity and prevented infections that could be harmful to human consumers (6, 9). In humans, dietary additives or high fat diets were also described to change intestinal adaptation and enhanced the growth of the intestinal villi after removal of parts of the bowl in resection operations (10), and it could be a useful strategy in treating humans with short gut syndrome (11–13).

The possible macroscopic and microscopic changes to the intestine and intestinal villi upon feeding acidified diets has not been studied in detail in ducks. Therefore, the present study aimed at studying the small intestine's morphology, histology and scanning electron microscopy in response to feeding acidified diets (through addition of organic acids to the standard diet) in ducks.

Material and methods

All procedures in the study were following animal care and use committee of the Faculty of Veterinary Medicine, Zagazig University.

Birds and study design

Mulard Ducklings (n = 30); a sterile breed of domestic ducks raised for meat production and is a hybrid between male Muscovy (*Cairina moschata*) and female Pekin (*Anas platyrhynchos domestica*) ducks, were purchased from a commercial duck hatchery. Upon arrival, the ducklings were weighed and randomly assigned to one of three groups (each group contained 10 ducklings) and reared for 52 days in Bird Nutrition Research Unit (Nutrition and Clinical Nutrition Department, Faculty of Veterinary Medicine, Zagazig University).

Ducklings feeding program consisted of standard starter diet (up to 21 days) and finisher diet (22-52 days). A commercial acidifier mixture was purchased, and it contained mainly formic acid and propionic acid. The three study groups received no (Control), 1% acidifier mixture in diet (1AM) or 2% acidifier mixture in diet (2AM). No forced feeding was done in this study and ducks had free access to water. After completing the rearing period, all ducks were weighed and then slaughtered, and samples were taken immediately.

Gross anatomical examination

Directly after slaughtering the study ducks, the intestine was removed and visually inspected for any apparent differences, then carefully straightened so the length of each small intestine segment (duodenum, jejunum and ileum) was easily measured. Duodenum was defined starting from its beginning after the gizzard (ventriculus) till the beginning of the mesojejunum (the jejunal mesentery), the jejunum was measured from the end of the duodenum till the end of the mesojejunum, while the ileum was measured from the end of the mesojejunum till the junction between the ileum and the two ceca. Also, the length of the two ceca (part of the large intestine) was recorded.

Histological examination

After performing the anatomical examination, samples from the middle of each part of the small intestine was taken, emptied from its contents by flushing with warm physiological saline solution and, then, immediately fixed in freshly prepared 10% neutral buffered formalin solution for at least 48 hours. Care was taken to obtain the samples from the same region in all study ducks. Afterwards, standard histological procedure was performed (dehydration in ascending grades of alcohol, clearing in xylene and embedding in paraffin wax). Finally, 5 µm sections were obtained, mounted, stained with Hematoxylin and Eosin stain (H&E) and examined under light microscopy.

The general structure (mucosa, submucosa and muscular layer) of each part of the small intestine was examined and the height (including the crypt of Lieberkühn) and width of the intestinal villi, and the thickness of the muscularis mucosa were measured using S-view computer software provided with the microscope camera (EHC10, S-VIEW Technology Co., Ltd, Zhejiang, China).

Scanning Electron Microscopy

For the Scanning Electron Microscopy (SEM), a two cm long intestine sample (from the part next to the samples obtained for histological examination) was cut open longitudinally, the cut was made very carefully not to touch the intestinal mucosa, and the mucosa was washed again with warm phosphate buffered saline. The SEM procedure followed previously published protocols for birds (14, 15). Briefly, the samples were immersed in 3% glutaraldehyde (in 0.1M cacodylate buffer, pH 7.4) at room temperature and transported to the SEM unit (Al-Azhar University, Cairo, Egypt). Then, the samples were cut into smaller pieces (approx. 5 mm^2) with sharp razor blades and fixed for an additional one hour in the same buffer. Then, the pieces were washed with 0.1M sodium cacodylate buffer (pH 7.4) and post-fixed with 1% osmium tetroxide (in ice-cold 0.1M sodium cacodylate buffer, pH 7.4) for two hours. Afterwards, the samples were washed with pure distilled water and dehydrated in ascending concentration of ethanol (50% to 100%, 2 times in each concentration for at least 20 min each). following this, the samples were critical point dried using liquid carbon dioxide (Autosamdri®-815, Tousimis, Maryland, USA), mounted on aluminum stubs with electrically conducting carbon mounting tabs and finally sputter-coated (SPI-Module™ Sputter Coater, Pennsylvania, USA). The arrangement of the villi and their surface epithelium were observed using JOEL scanning electron microscope (JSM-5500LV, JEOL Ltd., Tokyo, Japan) at 17-20 kV accelerating voltage.

Statistical analyses

The data collected was evaluated using ANOVA procedure, post hoc comparisons were applied, whenever appropriate, using Tukey's HSD test. All statistical procedures were performed using PASW statistics v.18 (SPSS Inc., USA). Statistical significance was considered at $P \leq 0.05$.

Results

Effects of acidifying mixtures on duck's final body weight

The addition of acidifying mixture to duck's diet (starter and finisher), greatly improved the final body weight of the ducks during the 52 days study period ($P<0.05$, Table 1). With highest duck's weight obtained in the group supplied with 2% organic acids, followed by 1% organic acids, and the least weight belonged to duck's that did not have organic acids in their diets.

Gross anatomical findings

After measuring the length of the different parts of the small intestine (duodenum, jejunum and ileum), the addition of acidifying mixtures (1% or 2%) resulted in longer duodenum, jejunum, ileum and ceca compared to the control group with no additives ($P<0.05$, Table 1). In addition, only in the ileum, the 2% acids mixture resulted in significant longer intestinal length than in the 1% group ($P<0.05$).

Histological findings

The addition of organic acids to the duck's diet resulted in significantly different length of duodenal, jejunal and ileal villi ($P<0.05$, Table 1). The shape and arrangement of the intestinal villi in duodenum, jejunum and ileum were also microscopically changed at 40X (Plate 1). At higher magnifications (100X, plate 1, and 400X, not shown) there were no observable difference in the lamina propria or the Crypts of Lieberkühn with H&E stain. The addition of organic acids to the diet increased villus length ($P<0.05$) and the villi were less wide than in ducks fed no organic acids in their diets. The longest and thinnest duodenal villi were observed in the 1% group, while the longest and thinnest jejunal and ileal villi were observed in the 2% group (Table 1). The intervillous space was similar in duodenum in all groups, and it was wider in the 1% and 2% groups compared to the control group with no additives in their diet (Plate 1, Figs. H, K, I, L, N, Q, O and R).

Table 1: Showing the final body weight, length of different parts of the small intestine (including villus length, width and muscularis thickness) and length of the ceca of studied Mulard ducks given no (control), 1% organic acids or 2% organic acids in their diets

	Control	1% organic acids in diet	2% organic acids in diet
Final body weight (gm)	2407±96.21 ^{c}	2690±84.23 ^{b}	2895±93.39 ^{a}
Duodenum length (cm)	29±2.58 ^{b}	33.33±2.17 ^{a}	34.67±2.26 ^{a}
Jejunum length (cm)	98.33±5.89 ^{b}	112.33±6.38 ^{a}	116.33±8.71 ^{a}
Ileum length (cm)	22.33±3.20 ^{c}	26.67±4.36 ^{b}	30.33±5.21 ^{a}
Cecum length (cm)	9.67±1.45 ^{b}	12.67±1.33 ^{a}	13.33±1.67 ^{a}
Duodenum villus height (µm)	1184.33 ±98.52 ^{b}	1613.00 ±131.55 ^{a}	1579.75 ±82.45 ^{a}
Jejunum villus height (µm)	883.00 ±84.21 ^{c}	863.25 ±110.98 ^{b}	901.00 ±174.27 ^{a}
Ileum villus height (µm)	393.00 ±40.05 ^{c}	671.88 ±107.03 ^{b}	797.43 ±97.03 ^{a}
Duodenum villus width (µm)	145.00 ±15.89 ^{a}	116.89 ±39.68 ^{c}	134.83 ±16.52 ^{b}
Jejunum villus width (µm)	225.44 ±49.02 ^{a}	206.00 ±38.05 ^{b}	171.67 ±16.50 ^{c}
Ileum villus width (µm)	176.00 ±18.69 ^{a}	162.00 ±16.81 ^{b}	143.71 ±21.41 ^{c}
Duodenum muscularis thickness (µm)	469.67 ±91.31 ^{a}	419.80 ±32.91 ^{b}	373.83 ±75.77 ^{c}
Jejunum muscularis thickness (µm)	319.50 ±58.52 ^{b}	347.67 ±54.38 ^{a}	364.00 ±46.25 ^{a}
Ileum muscularis thickness (µm)	233.50 ±25.32 ^{b}	254.33 ±28.08 ^{a}	258.33 ±39.55 ^{a}

Different superscript letters denote statistical significance ($P<0.05$). Values shown as mean ± standard deviation

Scanning Electron Microscopical findings

Results of the scanning electron microscopy showed the differences in shape and arrangement of the villi in different parts of the duck's small intestine in response to acidified diets (shown in Plates 2, 3, 4). Compared to the control group the duodenal villi were thinner, more abundant and interwoven between each other in the 1% group (Plate 2, Fig. D). While, in the 2% group they appeared thin, taller and less abundant (Plate 2, Fig. G). At higher magnifications (800X and 1500X) there were no obvious differences in the surface at the tip or sides of the duodenal villi between the study groups (Plate 2, Figs. B, C, E, F, H, I). In case of the jejunum, differences were more difficult to be noticed with the SEM (Plate 3). Only the sides of jejunal villi in the 2% group showed corrugations (Plate 3, Fig. H), these were less prominent in the 1% group and difficult to be observed in the control group. Differences in the ileum, between groups, were more pronounced than in the duodenum or jejunum. The 2% group showed more numerous ileal villi which are thinner and intermingled (Plate 4, Fig. G) with fairly corrugated sides at higher magnification (Plate 4, Fig. H). Whereas in the 1% group the ileal villi appeared less numerous than the 2% group and showed corrugated sides at higher magnification (Plate 4, Figs. D & E). At higher magnification (>1500X) the surface of the ileal villi in different groups showed no obvious differences (Plate 4, Figs. C, F, I).

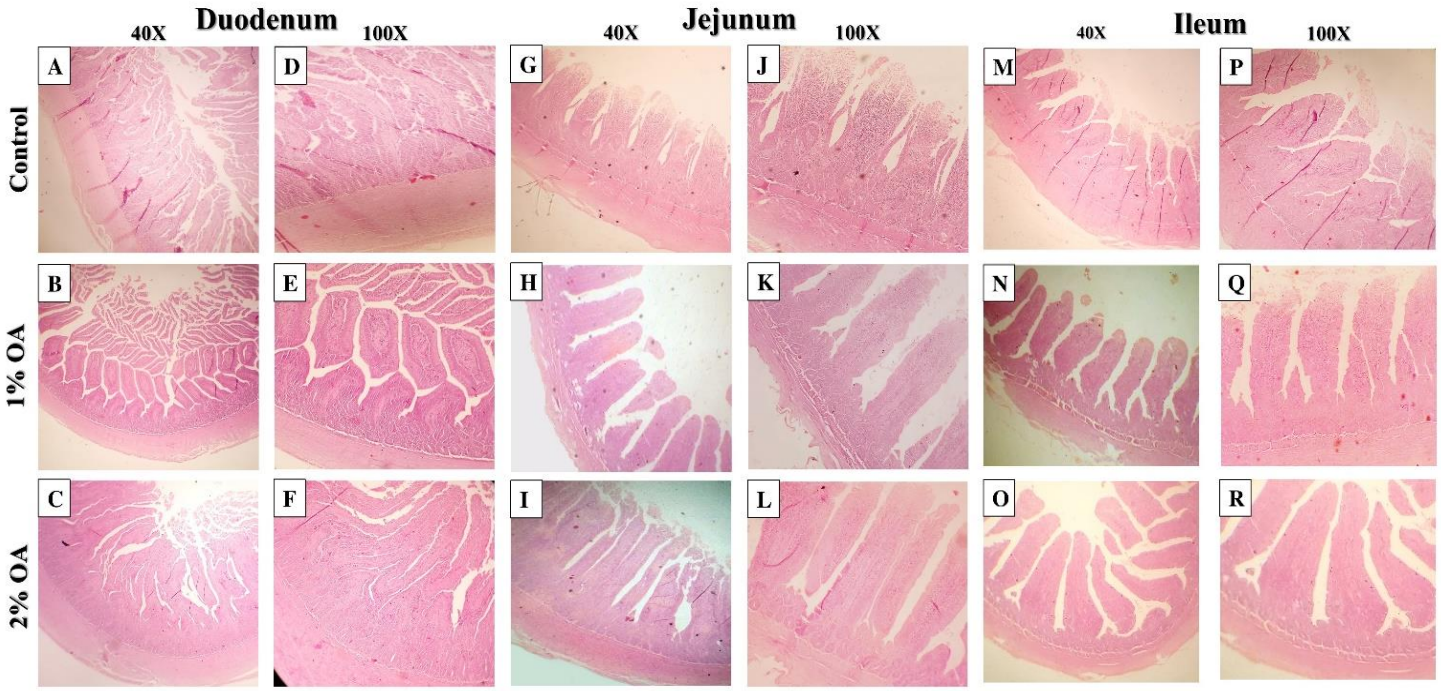

Plate 1: Photomicrographs A-R, showing the H&E sections of duodenum, jejunum and ileum in the study groups given no (Control), 1% organic acids (1% OA) or 2% organic acids (2% OA) in their diet for 52 days. Different length and arrangement of intestinal villi were noticed in response to the organic acids' administration is evident at 40X and 100X magnification

Discussion

Knowledge on duck's intestinal macro and microscopic anatomy is much less than the chicken in the literature, so far it has been given less attention. The histochemical developmental changes of the small intestine in ducks was described for the first time in 2011 (16). We here showed normal macroscopic and microscopic anatomy of the adult duck's small intestine, and possible changes that could arise from using modern feed additives; such as organic acids.

The present results showed that with changing the acidity of the diet, by incorporation of organic acids, the ducks weight improved (Table 1) while their feed intake was not changed significantly (data not shown). Similar improvements were detected in Aigamo ducks fed bamboo charcoal with vinegar (17) or natural zeolite including plant extract (18), in chicken fed organic acids (4) and pigs fed organic acids, reviewed in (19). Whilst, some studies in broiler chickens didn't find changes in final body weight; mainly because feed intake was not calculated and no normalization of bird's weight to its feed intake was done (6) or due to the use of single organic acid at a much lower concentration in the diet (7).

No damage was observed in the epithelium of the villi by SEM, nor visual differences was found, this underlines the safety of using up to 2% organic acids in the diet of ducks without causing any adverse effects. In chicken, organic acids use was also found to be safe on the intestine and did not cause damage to the villi (6).

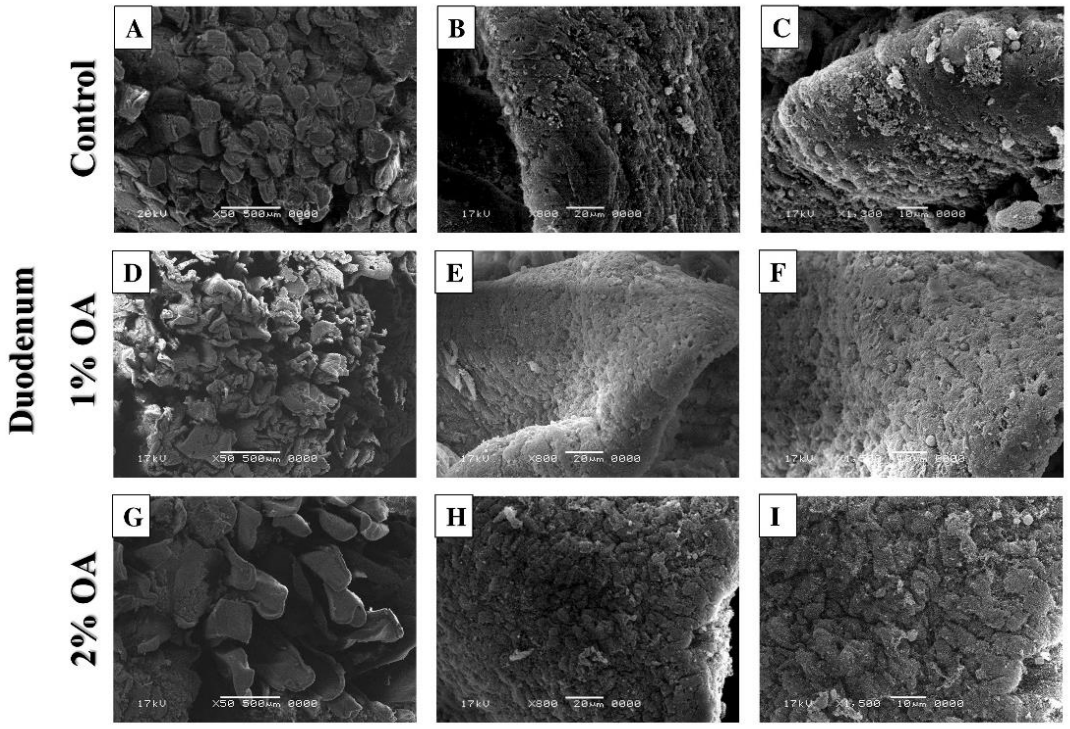

Plate 2: Photomicrographs A-I, showing the SEM scans of duodenum in the study groups given no (Control), 1% organic acids (1% OA) or 2% organic acids (2% OA) in their diet for 52 days. Different arrangement of intestinal villi was noticed in response to the organic acids' administration were noticed

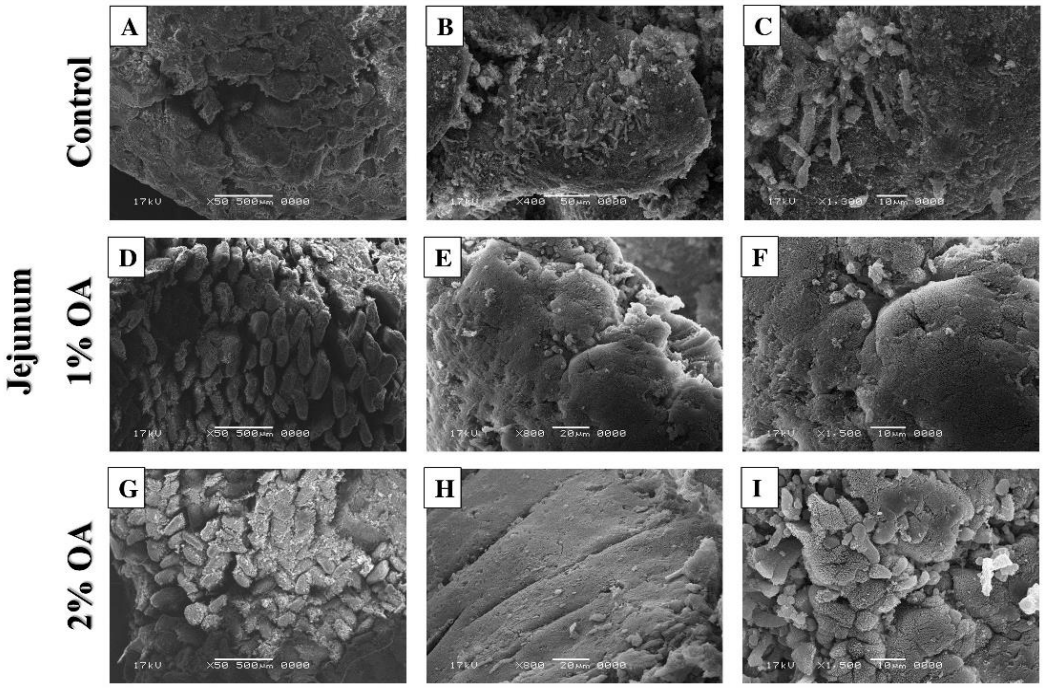

Plate 3: Photomicrographs A-I, showing the SEM scans of jejunum in the study groups given no (Control), 1% organic acids (1% OA) or 2% organic acids (2% OA) in their diet for 52 days. Different arrangement of intestinal villi was noticed in response to the organic acids' administration were noticed

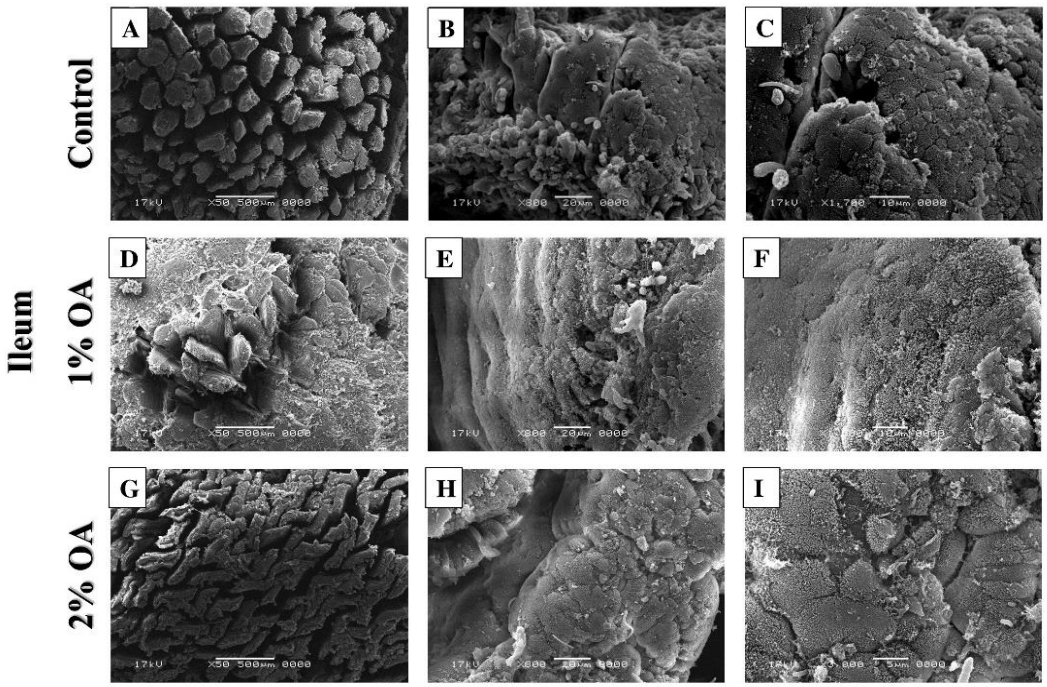

Plate 4: Photomicrographs A-I, showing the SEM scans of ileum in the study groups given no (Control), 1% organic acids (1% OA) or 2% organic acids (2% OA) in their diet for 52 days. Different arrangement of intestinal villi was noticed in response to the organic acids' administration were noticed

The length of the intestine in ducks, and poultry in general, is directly proportional to the absorption and utilization of digested feed (20). Consequently, our results showed that by adding organic acids in duck's diet, longer intestine with longer villi were obtained which resulted in substantially increasing the surface area of absorption, and therefore, increased feed utilization with achieving higher final body weight. Longer intestinal villi with organic acids administration were also recently reported in broiler chickens (4).

The ileum in poultry; ducks is no exception, has a characteristic ileal digestion (21), it was described to increase with addition of a single organic acid to the diet (7). The current results showed longer ileum and ileal villi with more organic acids in the diet, which implies that the utilization of feed is at its maximum, and this was reflected on the weight of reared ducks; as observed in our 2% organic acids group compared to the other groups. In addition, a higher dose of organic acids ensures that their favorable effects reach to the jejunum and ileum, thus guaranteeing maximum benefit from its actions throughout most of the small intestine; similar observations were previously reported in pigs (19).

From our results, the 2% group ducks had the highest final body weight and this group had longer duodenum, jejunum and ileum, also with longer villi, than the remaining groups. Although, it is difficult in this study to clearly determine whether the body weight was increased due to longer intestinal parts or the longer intestinal parts are a consequence to higher body weight, either ways the result is higher duck's weight and thus profitability in duck farming. In the 1% group these findings were also present but to a lower extent compared to the 2% group. This also highlights that the observed weight improvement and histomorphological changes in this study are dose dependent.

The arrangement of the villi was also different between groups with addition of organic acids to the diet. In the duodenum the villi became interwoven or zigzag-like and it was difficult to obtain a single straight villus in the H&E section (Plate 1, Figs. B & E) this was very clear with the 1% group and less clear in the 2% group compared to control ducks. While in the SEM the 2% group showed zigzag arrangement of jejunal villi (Plate 3, Fig. G). This could mean that different concentrations of organic acids can change the intestinal morphology. This interwoven or zigzag pattern of villus arrangement was mentioned in broiler chickens after giving organic acids in their diet (4); however, the authors failed to show any pictures of such arrangement in the published paper. The interwoven or zigzag arrangement of intestinal villi reported in the present study and in others suggests a more efficient nutrient absorption; as nutrients will have more contact time with the intestinal villi epithelium (22). Possible explanation to these changes in the arrangement of the villi could be due to either increased villus length (as seen in the present H&E results) or changes in the number of villi per square cm of intestine (as seen in the present SEM results). This was evident in both H&E stained sections and SEM images of the different intestinal parts; however, the exact mechanism needs further investigation.

It has been previously noticed that the intestinal villi shape and length changed in response to feed additives. For example, changes in villus height and cell mitosis rate was seen in response to dietary bamboo charcoal with vinegar (17) and to dietary natural zeolite including plant extract (18) in Aigamo ducks. Also, similar intestinal villi changes were seen in broiler chickens in response to addition of formic acid (7, 23), organic acid salts (24), organic acids (4) and aflatoxins (25) in their diet. In addition, similar differences were observed by comparing village chickens to commercial broilers in Malaysia (26).

Finally, from the previous studies and our results, one can hypothesize that any changes or even small amounts of additives to bird's diet can greatly alter the intestinal length and villus morphology. An important question could arise from this point, as changing a single constituent of the diet (addition of organic acids or simply changing the pH of the diet) caused changes in intestine's anatomy, histology and SEM within the same animal species. Therefore, could the

anatomical differences in the digestive system (macro and microscopic anatomy) between different species of birds be attributed to its feed habits rather than species-related differences? Answers to this question are unavailable in the literature and this needs further investigation.

Conclusions

The present results indicate that the addition of up to 2% organic acids to duck's diet increased small intestine's length and increased the length of intestinal villi without adverse effects on their microscopic structure. This evidently led to larger surface area for digestion and absorption of the feed, and, therefore, greatly improved the final body weight of the study ducks. It is, therefore, recommended to use 2% organic acids in duck's diet.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary data

Supplementary data is available upon reasonable request by e-mailing the corresponding author. The file contains detailed methodology used herein in addition to supplementary plates and tables.

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Original Research Article

THE IMPACT OF DIFFERENT STOCKING DENESITIES AND DIETARY PROTEIN LEVELS ON THE PERFORMANCE OF AFRICAN CATFISH (*Clarias gariepinus*) FINGERLINGS

Fawzy I. Magouz[1]; Malik M. Khalafalla[2], Eman M. Moustafa[3]* Amir S. Sadek[1]

[1]Department of Animal Production, Faculty of Agriculture, Kafrelsheikh University, [2]Department of Aquaculture, Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University, [3]Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt

*Corresponding author, E-mail: emantarek2002@yahoo.com

Abstract: The current experiment was randomly designed as a 3×2 factorial design to investigate the effects of two fixed factor (stocking density & dietary protein level) on growth performance, feed utilization, survival rate and physiological response of African catfish *(Clarias gariepinus)*. Fishes were allotted in 18 concrete (8×3×0.5 m^3; L×W×H) tanks at three different stocking density 30, 20 and 10 catfish fingerlings / m3 and fed on two different protein diets (25 and 30%). Each treatment was applied in three replicates. Fishes were fed 2.5% of biomass body weight twice/day. Results showed that growth performance, feed utilization, survival rate and hematological parameters were significantly improved with increased dietary protein level with low stocking density. The sixth treatment (high protein level 30% and lowest stocking density; 10 fish/m^3) exhibited the highest growth performance with no mortalities. Based on the results of the current study, it could be recommended to use protein level diet of 30% or more and stocking density of 10 catfish fingerlings/ m^3 to obtain high productivity in a short time with consequent decreased cost.

Key words: African catfish; *Clarias gariepinus*; dietary protein; growth parameters; Stocking density

Introduction

Fish is one of the most important alternative sources of dietary protein required to meet the increased human population demand in Egypt together with deceased other sources of animal protein (1). Aquaculture is considered a main key for bridging the national fish demand-supply gap (2). Increasing aquaculture production is clearly needed to meet this demand in the third millennium, because capture fisheries is showing precipitous decline due to habitat destruction, over fishing and pollution (3).

The utmost aquaculture goal is the production of high quality, high quantity, and low cost fish in short possible time. This can be achieved by careful species selection, good water quality, appropriate feeding and suitable stocking density (4).That is why it is important to determine the best carrying capacity of an aquatic environment in which we can keep certain numbers of fish in a given volume of the culture media resulting in the highest fish yield without negative

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effect on growth rate depending mainly on the amount and the quality of available food (5)

African catfish (*Clarias gariepinus)* is an endemic popular aquaculture species largely distributed in most African and Asian countries (6-10). It is widely cultured in freshwater ponds because of its high growth rate with excellent quality meat, ability to accept a wide variety of feed, resistance to diseases, easiness in reproduction and its ability to tolerate adverse environmental conditions, and high stocking densities under poly-culture conditions (7, 10-13).

The most critical aspect of aquaculture is to rear the early life stages of fish (fry and fingerlings) (14, 15) because fish at these stages are very sensitive to the different factors or determinants of production. Inadequate stocking densities and poor quality feed have a major role in poor growth and low survival rate of fry and/or fingerling stages resulting consequently in lower production

African catfish are carnivorous as well as predatory; having good special appetite for high protein diet. Therefore, dietary protein levels must be adjusted to meet their requirements. Moreover, stocking densities should be optimized to ensure that the available food is utilized more efficiently with maximum space utilization (16).

The present work was conducted to evaluate the effect of two different levels of dietary protein together with varying degrees of stocking density on the growth parameters, feed efficiency, nutrient utilization, hematological parameters and survival rate of African catfish fingerlings in concrete tanks.

Materials and methods

The present work was carried out in one of the commercial fish farms located at Kafr El-Sheikh Governorate, Egypt, in co-operation with the Animal Production Department, Faculty of Agriculture, Kafrelsheikh University, during the year 2017.

Fish

A number of 4320 African catfish fingerlings (*Clarias gariepinus*) with an average initial weight of (50 ± 2 g) were obtained from a private hatchery in Kafr El-Sheikh governorate. The fish were treated with potassium permanganate solution (3ppm) and acclimatized on the new environment (experimental conditions) for two weeks before the beginning of the experiment in concrete tanks. During the adaptation period, the fish were supplied with natural feeding (trash fish).

Experimental design

The experiment was randomly designed in a 3×2 factorial design. Three stocking densities were used with two levels of dietary protein. Thus, a total of six treatments run in triplicate were allotted into18tanks. Treatments (1-3) comprised of fingerlings fed with 25% CP and stocked at 30, 20, 10 fingerlings/m^3, respectively. However, Treatments (4-6) comprised of fingerlings fed with 30%CP and stocked at 30, 20, 10 fingerlings/m^3, respectively·

Catfish fingerlings (50 ± 2 g) were randomly stocked in 18 concrete tanks each was (8×3×0.5 m^3) capacity. All tanks were supplied with drainage water from drain El-Mohet through inlet PVC pipes (2 inch in diameter). Water outlet was found to be at a rate of 15 liter per minute. The fish were fed at a level of 2.5% of live body weight for about 90 days (experimental period).

Diets

Two tested diets containing 25 and30% protein levels were formulated from the local ingredients including fish meal, soybean meal, yellow corn, wheat bran, rice bran, wheat middling, sun flower oils, vitamins and minerals mixture, and Di-calcium phosphate to obtain control diet. The premix was added to all the experimental diets. The composition of the experimental diets was showed in table (1, A). The diets were prepared by thoroughly mixing the dry ingredients at first then mixed with oil. The diets were analyzed at The Central Laboratory of Food & Feed (CLFF), Agricultural Research Center, Ministry of Agriculture**.** Chemical composition and calculated gross energy of different experimental diets are presented in Table (1, B). First experimental diet (Diet1) consist of 25% crude protein and 408.14 kcal/100g

gross energy and the second experimental diet (Diet2) consist of 30% crude protein and 420.73 kcal/100g gross energy.

Feeding regime

Catfish were fed natural feeding for two weeks during the acclimatization period in the experimental tanks. After the two weeks of acclimatization, the catfish were fed the experimental diets at 2.5% from the total biomass daily for 90 days, and were applied twice a day (at 10:00 am & 14:00 pm). The feed amount was adjusted every seven days according to the new weight of the fish.

Proximate chemical analysis

Samples of the experimental diets as well as the experimental fish at the start and at the end of the experiments were obtained and then force dried, milled and deep frozen until determination the chemical analysis according to the methods described by (17).

Water quality management

Water quality parameters, such as temperature, dissolved oxygen, pH, alkalinity, hardness, ammonia, nitrate, nitrite and water salinity were estimated throughout the experiment. Water temperature was measured using thermometer. Oxygen level was measured daily at 8 o'clock by using oxygen meter (Model FE 247, EDT Instruments LTD. Dover Kent, UK). The pH was monitored using pH meter (Model Digi-sense, Cole-Parmer Instruments Co. Vernon Hills, IL. USA, Figure 1). Alkalinity was determined using test kit (Model WAT-DR. Code 4491-DR, LaMotte CO. Chestertown, Maryland, USA). Hardness was determined using test kit (Model HA-DT Cat. 20636-00, Hach Co. Loveland, Colorado, USA). Ammonia-N was estimated using test kit (Model NI, Cat. No. 22669-00, Hach Co.). Nitrite and nitrate were measured using test kits (Model NI-16 Cat. No. 20596-00 and Model NI-14 Cat.No. 14161-00, Hach Co., respectively). Determinations were carried out weekly according to APHA (18).

Determination of fish growth parameters

Growth and feed utilization were assessed by calculating average weight gain, average daily gain, specific growth rate, protein efficiency ratio, feed intake, feed conversion ratio, feed efficiency and survival rate:

Live body weight (LBW) was assessed in g for each individual group of each experimental treatment and recorded every 2 weeks (14 days).Total weight gain (TWG) (g/fish) = final body weight – initial body weight (19). Specific growth rate (SGR %/day) = [Ln final body weight- Ln initial body weight]×100/experimental period(d) (20). Average daily gain (ADG) = (W2 – W1) / t; where: W2 is the final weight, W1 initial weight and t is the time in days. Feed conversion ratio (FCR) = feed intake (g)/weight gain (g) (21). Protein efficiency ratio (PER) = weight gain (g)/protein intake (g). Protein productive value (PPV %) =100× (protein gain (g)/protein intake (g)). Survival rate (SR %) = total number of fish at the end of the experiment × 100 / total number of fish at the start of the experiment.

Hematological investigations

At the end of the experiment, fish in each group (3-4 fish) were weighed and blood samples were taken randomly from the caudal vein for blood analysis and differential leukocyte count. Anti-coagulated blood samples were prepared immediately for counting red and white blood cells, etc. Red blood cells count (RBCs$\times 10^6$/mm) and white blood cells count (WBCs$\times 10^3$/mm): were determined according to the method described by Stoskopf, (22). Hemoglobin concentration (Hbgm/dl) was estimated according to the method of Zinkl (23) and Packed cell volume (PCV%): was estimated by the microhaematocrite method described by Decie& Lewis (24).

Statistical Analysis

The obtained numerical data were statistically analyzed using SPSS (25) for two-way analysis of variance at 5% level of significance. When F-test was significant, least significant difference was calculated according to Duncan (26).

Results and discussion

Growth is the principal key of energy loss and/or gains in the aquatic environment; which

can be measured mainly by determination of the weight gain. Stocking density is a main factor significantly affecting the growth, feed utilization (FCR) and survival rate (SR) of the catfish fingerlings. High stocking densities act as stressors, fish aggregate for the same amount of feed and some of them cann't eat resulting in growth retardation (27) and/or decrease of feed utilization (28). Moreover, in high stocking densities, there is increased oxygen demand needed either for respiration or metabolism. Therefore, it is very important to adjust the stocking density with the carrying capacity of the aquaculture system.

Growth performance and survival rate

As shown in table 2, catfish fed on 30% protein level diet showed better final weights compared to those fed g on 25% protein level diet at the different stocking densities. The final weight was significantly increased with increased protein in diet ($P \leq 0.05$). The final weight of Treatment 1 (T1) was the lowest weight (214.3± 10.12) compared with the other treatments. While, final weight of treatment six (T6) was the highest weight (284.00± 14.32) ($P \leq 0.05$) compared with the other treatments. The average weight gain (AWG) was improved especially in groups fed on high protein level. While, the first treatment (T1) showed the lowest AWG (164.30±8.24) compared with other treatments. Similarly, average daily weight gain (ADG) of treatments (4-6) feeding on 30% protein was better than treatments (1-3) feeing on 25% protein diet in all variant stocking densities; with the highest value recorded in treatment 6 (2.60±0.63) and the lowest value in treatment 1 (1.83±0.42). Besides, the highest SGR (1.71±0.41) was observed in treatment 6. The results are similar to those reported by some authors (29-33), where they reported that increased protein concentration in the diet has a positive correlation with the final weight gain expressed as AWG, ADG and SGR.

On the other hand, results of the present study showed that the decreasing stocking density, the increased final weight. Decreased stocking density significantly affected the final weight gain, the highest stocking density in treatment 1 (30 fish/m^3)(214.30±10.12) gave the worst final body weight compared with treatment 3 (10 fish/m^3) (256.30±12.54), although both treatments feed on the same protein level diet 25%. These results also could be observed in fingerlings fed on 30% protein level diet, where treatment 6 (10 fish/$m^{3)}$ showed the best result of all growth parameters compared with other groups. This means that decreasing the stocking density gave a positive effect ($P \leq 0.05$) of final weight gain. These results agreed with many authors (34-38), where they found that increased stocking density resulted in less AWG, ADG and SGR.

Survival rate of the experimental fish was recorded. It was 100% in treatments 2, 3, 5 and 6 (low stocking density), but treatment 1 and 4 (highest stocking density) lost some fingerlings in the first days of experiment. This may be attributed to increased oxygen demand in high stocking treatments which may result in fish may succumb to suffocation. Survival rate increases with increased dietary protein level, this may be attributed to that low protein diets might have not met the nutrition requirements of catfish fingerlings and thereby leading to nutrient-deficient related mortalities (39, 40).

Water quality parameters

Water quality parameters of the experimental ponds were insignificantly affected by different treatments during the experimental period (90 days). As summarized in table 3, all water parameters for all experimental ponds showed that, dissolved oxygen not least 4 mg/ litter, toxic ammonia (NH3) no more than 0.6 mg/ litter, pH values between 8.2 and 8.5 degree, nitrate (NO2) no more than 0.2 mg/ litter, alkalinity 320, hardiness 150, salinity between 2000 and 4000 ppm (source of water was brackish water) and temperature about 27ºC. Water quality parameters observed in the current study were within the normal ranges required for normal growth of African catfish (9, 33, 39, 41-45); consequently any changes in the growth parameters may be attributed either protein level diet and/or stocking density.

Feed intake and nutrient utilization

As shown in table 4; holding all factors including the different stocking density of the present study, results showed that dietary protein has a marked influence on feed utilization. Results showed that increasing the level of protein in the diet increased significantly ($P \leq 0.05$) feed conversion ratio of fish. Diet 30% protein gave the best results ($P \leq 0.05$) of feed conversion ratio compared with diet 25% protein with different stocking densities; and the sixth treatment (1.00±0.09) gave the best feed conversion ratio (FCR) compared with the third treatment (1.13±0.11) (same of stocking density)while the first treatment (1.40±0.24) was the worst treatment compare with other treatments. Besides, increasing protein level in the diets affected significantly ($P \leq 0.05$) protein efficient ratio (PER) of fish. The same trend was observed with protein productive value (PPV); these results of feed utilization may be due to the impact of different protein levels together with different stocking densities. Protein efficiency ratio (PER) and protein productive value (PPV) were better generally with low stocking densities and high protein level diets. These results are in full agreement with some authors (35, 46-50).

Body composition of African catfish as affected by the experimental diets

Data concerning body composition responses of African catfish presented in table 5 revealed that the dry matter, crude protein, ether extract contents were increased by the time from the start to the end of experimental period (90) day, but the opposite was true for the ash percentage. Increasing protein level in the diet affected crude protein of body composition significantly ($P \leq 0.05$). Fingerlings in treatment 1 (25% protein) had low crude protein (66.92±5.36) ($P \geq 0.05$) as compared with the other treatments, while the sixth treatment (30% protein) (67.39±5.47) was the best in crude protein (CP) content ($P \geq 0.05$) compared with other treatments. However, dry matter (DM) in was low in treatment 1 (28.78±1.58) as compared with the other treatments, while sixth treatment (29.21±1.75) was the best treatment compared with the different treatments. Results also revealed that increasing level of protein in diet affected positively ($P \leq 0.05$) ether extract (EE) and gross energy (GE) of fish body.

Hematological investigation

As summarized in table (6), there was a significant increase in RBCs, Hb, PCV and WBCs in all treatments; the sixth treatment (30% protein and low stocking density) showed the best hematological parameters and treatment 1(25% protein and high stocking density) showed the worst hematological results. These results suggest that the physiological response of catfish fingerlings is improved when fed high protein level diet together with existing low stocking density.

In the present study, Growth depression observed in higher stocking densities groups may be attributed to reduced amount of adequate oxygen, followed by slowed down metabolism resulting in lower growth than in lower stocking density (27, 51). The decreased FCR at high stocking density (30 fish/m^3) may confirm that high stocking density reduced feed utilization efficiency. Due to competition for feed, there is low feed intake and consequently lower energy levels necessary for metabolism which is responsible to convert nutrients into fish flesh.

Growth depression observed in lower protein level diets has been observed in various fish species under captivity; this might be due to reduction in the available energy for growth (52, 53). Fish usually obtain energy from chemical breakdown of proteins than large animals that is why high protein is highly important in fish diet (54). Lowest growth in 25% protein level diet may be due to that most of protein was used for maintain life but unavailable for growth; however 30% protein level diet resulted in higher growth rate and better feed utilization in catfish fingerlings.

High focusing to body protein is of a great importance to meet the dietary requirements for tissue building, metabolism and repair; as a result of poor growth and poor feed utilization in fish group fed low protein level in the current study (55).This means the fingerlings survived in low stocking density and fed on 30% protein

level diet could use dietary protein more efficiently than fish survived in higher stocking densities and fed on 25% protein level diet.

Table 1A: Composition of the experimental diets

Raw Ingredients	Composition (%) experimental diets	
	Diet 1 (25% Protein)	Diet 2 (30% Protein)
Fish mail 62%	10	15
Soya bean 44%	27.5	35
Yellow Corn	10	10
Rice bran	24	21.5
Wheat middling	11	10
Wheat bran	14	5
Sun flower oil	2	2
Premix [1]	1	1
Di calcium phosphate	0.5	0.5
Total	100	100

(1)Premix Composition:- Each 3 kg contains , Vit A (1200000 i.u.), Vit D (300000 i.u.), Vit E (700 mg,) Vit K3 (500 mg,) Vit B1 (500 mg), Vit B2 200mg, Vit B6 (600mg), Vit B12 (3mg), Vit C 450mg, Niacin 3000mg, Methionine3000mg, Cholin chloride 10000mg, Folic acid 300mg, Biotin 6mg, Panthonic acid 670mg, Magnesiam salphate 3000mg, Copper sulphate 3000mg, Iron sulphate 10000mg, Zinc sulphate , 1800mg, Cobalt sulphate 300mg, Carrier upto 3000mg.

Table 1B: Proximate analysis of the experimental diets

Composition (%)	Diet 1 (25% CP)	Diet 2 (30% CP)
Dry matter	90.30	90.90
Organic matter	78.30	80.50
Crude Protein	25.45	30.40
Ether extract	5.40	5.00
Crude Fiber	6.60	6.40
Ash	12.00	10.40
Nitrogen free extract	50.55	47.80
Calculated energy value:		
*GE (kcal/100g)	408.14	420.73
**DE(kcal/g)	306.11	315.55

*GE (gross energy) was calculated according to NRC (1993) by factors of 5.65, 9.45 and 4.22 kcal per gram of protein, Lipid and carbohydrate, respectively. **DE (digestible energy) was calculated by applying the coefficient of 0.75 to convert gross energy to digestible energy.

Table 2: Growth performance parameters of African catfish fed different experimental diets

Treatment	Protein Levels %	Stocking density fish/ m^3	Initial BW(g)	Final BW(g)	AWG (g)	ADG (g)	SGR (%)	SR (%)
1	25	30	50.0±2.58	214.30±10.12^{c}	164.30±8.24^{c}	1.83±0.42^{c}	1.62±0.32^{ab}	99.80±5.54
2		20	50.0±2.44	227.00±11.24^{c}	177.00±9.54^{c}	1.97±0.51^{c}	1.40±0.38^{c}	100.0±0.00
3		10	50.0±2.64	256.30±12.54^{ab}	206.30±10.25^{ab}	2.29±0.61^{ab}	1.57±0.30^{b}	100.0±0.00
4	30	30	50.0±2.87	233.60±11.87^{bc}	183.60±8.98^{bc}	2.04±0.54^{bc}	1.45±0.24^{c}	99.90±4.87
5		20	50.0±2.67	247.00±12.54^{b}	197.00±9.68^{b}	2.19±0.55^{b}	1.52±0.29^{bc}	100.0±0.00
6		10	50.0±2.64	284.00±14.32^{a}	234.00±11.23^{a}	2.60±0.63^{a}	1.71±0.41^{a}	100.0±0.00

A,b and c mean the column bearing different letters differ significantly at 0.05 level

Table 3: Water parameters during the experimental period

Parameters	T1	T2	T3	T4	T5	T6	Standard no.
Temperature	27.5º	27.8º	28.1º	28.3º	27.7º	28.5º	20 : 32
Ph	8.2	8.4	8.3	8.5	8.2	8.3	6.5 – 8
Dissolved O_2	6	6	6	6	6	6	>4
NO_2	Nil	Nil	Nil	Nil	Nil	Nil	< 0.2
NH_3 (mg /l)	0.6 :0.9	0.6 :0.9	0.6 :0.9	0.6 :0.9	0.6 :0.9	0.6 :0.9	< 0.6
Alkalinity	320	320	320	320	320	320	<500
Hardiness	150	150	150	150	150	150	<500
Salinity ppm	2000 :4000	2000 :4000	2000 :4000	2000 :4000	2000 :4000	2000 :4000	0 – 5000

Table 4: Average feed intake, feed conversion ratio, protein efficiency ratio (%), feed efficiency and protein productive value of African catfish fed different experimental diets

Treatment	Protein Levels %	Stock density fish/ m^3	Feed in-take g/fish	Average weight gain (g)	FCR	PER	PPV
1	25	30	230.56±11.24	164.30±8.24^{c}	1.40±0.24^{c}	2.80±0.35^{c}	19.09±2.15^{b}
2		20	231.25±11.35	177.00±9.54^{c}	1.31±0.18^{bc}	3.01±0.57^{b}	20.56±2.22^{b}
3		10	233.33±11.57	206.30±10.25^{ab}	1.13±0.11^{a}	3.47±0.64^{a}	23.79±2.11^{a}
4	30	30	230.56±10.98	183.60±8.98^{bc}	1.26±0.17^{b}	2.62±0.38	17.96±1.98^{c}
5		20	231.25±10.89	197.00±9.68^{b}	1.17±0.12^{ab}	2.80±0.37^{c}	19.20±2.08^{b}
6		10	233.33±11.47	234.0±11.23^{a}	1.00±0.09^{a}	3.30±0.55^{ab}	22.56±2.34^{a}

A,b and c mean the column bearing different letters differ significantly at 0.05 level

Table 5: Body composition of African catfish as affected by the experimental diets (% on dry matter basis)

Treat. No	% On Dry matter basis				
	DM	CP	EE	Ash	GE** (Kcal/100g)
At the start of the experiment					
	25.00±1.15	62.80±4.56	14.10±1.22	20.95±0.38	497.14 ±11.5
At the end of the experiment					
T1	28.78±1.58	66.92±5.36	17.08±1.54	13.90±0.15	548.34±15.24
T2	29.12±1.64	67.13±4.87	17.02±1.36	13.91±0.21	548.31± 14.28
T3	29.11±1.52	67.36±5.24	16.94±1.54	13.74±0.32	548.94±13.47
T4	29.09±1.66	67.33±5.11	16.77±1.74	13.94±0.22	547.16±13.65
T5	29.19±1.87	67.36±5.22	16.77±1.64	13.87±0.19	547.50±13.78
T6	29.21±1.75	67.39±5.47	16.79±1.77	13.78±0.24	548.03±14.55

A,b and c mean the column bearing different letters differ significantly at 0.05 level.
**Gross energy was calculated according to NRC (1993) by using factors of 5.65, 9.45 and 4.22 Kcal per 1 gram of protein, lipid and carbohydrate, respectively

Table 6: Effect of protein levels and stocking denesity on the haematological parameters in catfish fingerlings

Treatment	Protein Levels %	Stocking density(fish / m^3)	Haematological parameters RBCs (x10/mm^3)	WBCs (x10^3/mm^3)	Hb (g/100ml)	PCV (%)
T1	**25**	30	2.48±0.05^d	75.86±1.12^b	7.62±0.41^c	21.00±0.5^a
T2		20	2.58±0.2^b	73.45±0.81^d	7.63±0.15^c	22.50±0.5^a
T3		10	2.68±0.14^b	74.23±1.3^c	7.64±0.33^c	22.00±0.5^a
T4	**30**	30	2.63±0.4^c	76.07±2.1^b	7.70±0.41^a	23.50±1.0^a
T5		20	2.72±0.2^a	72.51±0.5^e	7.79±0.5^{ab}	23.50±1.0^a
T6		10	2.83±0.08^a	67.23±0.59^f	7.82±0.28^a	24.00±0.19^a

RBCs= Red Blood cells; WBCs= White Blood cells; Hb = Hemoglobin; PCV = Packed Cell Volume

Conclusion

Both Stocking density and Protein level in fish diet have a significant effect on growth rate, feed utilization, physiological responses and survival rate *Clarias gariepinus* fingerlings in concrete tanks. Based on the results of the current study, it could be recommended to use protein level diet of 30% or more in combination with stocking density of 10 fish/ m^3 to obtain higher production in a short time.

Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

TRANSRECTAL ULTRASONOGRAPHY AND RECTAL PALPATION FOR JUDGING UTERINE AND CERVICAL INVOLUTIONS IN BUFFALO: A COMPARATIVE STUDY

Adel A. Ramoun[1]*, Essam A. Almadaly[1], Hamdy A. Hattab[2], Samy A. Darwish[3], Ismail I. EL- Kon[1]

[1]Department of Theriogenology, Faculty of Veterinary Medicine, Kafrelsheikh University, 33516, Egypt, [2]General Authority for Veterinary Services, Kafr EL-Sheikh, Egypt, [3]Mahallet Mousa Buffalo Research Station, Animal Production Research Institute, Agriculture, Research Center, Egypt

*Corresponding author: E-mail: ramoun2004@yahoo.com

Abstract: Uterine and cervical involutions were judged by transrectal ultrasonography (US) versus rectal palpations (RP) in buffaloes (*n = 26*). The diameters of the pregravid uterine horn (PGUHD) and cervix (CvD) were estimated by both transrectal US and RP every three days until gross uterine or cervical involution. Also, the US-measured PGUHD and CvD were recorded on the day of gross uterine or cervical involution on the basis of RP. The combined thickness of the myometrium and perimetrium of the pregravid uterine horn (PGUHMPT) and cervical wall thickness (CvWT) were parallelly estimated with US-measuring of PGUHD and CvD. The intervals to gross uterine ($P < 0.05$) and cervical ($P < 0.01$) involutions, on the basis of RP, were shorter than those on the basis of US. Both US-measured PGUHD and PGUHMPT, on the day of involution on the basis of US, were less than their US-measured counterparts on the day of gross involution on the basis of RP. Both US-measured CvD and CvWT, on the day of cervical involution on the basis of US, were less than their US-measured counterparts on the day of involution on the basis of RP. It is concluded that US- measuring of the pregravid uterine horn diameter and the combined thickness of myometrium and perimetrium as well as CvD and CvWT, is more accurate than RP-measuring of diameters of pregravid uterine horn and cervix for assessing the involution of the reproductive tract in buffaloes.

Key words: buffalo; involution; ultrasonography; cervical; combine thickness

Introduction

Uterine involution together with the earlier resumption of postpartum ovarian activity is prerequisites for having a successful reproductive performance in buffaloes (1). Days open is shorter in cows with smaller than those with the larger diameter of the pregravid uterine horn (2). Normal cervical and uterine involutions are essential for resuming ovarian activity (3). During the involution period, a crosstalk was detected between cervix and health status of the endometrium (4) from one side and with the resumption of the ovarian cyclicity from the other side such that the larger cervical diameter (CvD) associated with endometritis can result

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in abnormal follicular selection and abnormal ovarian cyclicity (5).

Before a cow is likely to conceive, it has to undergo cervical and uterine involutions (6). For the establishment of a new pregnancy in dairy cows, the uterus must return to its normal prepartum condition (7) and the cervix should close and regain its normal size and structure quickly (8).

Although rectal palpation (RP) remains the most commonly used method for assessing uterine involution in cows, it is less accurate than transrectal ultrasonography due to its high level of subjectivity (2) and variation among individual palpators (9,10).

Trans-rectal US is an alternative method for monitoring the dynamic changes in the reproductive system after calving which allows rapid, safe and accurate assessment of uterine involution and resumption of ovarian activity in cattle (10, 11). It gives information about cervical and uterine sizes, endometrial thickness and uterine contents, which are variables closely related to uterine involution (12). While the decrement in the diameter of the involuting uterus and cervix is expressed in ≤ 0.5 cm at least in the case of RP, it is estimated by millimeters in case of the transrectal US (13). The transrectal US is utilized not only in the follow up of puerperium but also shares in making reproductive management decision in many reproductive areas such as selection of animals to be kept in or culled from the herd, increasing the likelihood of the reproductive success and pregnancy diagnosis (14). The hypothesis of the work was that the US examination would be expected to be more accurate than RP for judging uterine involution thereby help to optimize reproductive management, ensuring higher future fertility, therefore the aim of the present work was to test the efficiency of transrectal US versus RP in judging cervical and uterine involutions in buffaloes.

Material and methods

Animals and management

The study was carried out in Mahallet Mousa Buffalo Research Station affiliated to Animal Production Research Institute, Agriculture, Research Centre, Egypt during the period extending from March to August 2017. Twenty-six healthy, 5-8 years old Murrah buffaloes, were enrolled in the present study. Their body condition score averaged 3.3 ± 0.4 (on a scale; 1 = lean to 5 = fatty). They had normal parturition and spontaneous placental expulsion within the first 12 hours after parturition. The calves were isolated after they had received colostrum and the buffaloes were milked twice daily. The animals were fed on a ration that met their maintenance and milk production requirements according to the requirements of Animal Production Research Institute (APRI 1997, unpublished data). Fresh water was available *ad libitum*. The Buffaloes were kept indoors in open yards, whereas half of the area was sheltered. This study was carried out under the Animal Welfare according to the regulations of Egyptian guidelines with approval granted by the Animal Ethics Committee of the Faculty of Veterinary Medicine, Kafrelsheikh University, Kafr EL-Sheikh, Egypt.

The judgment of uterine and cervical involutions

Rectal palpation

RP of the cervix and uterus was conducted by the same examiner every three days beginning on the third day postpartum. Uterine involution was considered complete when the diameters of the two uterine horns, at their bases, became nearly symmetrical and no further changes in the diameter of the pregravid uterine horn (PGUHD) could be detected during two successive RP of the uterus [6]. The cervix was considered involuted when the CvD, at three cm cranial to external OS, became stable in two successive RP (15). The PGUHD and CvD were recorded every three days and on the day of gross uterine or cervical involution. The interval to gross uterine or cervical involution on the basis of RP was recorded.

Ultrasonography

All transrectal US examinations were conducted by digital Ultrasonic Diagnostic Imaging System Model: D-P-30Vet. 10-2012 equipped with a linear probe, 7.5 MHz. All examinations were conducted by one examiner.

Cross-sectional images of PGUH, at its base, and cervix, at three cm cranial to external OS, were obtained by the transrectal US. When the image, cross section of either uterine horn or cervix, was not spherical, the diameter was estimated by averaging two 90° dimensions (2). Uterine involution was considered complete when uterine dimensions, PGUHD and the combined thickness of the myometrium and perimetrium of the pregravid uterine horn (PGUHMPT), became nearly stable in two successive transrectal US [16].

Cervical involution was considered complete when no further reduction in CvD/cervical wall thickness (CvWT) was detected in two successive transrectal US. The US-measured PGUHD and PGUHMPT were recorded when the uterine involution was considered on the basis of RP or US. Both the CvD and CvWT were measured and recorded every three days and on the day of a gross cervical involution on the basis of RP or US.The interval to gross uterine or cervical involution on the basis of transrectal US was recorded.

The rationale for measuring PGUHMPT rather than the thickness of either the whole of the uterine wall or endometrium by transrectal-US was due to the great folding of the endometrium into the uterine lumen and presence of still enlarged caruncles, especially during the early puerperium. The US-measuring of the endometrial thickness is more presumably beneficial for the diagnosis of endometritis rather than assessing the uterine involution.

The involution rate

The involution rate of PGUH

The involution rate (cm/ 3 days) of PGUH as indicated by the rates of the reduction in the PGUHD on the basis of RP or transrectal US examination was calculated by subtracting thePGUHD recorded on the days 6, 12, 18, 24, 30, 33...etc. from those recorded on the days 3, 9, 15, 21, 27, 30…etc. respectively, each of its previous one: e.g. (PGUHD on day 3- PGUHD on day 6), and so on until complete uterine involution. Also, the involution rate of PGUH as indicated by the rate of reduction in the PGUHMPT was calculated in the same manner as in the case of calculating the rate of reduction in the PGUHD.

The involution rate of the cervix

The involution rate (cm/ 3 days) of the cervix as indicated by the rates of reduction in the CvD on the basis of RP or the transrectal US was calculated by subtracting the CvD recorded on Days 6, 12, 18, 24, 30, 36, 42…etc. from those recorded on Days 3, 9, 15, 21, 27, 33, 39...etc. respectively, each of its previous one e.g. (CvD on day 3- CvD on Day 6). Also, the involution rate of the cervix as indicated by the rates of reduction in the CvWT was calculated in the same manner as in the case of calculating the rates of reduction in the CvD.

Statistical analysis

Data were analyzed with a statistical software program (GraphPad Prism version 5.0; GraphPad Software, San Diego, CA, USA).The means±SEM of the interval to gross uterine or cervical involutions on the basis of RP were compared to those recorded on the basis of transrectal US by using t-test. Also, by using t-test, the US-measured PGUHD and PGUHMPT or US-measured CvD and CvWT on the Day that either of the pregravid uterine horn or the cervix was considered involuted on the basis of RP were compared with their counterparts measured by transrectal US on the Day of uterine or cervical involutions on the basis of transrectal US. The involution rate of PGUH as indicated by the rate of reduction in either of PGUHD or PGUHMPT was examined by repeated measures ANOVA followed by Bonferroni's multiple comparison tests. Also, the involution rate of the cervix as indicated by the rates of reduction in CvD or CvWT was examined by repeated measures ANOVA.

Results

Intervals to gross uterine and cervical involutions on the basis of RP and US

The interval to gross uterine involution as being detected by RP was shorter ($P < 0.05$) than its counterpart detected by the transrectal US. The interval to gross cervical involutions as being detected by RP was shorter ($P < 0.01$) than its counterpart detected by transrectal US (Table 1).

The US- measured PGUHD and CvD on the day of gross uterine and cervical involutions on the basis of RP and US

The US-measured PGUHD and PGUHMPT, recorded on the day of a gross uterine involution on the basis of RP, showed increases at $P < 0.01$ and $P < 0.001$ respectively compared with their counterparts recorded on the day of a gross uterine involution on the basis of transrectal US. Also, the US-measured CvD and CvWT recorded on the day of a gross cervical involution on the basis of RP showed increases ($P < 0.05$) compared with their counterparts recorded on the day of a gross cervical involution on the basis of transrectal US (Table 2).

The Involution rate of pre-gravid uterine horn

The involution rate of PGUH as being indicated by the rates of reduction in RP-measured PGUHD throughout involution period showed a decrease ($P < 0.05$) among interval: 3 to 6, 6 to 9 and 9 to 12 and no difference ($P \geq 0.05$) among intervals: 9 to 12, 12 to 15, 15 to 18, 18 to 21, 21 to 24, 24 to 27 and 27 to 30 (Fig. 1). The involution rate as being indicated by the rates of reduction in the US-measured PGUHD throughout the involution period showed a decrease ($P < 0.05$) among intervals: 3 to 6, 6 to 9,9 to 12, 12 to 15, 15 to 18, 18 to 21, 21 to 24, and no difference among intervals: 24-27, 27 to 30, 30 to 33 and 33 to 36 (Fig. 1).The involution rate of PGUH on the basis of rates of reduction in PGUHMPT showed significantly ($P < 0.05$) decrease among intervals: 3 to 6, 6 to 9, 9 to 12, 12 to 15, 15 to 18, 18 to 21, 21 to 24 and 24 to 27 days and no difference among intervals: 24 to 27, 27 to 30 and 30 to 33 respectively (Fig. 3).

The involution rate of the cervix

The involution rate of the cervix as being detected by the rates of reduction in the RP- measured CvD showed a difference ($P < 0.05$) among interval 3 to 6, 6 to 9 and 9 to 12, and no difference among intervals: 9 to 12, 12 to 15, 15 to 18, 18 to 21, 21 to 24, 24 to 27, 27 to 30 and 30 to 33**.** On the other hand, the involution rate as being detected by the rates of reduction in the US-measured CvD showed differences ($P < 0.05$) among intervals: 3 to 6, 6 to 9, 9 to 12, 12 to 15, 15 to 18, 18 to 21, 21 to 24, 24 to 27, 27 to 30, and 30 to 33 and no difference ($P \geq 0.05$) among intervals: 33 to 36, 36 to 39 and 39 to 42 (Fig. 2). The involution rate of the cervix on the basis of the rates of reduction in CvWT showed a significant decrease ($P < 0.05$) among postpartum intervals: 3 to 6, 6 to 9, 9 to 12, 12 to 15, 15 to 18, 18 to 21, 21 to 24, 24 to 27, 27 to 30, 30 to 33 and 33 to 36 and no difference ($P \geq 0.05$) between intervals from 36 to 39 and 39 to 42 days (Fig. 4).

Table 1: Intervals to gross uterine and cervical involutions detected on the basis of rectal palpation and trans-rectal ultrasonography

Parameter	Method of Judgment	
	Rectal palpation (RP)	Ultrasonography (US)
Interval to gross uterine involution	24.8 ± 0.96	29.3 ± 0.89*
Interval to gross cervical involution	30.5 ± 0.92	37.0 ± 0.10**

Means within the same row and bearing different superscripts were significantly different at * $P < 0.05$;** $P < 0.01$. The interval to gross uterine involution as being detected by RP was shorter ($P < 0.05$) than its counterpart detected by transrectal US. The interval to gross cervical involutions as being detected by RP was shorter ($P < 0.01$) than its counterpart detected by transrectal US.

Table 2: The ultrasound- measured PGUHD and CvD as well as PGUHMPT and CvWT on the days of gross uterine and cervical involutions on the basis of RP and US

Day of involution	Uterine dimensions		Cervical dimensions	
	PGUHD	PGUHMPT	CvD	CvWT
Based on RP	$2.34 \pm 0.06^{**}$	$0.64 \pm 0.02^{***}$	$2.01 \pm 0.06^{*}$	$0.52 \pm 0.02^{*}$
Based on US	2.09 ± 0.04	0.47 ± 0.01	1.72 ± 0.08	0.43 ± 0.02

Means within the same column and bearing different superscripts are significantly different at * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

PGUHMPT means ultrasound –measured combine thickness of both myometrium and perimetrium of the pregravid uterine horn; PGUHD means the diameter of the pregravid uterine horn; RP means rectal palpation, US means ultrasonography. Both the US-measured PGUHD and PGUHMPT, recorded on the day of gross uterine involution on the basis of RP, showed increases at $P < 0.01$ and $P < 0.001$ respectively compared with their counterparts recorded on the day of gross uterine involution on the basis of transrectal US. Both the US-measured CvD and CvWT recorded on the day of gross cervical involution on the basis of RP showed increases ($P< 0.05$) compared with their counterparts recorded on the day of gross cervical involution on the basis of transrectal US.

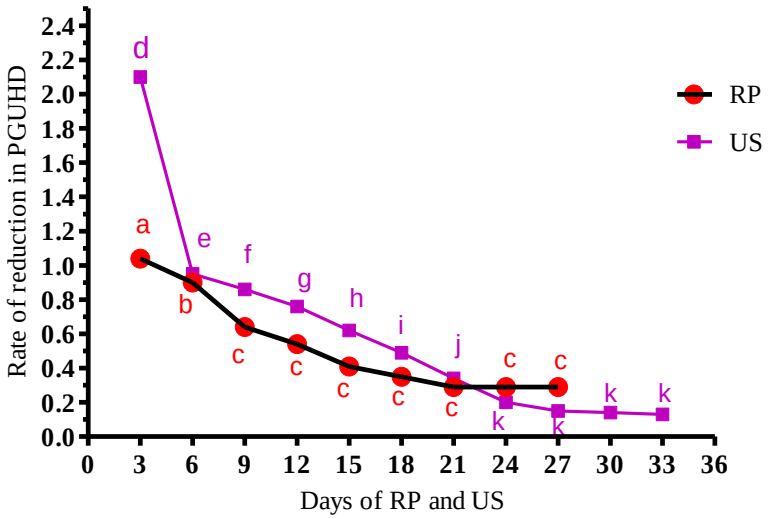

Figure 1: Rate of reduction in the PGUHD per three days interval throughout involution period on the basis of RP and US examinations in buffaloes. PGUHD = Pre-gravid uterine horn diameter; RP=Rectal palpation; US=Ultrasound examination. For rectal palpation, values carrying different letters from a - c differ at $P < 0.05$. For ultrasound examination, values carrying different letters from d – k differ at $P < 0.05$. The rate of reduction in the RP-measured PGUHD shows a decrease ($p < 0.05$) in the 3rd interval compared with either 2nd or 1st and in the 2nd compared with the 1st interval but shows no difference ($P > 0.05$) among the rest of intervals from 3rd (9-12) till 8th interval (24-27). On the other hand, the rate of reduction in the US-measured PGUHD shows a decrease ($p<0.05$) among intervals from 1st (3-6) till the 7th (21-24) but no difference are observed among intervals from 7th till 10th (30-33).

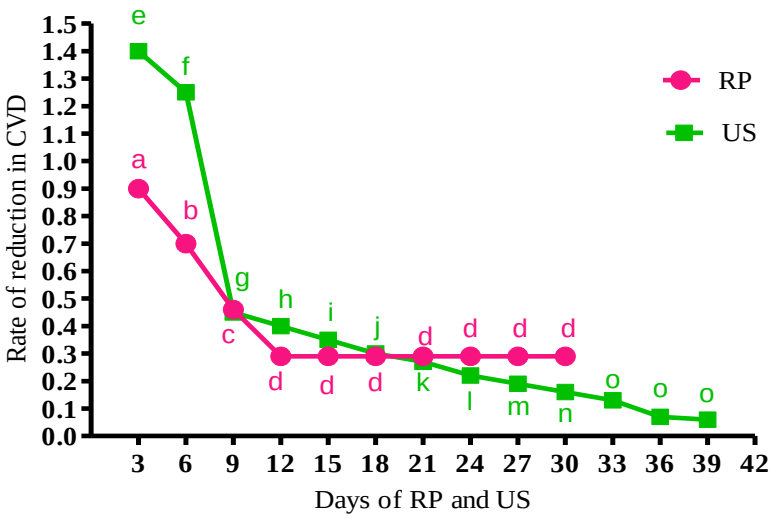

Figure 2: Rate of reduction in the CvD in buffaloes throughout the involution period on the basis of RP and US examinations. CvD = Cervical diameter; RP = Rectal palpation; US = Ultrasound examination. For rectal palpation, values carrying different letters from a - d differ at $P < 0.05$. For ultrasound examination, values carrying different letters from e – o differ at $P < 0.05$. The rate of reduction in the CvD on the basis of RP shows a decrease ($p<0.05$) among intervals: 3-6, 6-9 and 9-12 and no difference ($p \geq 0.05$) among intervals from 3rd (9-12) till 9th (27-30). On the other hand, the US-measured CvD shows among 1st (3-6) till the 10th (30-33) intervals and no difference ($p \geq 0.05$) among intervals from 10th till 13rd (39-42).

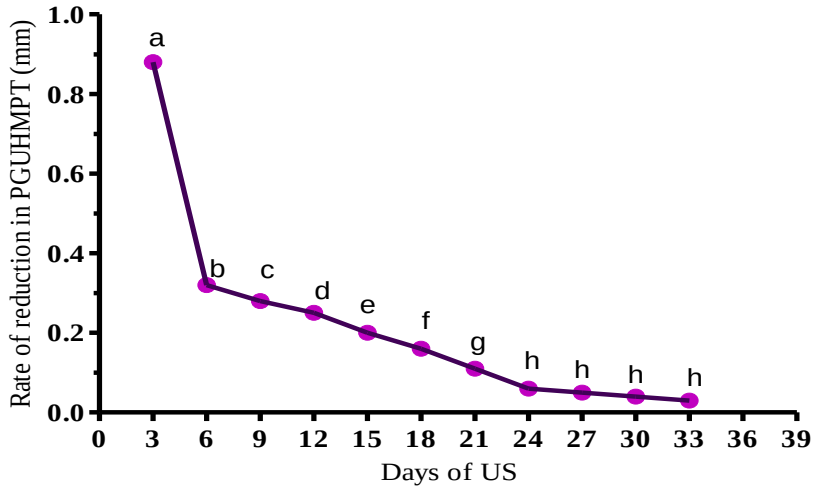

Figure 3: The rate of reduction in the PGUHMPT per 3 days throughout the involution period on the basis of US in buffaloes. US = Ultrasound examination; PGUHMPT= combine thickness of the myometrium and perimetrium. *Values carrying different letters differ at $P < 0.05$. The PGUHMPT shows a decrease ($p < 0.05$) among intervals from the 1st (3-6) till the 8th (24-27) and no difference among the intervals from 8th till 10th (30-33).

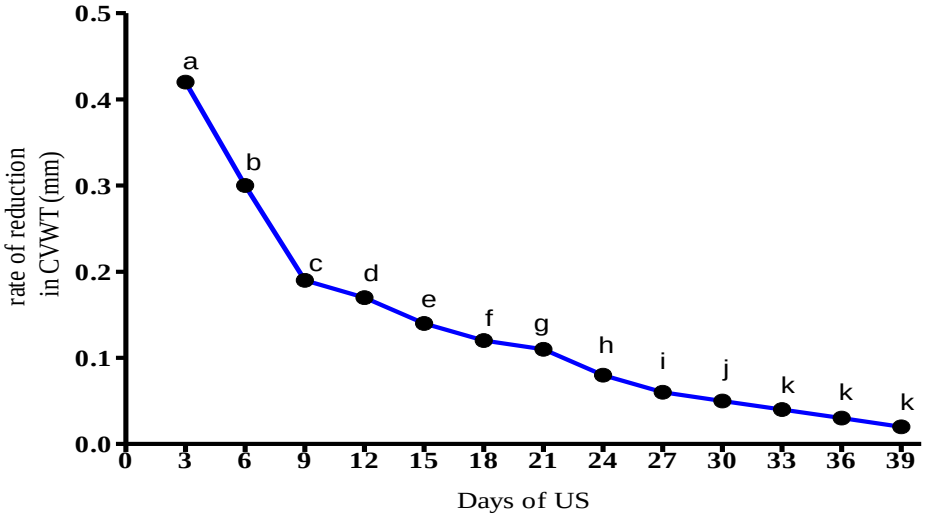

Figure 4: Rate of reduction in CvWT per 3-days throughout involution period by transrectal US in buffaloes. US = Ultrasound examination, CvWT= Cervical wall thickness. Values carrying different letters differ at $P < 0.05$. The CvWT shows a significant decrease ($P < 0.05$) among postpartum intervals: 3 - 6, 6 - 9, 9 - 12, 12 -15, 15 -18, 18 - 21, 21 - 24, 24 - 27, 27 - 30, 30 - 33 and 33 - 36 and no difference ($P \geq 0.05$) was detected between intervals from 36 - 39 and 39 - 42 days.

Discussion

The aim of the present study was to compare the efficiency of RP and the transrectal US in judging cervical and uterine involutions in buffaloes. On the basis of RP, the interval to gross uterine involution (24 days) may come in concurrence with previous studies (17, 18) in buffaloes. On the other hand, longer intervals to gross uterine involution, 24-56 days (19); 24-44 days (20) and 21-74 days (21), were recorded in buffaloes. Also, shorter intervals, 14-29 days (22) and 20 days (23) were recorded in cows on the basis of RP.

On the basis of transrectal US, the interval to gross uterine involution (30 days) in the present study, more or less, coincides with those recorded in previous studies: 30 days (24); 28 days (11); 31 days (25) and 27 days (17).

Matching the interval to gross uterine involution either on the basis of RP or the transrectal US, recorded in the present study, with their counterparts in previously mentioned studies herein revealed that while greater variations were observed in case of RP, lesser variations were noted in case of transrectal US. This observation indicates the higher accuracy of transrectal US compared with RP for judging uterine involution.

However, the wide variations between the interval to gross uterine involution on the basis of RP in the present study and intervals recorded in the previous studies might be due to the variations among the criteria used to evaluate the uterine involution and/or variations among individual palpators during measuring the dimensions of the involuting uterus (9). Also, Okano and Tomizuka. (1987) (16) attributed the inaccuracy in estimating the uterine dimension by RP to the variations in fingers width among palpators and nature of the postpartum uterus which is being relaxed and flat especially during early puerperium. However, the previously mentioned limitations, that affect the efficiency of RP in estimating uterine dimensions, are not present in case of transrectal US.

The interval to gross cervical involution on the basis of RP coincides with that (31.1) recorded byEl-Fouly et al. (1976) (26)and Usmani et al. (2001) (27) in buffaloes but was being longer than that, 25, recorded by Atansov et al. (2012) (10)in buffaloes. However, the disagreement between the current study and the study of Atansov et al. (2012) (10) may be attributed to individual variations among palpators in the two studies. The increase in the interval to gross uterine or cervical involution on the basis of transrectal US compared with RP is believed to

be expended in estimating the lesser differences (in terms of mm) in diameter of either pregravid uterine horn or cervix which would be supposed to still undergo involution on the basis of transrectal US. On the other hand, the reductions in the diameters (in terms of ≤ 0.5 cm) between successive RP were greater thus required a shorter time to reach stability.

Moreover, the US scanning of both uterus and cervix during assessing the involution gives more diagnostic criteria that cannot be accurately detected by RP such as the wall thickness of cervix and pregravid uterine horn as well as smaller amounts of intrauterine fluids (Lopez-Helguera et al. 2012) (12). Doubtless, the combining between the US- measured PGUHMPT or CvWT and corresponding PGUHD or CvD as diagnostic criteria, for assessing uterine and cervical involutions will be better than using either of them solely especially in case of assessing uterine involution.

However, US measuring of the PMGHMPT may be more accurate than measuring PMGHD because the PGUH in some cases may be compressed under linear probe while it is being positioned over it giving false results. Thus, measuring PMGHMP could be efficiently used as a complementary parameter to PMGHD for assessing uterine involution.

The benefit of assessing the involution rate of PGUH and cervix on the basis of detecting the rate of reduction in their diameters by RP or transrectal US was to check the efficiency of RP and transrectal US in determining the time when the greatest reduction in the uterine or cervical size had occurred as well as the normalcy of the involution process at any time throughout the involution period. Cengic et al. (13) reported that the speed of uterine involution could be monitored by detecting the decrease in the uterine horn diameter and its wall thickness.

The results of the present study indicated that the greatest rate of reduction in the PGUHD had occurred during the intervals 0-9 days on the basis of RP and 0-15 days on the basis of transrectal US. Afterward, the rates of reduction in the successive RPs or US examinations became relatively smaller. This indicates that the higher efficiency of US compared with RP in determining the time in which the greatest reduction in the size of PGUH had occurred. However, the pattern of reduction in US-measured PGUHD throughout involution period may come in coincidence with that described by Atansov et al. (10). Matching the involution rates between RP and transrectal US methods revealed that although there were no differences among rates of involution beyond 9th day postpartum in the case of RP, there were differences among all transrectal US examinations conducted from 3rd until 27th day except during the interval from 18th to 21st day indicating the higher efficiency of transrectal US in assessing involution rates throughout the involution period.

Matching the involution rate of the cervix as indicated by the rates of reduction in the CvD on the basis of RP with their counterparts on the basis of transrectal US revealed that although the involution rate detected by RP and the transrectal US on the Day 9 became similar, it abruptly decreased in case of RP to 0.29 cm/3 days on the 12th day and remained so until the end of involution period. On the other hand, in the case of transrectal US, it gradually decreased significantly until reaching comparable value, 0.30 cm/ 3days, on the 18th day. However, these results indicated the subjectivity of RP and higher efficiency of transrectal US in the follow up of the cervical involution.

The higher reduction rates (1.40 and 1.25 cm/3 days recorded during postpartum intervals 3 to 6 and 6 to 9 respectively) in US-measured CvD indicate that the greatest cervical involution occurs within the first postpartum week. These results partially agree with Atansov et al. (10) who found that the mean CvD decreased by more than 45% between the first and seventh days postpartum. Also, the results of the present study agree with the same authors beyond day 19 whereas CvD regressed slowly until the 25th day while non-significant differences were detected in CvD between the 25th and 34th day. However the overall pattern of the rates of reduction in the US-measured CvD may be comparable with that recorded by Pariksh et al. (1) in Buffalo who reported that the reduction in the

CvD was faster until the 15th day, marginal from the 16th to the 25th day and became negligible beyond the 25th day postpartum.

Regarding the involution rate of the PGUH on the basis of rates of reduction in PGUHMPT, it was noted that the greatest rate of reduction (0.88 cm/3 days) was recorded in the period from the 3rd to the 6th day postpartum indicating that the greatest reduction in the PGUHMPT had occurred during the first week. Afterward, it abruptly decreased to 0.32 cm between 6th and 8th day. Later on, it was regularly decreasing (at 0.04 cm) from 0.32 between 6th and 8th to 0.16 cm in the interval between 18th and 20th day. This indicates that the involution rate became slower as the time became farther from parturition. However, beyond day 21, the involution rate was similar and became nearly negligible.

Regarding the involution rate of the cervix on the basis of the rates of reduction in CvWT, it was noted that the greater rates of reduction were recorded in the intervals: 3 to 6 and 6 to 9 days. Afterward, although the differences between the rates of reduction among succeeding the transrectal US were significant, the decrease in the CvWT was slow. Beyond day 30, the rates of reduction among 3 successive transrectal US became similar and nearly negligible. This may be explained in light of the structural components of the cervix, which is mainly formed from connective tissues rather than smooth muscles as in the case of the uterus.

It is concluded that US- estimation of the diameter and the combined thickness of myometrium and perimetrium of the pregravid uterine horn as well as CvD and CvWT thickness is more accurate than RP-measured diameters of pregravid uterine horn and cervix for assessing the involution of the reproductive tract in buffaloes.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

METHICILLIN RESISTANT *Staphylococcus aureus* (MRSA) IN CAMEL MEAT: PREVALENCE AND ANTIBIOTIC SUSCEPTIBILITY

Waleed R. El-Ghareeb[1,2,*], Faisal S. Almathen[1], Mahmoud M. Fayez[3], Raed A. Alsultan[4]

[1]Department of Veterinary Public Health and Animal Husbandry, College of Veterinary Medicine and Animal Resources, King Faisal University, 400 Al-Hasa, 31982, Saudi Arabia, [2]Food Control Department, Faculty of Veterinary Medicine, Zagazig University, 44519, Egypt, [3]Veterinary Serum and Vaccine Research Institute, Cairo, Egypt, [4]Ministry of Environment, Water and Agriculture, Saudi Arabia

*Corresponding author, E-mail: welsaid@kfu.edu.sa

Abstract: This study is an initiative study intended to investigate the prevalence and antibiotic susceptibility of *Staphylococcus aureus* and MRSA obtained from fresh camel meat retailed in Al-Hasa, Saudi Arabia. The survey has been conducted for five months where fresh camel meat were microbiologically analyzed to isolate *S. aureus* using culture media and VITEK2 technique. The Identification of *S. aureus* was done by DNA expression of gene specific 16S rRNA, while mecA gene expression was recognized in the identified MRSA isolates using PCR. The recorded prevalence of *S. aureus* was 10.7 % (20/187) from raw camel meat. Three isolates out of twenty *S. aureus* isolates were confirmed to be MRSA. Susceptibility to 10 antimicrobials was estimated using the disc diffusion method. These results suggested that MRSA is found in camel meat retailed at Al-Hasa, Saudi Arabia. We recommend that, surveillance protocol should be embraced in meat safety and public health programs.

Key words: camel meat, *S. aureus*, MRSA, antimicrobial resistance

Introduction

Staphylococcus aureus is an important pathogen causing food-borne illness worldwide and causes serious diseases in human being (1). The main sources of infection could be the inadequate personnel hygiene, unhygienic handling and storage of food infected with staphylococci. Frequently associated foods are meat, dairy products, cream-filled bakery items and salads (2). Methicillin-resistant *S. aureus* (MRSA) appeared as a dangerous agent for different patients and especially in those with impaired immune system. MRSA strains are actually widespread human isolate but are unusual animal isolates (3). MRSA firstly recognized as nosocomial infection. Later in the community and livestock. It has a continuous and dynamic epidemiology. Human may acquire MRSA infection via contact with contaminated environment or contaminated marketed meat (4, 5). Resistance in MRSA is mediated by the gene mecA, which located on the chromosome in staphylococcal cassette chromosome mec (SCCmec), and this encodes penicillin-binding protein (PBP) 2a with a low affinity for beta-lactams (6).Transmission of MRSA from food

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to people causes a serious problem especially for the immunocompromised people. *S. aureus* was identified in 42 out of 176 raw meat (beef, poultry and pork) over a one-year survey (7) and they did not find any evidence indicating vancomycin, or methicillin-resistance. *S. aureus* also isolated from the wild animal carcasses (2.0% prevalence) and authors found no methicillin-resistance (8). Several studies in Saudi Arabia were conducted to investigate the prevalence, risk factors and genetic distribution of MRSA isolated from healthy and clinical human and animal cases (9-14). However, rare reports were recorded with regard to the prevalence of MRSA in food especially camel meat. To the best of our knowledge, few studies were conducted to isolate and identify MRSA from camel meat allover Arabian countries (15, 16).This study was done to investigate the MRSA prevalence in camel meat samples retailed to the public at Al-Hasa, Saudi Arabia. mec-A gene expression was carried out in identified *S. aureus* isolates using PCR. Antimicrobial resistance profile for the *S. aureus* isolates were further analyzed. This study is one of the very few studies regarding the prevalence and genetic characters of MRSA from camel meat in Saudi Arabia.

Materials and methods

All experiments followed the ethical and scientific principles adopted by King Faisal University, Saudi Arabia.

Collection of samples

A total of 187 minced camel meat samples were collected randomly from supermarkets in Al-Hasa province, Saudi Arabia in a 5 months period. The collected samples were taken directly in sterile polyethylene bags (icebox) to the Meat Hygiene laboratory at the Veterinary Public Health and Animal Husbandry Department, College of Veterinary Medicine, King Faisal University for the subsequent microbial culturing and identification.

Isolation and identification of coagulase positive S. aureus

For investigation of coagulase positive *S. aureus*, after sample digestion in a Stomacher® 400 Circulator, an inoculum of 0.1 ml of serial dilution 10^{-1}&10^{-2} was evenly surface distributed on Baird Parker agar base supported with egg yolk tellurite emulsion Agar (Oxoid CM0275) according to (17). After incubation at 37°C for 48 hours, counting of all typical colonies using colony counter was performed. For confirmation, five typical colonies were selected and transferred to brain heart infusion broth tubes (BHIB) (Oxoid CM1135) for subsequent culture and preservation. Presumptive colonies were transferred and subcultured on Mannitol Salt Agar (Oxoid CM0085) and then incubated for 24 hrs at 37°C. Gram stain, catalase, mannitol fermentation, DNAs and coagulase tests were applied on suspected colonies to identify coagulase *S. aureus* (18). In addition, VITEK 2-compact was used to identify and differentiate staphylococci species (BioMérieux, Rev 03, 2004).

Screening for MRSA

Screening of All *S. aureus* isolates for their methicillin resistance was done by the standard disc diffusion procedure described by Clinical and Laboratory Standards Institute (19) using cefoxitin (30 μg).

DNA Extraction

Staphylococcus aureus isolates were grown in BHIB at 37º C for 18 hours. The cells were harvested by centrifugation at 8000 rpm for 20 minutes at 4º C and washed twice by phosphate-buffered saline. The pellet was suspended in 1 ml of 10 mMTris-HCl (pH 8) containing lysozyme (2.5 mg/ml) and incubate at 37º C for 2 hours. Genomic DNA was extracted using QIAamp DNA Mini Kit (QIAGEN) according to the instructions from manufacturer.

DNA amplification by Polymerase Chain Reaction (PCR):

Detection of Staphylococcus 16S rRNA

PCR was used for detection of Staphylococcus 16S rRNA using primers in (Table 1), according to (20).The PCR was performed in a 25ul volume, where 2 ul of the extracted DNA (100-ng) was added to 12.5 ul of oasig ™2X

qPCR Master mix , 1 ul (0.5 mM concentration) of each primer set and 8.5 ul PCR grade H2O.PCR amplifications were performed in a MyGenie 32 Thermal Block (BIONEER as follows: Initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 2 min and ended with final extension for 10 min at 72°C and finally maintained at 4°C.

Detection of mecA-gene

The polymerase chain reaction (PCR) was used for detection of mecA-gene in antibiotic resistance strains using primers in (Table 1), according to (21). In a final volume of 50 ul, three ul of DNA template was added to 25 ul of oasig ™2X qPCR Mastermix; 1 ul (0.25 mM concentration) of each primer set and 20 ul PCR grade H2O. DNA amplifications were carried out in a MyGenie 32 Thermal Block (BIONEER as follows: Initial denaturation at 94°C for 5 min followed by 36 cycles of denaturation of 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min with a final extension at 72°C for 5 min. Strain MF01 (GenBank, KY647024.1) was used as a positive control for mecA gene.Ten microliters of PCR products (16S rRNA and mecA- genes) was analyzed by 1% agarose gel electrophoresis. A 100 bp molecular weight DNA ladder (Gel Pilot Plus, QIAGEN) was used for the validation of length of the amplified products.

Antimicrobial susceptibility

All isolates were tested using 10 antimicrobial agents: Penicillin G (10U), ampicillin (10 µg), tetracycline (30 µg), amoxicillin-clavulanic acid (20/10 µg), ciprofloxacin (5 µg), cefoxitin (30 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), erythromycin (15 µg), clindamycin (2 µg), gentamicin (10 µg), Antimicrobial sensitivity was monitored with the standard disk diffusion assay .The zone of inhibition was interpreted according to CLSI guidelines (19). *S. aureus* ATCC 25923 was used as reference strain.

Results

Primers for both staphylococcus 16S rRNA and mecA-gene were tabulated in table (1). Identification of *S. aureus* was done by DNA expression of gene specific 16S rRNA gene (figure 1).

Figure (2) clarified that, *S. aureus* was present in 20 out of the 187 samples (10.7%). Of the 20 *S. aureus* isolates, three (15 %) were found to be MRSA, therefore, Figure 2 shows the prevalence of MRSA strains among total samples was only three (3/187) (1.6%).

Identification of MRSA was done by DNA expression of gene specific mec-A gene (figure 3).

Table (2) showed different patterns of Antimicrobial susceptibility of *S. aureus* isolates. 35% were resistant to β-lactam, 50% were resistant to penicillin, 65% were sensitive to ciprofloxacin, 90% were sensitive to Erythromycin and Clindamycin, 85% were sensitive to Cefoxitin, 80% sensitive to Gentamicin, 60% were resistant to Penicillin G, 75% were sensitive to Trimethoprim and finally 75% were sensitive to tetracycline.

Figure (4) declared the percentages of *S. aureus* resistant strains among the identified isolates using the disk diffusion method according to Clinical and Laboratory Standards Institute guidelines. Results showed a higher resistant level for both penicillin and Ampicillin (60% and 50%, respectively). As shown in table (3), only two isolates (10%) out of all analyzed strains exhibited single resistance to penicillin. MRSA isolates presented antimicrobial multi resistance.

Table 1: Primers for both Staphylococcus 16S rRNA and mecA-gene

DNA target	Primer pair	Size (bp)	Reference
Staphylococcus 16S rRNA	5'GTT ATT AGG GAA GAA CAT ATG TG-3' 5'CCA CCT TCC TCC GGT TTG TCA CC-3'	750	Jaffe *et al.*, 2000
mecA-gene	5' AAAATCGATGGTAAAGGTTGGC-3' 5' AG TTCTGCAGTACCGGATTTGC-3'	530	Murakami *et al.*, 1991

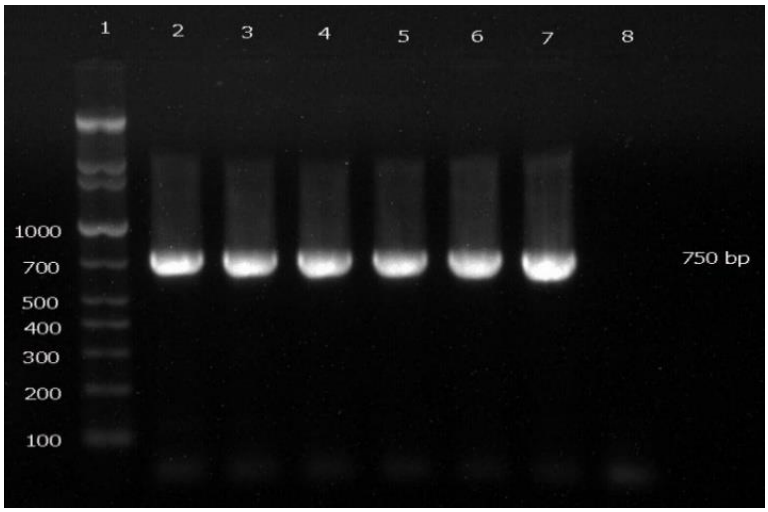

Figure 1: Agarose gel electrophoresis of amplified 750-bp DNA fragment. Lanes: 1 DNA ladder molecular weight marker (GelPilot), 2-6 staphylococcus isolates, 7 ATCC 12600

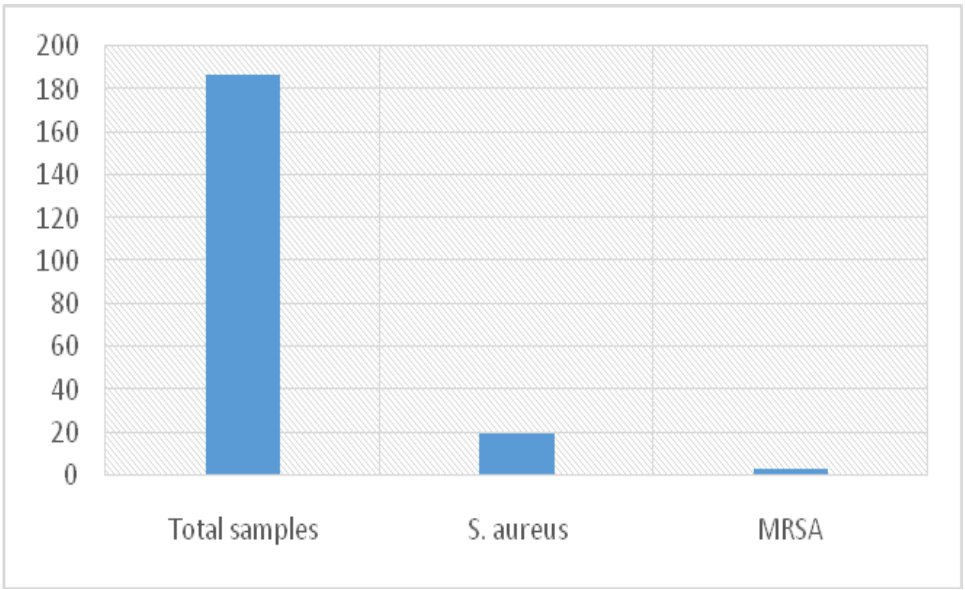

Figure 2: Prevalence (%) of *S. aureus* and MRSA in camel meat samples

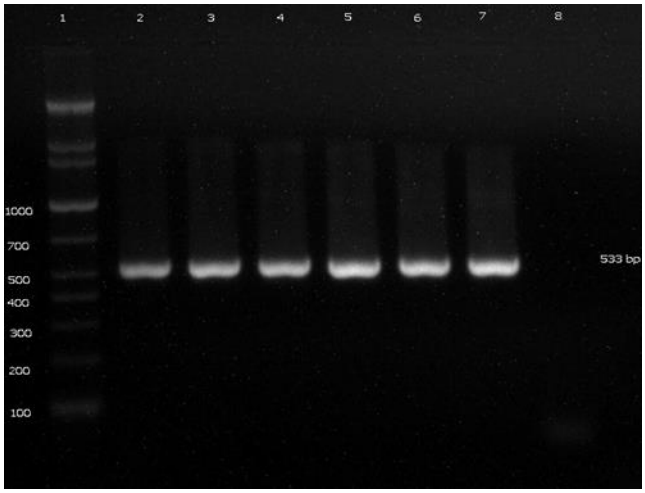

Figure 3: Agarose gel electrophoresis of amplified 533-bp DNA fragment. Lanes: 1 DNA ladder molecular weight marker (GelPilot), 2-6 MRSA isolates, 7 Strain MF01, 8 Negative control.

Table 2: Antimicrobial susceptibility of *S. aureus* isolates (n= 20):

% S	% I	% R	Antibiotic class	Antimicrobial agent
65	0	35	β-lactam+Inhibitors	Amoxicillin/Clavulanic acid (AMC)
50	0	50	Penicillins	Ampicillin (AMP)
65	5	30	Quinolones	Ciprofloxacin (CIP)
90	0	10	Lincosamides	Clindamycin (CLI)
90	0	10	Macrolides	Erythromycin (ERY)
85	0	15	Cephems	Cefoxitin (FOX)
80	0	20	Aminoglycosides	Gentamicin (GEN)
40	0	60	Penicillins	Penicillin G (PEN)
75	0	25	Folate pathway inhibitors	Trimethoprim/Sulfamethoxazole (SXT)
75	0	25	Tetracyclines	Tetracycline (TCY)

R= Resistant, T=Intermediate, S=Sensitive

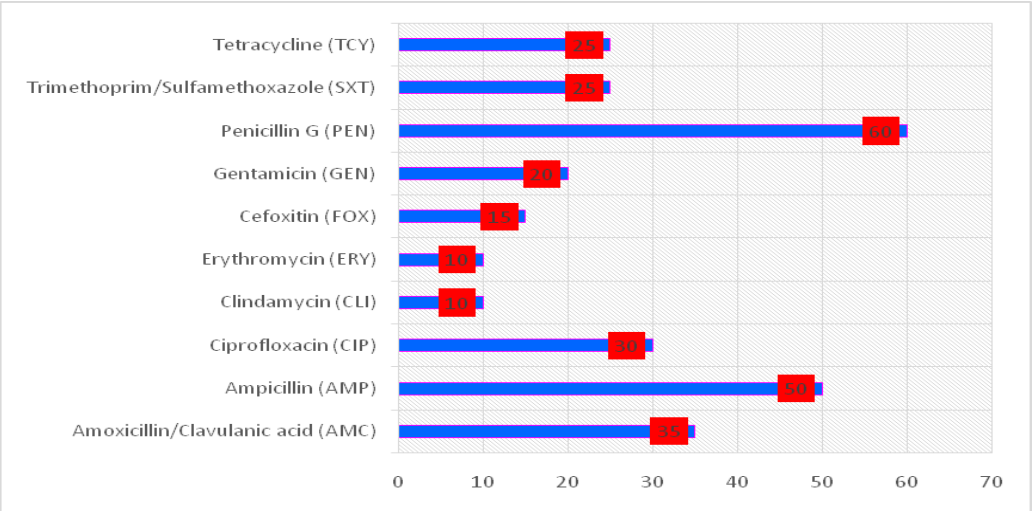

Figure 4: Percentages of *S. aureus* resistant strains among the identified isolates using the disk diffusion method according to Clinical and Laboratory Standards Institute guidelines

Table 3: Antibiotic resistance profile (number of S. aureus isolates= 20)

%	N. of isolates	Resistance profile									
40	8										
10	2	PEN									I
5	1	PEN	AMP		GEN						II
5	1	PEN	AMP	CIP							III
5	1	PEN	AMP	CIP	GEN	TCY					IV
10	2	PEN	AMC	AMP							V
5	1	PEN	AMC	AMP	CIP	SXT					VI
5	1	PEN	AMC	AMP	CIP	CLI	GEN	SXT	TCY		VII
5	1	FOX	PEN	AMC	AMP	CIP	GEN	SXT	TCY		VIII
5	1	FOX	PEN	AMC	AMP	ERY	CIP	SXT	TCY		IX
5	1	FOX	PEN	AMC	AMP	ERY	CIP	CLI	SXT	TCY	X

Discussion

As a major meat species marketed in Saudi Arabia, camel meat was used for determination of the prevalence of *S. aureus*& MRSA and antimicrobial susceptibility profile of isolates. In a survey conducted for 5 months, 187 raw camel meat samples (retailed in Al-Hasa, Saudi Arabia) were analyzed. Identification of *S. aureus* was done by DNA expression of gene specific 16S rRNA (figure 1).

Figure (2) clarifies that, *S. aureus* was present in 20 out of the 187 samples (10.7%). The prevalence of *S. aureus* in different foodstuffs and meats varied in the previous studies. They were reported 20.5% *S. aureus* from beef at USA (22).

MRSA has been already isolated and identified from retail meat worldwide, and the possible human transmission existed (23-26 and 22). The reported prevalence varied which indicated that contamination by MRSA in different types of meat varied in the different localities. Of the 20 *S. aureus* isolates, three (15 %) were found to be MRSA, therefore, Figure 2 shows the prevalence of MRSA strains among total samples was only three (3/187) (1.6%) and this result is very low in comparison with that obtained by (16) who found that the prevalence of MRSA in camel meat retailed in Riyadh was (20%) while the prevalence of MSSA was (28%). This difference may be due to variations in hygienic levels, preparation and handling of meat. While our result was nearly similar to (22) and (27) who reported that 1.3% and 1.2%, respectively were positive for MRSA from meat retailed in USA.

Meat play a significant role in transmission of antimicrobial resistance from livestock and food animals to human being, and antibiotic resistance usually has a great concern in hospital infection (nosocomial infections). Antimicrobial resistance may transfer via 3 ways: Through antimicrobial residues in food and meat, or consumption of resistant parts of original food microflora and transfer resistance to pathogenic microorganisms (28-31).

The antimicrobial resistance was higher for Penicillin G and Ampicillin (60 and 50%, respectively). In addition, 15% of tested *S. aureus* strains were methicillin-resistant while 85% were sensitive to cefoxitin (table 2). Another study showed antibiogram sensitivity differences in *S. aureus* isolated from food (7).

It was clear that MRSA isolates were resistant to all antimicrobials used.

Figure 4 showed a higher resistant level for both penicillin and Ampicillin and this result is similar to that stated by (32) in chicken giblets. Only two isolates (10%) out of all analyzed strains exhibited single resistance to penicillin. MRSA isolates presented antimicrobial multi resistance (7 and 32); similar result was found in our study (table 3).

Conclusion

From the above, it could be concluded that 20 *S. aureus* positive isolates were identified out of 187 camel meat samples (10.7% prevalence). Three were resistant to methicillin (1.6 % prevalence), 35% of *S. aureus* isolates were resistant to β-lactam antibiotic. MRSA isolates were resistant to all antimicrobials tested, and 15 % of tested *S. aureus* strains were methicillin-resistant. Our results indicated low MRSA prevalence in camel meat, which pointed to the limited risk of transmission via meat. However, attention should be paid to the safety along food chain.

Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

CORTICOSTEROID-INDUCED OSTEOPOROSIS AND OSTEONECROSIS: ROLE OF OXIDATIVE STRESS

Reham H. Alattar[1*], Abdel Alim F. Abdel Alim[2], Sabry M.A. Abdelmetal[2], Sayed A. Abdel Aziz[2]

[1]Zagazig University student's Hospital, Zagazig University, Egypt, [2]Pharmacology Department, Faculty of Veterinary Medicine, Zagazig University, 44511, Egypt

*Corresponding author, E-mail: reham_attar2013@yahoo.com

Abstract: Glucocorticoids (GC) play a significant role in body metabolism. In the last few years, advances and highlights have been made to understand the role of oxidative stress induced by corticosteroids in the pathogenesis of osteonecrosis (ON) and osteoporosis (OP) and the door for digging in GC mechanistic has been opened by the newly detection of high-affinity receptors for glucocorticoids and calcitriol in bones. The harmful free radicals produced by corticosteroid administration are strong emitters of many regulatory cytokines such as tumor necrosis factor (TNF), nuclear factor kappa β (NF-kB) and interleukins. For this, a great attention has been directed toward the possibility of using a novel free radical scavenger like natural antioxidant, e.g. ginseng, that can be helpful in the management of ON and OP. The use of antioxidants for the management of osteoporosis characterized by many improvements in the way of control the incidence rate of ON and OP. Many antioxidants have an anti-osteoporotic effect, with an overall redox state maintenance. Also, the lipid peroxides are alleviated and the intraosseous vascular integrity within the bone marrow are repaired. Moreover, the oxidative damage of DNA is contoured. The objective of this review is to highlight the role of oxidative stress in the pathogenesis of corticosteroid-induced osteonecrosis (ON) and osteoporosis (OP) and studying the possibility of using a novel free radical scavenger, a natural antioxidant, e.g. Ginseng, that can be helpful in the management of OP & ON.

Key words: osteoporosis; osteonecrosis; corticosteroids; oxidative stress; ginseng

Introduction

Osteoporosis (OP) which is a widespread disease characterized by an abnormal mass, tissue, fragility and fracture risk of bones (1). Although, it is well known that impairment in the blood supply of the bone and administration of corticosteroids can lead to osteonecrosis, the actual mechanisms of steroid hormone action on bone and how glucocorticoid-induced osteoporosis (GIOP) and how the cellular composition of bone is altered is still obscure. Secondary osteoporosis and fracture may occur in patients receiving corticosteroids either in high-doses or chronic therapy, which is considered as one of the major risk factors for ON (2). The oxidative stress was found to inhibit the differentiation and mineralization of bone and consequently induces necrosis of osteoblasts (3), and increases the expression of

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cytokines in the bone and thus induces osteoporosis. The effects of free radicals on the bone can be countered by the use of a proper antioxidant in a way that can prevent osteoporosis.

The main topics which will be discussed in the review are:

1. Glucocorticoids.
2. Free radicals involved in osteoporosis and osteonecrosis and the possible role of antioxidants.
3. Involvement of oxidative stress in bone pathophysiology: Apoptosis and Caspases.
4. Steroid-induced OP- animal models.
5. Osteoporotic biomarkers.

1. Glucocorticoids

Owing to the potent anti-inflammatory effects of glucocorticoids, they are widely used as an essential mendicant (4). The prolonged use of these drugs may provide adverse effects not only on the metabolism of proteins, lipids and carbohydrates, but also has immunosuppressive effects.

Hyperglycemia is one of the most important effects that may play a role in the stress response and osteoporosis induced by glucocorticoids. The age-associated loss of bone mass and strength or the aging skeleton can be contributed to the inflammatory cytokines, but without clear molecular declare (5). In human subjects, GIO occurs in two phases: a rapid early phase in which bone mineral density falls, occurs 5–15% per year, probably due to excessive bone resorption. A slower phase, characterized by a more slowly decline of bone mineral density, and occur 2% per year, which mainly attributed to bone formation defect (6).

1.1 Glucocorticoids mechanism

The mechanism of glucocorticoids action is found to be mediated by glucocorticoid receptors (GCR), belong to the nuclear steroid hormone receptor family, expressed in bone cells (7). In spite of the recent success in the molecular biological techniques, the precise molecular mechanism of steroid hormone action has remained obscure. These receptors include cytosolic glucocorticoid receptor (cGCR) for both the classical genomic and non-genomic mechanism of glucocorticoid action and membrane-bound glucocorticoid receptor (mGCR) for the mediated non-genomic effects. While, the interactions with cellular membranes result in a non-specific, non-genomic effects caused by direct interaction with the cell membranes (8). Seven members of steroid receptors are recognized: estrogen receptor a & b, estrogen-related receptors 1 & 2, and the receptors for mineralocorticoids, androgens and progesterone (9, 10). The gene encoding the human GR (hGR) is located on chromosome 5 in loci 31-32 (5q31-32) (11). Three main functional domains are included in each receptor; N-terminal domain, DNA-binding domain (DBD), and ligand-binding domain (LBD). Two distinct receptor forms for glucocorticoids in human, arise from alternative splicing of exon 9: hGR-α and hGR-β. While the classical signaling and modulation of gene expression are attributed to α- receptor form, while β-form may function only as a dominant negative inhibitor of α-signaling (8).

The effects of glucocorticoids on bone differ according to the developmental stage i.e. from the beginning stage to the late stage, from human to animal species and moreover in a cell-type-specific manner (3). While GCs appear to be essential for the early osteoblastic stage, responsible for the differentiation of osteoblasts into bone-forming osteoblasts, act as inhibitory at the very late stages of osteoblast differentiation and osteocytes (12). In bone, local activation of the inactive cortisone into the active cortisol by 11β-hydroxysteroid dehydrogenase is a pivot point, is responsible for function of osteoblasts, differentiation, proliferation and. One of the most important factors that cause increases in the bone turnover, loss of bone mass and fracture risk is the changes in the levels of steroid hormone as occurs in the adulthood of either sex. In osteoblasts and osteoclasts, estrogen receptor α (ERα) has a protective effect on bone (14, 15).

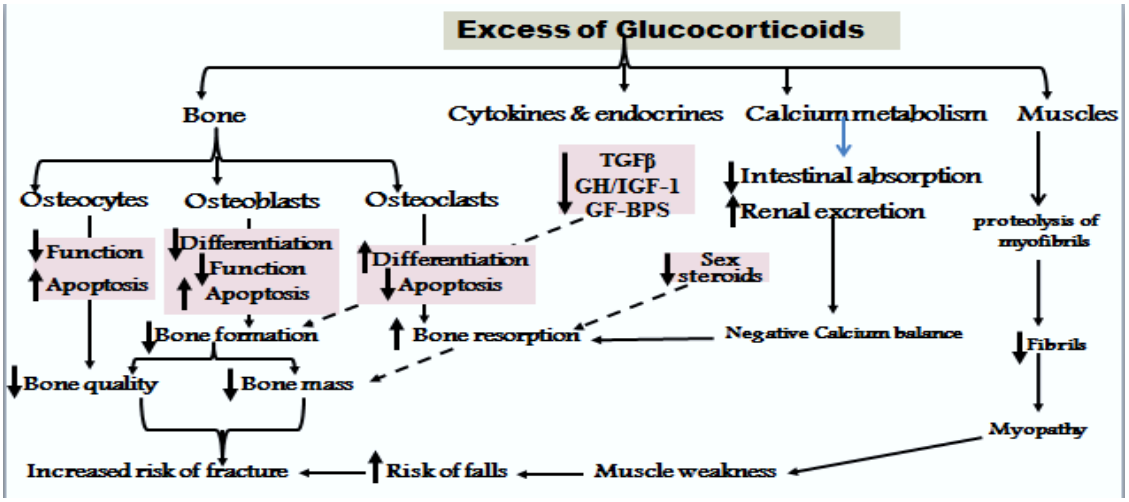

Figure 1: Pathophysiology of glucocorticoid-induced osteoporosis, adapted from (12, 13)

2. Free radicals involved in osteoporosis and osteonecrosis

The proper bone modeling is controlled mainly by the function of two types of bone cells, osteoclasts (bone formation) and osteoblasts (bone resorption), and OP occurs when the balance between both functions is disrupted (16-18) . This balance is affected by many risk factors including high-doses of corticosteroids, alcohol abuse, genetic, race, hormonal, mechanical and nutritional factors.

2.1 Free radicals

The presence of unpaired electron(s) in atomic or molecular orbitals makes the molecule unstable and highly reactive and considered a free radical. As shown in table (1), free radicals are usually, but not always oxygen, which include reactive oxygen species (ROS) and reactive nitrogen species (RNS) (19) . Numerous agents and factors can induce significant generation of ROS, including drugs as anticancer, diseases as diabetes and environmental as UV irradiation, pollutants and toxicity (12).

As mitochondria is the major generator of ROS. In cells, the main source of $O_2^{\cdot-}$ is the leakage of electron(s) from the mitochondrial ETC, and therefore the damage occurs mainly for mtDNA leading to apoptosis (20). Under physiological conditions, H_2O_2, but not $O_2^{\cdot-}$ is produced by the peroxisomes (21). Oxygen free radicals can also be produced by NADPH oxidase on osteoclasts, and xanthine oxidase and NOS (22, 23). Also, treatment with interferon (IFN)-γ, which act as a stimulator of the activity of NADPH-oxidase enzyme lead to liberation of free radicals.

2.3 Hyperglycemia-induced oxidative stress

Various mechanisms were adopted to describe the hyperglycemia-induced oxidative stress. One mechanism is the increased production $O_2^{\cdot-}$ by the disrupted ETC, which is increased according to diabetic complication state. Also, the oxidative stress is increased in diabetes because of the accelerated polyol/sorbitol pathway and the decrease in NADPH availability and thereby GSH depletion (24-26).

2.4 Oxidative stress

Although, free radicals have been shown to play beneficial roles in biology, especially when present in physiological concentrations, may act as a second messenger in some of the signal transduction pathways. When the production of these free radicals is greater than the ability of the cell to detoxify, an oxidative stress is developed and oxidative damage of many biological molecules, especially proteins and nucleic acids occurs (27, 28). The levels of G-SH, is decreased, while the lipid peroxides (LPO) and advanced glycation end product (AGE) are accumulated (28-30).

Table 1: Different reactive species

Reactivity / Remarks	Half life (in sec)	**Symbol**	**Reactive species**
I. Reactive oxygen species			
Generated in mitochondria , in cardiovascular system and others, very highly reactive , generated during iron overload and such conditions in our body	10^{-4} s 10^{-9} s	$O_2^{\cdot-}$ $^{\cdot}OH$	- Superoxide - Hydroxyl Radical
Formed in our body by large number of reactions and yields potent species like ^{+}OH	Stable	H_2O_2	- Hydrogen Peroxide
Reactive and formed from lipids, proteins, DNA, sugars etc.	S	$ROO^{\cdot}$	- Peroxyl radical
During oxidative damage			
Reacts with transient metal ions to yield reactive species	Stable	ROOH	- Organic hydroperoxide
Highly reactive , formed during photosensitization and chemical reactions	10^{-6} S	$^1O_2$	- Singlet oxygen
Present as an atmospheric pollutant , can react with various molecules, yielding $^1O_2$	S	O_3	- Ozone
II. Reactive nitrogen species			
Neurotransmitter and blood pressure regulator, can yield potent oxidants during pathological states	S	$NO^{\cdot}$	- Nitric oxide
Formed from NO_2 and	10^{-3} s	$ONOO^{-}$	- Peroxynitrite

The induction of OP and/or ON by the accumulated free radicals can be explained by several axes. An important axis occurs by inhibition the differentiation of two vital cell lines, the marrow stromal M2-10B4 cell line, and the pre-osteoblastic cell line. In this mechanism, the early differentiation marker, alkaline phosphatase (ALP) was found to be markedly increased and the mineralization in these cell lines is lowered (31). Another one, is the foundation that metallothionein (MT) has a protective effect against H_2O_2-induced inhibition of the differentiation of osteoblasts and has the ability to scavenge $^{\cdot}OH$ & $O_2^{\cdot-}$ free radicals by mechanisms similar to G-SH and superoxide dismutase, respectively (32-34).

It has been reported that the free radicals is a strong emitter of the expression of many cytokines and many of these cytokines are involved in bone osteoporosis including TNF-α, NF-κB, IL6 and IL-1 (35). The production of these signals is regulated by steroids. While TNF-α and IL-1 are a powerful bone resorption effectors by increasing the number of osteoclasts, IL-6 is an osteotropic (36, 37). NF-κB is involved in the regulation of cell growth, differentiation, death and development of bone and is a downregulator of the differentiation of osteoblasts (38-40).

2.5 Role of antioxidants in osteoporosis

Oxidative stress arise when the production of the radicals oxygen and non-oxygen free radicals exceed the capacity of the antioxidants to overcome. A proper antioxidant (enzymes and non-enzymes) is essential to protect against free radicals (41, 42). Enzyme antioxidants include: superoxide dismutases, glutathione peroxidases, catalase, glutathione S-transferase, glutathione reductase, peroxiredoxins, glutaredoxin and thioredoxin Non-enzyme antioxidants include: Reduced glutathione (G-SH), tocopherols and β-carotenes (43).

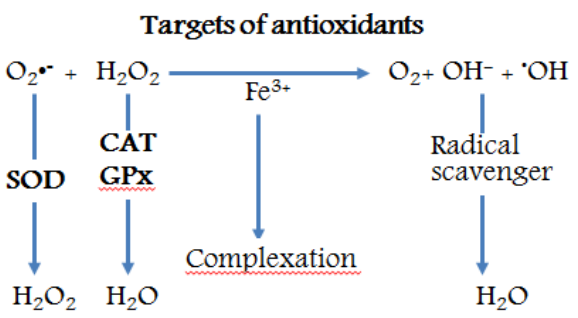

Figure 2: Possible mechanisms of antioxidants adapted from (44)

In the post-menopause, the bone mineral density is decreased, the susceptibility for OP is increased and the antioxidants are depleted. Such condition can be alleviated by regular intake of exogenous antioxidant (45). The metabolism of calcium is dramatically affected, because of a decrease in the gastrointestinal calcium absorption and increase in the renal calcium loss. These manifestations are recorded in glucocorticoid administration. It was reported that co-administration of the antioxidants tocopherol or ascorbate can withstand the development of corticosteroid-induced ON (46-48). In addition, a secondary hyperparathyroidism has been suggested as a determinant effector of bone (49).

3. Osteoporotic biomarkers

It have been suggested that osteoporosis is associated with several biochemical markers that can be taken as a monitor for the oxidative stress-induced OP and ON to follow-up and the possible ameliorative effect of a natural antioxidant.

4. Antioxidants

4.1 Genistein (Giensing or Ginseng)

Ginseng, one of the most popular oriental medicinal herbs, widely used as an herbal remedy for various physiological and pathological disorders. White and red ginseng have been used as an excellent stimulant for the immune and antimicrobial defenses (51). Genistein is not only an effective scavenger of free radicals *in vitro*, but also a strong inhibitor of cell-derived H_2O_2 formation *in vivo*. It has an antioxidant capacity that may exceed that of acrobate and tocopherols (52).

4.2 Ginseng as a hypoglycemic effector

An proposed and accepted anti-osteoporetic effect of ginseng is the hypoglycemic effect. It has been reported that the blood glucose level and the glycogen content are improved and controlled in ginseng-supplemented diabetics. One possible mechanism of this hypoglycemic effect of ginseng is the antioxidant scavenging effect, enhancement of the aerobic glycolysis through stimulation of β-adrenoreceptor and increase of various rate-limiting TCA enzyme activities (53). Ginseng supplementation significantly reduce the incidence of ON in the most studied corticosteroid- induced ON animal models, characterized by a marked increase in the GSH content and MDA levels decreased. Thus providing a fact that oxidative stress and ON was inhibited by ginseng (16, 28).

5. Involvement of oxidative stress in bone pathophysiology

The pathophysiology of the oxidative stress varies according to the load of the stress and the provided antioxidant capacity. Oxidative stress in low levels may function as a signal mediator for cell growth and cellular signaling (54). In contrast, higher oxidative stress is harmful, causing cellular injury and involved in various disease pathogenesis. The damage include enzymes, structural proteins, lipids, and DNA are seriously affected in a way can lead to apoptosis (55). Bone architecture and osteoblast / osteoclast balance is also altered (28).

6. Apoptosis

The programmed cell death (PCD), apoptosis, is actually essential for the normal development and homeostasis and the removal of the damaged, infected and potentially neoplastic cells (56). Glucocorticoids are potent inducers of apoptosis in many cells(30). GCs have proapoptotic effects on osteoblasts and osteocytes due to activation of caspase-3, the key mediator of apoptosis (56). As previously mentioned, apoptosis-induced by intake of corticosteroids varies according to the form of the receptor present; glucocorticoid receptors α-C (GRα-C) is a potent apoptotic inducer than GRα, while GRα-D is less (57).

6.1 Caspases

Caspases are major player in the cell death by apoptosis. These cysteine-dependent caspases are affected by many factors especially the redox state of the cell. As shown in figure (3): The function of glucocorticoid receptors can propagate signals either for pro-apoptotic genes or for anti-apoptotic genes that can either lead to apoptosis or cell survival, respectively depending on the cell type or tissue (58, 59).

Table 2: Biomarkers can be used to follow-up the oxidative stress-induced osteoporosis, adapted from (50).

Biomarkers	
1. Markers of bone formation	-ALP: An osteoblastic differentiation marker. -Serum measurement of total and bone-specific alkaline phosphatase (BALP) - Osteocalcin (OC), -Type I collagen extension peptides.
2. Assessment of bone resorption	- Urine specimens were assayed for markers of bone resorption: N - telopeptides (NTX), free pyridinolines (Pyr), free deoxypyridinoline. - Measurement of fasting urinary calcium and hydroxyproline, -Urinary hydroxylysine glycosides, urinary excretion of the pyridinium cross-link and bone - specific alkaline phosphatase. - Osteoclast enzymes: .Tartarate-resistant acid phosphatase (TRACP) andCathepsin K.
3. Biochemical determinations	-The lipid peroxide marker, MDA. -Calcitonin. -DNA damage marker, 8-OHdG. -Antioxidant status: Reduced blood glutathione (G-SH). -The antioxidant enzymes: SOD, Catalase and Glutathione peroxidase (GPX). -Endothelin (ET) in plasma. -PTH, Calcium & Phosphorus.
4. Immunohisto-chemical investiga-tions	-The presence/absence of advanced glycation end-product expression. -Proinflammatory cytokines: (IL-1α, 1β, 6, TNF- α). -Anti-inflammatory: (IL-4, 10). -Bone-marrow-specific cytokines: (IL-7 & 11). -Caspase: 1, 3, 7 & 9. -Caspase-activated DNase (CAD). -Adipogenic transcription factor (PPARγ). -TGF-β2. - Vascular endothelial growth factor (VEGF). - Tumor necrosis factor-α (TNF- α). - Tumor necrosis factor-receptor-1 (TNF-R1). -The pro-apoptotic Bcl-2 (Bim). -The pro-apoptotic proteins (Bax/Bak). - NF-κB: For osteoblastic differentiation of BMSCs.
5. Determina-tion the gene ex-pression	-Sphingomyelin Synthase-2. -PTH receptor-1.

Table 3: The protective role of different antioxidants in osteoporosis, adapted from (44).

Antioxidant	Effect
Reduced glu-tathione (G-SH)	Reduced glutathione is important for the maintenance of the cytoskeleton, stabi-lizes the lysosomal membrane and suppresses injury to the vascular endothelium and wall by inhibiting an increase of lipoperoxides.
Vitamin E	Vitamin E deficiency impairs calcium transport via the intestine and reduces bone den-sity, free radical scavenger and has been shown to offer protection against free radical-associated diseases, such as atherosclerosis, cancer and osteoporosis.
Vitamin C	Enhances collagenogenesis, possible effect on bone formation, the antioxidant effect on osteoporosis is under investigation
Vitamin A	Excess vitamin A intake has been associated with accelerated bone loss, but this re-quires further investigation.
Genistein	Free radicals scavenger, inhibitor of cell-derived H_2O_2 formation.
Ginkgo biloba	Free radical scavenger.

Intrinsic as well as extrinsic apoptotic pathways are suggested as possible mechanisms of GC-induced apoptosis. The activation of the intrinsic apoptotic pathway is the classic mechanism (60). Mitochondrial-dependent or mitochondrial-independent mechanisms are involved in the extrinsic apoptotic pathway. Glucocorticoid signaling increases the expression of the pro-apoptotic Bcl-2 family member Bim, which can activate the pro-apoptotic proteins Bax/Bak to disrupt mitochondrial membrane potential, resulting in the release of cytochrome c and other apoptogenic proteins. This leads to caspase 9 activation and subsequent effector caspase 3 activation and apoptosis occurs. Other factors that may influence the intrinsic pathway during GC-induced apoptosis include up-regulation of other pro-apoptotic proteins such as Bad and Puma, or down regulation of anti-apoptotic proteins such as Bcl-2 or Bcl-xL. A major checkpoint in this pathway is the ratio between pro-apoptotic (Bax) to anti-apoptotic (Bcl-2) members, which is something unique for apoptosis. Caspase-3 and caspase-7 cleave this ICAD, resulting in the release of active CAD (61).

6.2 Steroid-induced OP- animal models

Several animal models are proposed to describe the pathogenesis of corticosteroid-induced ON and OP, differing according to the animal used (rabbits or mice or rats), the steroid drug, the dose used either acute or chronic doses and the period of the experiment (38).

Natural antioxidants could be applied as a functional material to prevent diseases caused by oxidative stress. The ginseng can significantly decrease the incidence of osteoporosis and osteonecrosis in the steroid-treated animal models e.g. rabbit. The oxidative stress is inhibited and the vascular endothelial dysfunction is ameliorated. The precise mechanism still requires a further *in vivo* study.

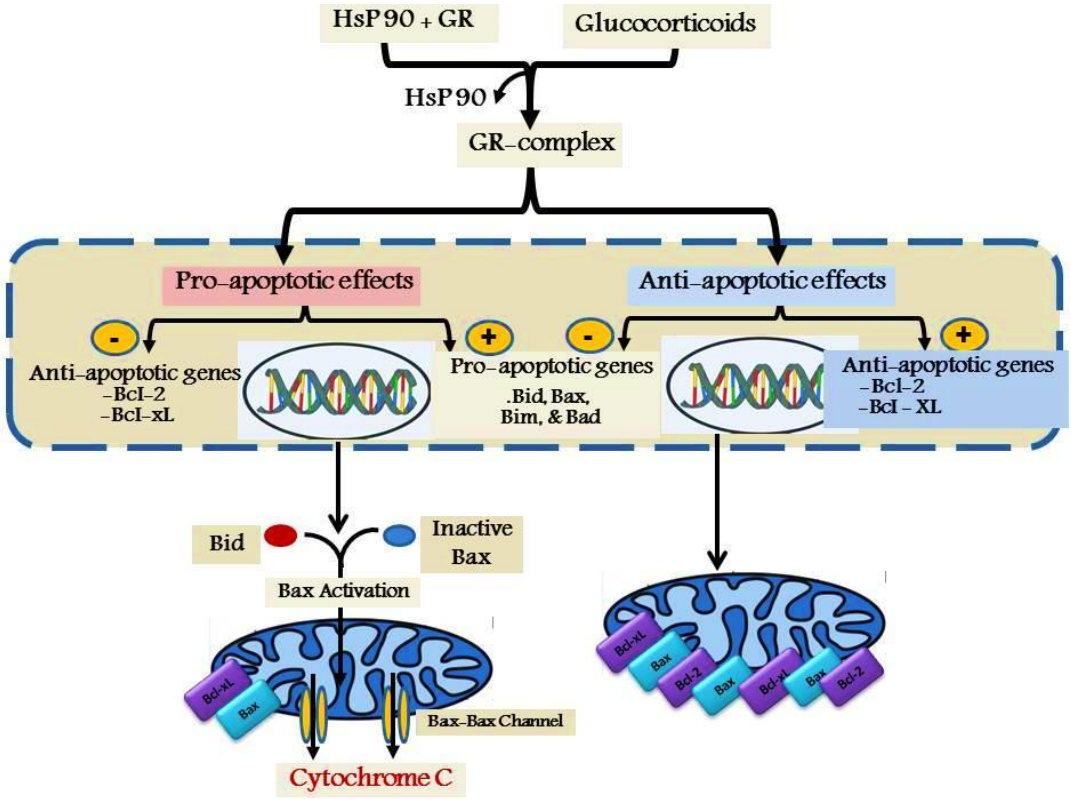

Figure 3: Glucocorticoid signaling through GR, adapted after (29)

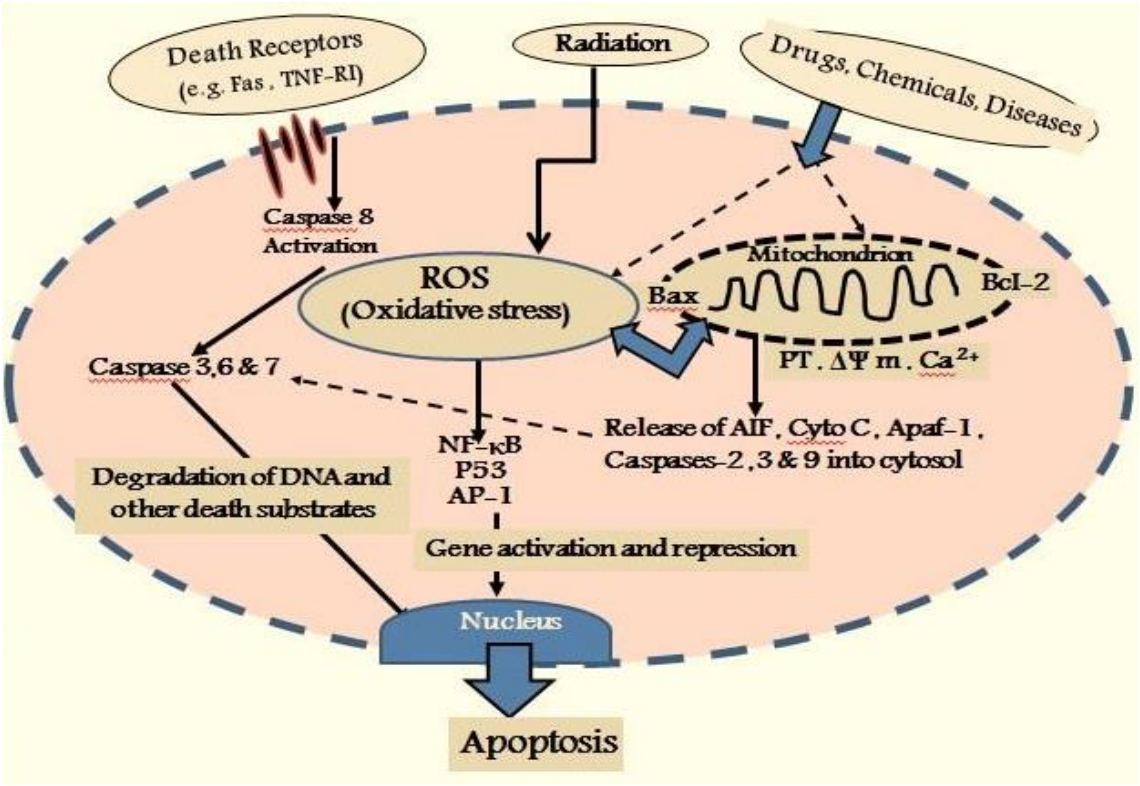

Figure 4: Schematic model of mammalian cell death pathway, adapted from (56)

Table 4: Experimental animal models for steroid-induced OP

Animal	Drug	Dose	Exp. Period	Biomarkers
Male NewZealand white rabbits	Methylprednisolone	4 mg/kg B.Wt.	14 days ref [36]	GSH and LPO
Female Japanese white rabbits	Methylprednisolone	4 mg/kg, I.M. once	Sampling at days: 3rd , 5th & 14th, Ref [36]	Histopathology, Biochemical and immunohistothemisty
Female Japanese white rabbits	Methylprednisolone	40 mg/kg, I.M. once	Sampling at days: 1st, 3rd , 5th & 14th Ref. [18]	Histopathology, Biochemical and immunohistothemisty
Male Japanese white rabbits	Methylprednisolone acetate	20 mg/kg, I.M. once	-2- 10 weeks , Ref [62]	Histopathology, hematological and immunohistothemisty
Male adult Newzealand white rabbits	Methylprednisolone	20 mg/kg, I.M. once	Sampling at days: 3rd , 5th , 7th & 14th ,Ref[63].	Histopathology, Biochemical and immunohistothemisty
Rats	Slow-release Prednisone pellets, S.C. implanted	1.5 mg/ Kg B. Wt.	6 months Ref[60]	Histopathology, Biochemical and immunohistothemisty

Conclusion

Finally, we can conclude that, the causes of osteonecrosis and osteoporosis are multifactorial. Both *in vitro* and *in vivo* data have recently shown that steroid-associated oxidative injury exerts a crucial role in the development of OP and ON. Several antioxidants have been reported to effectively suppress the development of this illness. Soon after steroid administration, attenuation of the protective antioxidative capability occurs, leading to tissue and

protein modifications, especially in bone tissues, vascular endothelial dysfunction, which contributes to local intravascular thrombosis and circulatory disturbances, concomitantly with the development of osteoporesis. It is of a great clinical significance if natural products without side effects on the human body were able to indirectly increase the intravital antioxidant defense system and directly eliminate excessive ROS.

Conflict of interest

None of the authors have any conflict of interest to declare.

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Original Research Article

ALLEVIATION OF CHRONIC HEAT STRESS IN BROILERS BY DIETARY SUPPLEMENTATION OF NOVEL FEED ADDITIVE COMBINATIONS

Saad I. Al-Sultan[1], Sherief M. Abdel-Raheem[1, 2]*, Sherief M.S. Abd-Allah[3], Abobakr M. Edris[1,4]

[1]Department of Veterinary Public Health and Animal Husbandry, College of Veterinary Medicine, King Faisal University, 400 Al-Ahsa ,31982, Saudi Arabia, [2]Department of Animal Nutrition and Clinical Nutrition, Faculty of Veterinary Medicine, Assiut University, 71526, Egypt, [3]Department of Food Hygiene (Meat Hygiene), Faculty of Veterinary Medicine, Assiut University, 71526, Egypt, [4]Food hygiene and control Department, Faculty of Veterinary Medicine, Benha University, Egypt

*Corresponding author, E-mail: sdiab@kfu.edu.sa

Abstract: Heat stress causes economic losses via decreasing feed intake, nutrient digestion, weight gain, feed conversion, immunity, carcass quality and increasing mortality in broilers. The aim of the study was to assess the ability of dietary additive combinations to ameliorate the detrimental effect caused by chronic heat stress (32 ± 2ºC for 24 h per d) on performance, carcass traits, metabolic status and economic efficiency of broiler chickens. A total of 420, one-day old Cobb-500 broiler chicks were assigned into seven treatment groups (n=60) of five replicates. Each replicate contained 12 unsexed chicks. The control groups did not receive any supplementation to the basal diet (thermoneutral control (TNC) and heat stress control (HSC) groups). The other groups received control diet supplemented with 1 % cumin plus 1 % turmeric powders (T1); 1.5 g/kg potassium chloride plus 2 g/kg sodium bicarbonate (T2); 1000 ppm propolis plus 15000 IU vitamin A (T3); 1200 ppb chromium plus 500 ppm vitamin C (T4) ; 1200 ppm betaine plus 500 ppm vitamin E (T5). The results indicated that at 42 d of age, all dietary additive combinations improved the growth performance indices, carcass traits, concentrations of serum antioxidant enzyme biomarkers, stress biomarkers and economic efficiency in comparison to HSC group. Based on the obtained results, it could be concluded that dietary supplementation with betaine and vitamin E followed by chromium and vitamin C combinations offers a good management practice for alleviating heat stress related depression in the performance of broiler chickens.

Key words: heat stress; broilers; vitamin C; betaine; chromium; propolis; turmeric

Introduction

The most obvious constraint on poultry production in Saudi Arabia, especially during summer months is high temperature with high humidity, which causes severe stress on birds and reduces performance. The continuous challenge for the poultry production in tropical and subtropical region all over the world remains heat stress. Hot environments making several alterations in blood hormones, glucose levels, leukocytes, electrolytes and organ functions (1).

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Heat stress causes economic losses via decreasing feed intake, nutrient digestion, weight gain (WG), egg production, feed conversion (FC), immunity, carcass quality and increasing mortality (2).

Heat stress trigger the hypothalamo-hypophyseal adrenocortical axis to secrete corticosteroids (3). The catabolic effects of higher blood corticosteroids elevate the free radicals through changing oxidative metabolism, producing huge damage of cellular functions and cell membrane, which causing muscle degeneration and growth retardation (3). The greatest part of the energy production is directed to thermoregulation mechanisms, which develop stress-induced immunosuppression. The immune suppressed birds are predisposed to numerous dangerous diseases associated with high mortality rates (4)

Dietary modifications by the use of commercial feed additives are considered the most favored and practical ways to mitigate the effect of heat stress in poultry (5). Therefore, dietary inclusion of some selected feed additives could also be effective for the preservation of growth and immunity for broilers during periods of heat stress. Many previous studies illustrated the beneficial effect different dietary supplements on the performance of broilers under heat stress independently (6, 7, 8, 9, 10, 11, 12). These studies revealed that Vitamin A, E, C, play important roles in the performance and immune function of poultry under heat stress. Betaine is a methyl donor and it has methionine sparing effect, help in maintaining cellular water balance, protect the integrity of cell membrane and improve broiler performance and to overcome stress. In addition, the supplementation of dietary electrolytes increase tolerance to heat stress improve performance of broilers under heat stress. Electrolyte prevent acid base balance disturbances and decrease the occurrence of respiratory alkalosis during heat stress. Herbal additive like cumin, turmeric and propolis very rich in flavonoid and aromatic acids and they have antioxidant, anti-inflammatory, antibacterial, antifungal activity and have a positive effect on broiler performance under heat stress. However, few studies have been conducted to detect the best combination at the recommended concentrations suggested by previous studies to mitigate the decline in performance caused by heat stress in broiler chickens. Therefore, the aim of the current study was to detect the best commercial additive combinations to ameliorate the detrimental effect caused by chronic heat stress on performance, carcass traits, metabolic status, antioxidant capabilities and economic efficiency of broiler chickens.

Material and methods

Experimental animals and protocol

The current experiment was accomplished according to King Faisal University Animal Care and Use Committee and the experimental protocol was approved by the Deanship for Scientific Research with reference number (Decision No: KFU-REC/2017-04-01).

The experiment was carried out on 420 one-day-old broiler chickens (Cobb - 500) purchased from a marketable hatchery (Al-Ahsa, KSA). Birds were housed in pens with wood shavings floor with 10 birds/m2at the Research and Agriculture Station, King Faisl University, Al-Ahsa, Saudi Arabia from 1 to 42 d old. Water and feed provided ad libitum during 24 hours. Constant observation for health status and behavior usually carried out for broiler chickens. The temperature was kept at 32 ± 2 °C with 65 % relative humidity (RH) for 24 h for all treatment groups except thermoneutral control (TNC). For maintaining the desired temperature and relative air humidity air conditioners, heaters with thermostats, and air circulators were applied. The thermoneutral control group (TNC) were held at thermoneutral chamber with normal ambient temperature (22 ± 4 °C) and relative humidity (RH) of commercial management program (55±3 %, RH) at the same experimental place.

The experimental design

A total of 420 one-day-old unsexed broiler chickens (Cobb - 500) randomly allocated to 7 experimental groups (60 chick/each). Each group consisted of 5 replicate pens with 12

chicks each. The control groups did not receive any supplementation to the basal diet (thermoneutral control (TNC) and heat stress control (HSC) groups). The other groups received control diet supplemented with 1 % cumin plus 1 % turmeric powders (T1); 1.5 g/kg potassium chloride plus 2 g/kg sodium bicarbonate (T2); 1000 ppm propolis plus 15000 IU vitamin A (T3); 1200 ppb chromium (Cr) plus 500 ppm vitamin C (T4) ; 1200 ppm betaine (Bet) plus 500 ppm vitamin E (VE) (T5). According to NRC (13) guidelines, fourteen experimental diets, seven in each feeding phase (starting and growing) were formulated. The basal diets contained 23% and 20 % protein and 3200 kcal of ME/kg for starter and grower diets, respectively. Composition and chemical analysis of broiler basal diets are presented in (Table 1). Proximate composition of feed ingredients were performed according to AOAC (14) and the experimental diets were formulated based on these values. The selected feed additives were thoroughly mixed with small amount of feed on the expense of corn, then homogenized with the total amounts and added to the diet. Prophylactic vaccination against viral diseases was carried out. All chicks were immunized with eye drops by weak Newcastle virus vaccine at age of 7 days and with a live attenuated freeze-dried vaccine (Nobilis® Ma5 + Clone 30) at the age of 20 days.

Broiler performance

Feed consumption and body weight for each pen were recorded weekly during the whole experiment. Average daily gain, feed intake and feed conversion ratio were calculated during starter (1 to 21 d), grower (22 to 42 d), and overall (1 to 42 d) phases.

Blood Sampling

Ten birds from each group were used for collection of blood samples at days 21 and 42.The samples were collected into two tubes, one contained EDTA (as anticoagulant) and the other had no anticoagulant. Samples were centrifuged at 3500 g for 15 min for gathering plasma and serum, respectively.

Determination of New castle serum antibody titre

The log10 of serum antibody titer of new castle disease was determined by using commercial ELISA kits (IDEXX® Laboratories, B.V., Netherlands) according to the recommendation of the supplier.

Determination of blood antioxidant enzymes and oxidative stress biomarkers

The activities of antioxidant biomarkers (glutathione peroxidase (GSHPx), superoxide dismutase (SOD) and malondialdehyde (MDA) were analyzed by spectro-photometrical method with commercial kits obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All procedures were achieved according to manufacturer's instructions. Plasma corticosterone (CS) levels was assayed by the use of ELISA kit (DetecteX® Corticosterone, EIA kit, Arbor Assays®, USA), according to procedures of Quintero-Filho et al. (3).

Determination of blood biochemical profile

Serum glucose, total protein, albumin, total cholesterol and globulin levels were analyzed spectrophotometrically by using commercial kits from Spinreact company ® (Spain) and according to manufacturer's instructions.

Heterophil/lymphocyte (H/L) ratio

At the end of the experiment d 42, 10 birds from each group were used for determination of H/L ratio. Smears were prepared using the standard two-slide wedge procedure for differential leukocyte counts. Subsequently, blood films were air dried and stained with Wright-Giemsa Quick stain. The H/L ratio was calculated according to method adopted from (15)

Carcass characteristics and relative organ weight

At 42 day of age, 10 bird from each treatment (2 from each pen) were randomly selected and humanely slaughtered according to Islamic method for carcass traits characteristics. The gizzard, bursa of Fabricius, liver, spleen, ab-

dominal fat, heart, thymus, pancreas, and stomach were removed and blot dry weighed. Relative organ weight was calculated as a percentage of body weight.

Economic efficiency

Economic efficiency was estimated according to Wealleans et al., (16). Economic efficiency (EE) = (Net revenue/ total costs) ×100. Relative economic efficiency (REE) = Economic efficiency/economic efficiency of the positive control (17)

Statistical analysis

Data analysis was accomplished by one-way ANOVA using the procedure of SPSS software version 19 (18). Data were tested for normality before analysis by the use of the Kolmogorov–Smirnov test. Duncan is multiple range test (19) was used to detect the differences among means and significance level was set at $P<0.05$.

Results

The effect of diet supplementation with novel feed additives on growth performance of 42-day broilers was presented in (Table 2). The results presented in (Table 2) indicated the presence of statistically significant differences ($P< 0.05$) in the broiler growth performance indices such as body weight, feed intake, body weight gain and feed conversion ratio between the different treatment groups because of the dietary inclusion of all supplements. All dietary additive combinations induced a significant improvement in body weight gain, feed intake, feed conversion rate in birds of treatment groups during the entire period of the study (d 0 –42). On the other side, chronic heat stress (HSC group) induced a significant reduction in body weight, weight gain, feed intake, feed conversion efficiency and mortality during the entire period of the study. It is interesting to note that, dietary supplementation with the combination of 1200 ppm betaine and 500 ppm vitamin E followed by 1200 ppb chromium and 500 ppm vitamin C showed the most favorable and the best results regarding all growth performance indices of broilers subjected to heat stress in comparison with other treatment groups.

All the dietary supplements significantly increased ($P<0.05$) the carcass weight, dressing weight, dressing percentage or carcass yield and relative liver, heart, gizzard weights in comparison with the HSC group (Table 3). The greater relative organ weights were recorded in T5, T4, T3 when compared with T1 and T2. The major impact of the heat stress on the carcass traits was showed in the increase of the relative abdominal fat in the HSC to about 1.98 %, which decreased to 1.03 and 1.12 % in birds receiving both the combination of betaine and vitamin E and the chromium and vitamin C, respectively. The highest dressing percentage value (70.27%) was observed in birds of T5 group in comparison with other treatments groups. The lowest dressing percentage was recorded in the heat stressed control group (68.42 %). The greater response of the dietary supplements on carcass traits of broilers under chronic heat stress was obtained by dietary supplementation of betaine and vitamin E combination.

Heat stressed broilers had higher ($P<0.05$) levels of blood glucose, total cholesterol and albumen and lower ($P< 0.05$) level of total protein and globulin when compared with all treated groups and TNC (Table 4). The blood levels of triglycerides and total cholesterol were significantly reduced ($p <0.01$) in all treated groups in comparison with heat stressed control (HSC).

In comparison with the heat stressed control, the dietary inclusion of all supplement combinations in broiler diets significantly increased ($P < 0.001$) the activities of antioxidant enzymes (SOD, GSH-Px,) and TAOC but reduced ($P < 0.001$) the MDA content in the serum of chicks at the ages 42 d (Table 4). Heat stress reduce blood antioxidant status in broilers and increased the oxidative stress indicators. It was noted that dietary supplementation with selected additive combinations had decreased the concentrations of both H: L ratio and corticosterone significantly ($P<0.001$) of broilers under heat stress (Table 4) in comparison with HSC group. The concentrations of ND antibodies titer increased significantly ($P<0. 001$) at 21 and 42 d of age by dietary additives in treatment groups in comparison with heat stressed

control and the highest values were noticed in birds of T5, T4 and T3 groups, respectively. This research extends our knowledge that dietary supplementation of betaine and vitamin E, chromium and vitamin C, propolis and vitamin A combinations are better option for enhancing serum antioxidant status and immune response (ND antibodies titer) for broilers exposed to chronic heat stress.

The economic efficiency of 6 weeks old broiler chicks fed diet fortified with different supplement combinations under heat stress are shown on (Table 5). The dietary additive supplementation increased (P <0.001) the net return and economic efficiency in all treated groups in comparison with HSC group. Concerning the economic feed efficiency and the relative economic efficiency, the highest values were recorded for the treatment groups (T5, T4, T3 and T2) in comparison with T1 and HSC groups.

Table 1: Ingredients and analyzed chemical composition of basal diets

Item* (% unless noted)	Starter diet	Grower and finisher diet
Ingredient, %		
Corn	54.64	62.55
Corn gluten meal (60%)	4.11	3
Soybean meal, 44%	32.5	26.4
Sunflower oil	4.4	3.7
Limestone	1.5	1.5
Dicalcium phosphate	1.8	1.8
Salt	0.4	0.4
Vitamin - mineral premix	0.3	0.3
DL-methionine	0.2	0.2
L- lysine	0.1	0.1
Antioxidant	0.05	0.05
Total	100	100
Chemical analysis		
Dry matter	90.11	89.94
Crude protein	23.01	20.05
ME(Kcal/kg diet) [b]	3200	3200
Calcium	1.08	1.06
Non-phytae phosphorus	0.45	0.43
Na	0.20	0.17
Lysine	1.25	1.07
DL-Methionine	0.58	0.53
Methionine+cystine	1.05	0.87
Crude fiber	2.96	2.95
Crude fat	6.38	6.04

*Ingredient and nutrient composition are reported on as-fed basis.

[a] The vitamin and mineral premix provided per kg of diet: vitamin A, 4000000 IU; vitamin D3, 667000 IU; vitamin E 3334 mg; vitamin K3, 1167 mg; vitamin B1, 334 mg; vitamin B2, 1667 mg; vitamin B3, 3334 mg; B6, 500 mg; vitamin B12 33.4 mg, Folic acid, 334 mg; Biotin, 17 mg; Iron, 10 ; Copper, 2.167; Zinc, 18.334; Manganese 20.0; Iodine, 0.167; Cobalt, 0.034 and Selenium, 0.034.

[b] ME Calculated based on NRC (1994) feed composition tables

Table 2: Effect of dietary treatments on body weight (BW), feed intake (FI) and feed conversion ratio (FCR) of boilers under thermoneutral (TN) and heat stress (Mean± Pooled SE)

Item	TNC	HSC	T1	T2	T3	T4	T5	SEM	P
Initial BW (day1)	40.56	41.23	40.86	41.25	42.07	41.33	40.07	0.23	0.332
0 to 21 day									
BW (day 21 W3)	904.19^{a}	417.82^{d}	621.73^{b}	608.06^{b}	599.33^{b}	572.40^{c}	625.31^{b}	13.39	<0.001
Feed intake (g/bird)	1191.04^{a}	866.2^{e}	1077.37^{b}	1013.98^{cd}	1027.17^{c}	1001.76^{d}	1019.69^{cd}	9.05	<0.001
BW gain (g/bird)	863.62^{a}	376.59^{e}	580.86^{bc}	566.81^{bc}	557.27^{c}	531.07^{d}	585.31^{b}	13.41	<0.001
FCR (g/g)	1.38^{d}	2.32^{a}	1.86^{bc}	1.83^{bc}	1.84^{bc}	1.89^{b}	1.74^{c}	0.02	<0.001
22-42 day									
Feed intake (g/bird)	3475.15^{a}	2431.59^{c}	2612.92^{b}	2478.13^{c}	2512.65^{c}	2486.72^{c}	2479.62^{c}	34.7	<0.001
BW gain (g/bird)	1740.81^{a}	1045.52^{d}	1142.93^{c}	1151.5^{c}	1169.67^{c}	1217.33^{b}	1177.81^{c}	21.07	<0.001
FCR (g/g)	1.99^{d}	2.33^{a}	2.29^{a}	2.16^{b}	2.15^{b}	2.05^{cd}	2.11^{bc}	0.01	<0.001
0-42 day									
Final BW (day 42)	2645.00^{a}	1463.35^{d}	1664.66^{c}	1759.56^{c}	1769.00^{b}	1789.73^{bc}	1803.13^{bc}	21.64	<0.001
Feed intake (g/bird)	4666.18^{a}	3297.79^{d}	3690.28^{b}	3492.11^{c}	3539.81^{c}	3488.49^{c}	3499.31^{c}	42.27	<0.001
BW gain (g/bird)	2604.43^{a}	1422.11^{d}	1723.8^{c}	1718.31^{c}	1726.93^{c}	1748.4^{bc}	1763.13^{b}	33.33	<0.001
FCR (g/g)	1.79^{d}	2.32^{a}	2.14^{b}	2.03^{c}	2.04^{c}	1.99^{d}	1.98^{d}	0.01	<0.001
Mortality, %	1.75	12.77	4.44	3.88	4.88	3.33	2.22	1.09	0.08

BW, body weight, FCR, feed conversation ratio = feed intake/ body weight gain
a, b, c, d, e means with different letters in the same row are significantly different at P<0.05.
TNC, thermoneutral control, HSC, heat stress control; basal diet supplemented with:1 % Cuminand1 % turmeric powder (T1) ; 1.5 g/kg potassium chloride and 2 g/kg sodium bicarbonate (T2) ; 15000 IU vitamin A and 1000 ppm propolis (T3); 500 ppmvitamin C and 1200 ppb chromium (T4); 1200 ppm betaine and 500 ppm Vitamin E (T5).

Table 3: Effect of dietary additives on carcass characteristics and relative weight (% of live weight) of internal organs of broilers under thermoneutral (TN) and heat stress (Mean± Pooled SE)

Item	TNC	HSC	T1	T2	T3	T4	T5	SEM	P
Preslaughter weight	2665.00^{a}	1621^{e}	1722^{d}	1730^{d}	1788.00^{b}	1798.00^{bc}	1831.2^{b}	56.21	<0.001
Carcass weight	1872.6^{a}	1109^{e}	1206^{d}	1210^{d}	1247^{c}	1258^{c}	1287^{b}	40.29	<0.001
Dressing, %	70.28^{a}	68.42^{c}	70.08^{ab}	69.94^{ab}	69.74^{b}	69.97^{ab}	70.27^{a}	0.33	<0.001
Liver, %	2.78^{a}	2.15^{c}	2.62^{b}	2.46^{b}	2.5^{b}	2.68^{b}	2.74^{a}	0.04	0.04
Heart %	0.76^{a}	0.45^{d}	0.71^{b}	0.65^{c}	0.62^{c}	0.72^{b}	0.75^{b}	0.11	0.001
Gizzard, %	1.73^{a}	1.35^{c}	1.54^{b}	1.52^{b}	1.55^{b}	1.58^{b}	1.61^{b}	0.02	0.01
Abdominal fat, %	1.65^{b}	1.98^{a}	1.23^{d}	1.48^{bc}	1.39^{cd}	1.12^{d}	1.03^{e}	0.05	<0.001

TNC, thermoneutral control, HSC, heat stress control; basal diet supplemented with: 1 % Cumin and 1 % turmeric powder (T1) ; 1.5 g/kg potassium chloride and 2 g/kg sodium bicarbonate (T2) ; 15000 IU vitamin A and 1000 ppm propolis (T3); 500 ppm vitamin C and 1200 ppb chromium (T4); 1200 ppm betaine and 500 ppm Vitamin E (T5)

Table4: Effect of dietary additives on blood biochemical profile, oxidative stress biomarkers, and ND antibody titre (Mean± Pooled SE)

Item	TNC	HSC	T1	T2	T3	T4	T5	SEM	P
Glucose, mg/dl	221.67^{d}	281.69^{a}	240.27^{c}	251.16^{b}	230.48^{cd}	224.61^{d}	226.81^{d}	3.533	<0.001
Total cholesterol, mg/dl	126.83^{e}	174.92^{a}	153.08^{b}	146.17^{c}	138.08 ^{d}	135.25^{d}	121.25^{e}	2.888	<0.001
Total protein, g/dl	3.79^{a}	2.79^{e}	3.24 ^{cd}	3.37 ^{c}	3.47^{bc}	3.62 ^{b}	3.94^{a}	0.062	<0.001
Albumen, g/dl	1.84^{a}	1.86^{a}	1.72 ^{ab}	1.63 ^{b}	1.62 ^{b}	1.62^{b}	1.53^{b}	0.028	0.004
Globulin, , g/dl	1.95^{b}	0.93	1.52^{d}	1.73^{c}	1.85^{bc}	2.00 ^{b}	2.41 ^{a}	0.075	<0.001
Triglycerides, mg/dl	99.17^{a}	94.92^{a}	86.72 ^{b}	82.29 ^{bc}	77.02 ^{d}	85.96^{b}	80.86 ^{bc}	1.59	<0.001
SOD, U/ml	152.37^{bc}	127.80 ^{d}	152.60^{bc}	154.4^{abc}	148.4^{c}	160.60^{a}	155.60^{ab}	1.77	<0.001
GSH-PX, n mol/ml	11.02^{a}	5.98^{e}	7.48 ^{d}	8.08 ^{cd}	8.62 ^{c}	9.52 ^{b}	8.19 ^{c}	0.266	<0.001
MDA, nmol/ml	6.90^{e}	11.77^{a}	9.91^{b}	9.10 ^{c}	7.98 ^{d}	7.06 ^{e}	8.92^{c}	0.273	<0.001
TAOC, U/ml	8.02^{e}	8.42^{e}	9.64^{d}	10.32 ^{c}	10.69^{bc}	12.05^{a}	10.85 ^{b}	0.233	<0.001
Heterophils	21.51^{e}	28.9 ^{a}	23.88 ^{ab}	24.5 ^{b}	22.8 ^{bcde}	23.1 ^{abcd}	22.02 ^{de}	23.75	<0.001
lymphocytes	77.23^{abc}	71.34^{d}	76.30^{cd}	76.94 ^{abc}	77.56 ^{ab}	76.60 ^{bc}	77.80 ^{a}	0.365	<0.001
H/L ratio	0.28^{d}	0.41^{a}	0.31 ^{b}	0.32^{a}	0.29^{cd}	0.30 ^{bc}	0.28^{d}	0.006	<0.001
Corticosterone, ng/ml	10.92^{a}	10.46^{b}	9.57^{c}	9.51^{c}	9.52^{c}	9.13^{d}	9.07^{d}	0.11	<0.001
ND titer (log 10)									
21 days	1.95 ^{g}	2.27 ^{f}	2.47 ^{e}	2.58 ^{d}	2.76 ^{c}	2.87^{b}	3.11^{a}	0.06	<0.001
42 days	3.14^{e}	3.51^{d}	3.67^{c}	3.74 ^{bc}	3.86^{b}	4.44^{a}	4.48^{a}	0.08	<0.001

$^{a, b, c, d, e}$ means with different letters in the same row are significantly different at P<0.05.ND= Newcastle disease
GSHPx, glutathione peroxidase; SOD, total superoxide dismutase and; MDA, malondialdehyde; TAOC, total antioxidant capacity, H/L heterophile/lymphocyte
TNC, thermoneutral control, HSC, heat stress control; basal diet supplemented with: 1 % Cumin and 1 % turmeric powder (T1) ; 1.5 g/kg potassium chloride and 2 g/kg sodium bicarbonate (T2) ; 15000 IU vitamin A and 1000 ppm propolis (T3); 500 ppm vitamin C and 1200 ppb chromium (T4); 1200 ppm betaine and 500 ppm Vitamin E (T5)

Table5: Economic analysis of6-week broilers fed different dietary treatments (Mean± Pooled SE)

Item	TNC	HSC	T1	T2	T3	T4	T5	SEM	P
Cumulative feed intake (g/bird)	4666.18^{a}	3297.79^{d}	3690.28^{b}	3492.11^{c}	3539.81^{c}	3488.49^{c}	3499.31^{c}	42.27	<0.001
Feed cost / bird (SR)	5.6^{a}	3.96^{d}	4.42^{b}	4.19^{c}	4.25^{c}	4.18^{c}	4.19^{c}	0.02	<0.001
Total Cost per bird (SR)*	8.6 ^{a}	6.96^{d}	7.42^{b}	7.19^{c}	7.24^{c}	7.18^{c}	7.19^{c}	0.02	<0.001
Total return (SR/bird)	31.25^{a}	17.06 ^{d}	20.68 ^{c}	20.61 ^{c}	20.72 ^{c}	20.98 ^{bc}	21.16^{b}	0.4	<0.001
Net profit (SR/bird)	22.65^{a}	10.11	13.26	13.42	13.47	13.79	13.95	0.35	>0.05
Economic feed efficiency	263.47^{a}	145.32^{e}	178.45^{d}	186.78^{c}	185.94^{c}	191.94^{b}	193.91^{b}	3.23	<0.001
Relative economic efficiency	1^{a}	0.56^{e}	0.68^{d}	0.72^{c}	0.71 ^{c}	0.73 ^{b}	0.75^{b}	0.01	<0.001

SR, Saudi riyal
TNC, thermoneutral control, HSC, heat stress control; basal diet supplemented with: 1 % Cumin and 1 % turmeric powder (T1) ; 1.5 g/kg potassium chloride and 2 g/kg sodium bicarbonate (T2) ; 15000 IU vitamin A and 1000 ppm propolis (T3); 500 ppm vitamin C and 1200 ppb chromium (T4); 1200 ppm betaine and 500 ppm Vitamin E (T5).
*Total cost include (chick price, light, electricity, workers, vaccinations and bedding)

Discussion

The current study confirmed the detrimental impacts of heat stress on broilers performance, carcass traits, blood metabolites, antioxidant capabilities and economic efficiency. The harmful effects of heat stress on growth performance indices detected in the current experiment were consistent with previous studies (8, 10, 11). Heat stress reduces feed intake as an adaptation defense mechanism started by birds to reduce metabolic heat production (20). Heat stress increases free radical generation and lipid peroxidation, which impair growth performance. The improved broiler performance by the selected additive combination in the current studies are matching the findings of earlier studies (6, 7, 8, 9, 10, 11, 12).

Antioxidant vitamins (C and E) had a beneficial effect in relieving heat stress in broilers through their antioxidant effects (21). Betaine is crucial to be supplied during heat stress, as poultry cannot synthesizes sufficient amount and poultry, feed considers as a poor source (22). This mixture vitamin E and betaine (Bet with VE) resulted in complete reduction of the negative effect of chronic heat stress (CHS) on broiler performance. The synergetic effect of Bet and vitamin E may reveal the different mode of action of both. The supplementation of betaine and vitamin E elicit the best significant effect on growth performance and this finding could have important implications in poultry production in subtopic and tropical regions. The potential effect of betaine represented by improving the digestibility of specific nutrients as energy and protein through enhancing the structural and functional characteristics of intestinal epithalia (23). Moreover, dietary supplementation of betaine and vitamin E combination provides a more potent antioxidant effect, and spares essential amino acids like choline and methionine (12, 23). A positive effect was detected between the dietary combination vitamin C and Cr as evidenced by their potent antioxidant property against oxidative stress in the current study and confirm the finding of previous studies (5, 24, 25). There are several explanation for this result, vitamin C and chromium supplementation increased serum Vit C, Cr, Vit E, Fe, Zn, Mn and improved nutrient digestibility in laying hens under cold stress (24). The rate of ascorbic acid uptake into the cells was decreased during heat stress because of the decrease in insulin level and hyperglycemia (25). Chromium indirectly increases the intracellular availability of Vit C by intensifying the action of insulin (24). The positive impact of the combination of vitamin A and propolis could be due to the role played by vitamin A as an effective radical-trapping antioxidant (26). Heat stress reduce the conversion of carotene to vitamin A and the concentration of vitamin A decreases during stress conditions so much vitamin A needed during heat stress (26).

The observed beneficial effects of cinnamon and turmeric in the current study could be attributed to their stimulation of appetite, feed intake, digestive enzyme secretions, immune response and the antioxidant actions (27). Increased growth performance and carcass traits by KCl supplementation could be ascribed to decreased body temperature by more water ingestion after electrolyte supplementation that caused divert more energy toward BW gain. In accordance with the present results, previous studies have demonstrated that, the addition of KCl and NaHCO3 during heat stress, at levels of 1.5% and 0.5% respectively, improved final weight, feed efficiency and improved ($P<0.05$) serum potassium and bicarbonate level through providing of potassium and bicarbonate ions (2).

It has been established that heat stress alter the carcass composition of birds through increasing fat deposition and reducing body protein content (6). The improved carcass characteristics due to the dietary additive in the current study are in line with the finding of previous studies (6, 28, 29) who reported that supplementation of propolis, ascorbic acid, chromium, and turmeric significantly increased the dressing percentage and improved crass characteristics in chicken reared under heat stress. The increase in dressing percentage in bird receiving vitamin E and Bet may be due to the osmotic effects of Bet, which increases water re-

tention and improving the digestibility of energy and protein through enhancing the structure and function of intestinal epithalia and sparing essential amino acids (23). The present findings seem to be consistent with other researches which found that dietary inclusion of vitamin C (5, 24), or Cr (24) improved all carcass characteristics in broilers. Another study illustrated that Cr supplementation increased carcass yield and decreased abdominal fat in heat stressed chickens (28).

The increase in glucose concentration in the current study could be ascribed to the increase in glucocorticoids that can result from heat stress as previously explained by (3). Glucocorticoids stimulate gluconeogenesis from muscle tissue proteins. Heat stress increased the plasma level of glucose and cholesterol and decreased total protein levels (30). Both Ascorbic acid and chromium supplementation enhance the utilization of corticosteroids released during stress (24), thus playing a crucial role in response to stress. Vitamin C mitigates the negative effects of stress related depression in poultry performance through reducing synthesis and secretion of corticosteroids (26). The increase in blood lipids under heat stress caused by compensation of energy needed due to the reduction in feed intake through lipolysis of body lipid which resulting in increasing the blood cholesterol and triglyceride levels (30).

The improved in blood oxidant and antioxidant biomarkers caused by dietary additives was in line with the results of previous studies (4, 7, 10, 21). These studies reported that dietary supplementation of chromium, betaine, vitamin E, C, betaine and propolis significantly improved primary and secondary immune response ($P<0.01$), and improved H/L ratio ($P<0.05$). This finding supports previous research of Niu et al. (4) dietary supplementation of Vitamin improved performance and immune competence of broilers under HS.

The improvement in economic efficiency in all treated groups after dietary supplementation of different additive combinations may be attributed to the ability of these additives to increase broiler performance, feed efficiency and their ability to stimulate bird immunity and to reduce mortality rate. This result match those observed in earlier studies (7, 31).

Conclusion

Based on the obtained results, it could be concluded that dietary supplementation of broiler under chronic heat stress with selected additive combinations improved the growth performance, carcass traits, concentrations of serum antioxidant enzyme biomarkers and economic efficiency. From the tested dietary additive combinations, the use of betaine and vitamin E or chromium and vitamin C together gave the best results in terms of improved growth performance, feed efficiency, immune response, antioxidant status and profitability of broilers under chronic heat stress. From the findings of the current study, it could be recommend the use of dietary either betaine and vitamin E or chromium or vitamin C combinations as a good management practice for mitigating heat stress related depression in the performance of broiler chickens.

Conflict of interest

The authors declare that they have no conflict of interest

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Original Research Article

TREATMENT AND OUTCOME OF HORSES WITH CUTANEOUS PYTHIOSIS, AND META-ANALYSIS OF SIMILAR REPORTS

Hoda Elkhenany[1]†*, Shady Nabil[1, 2]†, Howaida Abu-Ahmed[1], Hassan Mahmoud[3], Ahmed Korritum[1], Hoda Khalifa[4]

[1]Department of surgery, Faculty of veterinary medicine, Alexandria University, Alexandria, Egypt, [2]Brooke hospital for animals, Mansoura, Egypt, [3]Department of Information systems, Faculty of computers and informatics, Banha University, Egypt, [4]Department of Histology, Faculty of medicine, Alexandria University, Alexandria, Egypt
† Equal contributor

*****Corresponding author, E-mail: hoda.atef@alexu.edu.eg

Abstract: Pythiosis is reported to be one of the most life-threatening infections of people and animals in tropical, subtropical and temperate areas worldwide. Infection can result from ingesting zoospores of *Pythium insidiosum* organism or from contact with water contaminated with zoospores. The ingestion of zoospores may result in enteric pythiosis, whereas cutaneous contact with zoospores/oomycete may result in cutaneous pythiosis. Here, we reported the clinical details of 10 horses with pythiosis introduced to our clinic. These horses were treated by excision of the lesion and application of an antifungal cream to the resulting wound. Also, we performed a meta-analysis of 214 horses, of 18 reports, affected with cutaneous pythiosis and analysed the influence of the horse's sex, age, lesion site, and treatment on outcome. Treatment of horses in these 18 reports varied from surgical excision, topical, regional or systemic administration of an antifungal drug, immunization, or a combination of treatments. Horses with the most successful outcome were those treated by excision of the lesionc oupled with systemic or topical administration of an antifungal drug. Horses treated by immunization responded the poorest.

Key words: pythiosis; skin lesions; equine; surgery; *Pythium insidiosum*

Introduction

Equine cutaneous pythiosis (ECP) is a granulomatous ulcerative disease caused by*Pythium insidiosum*, an aquatic fungus-likepathogen (1, 2). The cutaneous lesion caused bythis pathogen has been described by some cliniciansas tumor-like, rounded, large, nodular granulomatous containing necrotic tissue and "irregularly shaped yellow-tan to grey, gritty, coral-like masses commonly calledkunkers or "leeches" (1). Kunkers, or "leeches"are composed of sequestered vessels, dead eosinophils, and Pythium hyphae and range in size from that of a grain of rice to several centimeters wide and several centimeters long (1). Kunkers can sometimes be found in dressings, when bandages are removed (2, 3). An epidemiological survey found the incidence of mortality of horses caused by pythiosis to be 1.3% in the Brazilian Pantanal region and 2.3% in the Brazilian Cerrado region. The case fatality rate for infected horses was 23- 45.5 (4).

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Because the cell wall of the oomycete of *Pythium insidiosum* is comprised primarily of cellulose and ß-glucan, an essential component of the cell wall, researchers have suggested that administration of an inhibitor of b-glucan synthesis, such as caspofungin, could be an effective treatment of animals for pythiosis (5, 6). The effectiveness of caspofungin in resolvingcutaneous pythiosis was tested by intraperitoneal injection in an experimentally induced pythiosis rabbit model, and resultsr evealed reduction in the growth of the lesions, but the lesions reappeared after treatment was stopped (7).

Radical surgery, including amputation of anaffected limb, is a common and effective treatment for pythiosis of human patients and animals(3, 8-10). Immunotherapy has been effective in treatment of human patients and animals for pythiosis, but results of immunotherapy vary (3, 11, 12). Combining surgical therapy with immunotherapy is more effective than is immunotherapy alone or surgery alone (4, 13).

The aims of this study were to evaluate the effectiveness of topic antifungal cream application after surgical management in 10 horses suffering from cutaneous infection of *Pythium insidiosum*. We also, performed a meta-analysis of 214 horses, in 18 reports, affected with cutaneous pythiosis to evaluate the effectiveness of different methods of treatment. This analysis also examined the influence of age, sex, pregnancy and the site of the lesion on the outcome of the infected horses.

Materials and methods

Case report history and clinical signs

Ten horses, all of which were from the Delta region of Egypt, were presented to the Brooke Hospital for Animals (BHA) between July and September 2017 for treatment for cutaneous lesions of pythiosis. These horses had one or more exudative, ulcerative, edematous, granulomatous lesions (Fig. 1A). The presumptive diagnosis of pythiosis, based on the appearance of the lesion, was confirmed by histological examination of tissue excised from the lesion, using H&E and Masson's trichrome staining. The surface area (A) of each lesion was calculated using following equation: (A= $\pi \times r(a) \times r(b)$, where $r(a)$ was the half (½) of the longitudinal diameter and $r(b)$ was the ½ of transverse diameter of the lesion.

The age, sex, pregnancy status of females, site, and size of the lesion of each horse were recorded. Also recorded the date where the horse was first examined, the date of 1st surgery, date of recurrence, and the date at which the disease was considered to be resolved. Consent forms for treatment were signed by all owners.

Surgical excision

The horses were sedated with xylazine HCl (1.0 mg/kg, IV; Xylaject, Adwia, Egypt) and anesthetized with propofol (2 mg/kg, IV; Deprivan, Astra Zeneca, UK). The horses were then positioned in lateral or dorsal recumbency, depending on the site of lesion.

The lesion was excised with a scalpel, after preparing the lesion and surrounding skin for surgery, then we used curette to remove the superficial layer of the skin in case of large size lesions. An antifungal cream containing terbinafine HCl (Lamisil AT 1% Cream; Novartis, Switzerland) was applied topically after surgery once daily for one week and covered with bandage. All horses were administered metronidazole (20 mg/kg, IV; Amrizole, Amriya pharm, Egypt), flunixin meglumine 0.50 mg/kg, IV; Flunix, Norbrook, U.K) and Procaine penicillin G (4 mg /100 kg, IM; Norocillin, Norbrook, U.K) for 5 days.

Medline database analyses

Our meta-analysis of studies of various treatments for human patients and animals for pythiosis included 37 studies. We divided these studies into *in vitro* and *in vivo* preclinical studies (n=19) and clinical studies (n=18). Twelve preclinical studies tested the sensitivity of *P. insidiosum* to various antifungal drugs, minerals, and herbal either *in vitro alone (n=7), in vivo alone (n=1) or both in vitro and in vivo (n=4)*. Sev en preclinical studies were conducted to identify the molecular, proteomic, and genomic makeup of *P. insidiosum*.

We computed the significance between each treatment group, 95% confidence interval and sample odd ratio using Fisher exact test statistics using R program, ver.3.5.1

Results

Surgical removal followed by application of an antifungal drug

The 10 horses presented because of lesions caused by *P. insidiosum* were adults 3 to 11 years old (mean, 6.5±2.6 years), weighing 300 to 360 kg (mean, 324±18.3 kg). Three were male and 7 were mares. Four of the mares were pregnant. The cutaneous lesions were located on abdomen (n=7; the lesion of one extended caudally to include the mammary glands), shoulder (n=1), thigh (n=1), and fetlock (n=1). The cutaneous lesions were ranged in size from 27 to 297 mm^2 (mean, 147.64± 123.1; Fig. 1).

The results of surgical excision and topical antifungal cream application showed that this protocol was effective in 7 out of 8 cases (87.5%). One of the horses was lost to follow-up, and another one died after the second surgery as the lesion extended to the udder and animal was emaciated with very big lesion at the time of introduction. Four of the horses recovered after excision of the lesion, but for the other four horses, signs of recurrence of the lesion appeared after surgery. These 4 horses again underwent excision of the lesion 12 to 45 days (mean 22 days) after the first surgery. The average total cure time was 56.57± 19.3 days from the time of first surgery until the day of full recovery.

Microscopic examination of histological sections stained with H&E or Masson's Trichome stain revealed fragments of hyphae surrounded by well-developed fibro-cellular capsule, connective tissue rich in collagen fibers, fibroblasts surrounded by esosinophils and radiating, esosinophilic material creating the histological appearance of the Splendore-Hoeppli phenomenon (Fig. 2).

The outcome of Medline database analysis of preclinical studies

The antifungal drug, terbinafine HCl, was used to treat the horses for pythiosis, which is a fungal-like disease. In one *in vitro* study of the effects of various antifungal drugs on *P. insidosum*, miconazole was found to be the drug most effective against *P. insidiosum*, followed by ketoconazole (14). In other in vitro studies, some antifungal drugs were found to be ineffective against *P. insidiosum (15)*. In one study, the antifungal drug caspofungin (lipopeptide) was found to have a limited fungistatic effect against *P. insidiosum* (7). In another study, the fungistatic effect of caspofungin on *P. insidiosum* was found to be similar to that resulting from immunotherapy but that treating a horse with caspofungin was more expensive than was treating a horse using immunotherapy (16). The efficacy of caspofungin against *P. insidiosum* was compared with that of other antifungal drugs in two *in vitro* studies, and in neither study was caspofungin found to be as effective as the other drugs (17, 18). Mefenoxam was found to have the highest fungal eradication (< 90%) in a study comparing the efficacy of caspofungin in resolving infection caused by *P. insidiosum* with that of itraconazole, posaconazole, voriconazole, terbinafine, and mefenoxam, Brown et al. (17). Argenta et al. (18) used caspofungin in combination with terbinafine, itraconazole, ibuprofen, or fluvastatin, and found the best combination for inhibiting growth of *P.insidiosum* to be terbinafine, itraconazole, and fluvastatin. Valente et al. (19) recently reported that plant-derived essential oils (EOs) and itraconazole worked synergistically to resolve infection caused by *P. insidiosum in vitro*. Some herbal extracts, such as propolis and garlic, which are known for their antifungal and antibacterial effect, have shown efficacy against *P. insidiosum in vitro* (20, 21).

The results of Zanette et al. (2013) study showed that an iron chelator, deferasirox, could ameliorate anemia caused by thalassemia but could not resolve lesions of pythiosis (22). They also reported that administering deferasirox in combination with immunotherapy resulted in dissemination of pythiosis to the lung. Zanette et al. (2015) treated rabbits for pythiosis with deferasirox in combination with the antifungal drug, micafungin, and reported and incidence of resolution of 88.2% (23).

Sekhon et al. (1992) reported that the polyene antibiotics, like amphotericin B, were not effective against *P. insidiosum* (14), whereas another class of antibiotics as Macrolides and tetracycline were reported to be effective against *P. insidiosum* (24). These antibiotics exert their effect on *P. insidiosum* by inhibiting protein synthesis and inhibition of amino acid transport (25). Another study examining the effect of various antibiotics with antifungal activity against *P. insidiosum* found that gentamicin, neomycin, paromomycin, and streptomycin each had an inhibitory and fungicidal effect on at least 62.5% of all P. insidiosum isolates from equine pythiosis; tigecycline inhibited growth of 70.8% of the isolates (26).

In vitro studies examining the effect of various drug on *P. insidiosum* isolated from equine lesions are summarized in Table 1.

The outcome of Medline database analyses of clinical studies

Eighteen clinical studies involving 214 horses infected with *P. insidiosum* from 7 countries, including Australia (n=5 horses), Brazil (n=106), Colombia (n=1), Costa Rica (n=39), Egypt (n=43), Thailand (n=1), and the USA (n=19), were evaluated. The youngest adult horse (above one year old) in these studies was 2 years old, and oldest was 25 years old (mean, 11.45±6.3) and foals (up to one year old) at ages ranged from 3 to 4 months with susceptibility rate only 6% in comparison to 94% for adults. The number of mares in the study group was ab out 2.5 times greater than the number of males. Twenty one of 59 mares (38%) for which information about pregnancy status was available were pregnant (Fig. 3).

Cutaneous lesions of pythiosis were most commonly found on the limbs (54.12%) followed by abdomen (25.88%). Less common locations of lesions included the mammary glands, the thorax, including the withers. Some horses had lesions of pythiosis in the nasal cavity or intestine.

Horses in these 18 clinical studies were treated by surgery, immunotherapy, injection of an antifungal drug into the lesion, or combination these treatments. In a report of 33 horses treated for pythiosis by surgery alone, 73% of horses had complete resolution of the lesions (27). Surgical excision of the lesion, coupled with oral administration sodium iodide for 2 weeks resulted in complete resolutions of lesions on the back and prepuce of a 3-monthold foal, but the time of follow-up for this foal was only 3 months (28). Statistical comparison between surgical approach alone and the empirical methods (developed by nonveterinarian where they used acids and another drugs combination) revealed a significant difference ($P<0.05$, 95% Confidence interval= 1.09-23.59 and odd ratio= 4.75) between treatments (Figure 3D). Eighty percent of horses in our study had complete resolution of cutaneous pythiosis after they were treated by surgical excision of the lesion and by topical application of an antifungal cream to the resulting wound. In another study of horses affected with cutaneous pythiosis reported that 8 0% of horses had complete resolution of lesion after local injection with amphotericin B (8). When the antifungal drug, amphotericin B, administered by intravenous regional limb infusion (IV-RLP) after surgical excision to resolve lesions of pythiosis located on the distal portion of the limb, 92% of horses had complete resolution of infection (29). When amphotericin B administered in combination with 10% DMSO, 100% of horses had complete resolution of infection (30). The complete infection resolution was significantly higher among horses that received surgery and I V-RLP with amphotericin B than among horses that received surgery alone ($P<0.0067$, 95% confidence interval=1.39-24.06 and odd ratio=5.14). The complete resolution of infection was significantly higher among horses that received IV-RLP with amp hotericin B, with or without DMSO, than among horses that were treated empirically ($P<0.00001$, 95% confidence interval= 4.35- 154.2 and odd ratio=22.47) (Fig. 3). Immunotherapy alone was used to treat 36 horses for pythiosis in 3 studies (4, 12, 13).

Immunotherapy combined with surgery was used to treat 24 horses for pythiosis in 4 studies (3, 4, 11, 31). There was no significant difference in incident of resolution between those

horses that were treated with immunotherapy alone and those horses treated with surgery in addition to immunotherapy (P = 1, 95% confidence interval=0.246-3.80, and odd ratio=1). The outcome of horses in the group that received only immunotherapy was significantly better than the outcome of 15 horses treated empirically (P<0.001, 95% confidence interval=1.78-42.87 and odd ratio=7.84). The outcome of horses were treated with a combination of immunotherapy and surgery was also significantly higher than the outcome of horses treated empirically (P<0.01, 95% confidence interval=0.00-116.7 and odd ratio=0) (Fig. 3B).

One report described using photoablation with a neodymium: *yttrium-aluminum garnet* laser after excising the lesion of two horses to induce thermal necrosis of infected tissue that surgeons failed to remove at time of surgery (32). The authors reported that the lesions had not recurred after one year.

The results of all the published studies of horses undergoing treatment for pythiosis, including the number of horses in each study, age, sex, methods of treatment, and the incidence of resolution are summarized in Table 2.

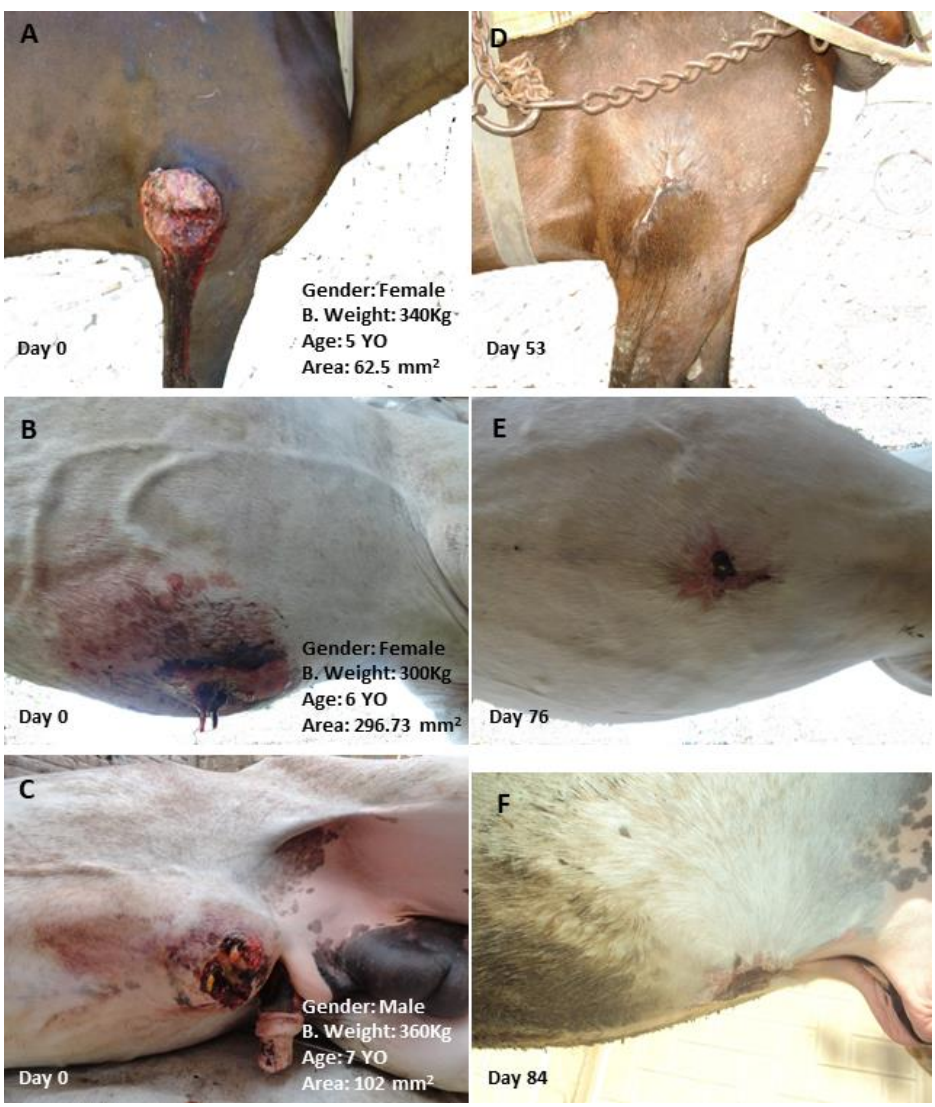

Figure 1: Representative clinical cases diagnosed as cutaneous pythiosis, before surgical intervention (A, B and C) and after complete recovery (D, E and F). Each case has a brief information about the case as the gender, weight, age and the surface area of the lesion on the lower left corner of the image (A, B and C) also the day of the recovery at the lower right corner in (D, E and F) images

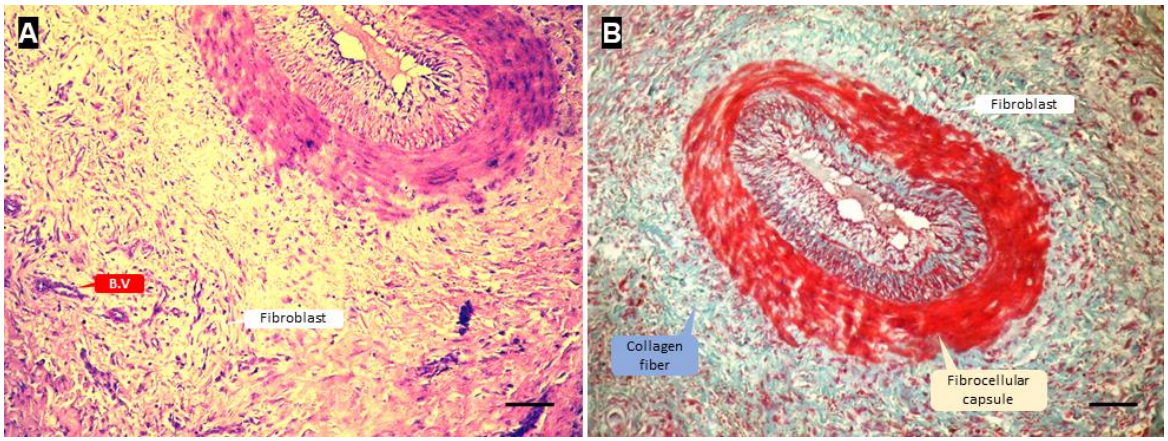

Figure 2: Representative histological section of skin lesion stained with H&E (A) and Trichome Stain (B) showing the characteristic Splendore-Hoeppli like reaction. Scale bar = 500 µm

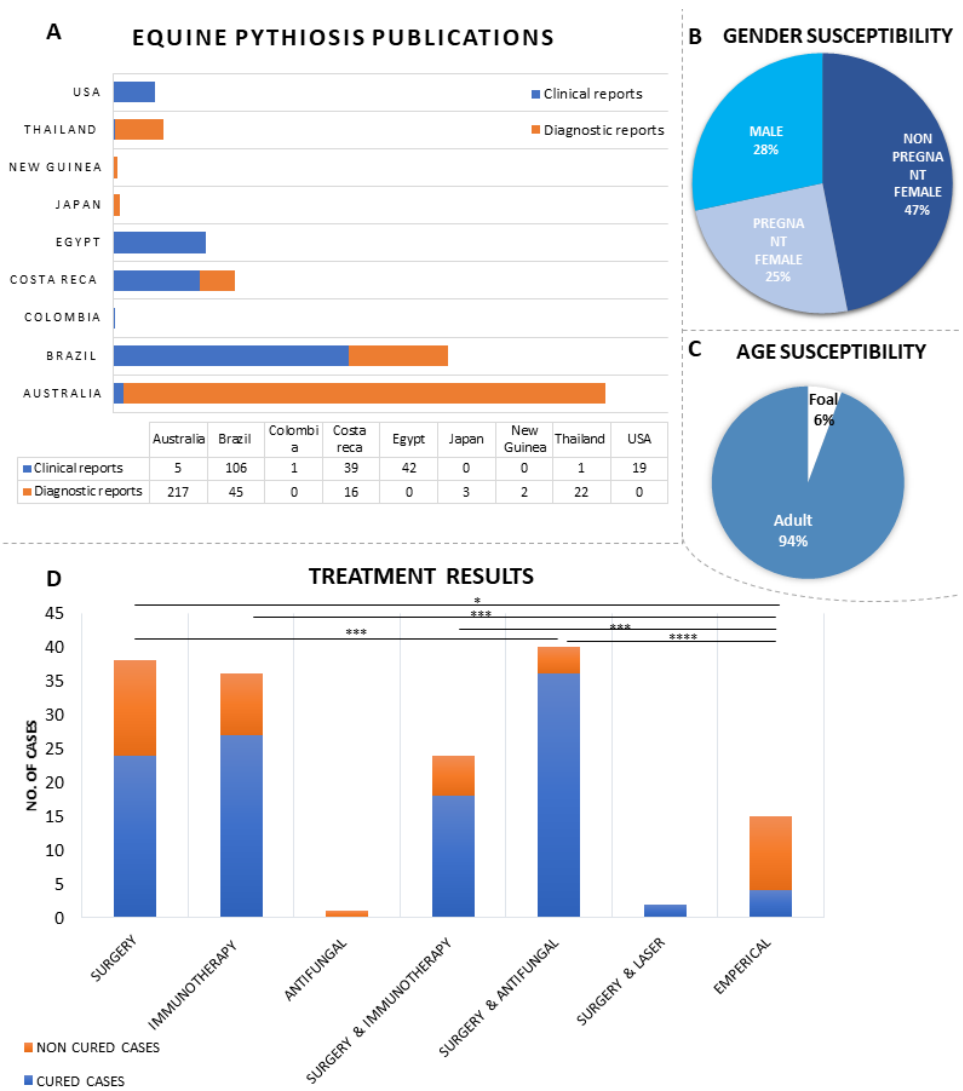

Figure 3: MEDLINE database analysis of clinical cases. (A) Publications around the world, bars represent no. of animals in all studies published/country (B) Gender susceptibility, (C) Age susceptibility and (D) MEDLINE database analysis comparing between different treatment approaches that have been used to treat equine pythiosis, *P<0.05, ***P<0.001, ****P<0.000001

Table 1: Summary of published preclinical studies *in vitro* and *in vivo* using different antifungal drugs, minerals and herbals to eradicate *P. insidiosum*

Drugs	Drug under investigation	In vitro/ In vivo	Animal M/F /Age	Results	REF
Antifungal drugs	Amphotericin B, hamycin, 5-fluorocytosine, fluoconazole, itraconazole, ketoconazole and miconazole	In vitro	-	P. insidiosum were sensitive to fluconazole, ketoconazole, and miconazole and miconazole was the strongest one.	(14)
	Terbinafine, itraconazole, caspofungin, ibuprofen and Fluvastatin	In vitro and In vivo	Rabbit (M/3m)	Terbinafine, itraconazole and Fluvastatin combination resulted in the lowest hyphae number.	(18)
	Caspofungin	In vitro and In vivo	Rabbit (M&F/3 m)	The results showed that caspofungin has limited fungistatic activity against P. insidiosum.	(7)
	Immunotherapy and caspofungin	In vivo	Rabbit (M&F/3 m)	No significant difference between both treatments but the cost of the immunotherapy is lower than caspofungin.	(16)
	Itraconazole, posaconazole, voriconazole, terbinafine, caspofungin, and mefenoxam.	In Virto	-	Mefenoxam had a profound effect on P. insidiosum at concentration 1µg/ml with >90% inhibition rate	(17)
Iron chelator	Immunotherapy, Iron chelator deferasirox and combination of both	In vitro and In vivo	Rabbit (F/3m)	Deferasirox alone did not result in any significant enhancement for the lesion from the control. However, it could ameliorate the anemia. The combination between the immunotherapy and iron were the worse than the immunotherapy alone.	(22)
	Micafungin with or without deferasirox	In vitro and In vivo	Rabbit (F/3m)	Micafungin alone is not effective against P. insidiosum but effective when combined with deferasirox cure rate 88.2% however, disease dissemination in the lung were reported.	(23)
Herbal extracts	Terbinafine or itraconazole with Melaleuca alternifolia, Mentha piperita and Origanum vulgare essential oils (EOs)	In vitro	-	The antimicrobial combinations using EOs with terbinafine or itraconazole can be an attractive therapeutic option for controlling P. insidiosum infections.	(19)
	Propolis and geopropolis in a final volume of 1.0 mL	In vitro	-	Propolis was more efficient than geopropolis in inhibiting P. insidiosum.	(20)
	Garlic extract	In vitro	-	garlic extract has strong inhibitory activity toward P. insidiosum	(21)
Antibiotics	Macrolides and tetracycline antibiotics	In Virto	-	Macrolides and tetracycline antibiotics had an effective inhibitory effect for the P. insidiosum	(24)
	Aminoglycoside antibiotics and tigecycline	In Virto	-	Tigecycline showed the lowest MIC (0.25 to 2 mg/liter) and MFC (1 to 8 mg/liter) range values. The in vitro susceptibility observed to tigecycline makes this drug a good option for management of pythiosis	(26)

Table 2: Summary of published studies on clinical studies including the number of animals under investigation in each study, age, gender, methods of treatment and the recovery rate

REF	Place of study	Site of infection	Cases No	Gender	Age	Treatment approach	Result
(4)	Brazil	Cutaneous	76	-	75 Adult	-Immunotherapy -Surgery and Immunotherapy -Empirical	Immunotherapy showed 79.4% efficacy however, combination of immunotherapy and surgery resulted in the highest recovery rate 84.6%
(30)	Brazil	Distal limb	15	7 F 8 M	4M-15Y	IRP of amphotericin B in a 10 % DMSO	Complete resolutions of the lesion between 6 to 9 weeks after a single treatment
(29)	Brazil	Distal limb	12	8 F 4 M	4M-10Y	Intravenous regional perfusion (IRP) of amphotericin B	Complete lesion resolution in 92% of case after 35 to 60 days
(12)	Brazil	Cutaneous	1	M	-	Immunotherapy	Lesion disappeared after 5 doses immunotherapy and the recurrence was detected 2 years later in the abdomen
This study	Egypt	Cutaneous	10	7 F 3 M	3-11Y	Surgical excision followed by local antifungal injection	Most of them required a 2nd surgical intervention 80% Cure rate
(27)	Egypt	Cutaneous	33	29 F 4 M	3-9Y	Surgical excision	Surgical excision resulted in 73% cure rate
(8)	USA	Cutaneous	10	-	-	Surgery followed by Amphotericin B IV and local	80% Cure rate
(31)	USA	Cutaneous	3	F	1.5Y-10Y	2 cases surgical excision 1 case surgery and immunotherapy	The treatment failed and all of them were died. mammary part, nostrils, both limbs and maxillary region, necropsy showed granulomatous lesion in lung and liver
(39)	USA	Cutaneous, extend to bone	1	F	9Y	-Antimicrobial and sodium iodide, IV injection -Surgical excision and regional antifungal injection	-Treatment with antimicrobials for 12 days, debridement, and i.v. administration of sodium iodide resulted in disease progression. - Surgical excision and regional antifungal injection were the most effective.
(32)	USA	Cutaneous	2	-	-	Surgery followed by Photoablation	Complete cure and no recurrence detected for up to 1 year
(13)	USA	Limb	1	F	18Y	Immunization	The case did not respond to vaccine and euthanized after 5 weeks
(28)	USA	Cutaneous	1	M	3M	Surgery followed by sodium iodide oral	Complete healing
(41)	USA	Enteric pythiosis	1	M	2Y	Surgery	No report about the recovery
(3)	Costa reca	Cutaneous	39	-	-	Surgery and Immunotherapy	Only five horses were treated. 3 of them respond to the treatment however the other 2 cases did not respond as they were chronic pythiosis
(11)	Australia	Cutaneous	5	-	-	Surgery, antifungal, Immunization	Surgery and antifungal were ineffective, Vaccine resulted in 1 completely cured, 3 developed osteitis and one died before completing vaccine course
(42)	Colombia	Enteric pythiosis	1	F	8Y	-	-
(43)	Thailand	Nasal cavity	1	F	4Y	Surgical removal, local injection of mix of Ampho. B, DMSO and ringer lactate then topical application of antifungal cream	-Complete healing and recovery after 3 Weeks

Discussion

Horses that develop pythiosis reside most commonly in a tropical, subtropical, or temperate region (33), and consequently, horses in most of the published reports of pythiosis were located in Australia, Brazil, Colombia, Costa Rica, Egypt, Thailand, and southern states in USA.

The presence of kunkers within granulation tissue is a grossly observable characteristic of cutaneous lesions of pythiosis, and the Splendore-Hoeppli phenomenon, an immunological reaction surrounding the causative agent, is a histologically observable characteristic.

Infection caused by *P. insidiosum* stimulate T-helper 2 (Th2) cells in the infected host, which causes eosinophils and mast cells to surround the hyphae of *P. insidiosum.* Degranulation of these cells leads to a Splendore-Hoeppli reaction. This reaction in horses is so intensive that kunkers develop (34).

Drugs interfering with the biosynthesis of ergosterol, like the azoles (itraconazole, ketoconazole, miconazole, and fluconazole), terbinafine, and amphotericin B, change the permeability of the cell membrane, causing lysis fungal cell. B ecause the cell membrane of the *P. insidiosum* is composed mainly of cellulose and ß-glucan, thus the above mentioned drugs p redictable to have little effect on *P. insidiosum*. Nevertheless, people and horses infected with *P. insidiosum* have been treated su ccessfully with drugs that interfere with the biosynthesis of ergosterol (35-37).

P. insidiosum has been reported to be sensitive to inhibitors of biosynthesis of ergosterol and β-glucan (6, 7, 17, 18, 37, 38). The efficacy of various antifungal drugs in the treatment of horses for pythiosis varied widely among the studies and depended on whether ergosterol was present in the cytoplasmic membrane of the oomycete (14, 34).

Not all antifungal drugs used to treat horses for pythiosis in these studies were effective against *P. insidiosum*. Caspofungin, for example, was found to be ineffective against *P. insidiosum*. Mefenoxam or a combination of antifungal drugs, such as terbinafine, itraconazole and fluvastatin, however, may be effective in inhibiting fungal growth and may provide a high incidence of resolution of infection. Data suggest that injecting the antifungal drug locally into the lesion or administering it by intravenous regional limb perfusion incr eases the likelihood of resolving infection (8, 29, 39). Adjuvants, such as essential oils, iron chelators and DMSO have been combined with antifungal drugs to increase the efficacy o f the antifungal drugs, in preclinical and clinical studies (19, 23, 30).

The *in vivo* effect of a drug may be different than that expected based on *in vitro* testing of that drug. For instance, Sekhon et al. (14) showed that amphotericin was not effective against P. insidiosum, but Doria et al. (2012) reported that 92% of horses experienced resolution of lesions caused by *P. insidiosum* on one or more limbs when amphotericin B was administered by IV-RLP (29).

The herbal extracts, propolis and garlic, appeared to be effective against *P. insidiosum in vitro*, though we can find no reports describing the use of these herbs clinically (20, 21).

Adult horses have been reported to be 8 times more susceptible than foals to infection caused by *P. insidiosum* (4), but meta-analysis of the studies we report shows that adults appear to be 16 times more likely than foals to become infected with *P. insidiosum*. Meta-analysis of susceptibility according to gender showed that the females are more likely than males to be presented because of infection caused by P. insidiosum (72% vrs 28%). Mendoza and Alfaro (1986), on the other hand, reported that pythiosis had no sex or age predilection (3).

Regarding the treatment, based on the meta-analysis, we found that the best approach to be surgical excision coupled with application of an antifungal drug topically or, if the lesion was located on the distal portion of the limb, by IV-RLP. There was no significant difference between the efficacy of immunotherapy used alone and the efficacy of immunotherapy used in conjunction with surgery. This can be explained by the short follow-up periods after the immunotherapy. Longer follow-up revealed that immunotherapy alone resulted in recurrence after 2 years as reported by Santos et al.

(12). This may be explained by the progressive decrease in IgG antibody titers after administration of immunotherapy, which could result in titers too low to protect the host after one year (40).

Conclusion

The excision of a cutaneous lesions of pythiosis was proved to be an effective treatment of affected horses, especially when combined with local or regional administration of an antifungal drug. Also, combining DMSO with the antifungal has revealed a higher cure rate. Because propolis was shown to be effective against P. insidiosum in vitro, we suggest testing its efficacy clinically. Including propolis in the treatment protocol is expected to be more efficient as it has antibacterial and antifungal in addition to its high regenerative capacity. Also, photoablation was suggested as effective treatment for horses with cutaneous pythiosis, but more studies are needed to prove its efficacy.

Conflict of interest

The authors declare that they have no conflict of interest.

Limitations

Some studies did not include enough data about the sex, age, and pregnancy status, so our analysis was based only on the studies that included that data.

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Original Research Article

USING OF ANIMAL BY PRODUCTS AS AN ALTERNATIVE PROTEIN SOURCE IN NILE TILAPIA (*Oreochromis niloticus*) DIETS

Malik M. Khalafalla, Rady A. Mohamed, Mohamed M. Zayed, Ahmed A. Metwally*

Department of Aquaculture, Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University, Kafr El-sheikh, Egypt

*Corresponding author, E-mail: mbd_11@yahoo.com

Abstract: Four diets were formulated to include different sources of animal by-products as a protein source in Nile tilapia (5.79 ± 0.05 g) diets. The first group fed diet containing fish meal (T1), while the 2nd, 3rd and 4th groups fed a mixture of poultry by-product meal (PBM), animal by-product meal (ABM) and kitchen waste meal (KWM) at 50:25:25 (T2); 25:50:25 (T3) and 25:25:50 (T4) ratio, respectively. All diets were formulated to be isonitrogenous and isolipidic. After 120 days, apparent digestibility and growth parameters were measured. The obtained results revealed that, the chemical composition of fish meal had the highest value of protein followed by PBM and KWM. Fish meal had higher essential amino acids content, except for the threonine and arginine amino acids, followed by PBM and ABM. The highest apparent digestibility of crude protein and ether extract were recorded in T1 and T2 followed by T4 and T3 groups. T1 and T2 groups exhibited significantly (P<0.05) the highest growth performance over fish fed T3 and T4 diets. It could be suggested that using of waste protein sources especially containing high PBM as a replacement for fish meal to reduce the feed cost per Kg fish Production.

Key words: animal by products; aigestibility; aish meal; arowth; Nile tilapia

Introduction

Nile tilapia (*Oreochromis niloticus*) has been widely cultured in Egypt due to its fast growth, efficient feed conversion and high market value (1, 2). In Egypt, the cost of feeding fish is about 70% from the total cost of farming (3). The high prices of the pelleted feeds resulted in decreasing the profit of tilapia farming (4). Also, the high prices and low availability of fish meal resulted in increasing the cost of pelleted feeds. Fish feed accounts for more than 50% of the total cost of fish farming.

For long time, fish meal has been served as major protein sources in manufacturing aquafeed due to its balanced amino acid profile and palatability (5). However, the resource shortages and rising price of fish meal seriously restrict the use of fish meal in aquaculture. In this light, much efforts have been conducted to seek the sustainable supplies of protein sources to substitute fish meal.

The plant protein sources have been limited used due to their relative low protein content and palatability, the presence of anti-nutritional factors and unbalanced amino acids profiles (6), and animal protein sources are also potential to replace fish meal in aquafeed because of their

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characterization in high protein content, total digestible dry matter and lack of anti-nutritional factors (7).

Animal by-products are used widely in aquafeed industry as an animal protein source due to its high nutritional value, palatability as well as the increased digestibility (8). Poultry wastes can be treated to preserve their nutrients and finally include them into fish diet which would reduce the cost of feeding as well as the level of environmental pollution (9).

This study was conducted to evaluate the possibility of replacing fish meal by mixture of poultry by-product, animal by-product and kitchen waste meals as an alternative protein sources and to study their chemical composition, as well as, their effects on digestibility coefficient, growth performance and nutrient utilization of Nile tilapia *(O. niloticus)*.

Materials and methods

Experimental waste by products

Experimental by products of poultry by-product meal (PBM), animal by-product meal (ABM) and kitchen waste meals (KWM) were collected from the poultry shops, animal slaughter houses and large restaurants, respectively. These wastes were boiled for 15 minutes to destroy any presence of *Escherichia coli* and *Salmonella sp.* by drying at temperature over of 60°C for 28 h, then, all ingredients were powdered in an electrical grinder, passed through a 0.5 mm sieve and mixed. All ingredients were processed into dry sinking pellet form with 1 mm diameter. Samples of ground waste were then taken to the laboratory for proximate composition analysis as shown in Table (1).

Experimental design

Four diets were formulated; the first treatment (T1) was kept as (Control) in which the fish was fed the basal diet with fish meal as a protein source, while in the other treatments, the fish meal (FM) in the basic diet was full replaced with a mixture of PBM, ABM and KWM in the following proportions; the second treatment (T2**)** 50, 25 and 25 %; the third treatment (T3), 25, 50 and 25 %; while the fourth treatment (T4), 25, 25 and 50 %, respectively and these ingredients were mixed after processing until obtaining a homogenous mixture (10). Each diet was fed to three replicates.

The diets were given according to their live body weight (BW) of the fish (3% of BW) and offered in two equal portions at 10.00 a.m. and 16.00 p.m. About 30 min after each feeding, rubber siphon was used to remove any feed residues from the aquarium tanks and maintain the water quality. The amount of feed given was constantly monitored so as not to over or underfeed them.

Experimental fish

A total number of 360 Nile tilapia (*O. niloticus*) fingerlings with an initial weight of 5.60-5.92 g were collected from private hatchery, Tollumbat No.7 in Riyad City, Kafrelsheikh Governorate, Egypt. Fish were randomly allocated into 12 glass aquaria (84 x 40 x 40 cm^3) (30 fish per aquarium) and acclimatized on the new environment (experimental conditions) for one week before the beginning of the experiment.

Growth parameters

Growth performance and feed utilization items were assessed by calculating average weight gain, average daily gain, specific growth rate, protein efficiency ratio, feed intake, feed conversion ratio, feed efficiency and survival rate.

Proximate chemical analysis

Chemical analysis of feed ingredients, experimental diets by following the standard methods, while amino acid analyzer was calibrated using a standard solution (AA-S-18, Sigma) according to (11). Apparent digestibility coefficients (ADC) of nutrients in the diets were determined using Cr_2O_3 as a marker (5g/kg) and was calculated according to (11).

Statistical analysis

Duncan multiple range test was used to detect the significant differences between the means of treatments (12). All analysis was performed using SAS (version 9.1 2004 SAS Institute, Cary, NC, USA) (13).

Results and discussion

As shown in Table (2), the CP; EE and ash contents were significantly varied ($P<0.05$) between different treatments. The FM had the highest value of CP followed by the PBM while the ABM had the lowest value. These results are supported by (14), who observed that, the values of FM protein were ranged between 64.31and 71.00 %. Conversely in the present study, the highest level of EE was in ABM followed by KWM while the FM had the lowest level of EE. The result agrees with (15) who observed that the EE of FM was ranged between 6.90 and 12.77%, respectively.

The results showed that, the KWM had the highest value of ash however, the lowest value was recorded in the FM. While, both PBM and ABM were intermediate. There were similar values for DM content in the fish fed for all dietary treatment ($P > 0.05$). Similar results were obtained by (16), who obtained that, the dry matter percentage was 93.16 and 94.23; crude protein was 70.44 and 52.12; crude fat was 7.36 and 23.47 and ash was 11.18 and 18.34 in FM and PBM, respectively. The opposite studies showed no significant variation in the case of crude protein between FM and PBM values (17). Moreover, (18) indicated that PBM is a suitable replacement of FM in practical formulation diets for tilapia.

Generally, the composition of PBM depends on processing conditions and the source of raw materials (19). So, there are some PBM have very high protein content (75-90%) with low contents of ash (less than 10%) and fat (less than 15%). Opposite values were reported by (20), who showed that the low quality of PBM had protein content (55-75%) higher levels of ash (up to 15%) and fat (more than 15% and up to 30%).

The present data revealed that, the essential amino acids (EAA) profile was significantly higher in the basal diet (control) except both threonine and arginine were higher in ABM and KWM, respectively. The lowest level of EAA found in KWM (Table 3). The chemical score of the free essential amino acid of the experimental FM, PBM, ABM and KWM were low for methionine, iso-leucine, and phenylalanine, while the highest value recorded for arginine and lysine. Similar results were obtained by (21). Additionally, (22) showed that the EAAs in FM and PBM were 5.97 and 6.09 for arginine; 2.06 and 1.98 for histidine; 3.73 and 3.89 for isoleucine, 6.78 and 7.04 for leucine; 12.78 and 12.17 for lysine; 2.92 and 2.74 for methionine; 4.05 and 4.17 for phenylalanine; 3.88 and 3.65 for threonine; 0.43 and 0.42 for tryptophan and 4.59 and 4.84 g /kg diet for valine, respectively.

However, PBM is deficient in one or more essential amino acids (16). But, the level of amino acid profiles of PBM is relatively like FM (23) making the ingredient a valuable protein source for many species. However, at replacing of FM with PBM may need to be supplemented with some essential amino acid such as lysine, methionine and threonine to make the best growth performance and body composition of fish.

The deficiency of EAAs results in poor utilization of dietary CP and reduces growth and feed efficiency. By estimating the level of EAAs in the tested ingredients, it was clear that, the level of all the essential amino acids was higher than the required level for fish feeding.

As shown in Table (3), the highest values of non-essential amino acids in tested different ingredients were recorded in FM except glycine and tyrosine. In contrast, the lowest value of non-essential amino acids was found in KWM and PBM had the highest value of tyrosine and aspartic acids while glycine showed the highest value in ABM. Similarly, (24) who found that amino acids of Nile tilapia fed FM, PBM or porcine by-product meal had similar amino acids profile.

The present results showed lower value of non-essential amino acid than those reported by (22), who reported that the non-essential amino acids value in FM and PBM were for alanine 5.98 and 5.85; aspartic 8.36 and 7.67; cystine 0.95 and 0.94; glycine 8.54 and 8.37; glutamate 13.37 and 13.53; proline 5.35 and 5.80; serine 3.75 and 3.68; tyrosine 2.80 and 2.70 g /Kg, re-

spectively. In opposite, FM could only be replaced with PBM at a level which did not exceed 50%.

Table 1: Ingredients and chemical composition (%) of the experimental diets

Ingredients	Treatments			
	FM	PBM	ABM	KWM
Fish meal	10. 00	00.00	00.00	00.00
Poultry by-product meal	00.00	5.00	2.50	2.50
Animal by –product meal	00.00	2.50	5.00	2.50
Kitchen west meal	00.00	2.50	2.50	5.00
Soybean meal	50 .56	50.50	50.60	50.75
Yellow com	32.50	32.35	32.20	32.00
Com oil	4.38	4.50	4.43	4.38
Limestone, ground	1.52	1.60	1.71	1.80
Methionine	0.24	0.25	0.26	0.27
Premix*	0.30	0.30	0.30	0.30
Chromic oxide	0.50	0.50	0.50	0.50
Total	100	100	100	100
Proximate analysis of the experimental diets (% DM)				
Dry matter (DM)	91.57±0.11	91.69±0.07	91.60±0.07	91.48±0.12
Crude protein (CP)	35.20±0.15	35.03±0.14	35.16±0.25	34.67±0.45
Crude fat (EE)	10.17±0.21	10.33±0.14	9.85±0.08	10.57±0.65
Ash	12.18±0.16	12.54±0.55	12.70±0.28	12.48±0.21
Crude fiber (CF)	4.95±1.50	5.13±1.48	5.59±0.82	5.75±0.92
Nitrogen-free extract (NFE)	37.50±0.32	36.97±0.61	36.70±0.59	36.53±0.50

*Each 1 kg contains , Vitamin A, 200,00000 IU: Vitamin D3, 400,000 IU; Vitamin E, 5,00 mg; Vitamin C, 20,000 mg; Vitamin B1, 800 mg; Vitamin B2, 1,000 mg; Vitamin B6, 2,400 mg; Vitamin B 12, 40 mg; L-lysine, 3,000 mg; DL Methionine, 2,000 mg; Choline chloride, 5,000 mg; Niacinamide, 10,000 mg; Magnesium sulphate, 24,000 mg; Cobalt Sulphate, 80 mg; sodium selenite, 20 mg; potassium iodide, 240 mg; Calcium d pantothenate, 2,000 mg; Biotin, 150 mg, ferrous sulphate, 28,000 mg; Copper sulphate, 24,000 mg; Zinc sulphate, 24,000 mg; Manganese sulphate, 6,800 mg; Inositol, 5,000 mg.

Table 2: Chemical composition of ingredients waste meal used in experimental diets

Items	Treatments			
	FM	PBM	ABM	KWM
Dry matter	96.08± 0.15	95.68± 0.08	96.71± 0.85	96.11± 0.90
Crude protein	72.61± 2.35 ^{a}	58.31± 2.55 ^{b}	51.98± 3.05 ^{c}	52.98± 2.60 ^{c}
Crude Fat	9.61± 0.82^{c}	22.61± 1.05^{b}	25.06± 1.12 ^{a}	24.26± 0.90 ^{a}
Ash	12.09± 1.45 ^{c}	17.49± 2.15 ^{b}	19.28± 1.90^{ab}	21.63± 3.45^{a}

Means on the same row with different superscripts are significantly different (P<0.05).

Table 3: Essential and nonessential amino acids of FM, PBM, ABM and KWM used in experimental diets (%, wet/ wt.)

Items	Treatments			
	FM	PBM	ABM	KWM
Threonine	2.79±0.18^{b}	2.08±0.16 ^{c}	3.25± 0.21 ^{a}	2.00±0.21^{c}
Valine	3.72±0.21 ^{a}	2.80±0.21^{b}	3.06±0.30 ^{b}	2.56±0.19^{c}
Methionine	1.17±0.11 ^{a}	1.05± 0.01 ^{a}	0.52± 0.08 ^{b}	0.68±0.01 ^{b}
Isoleucine	2.71±0.23 ^{a}	1.97± 0.12^{b}	2.26±0.21 ^{b}	1.59±0.15 ^{c}
Leucine	5.53±0.31 ^{a}	3.74± 0.25^{c}	4.24±0.31^{b}	2.78±0.21 ^{d}
Phenylalanine	2.76±0.17 ^{a}	2.08± 0.14^{b}	2.62±0.15 ^{a}	1.67±0.14^{c}
Lysine	5.03±0.26 ^{a}	3.43± 0.22^{b}	3.87±0.23 ^{b}	3.67±0.31^{b}
Histidine	3.15± 0.20 ^{a}	2.92± 0.11 ^{a}	2.15±0.17 ^{c}	2.67±0.19 ^{b}
Arginine	3.56± 0.25^{b}	2.32±0.19 ^{c}	2.59±.020^{c}	4.08±0.31 ^{a}
Taurine	2.28± 0.14 ^{a}	1.95± 0.26^{b}	2.00± 0.16^{b}	1.61± 0.14 ^{c}
Aspartic	1.84± 0.21 ^{a}	1.96± 0.11 ^{a}	1.34± 0.08 ^{b}	1.05± 0.07 ^{b}
Serine	2.26± 0.14 ^{a}	1.82± 0.24^{b}	1.41± 0.10^{c}	1.68± 0.18 ^{b}
Glutamic	12.20± 0.41 ^{a}	8.98± 0.31 ^{b}	7.97± 0.31^{b}	11.07± 0.53 ^{a}
Glycine	2.37± 0.19^{b}	1.70± 0.17^{c}	3.90± 0.26 ^{a}	1.92± 0.24 ^{c}
Alanine	7.79± 0.31 ^{a}	5.29± 0.21^{c}	7.20± 0.34 ^{a}	6.31± 0.44^{b}
Cystine	0.12±0.01^{a}	0.07±0.002^{c}	0.09±0.001^{b}	0.07±0.001^{c}
Tyrosine	2.58± 0.24 ^{b}	2.93± 0.21 ^{a}	1.51± 0.22^{c}	2.12± 0.19^{b}
Proline	3.05± 0.31 ^{a}	2.65± 0.18^{b}	1.87± 0.13^{d}	2.24± 0.21^{c}

Means on the same row with different superscripts are significantly different (P<0.05).

Table 4: Apparent digestibility coefficients of the different diets

Items	Treatments			
	FM	PBM	ABM	KWM
Dry matter (%)	83.2±0.34^{a}	82.4±0.26^{a}	78.2±0.32^{c}	80.3±1.62^{b}
Organic matter (%)	85.3±0.14^{a}	85.2±0.35^{a}	80.9±1.27^{c}	83.1±1.24^{b}
Crude Protein (%)	89.8±0.24^{a}	88.9±0.52^{a}	81.2±0.65^{c}	84.3±1.52^{b}
Ether Extract (%)	96.6±0.45^{a}	96.5±0.23^{a}	87.2±0.65^{c}	93.4±0.65^{b}

Means on the same row with different superscripts are significantly different (P<0.05)

Table 5: Initial body weight, nutrient utilization and economical evaluation of Nile tilapia fed different diets (mean ± SD)

Items[1]	Treatments			
	FM	PBM	ABM	KWM
Initial body weight (g)	5.72±0.11[a]	5.92±0.22[a]	5.60±0.14[a]	5.92±0.22[a]
Final body weight (g)	99.25±0.07[a]	96.82±0.23 [a]	86.00±0.48[b]	83.32±0.06[c]
Body weight gain (g)	93.53±0.21[a]	90.90±0.32[b]	80.40±0.21[c]	77.40±0.10[d]
Gain in weight (g fish)	0.78±0.004[a]	0.76±0.010[a]	0.67±0.006[b]	0.65±0.004[b]
Specific growth rate	2.38±0.15[a]	2.33±0.05[a]	2.28±0.08[b]	2.20±0.05[b]
Total feed intake (g/fish)	180.9± 3.0 [a]	178.5±3.0 [b]	162.6 ±3.0[c]	157.2±3.0[d]
Av. Daily feed intake(g/fish)	1.51± 0.15 [a]	1.49± 0.15 [a]	1.36±0.15[b]	1.31± 0.15[c]
Feed conversion ratio	1.93 ±0.14[a]	1.96±0.07[a]	2.02±0.09[a]	2.03±0.31[a]
Crude protein (%)	32.23±0.15[a]	32.12±0.14 [a]	32.21±0.25 [a]	31.72±0.45 [a]
Protein intake (g/fish)	58.30±1.35 [a]	57.33±2.10 [a]	52.37±1.45[b]	49.86±2.15 [c]
Protein efficiency ratio	1.60±0.01[a]	1.59±0.05[a]	1.54±0.21[a]	1.55±0.07[a]
Survival ratio (SR %)	100 [a]	100 [a]	100 [a]	100 [a]
Economical evaluation[2]				
Costs (L.E)/ton	9000	7175	7275	7150
Relative to control (%)	100	79.72	80.83	79.44
Decrease in feed costs	00.00	20.28	19.17	20.56
Feed costs * (L.E)/kg WG	17.41	14.09	14.71	14.52
Relative to control (%)	100	80.93	84.25	83.16
Decrease in feed costs* (L.E)/kg WG	00.00	19.07	15.75	16.84
Kg Feed /kg weight	1.934	1.964	2.022	2.031
kg Weight / Kg feed	0.517	0.509	0.495	0.492

Values are the mean ± S.E. of triplicate groups of each treatment.
[1]Live body weight (LBW) in g of individual group of each experimental treatment was recorded every 2 weeks (14 days); Weight gain (WG) = final weight (g) – initial weight (g); Specific growth rate (SGR % /day) =100(lnW2−lnW1) / T; Average daily gain (ADG) = (W2 – W1) / t; Feed conversion ratio (FCR) = feed intake (g)/weight gain (g); Protein efficiency ratio (PER) = weight gain (g)/protein intake (g); Survival rate (SR %) = total number of fish at the end of the experiment × 100 / total number of fish at the start of the experiment. Where: W2 is the final weight, W1 initial weight and t is the time in days; ln = the natural log; T = period.
[2]Local price (L.E./Kg) for feed ingredients used FM (25); PBM (6); ABM (10) and KBW (5).

Apparent digestibility coefficients (ADC) of the different experimental tilapia fish diets are shown in (Table 4). The current data illustrated that the ADC of all studied parameters recorded the highest value in FM and PBM without any significant variation between them followed by ABM and KWM. These results are supported by (25), who demonstrated that, the ADC of commercial FM replacement diets with different level of PBM (0, 25, 50 and 100%) were organic matter 65.4, 67.5, 62.4 and 69.1 1; crude protein 97.2, 97.2, 96.7 and 97.2; lipid 85.3, 87.2, 87.0 and 89.9 %, respectively. In the same way, (26) reported that the ADC of DM, EE, CP and NFE were not altered by the inclusion of PBM.

The present results showed higher values than those reported by (27) where the ADC of diets containing mixture of ABM 25 (83.6% ± 1.15%), mixture of ABM 50 (79.21% ± 1.01%) and mixture of ABM 75 (78.7%± 1.57%) were not significantly different (P>0.05) compared

with the control diet (83.4% ± 5.21%). The high ash content in PBM could reduce protein digestibility in fish diet (28, 29).

As shown in Table (5) the average initial weight of experimental Nile tilapia fish was ranged between 5.60 and 5.92 g/fish without any significant differences between the different treatments. The highest final weight was measured in FM (99.25±0.07 g) followed by PBM (96.82±0.23 g) and ABM (86.00±0.48 g), and KWM (83.32±0.06 g). This means that the final weight in FM increased about 2.45, 13.35 and 16.05 % when compared with PBM, APM and KWM, respectively. In the same trend, ADG was significantly different between different diets and take the same direction of previous results whereas the highest value of ADG noted in T1 followed by T2, T3 and T4, respectively. Similarly, (26) reported that the highest growth performance was recorded in tilapia fish fed PBM. (30) showed that the higher final growth weight and ADG of fish may be due to the type of diet and its composition, level of essential amino acids, the higher initial weight of the stocked fish or to higher rates of the supplemental food offered to the cultured fish. On the contrary, the present results of the ADG was lower than reported by (31) who found that the ADG values was between 1.6 and 3.04 g d-1 after feeding the cultured fish.

PBM is rich of protein (65%) with ridiculous the essential amino acids (EAA) profile which resulted in increased growth in fish (32,33. Improved growth rate and daily gain may be attributed to improve protein composition and essential nutrients in the test diets (34). Moreover, (16) reported that the growth performance increased in fish fed diets in which up to 50% of the FM was replaced by PBM, similar to results in *Tilapia zilli* (35).

As well as, average feed utilization in terms of total feed intake (TFI), daily feed intake (DFI), feed conversion ratio (FCR), total protein intake (TPI) and protein efficiency (PE) are presented in Table (5). The present results showed that DFI and TPI recorded the highest values in FM and PBM without significant differences between them and significantly higher when compared with APM and KWM. While, there are no significant difference in FCR and PE between treatments. Also, TFI recorded the highest quantity in FM followed by PBM, ABM and KWM. Similarly, (36) reported that the average daily intake of fish fed FM diet did not differ from those of fish fed diets FM with 20 and 40% PBM ($P > 0.05$) due to the protein source, fish species and size, experimental period and culture systems. The major problem in feeding PBM is limited content of essential amino acids especially methionine, phenylalanine, and lysine (37). (31) reported that, the FCR was ranged from 1.17 to 1.6 for sex-reversed tilapia fed on 30% crude protein. In contrary, high FCR value (5.56 -7.77) were obtained by (38) in Nile tilapia.

In the present study, the overall survival rate was 100% during the experimental period. These results harmonized with those reported by many researchers (25;39; and 22).

Moreover, the one ton feed cost in the present study was reduced in all replacing treatments of FM by 20.28; 19.71 and 20.56 % for PBM; ABM and KWM, respectively and decreased feed costs/kg weight gain by19.07; 15.75 and 16.84%, respectively . In this trend, (40) reported that, replacement of FM by a mixture of plant protein sources significantly reduced incidence costs, as well as being of immediate importance for feed production in Egypt.

Conclusion

From the present study, it could be concluded that using waste protein sources especially containing high PBM as a replacement of FM would be a helping tool to reduce feed cost per Kg fish production.

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Original Research Article

MYCOLOGICAL EXAMINATION OF FISH FEED STUFF WITH SPECIAL REFERENCE TO MYCOTOXIN PRODUCTION

Maha M.Kholife[1, 2]*, Amgad A.Moawad[1], Amany M.Diab[3], El-keredy M.S. Abeer[4]

[1]Department of Bacteriology, Mycology and Immunology, Animal Health Research Institute, Kafr El Sheikh, Egypt, [2]Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt, [3]Aquatic Microbiology, Faculty of Aquatic and Fisheries sciences, Kafrelsheikh University. [4]Department of Biochemistry, Nutritional deficiency diseases and Toxicology, Animal Health Research Institute, Kafr El Sheikh, Egypt

*Corresponding author, E-mail: maha mahmod1988@gmail.com

Abstract: Mycotoxin contamination is considered as one of the most economic problems for livestock and feed industries. A total of thirty fish feedstuffs were collected for isolation and identification of fungi from fish farms in Kafr El Sheikh Governorate, Egypt. Five fungal genera were isolated from all examined feedstuffs. The most frequent isolated fungi was *Aspergillus* spp. (86.66%) followed by *Penicillium* spp. (23.33%), and *Fusariam* spp. (10%), *Mucor* spp. (6.66%) then *Rhizopus* spp. (3.33%). *Aspergillus niger* was the most prevailing genus (43.33%) followed by *Aspergillus flavus ((*30%), and *Aspergillus fumigatus* (6.66%), *Aspergillus versicolor* (3.33%) then *Aspergillus terrus* (3.33%). *Aspergillus ochraceus* was not found despite the presence of ochratoxin A (OTA) in low concentrations. Some mycotoxins were produced by more than one fungal species. Mycotoxins determination using HPLC revealed that 23.33% (7/30) of examined fish feed samples were positive, while 23 samples (76.66%) were mycotoxins free. In positive samples of mycotoxins, aflatoxins (AF) and OTA represented 23.33%, 10%, respectively. The estimated carcinogenic aflatoxins were AFB_1, AFB_2, AFG_1 with a percentage of 23.33%, 13.34%, 6.67%, respectively, but AFG_2 was not found. About 42.86% of the AFs producing fish feeds was higher than the permissible limit of aflatoxins (permissible limit is 20 ppb). The aflatoxigenic ability of the recovered nine *A. flavus* referred to 6 out of 9 (66.67%) were aflatoxin producers. Polymerase chain reaction (PCR) was carried out using the norsolonic acid reductase (*nor*), versicolorin A (*ver-1*) and O-methylsterigmatocystin (*omtA*) as aflatoxin producing genes of the isolated *A. flavus* strains. From the AF producing *A. flavus*, 50% was above the permissible limit. Therefore, the high contamination of fish feedstuffs with fungi, AFB_1 and low OTA in fish farms indicated potential risks to fish liveliness, derived products and the health of fish consumers.

Key words: fish feedstuff; fungi; aflatoxins; ochratoxin A

Introduction

Fish serve as a substantial source of human dietary protein worldwide, especially in African countries (1). Fish feed is considered the

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enormous cost item in the aquaculture industry and represents 40–50% of the total production costs in intensive culture systems (2). The low quality feed intake have adverse effects on animal health and productivity (3). Presence of mold in fish feeds indicates contamination probably due to improper selection of ingredients for manufacturing or an inadequate storage. Mold increase over a temperature range of 10-40 °C, pH range of 4-8 and humidity levels greater than 62% as well as more than 12-13% moisture. Fungal growth and production of their metabolites (mycotoxins) are related to extremes weather, improper storage conditions causing low feedstuff quality and bad feeding conditions (4). The most repeatedly isolated genus of fungi in feed was *Aspergillus* spp. followed by *penicillium* spp. and *Aspergillus flavus* is the most prevalent isolated fungi species (5).

Mycotoxins are a structurally diverse group of mostly small molecular weight compounds, produced by the secondary metabolism of fungi that grow in feeds, from the harvested products to the consumers. Mycotoxins occur sporadically both seasonally and geographically (6). Many reports indicated an economic losses from mycotoxicosis in fish from chronic infection as well as increasing feed conversion ratios and unforeseen outbreaks of fish mortality (7, 8). The remarkable mycotoxin types of concern produced by fungal genera *Aspergillus*, *Penicillium* and *Fusarium* include the aflatoxins, ochratoxin A, trichothecenes and fumonisins (9).

Aflatoxin was considered to have a great importance in aquaculture. Aflatoxin exerts carcinogenic effects in fish as in all animal species (10). The intake of moderate to high doses of aflatoxin by fish develop an acute intoxication, called acute aflatoxicosis, that generally leads to poor heath, fertility, productivity loss, weight gain reduction and immunosuppression (11). The main types of aflatoxins are B1, B2, G1, and G2 based on their fluorescence under UV light blue or green and relative chromatographic mobility during thin-layer chromatography (12). The main sources of AFB_1 contamination in aquaculture represented in pelleted fish feed due to the isolation and identification of fungi *Aspergillus* spp., *Penicillium* spp. in feed samples, as well as other several genera (13). Ochratoxin A (OTA) is produced by *Aspergillus* and *Penicillium* species (14).

For fungal examination, the slide culture technique is carried out for some mould species identification , which characterized by their restricted growth making difficulty to be identified using the wet mount slide method (15). The prepared samples were analyzed using a validated method by reversed-phase HPLC separation and fluorescence detection after post-column derivatization (16).

Herein, this investigation was conducted to examine and identify the most prevalent fungi in fish feeds as well as to determine aflatoxins and OTA in examined fish feed samples moreover the aflatoxigenic ability of isolated *Aspergillus flavus*.

Materials and methods

Fish feed samples collection

A total of 30 fish feedstuff samples were collected from ten different fish farms in Kafr El Sheikh Governorate in Egypt. Each representative fish feedstuff sample was thoroughly ground and mixed. The samples were examined for mycotic contamination and mycotoxin production. The samples after the dilution were inoculated into plates containing prepared media for isolation Sabouraud's dextrose agar (SDA) with chloramphenicol (0.05g/l) (17). Lactophenol cotton blue stain for fungal microscopic examination prepared as previously described (18). The examined samples of fish feeds and nine isolates of *A. flavus* were sent to laboratory for mycotoxins determination.

Isolation and purification of fungi

The dilution of the samples was carried out (19). Approximately 10 g of each sample were homogenized in a sterile mortar, diluted in 90 ml distilled water, and then 1 ml was transferred to tube containing 9 ml sterile distilled water. The tube was shaken and 1ml was removed into a sterile petri dish containing SDA with chloramphenicol (0.05g/l) using surface spread method and incubated at 25-28^{o}C for 7-10 days

under complete aseptic conditions. After incubation, the plates were examined visually and microscopically by making films. All the positive fungal cultures were purified by subculturing on SDA plates and incubated at 25-28 °C for 3-5 days. Stock isolates maintained in SDA slopes in refrigerator for further identification.

Identification of fungal isolates

The identification of the isolated fungi recovered from the examined fish feedstuffs included the macroscopical and microscopical examination. The identification of mold genera and species was carried out (18, 20) for genus Aspergillus and (21- 24) for the other mold genera. The macroscopical examination shed light on the rate and pattern of growth as color, texture, basal and surface mycelia. The microscopical examination of fungal colonies was carried out using both wet mount slide method and the micro slide culture technique. The slide culture method (23) was carried out in case of some mold species, which characterized by their restricted growth and failed to be detected using the wet mount slide method.

Estimation of mycotoxins in the examined fish feedstuffs

Qualitative and quantitative estimation of aflatoxins (AF)

Determination of aflatoxins in fish feed samples by high-performance liquid chromatography (HPLC) (16). The preparation of chemicals, standard Aflatoxin (AF) solutions of B_1, B_2, G_1 and G_2 were prepared (25). Extraction and clean-up procedures for HPLC analysis of the prepared samples (16) using a validated method by reversed-phase HPLC separation and fluorescence detection after post-column derivatization.

Qualitative and quantitative estimation of ochratoxin A

The samples were extracted (26) with few modifications. The sample (15 g) was blended (15 min) in 50 ml of acetonitrile - water (45:05, v/v), using high speed blending and then the extract was filtered through filter paper. About 5 ml of the filtrate was mixed with 50 ml of phosphate buffer saline (PBS) and filtered through a glass microfiber. Then 10 ml of the filtrate was passed through immunoaffinity columns. OTA was eluted from the column by passing 1.5 ml of methanol (HPLC grade) and collected in a vial. The eluate was evaporated until dryness at 40°C and residues were re-dissolved in 1 ml of mobile phase i.e. acetonitrile: water: acetic acid (47/51/2, v/v/v) for HPLC analysis. Stock standard solutions of each sample were prepared then the method was validated using The European Commission (27) as for guidelines. Selectivity was determined from retention time, ion ratios, and identification-points (IP) for each analyte. The permissible limit of aflatoxins and ochratoxin A were determined as previously described (30).

Screening of aflatoxigenicity test for isolated Aspergillus flavus strains

The technique using fluorescence of agar medium under U/V light (28) was performed for detection the toxigenic strains of *A. flavus* including the cultivation, the observation of fluorescence and the extraction of *A. flavus* toxins. In the cultivation, the identified isolates of *A. flavus* were inoculated at the center of a solidified fluorescence agar medium in glass Petridishes then the plates were incubated at 25°C for 10 days. The plates were examined under U/V illumination at 360 nm, starting from the 7^{th} day of incubation up to 10^{th} day for the detection of the fluorescence in the agar surrounding the colonies. Finally, the extraction of *A. flavus* toxins was carried out (29) whereas the toxic strains of *A. flavus* that illuminated in the fluorescence agar medium were inoculated in rice medium and incubated at 25°C for 15 days. At the end of incubation period, 25 ml of chloroform were added and the mixture was thoroughly homogenized for one minute. The homogenate was centrifuged at 3000 rpm for 10 minutes and the chloroform layer was decanted. The chloroform extraction was repeated only once. Furthermore, 1 ml ethanol, 3 g copper, 111 g hydroxide carbonate and 5 g anhydrous sodium sulphate were added to the chloroform extract, mixed well and filtered. The filtrate was

evaporated in rotatory vacuum evaporator to obtain the produced aflatoxins.

Polymerase Chain Reaction (PCR) for demonstration of aflatoxigenic strains of A. flavus

The demonstration of virulence factors including norsolonic acid reductase *(nor)*, versicolorin A (*ver-1)* and O-methylsterigmatocystin *(omtA)* as aflatoxin producing genes of the isolated *A. flavus* strains was carried out (31) using the primers in (Table 1).

The strains of identified *A. flavus* were grown for 72 hours under continuous shaking conditions in the Potato Dextrose Broth (32). The mycelium was then harvested by filtration, transferred to a mortar, frozen in liquid nitrogen and ground to a powder which was resuspended in a lysis buffer (50 mmol/L EDTA, 0.2% SDS, pH =8.5) and heated immediately at 68°C for 15 min. After centrifugation for 15 min at 15000 rpm, 10 ml volume of the supernatant fluid was transferred to a new centrifuge tube and 1 ml 4 mol/L sodium acetate was added. This solution was placed on ice for 1 h and centrifuged for 15 min at 15000 rpm. After centrifugation, the supernatant fluid was transferred to a fresh tube and extracted by genomic DNA extraction Kit.

The amplification was performed (33) on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany). All of the isolated *A. flavus* were examined for the presence of three important aflatoxin genes (*nor-1, ver-1* and *omtA*) enclosed in the aflatoxin biosynthesis pathway by multiplex PCR using the above mentioned 3 primer sets. PCR reaction was performed in 25 µL containing 2.5 µL 1 X PCR buffer, 0.75 µL 50 mM MgCl2, 0.5 µL 10 mM dNTPs, 2 µL of each primer, 0.2 µL *Taq* DNA polymerase (1 U/µL), 5 µL extracted DNA as template and 8.05 µL sterile distilled water. The PCR cycling protocol was applied as following: a total of 35 cycles was started with heating at 94°C for 5 min, and continued by denaturation for 30 sec at 94°C, annealing for 30 sec at 67°C, elongation for 30 sec at 72°C and a final extension of 10 min at 72°C. The amplified DNA fragments were analyzed by 1% of agarose gel electrophoresis (Applichem, Germany, GmbH) in 1x TBE buffer stained with ethidium bromide and visualized on UV transilluminator. A 100 bp DNA ladder was used as a marker for PCR products.

Determination of crude protein in fish feed samples

The protein content of fish feed samples was determined (34) using the Kjeldhal method in an attempt to find a relation between the incidence of mycotoxins produced by fungi in fish feedstuffs and the crude protein (C.P) content.

Results

Isolation and identification of fungi in fish feedstuff

The mycological analysis of examined fish feedstuffs demonstrated presence of five genera of fungi. The *Aspergillus* species was the most predominant fungi (86.66%), followed by *Penicillium spp.* (23.33%), and *Fusariam spp.* (10%), *Mucor* (6.66%), and then *Rhizopus* (3.33%) (Table 2). Percentages had been calculated in relation to the total number of examined samples (30 samples).

Aspergillus species in fish feed samples

Aspergillus species was the most prevalent isolated mold. *Aspergillus niger* was the most frequent (43.33%), followed by *A. flavus* (30%), and *A. fumigates* (6.66%), *A. versicolor* (3.33%) and then *A. terrus* (3.33%) (Table 2). The results also showed that nine isolates (30%) of *Aspergillus flavus* were recovered from the examined samples (Tables 2 and 3).

Mycotoxins analysis

Determination of the mycotoxins by HPLC in showed higher prevalence rate for aflatoxins in 7 samples (23.33%) out of 30 examined samples (Table 3). The negative samples to mycotoxins contamination were 23 (76.67%) in spite of showing fungal growth on the culture. OTA was found in three samples (10%).

From the aflatoxins producing samples, 42.86% were higher than the permissible limit of aflatoxins in fish feeds (permissible limit is 20 ppb). OTA producing samples were found in

low percentages. 66.67% of the detected OTA was higher than the permissible limit of OTA (permissible limit of OTA is 5 ppb). The estimated carcinogenic types of aflatoxins $AFB_{1,}$ AFB_2 , AFG_1 were (23.33% , 13.34%, 6.67%), respectively and AFG_2 was not found. AFB_1 was the predominant type of detected aflatoxins (23.33%) in 7 fish feed samples (Table 3). On the other hand, 3 samples (10%) of the examined fish feedstuffs contained aflatoxins but did not give *Apergillus flavus* on culture (Table 3). However, 3 samples (10%) contained OTA but did not produce *Aspergillus ochraceus* on culture. The negative OTA producing samples of fish feedstuff were 27 samples (90%). OTA was (10%) of total detected mycotoxins in the all examined fish feedstuffs but was higher than the permissible limit (5 ppb).

Crude protein determination of fish feedstuffs

The protein content in each fish feedstuff was determined (Table 3). The variations of c.p % were recorded pointing to the difficulty to demonstrate the relation between estimated mycotoxins produced by the isolated fungi and the protein content of fish feedstuffs.

Toxigenic ability of isolated Aspergillus flavus

As shown in (Table 4), six isolates of *A.flavus* (66.67%) produced aflatoxins at rate of 50% above the permissible limits while 3 isolates of *A.flavus* (33.33%) were negative to aflatoxins.

The multiplex PCR of *nor-1* at 400 bp, *ver-1* at 537 bp and *omtA* at 797 bp virulence genes was used for demonstration of aflatoxigenic strains of isolated *Aspergillus flavus* as shown in (Fig.1). PCR of *A.flavus* isolates screened that 3 isolates were negative for the aflatoxin producing genes and 6 isolates were positive. The percentages were calculated regarding to the total number of *A.flavus* isolated from the examined fish feedstuffs (9 isolates).

Table 1: Primers' sequences for PCR identification of aflatoxin producing genes of the isolated *A. flavus* strains

Primer	Target gene	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
nor-1	*aflD*	F-ACCGCTACGCCGGCACTCTCGGCAC	400	(31)
nor-1		R-GTTGGCCGCCAGCTTCGACACTCCG		
ver-1	*aflM*	F-GCCGCAGGCCGCGGAGAAAGTGGT	537	(31)
ver-1		R-GGGGATATACTCCCGCGACACAGCC		
omtA	*aflP*	F-GTGGACGGACCTAGTCCGACATCAC	797	(31)
omtA		R-GTCGGCGCCACGCACTGGGTTGGGG		

Table 2: Incidence of isolated mold in the examined fish feedstuffs

Fungal species	Number of fungi in examined samples	Frequency (%)
Aspergillus spp	26	86.66%
Aspergillus niger	13	43.33%
Aspergillus flavus	9	30%
Aspergillus fumigates	2	6.66%
Aspergillus terrus	1	3.33%
Aspergillus versicolor	1	3.33%
Penicillium spp	7	23.33%
Fusarium spp	3	10%
Mucor	2	6.66%
Rhizopus	1	3.3%

Percentages were calculated in relation to the total number of examined samples (30).

Table 3: Determination of aflatoxins (AF) and ochratoxin A(OTA) in fish feedstuff using (HPLC), crude protein (C.P%) and the isolated fungi in each examined fish feed sample

Serial No.	**C.P%**	**Isolated fungi**	**Aflatoxins (microgram/kg"ppb")**				**Ochratoxin A (ppb)**
			AFB_1	**AFB_2**	**AFG_1**	**AFG_2**	
1.	28.09%	*A.flavus,A.niger*	29.4	15.1	6.6	0.0	10.7
2.	30.89%	*A.niger*	4.6	0.0	0.0	0.0	0.0
3.	30.54%	*A.flavus,Rhizopus*	1.8	0.0	0.0	0.0	0.0
4.	25.375%	*A.niger,Mucor*	13.9	7.2	1.3	0.0	3.5
5.	26.022%	*A.niger*	2.0	0.0	0.0	0.0	0.0
6.	28.875%	*A.flavus,A.niger*	18.7	7.9	0.0	0.0	6.1
7.	24.1%	*A.flavus*	3.5	1.0	0.0	0.0	0.0
8.	25.03%	*A.fumigatus,A.niger*	0.0	0.0	0.0	0.0	0.0
9.	26.78%	*A.niger*	0.0	0.0	0.0	0.0	0.0
10.	28.22%	*A.niger,penicillium*	0.0	0.0	0.0	0.0	0.0
11.	33.51%	*A.niger*	0.0	0.0	0.0	0.0	0.0
12.	23.63%	*Penicillium*	0.0	0.0	0.0	0.0	0.0
13.	27.74%	*A.niger*	0.0	0.0	0.0	0.0	0.0
14.	21.96%	*A.niger*	0.0	0.0	0.0	0.0	0.0
15.	30.63%	*A.niger*	0.0	0.0	0.0	0.0	0.0
16.	30.89%	*A.niger*	0.0	0.0	0.0	0.0	0.0
17.	23.19%	*A.versicolor*	0.0	0.0	0.0	0.0	0.0
18.	26.25%	*Fusarium*	0.0	0.0	0.0	0.0	0.0
19.	24.5%	*Penicillium*	0.0	0.0	0.0	0.0	0.0
20.	25.38%	*Fusarium*	0.0	0.0	0.0	0.0	0.0
21.	25.38%	*Penicillium*	0.0	0.0	0.0	0.0	0.0
22.	29.3%	*Mucor*	0.0	0.0	0.0	0.0	0.0
23.	26.69%	*A.flavus*	0.0	0.0	0.0	0.0	0.0
24.	28.26%	*Penicillium, A. fumigatus*	0.0	0.0	0.0	0.0	0.0
25.	24.23%	*Penicillium, Fusarium*	0.0	0.0	0.0	0.0	0.0
26.	25.31%	*Penicillium*	0.0	0.0	0.0	0.0	0.0
27.	24.76%	*A.flavus*	0.0	0.0	0.0	0.0	0.0
28.	30.19%	*A.flavus*	0.0	0.0	0.0	0.0	0.0
29.	31.4%	*A.flavus*	0.0	0.0	0.0	0.0	0.0
30.	31.24%	*A.flavus*	0.0	0.0	0.0	0.0	0.0

Table 4: Determination of Aflatoxins using (HPLC) produced by *A.flavus* isolates (microgram/K "ppb")

Serial No.	*A. flavus* isolates	*A. flavus* isolates			
1	-ve	AFB_1	AFB_2	AFG_1	AFG_2
2	+ve	0.0	0.0	0.0	0.0
3	-ve	16.9	9.2	4.5	0.0
4	+ve	0.0	0.0	0.0	0.0
5	+ve	5.2	0.0	0.0	0.0
6	+ve	14.6	5.7	0.0	0.0
7	+ve	23.1	12.9	7.3	0.0
8	-ve	9.4	0.0	0.0	0.0
9	+ve	0.0	0.0	0.0	0.0

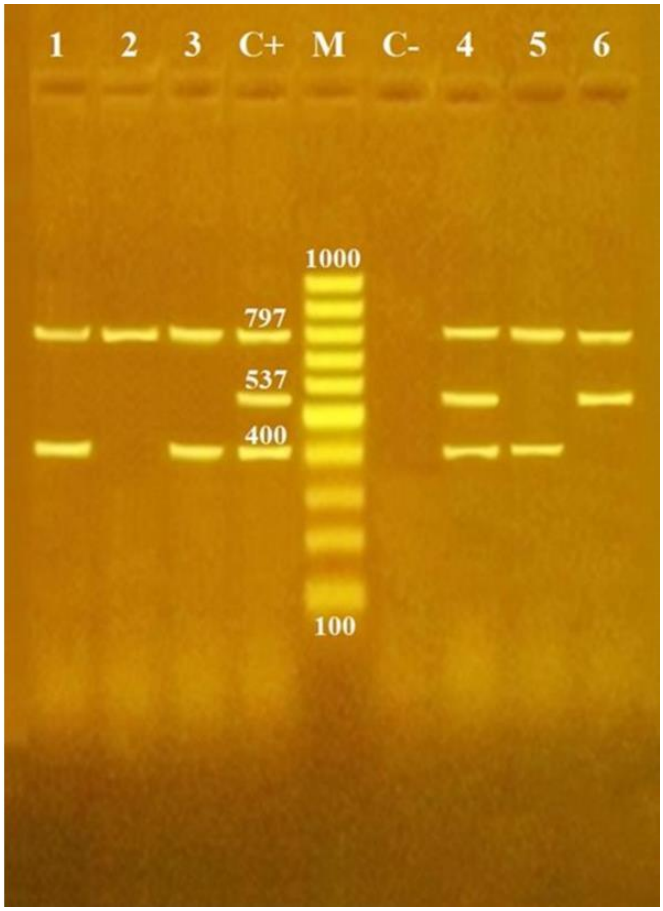

Figure1: Agarose gel electrophoresis of multiplex PCR of *nor-1* (400 bp), *ver-1* (537 bp) and *omtA* (797 bp) virulence genes for demonstration of aflatoxigenic strains *of Aspergillus flavus*

Lane M: 100 bp ladder as molecular size DNA marker
Lane C+: Control positive strain for *nor-1*, *ver-1* and *omtA* genes
Lane C-: Control negative
Lane 4: Positive *A. flavus* strain for *nor-1*, *ver-1* and *omtA* genes
Lanes 1, 3 & 5: Positive *A. flavus* strains for *nor-1* and *omtA* genes
Lanes 6: Positive *A. flavus* strain for *ver-1* and *omtA* genes
Lane 2: Positive *A. flavus* strain for *omtA* gene

Discussion

The significance of fish as an imperative wellspring of human protein, particularly in African nations is taken into consideration. The nearness of molds in fish feedstuff is a guide of tainting likely because of an unseemly feed preparing for assembling or insufficient stockpiling of fish feeds motivating the mycotoxin output. Mycotoxins are created by fungal growth causing injurious consequences for human.

In the present study, the isolation rate of fungi from the all 30 fish feedstuffs collected from different fish farms in Kafr El Sheikh governorate involved *Aspergillus* spp., *Penicillium* spp., *Fusariam* spp., *Mucor* spp. and *Rhizopus* spp. (86.66%), (23.33%), (6.66%), (10%), (3.33%), respectively .This is nearly similar to (35) in Brazil who demonstrated that *Aspergillus* spp. was the most frequent followed by *Penicillium* spp. in fish feed intended for fish farms. Similarly, (36) detailed that Aspergillus spp. prevailed alternate types of fungi in fish feedstuffs pursued by *Penicillium* and *Fusarium*. Morever, Nunes et al. (37) in Piaui, Brazil declared a higher rate of *Penicillium* spp. (83.3%) and *Rhizopus* spp. (23.3%) in the fish feed. In between the fungal species, *Fusarium* spp. was not recovered in fish feed by (35) while was isolated in the examined fish feedstuffs (10%). The rate of isolation of *Penicillium* spp. converted with Embaby et al. (38) who isolated *Penicillium* spp. in a frequency of (24.4%). In between *Aspergillus* species, *A .niger* was the most predominant nearly agreed with (35, 39) who isolated *A. niger* (36%),(40%) from fish feeds in Brazil and Qena in Egypt, respectively. Marijani et al. (40) isolated *A. niger* in a lower percentage (6%) from fish feed in Kenya, Tanzania, Rwanda and Uganda. Nunes et al. (37) did not isolate *A. niger* from the finished fish feed. Many previously investigations reported that the variations in mold species growth and their frequencies might be attributed to different weather conditions in combination with variant storage measures and manufacturing of fish feeds.

Mycotoxins detected by HPLC in the examined samples revealed the incidence of high aflatoxin contamination and low OTA. AFB_1 was found in all mycotoxin producing fish feed samples and AFG_2 were not found. Nearly similar results were reported by (37, 41) as AFB_1 in fish feed was (16.7%) and (28.5%) respectively. Also (39) recorded low concentrations of AFB_1, AFB_2 and AFG_2 differing with this study about AFG_2 which not found. The high frequency of AFB_1 in fish feed (55%) reported by (35) and (40). However, it is substantial to be taken in consider that fish aflatoxicosis has been mentioned in spite of the low concentration of 20 ppb of AFB_1 in feed as reported by (44) causing a high risk to consumers through mycotoxins residues in fish musculature fed on aflatoxins especially AFB_1 and ochratoxins containing fish feed stuffs. OTA recorded in low rate (10%) while (42) revealed that OTA containing samples were (25%). This may be attributed to (14) who reported that *A. niger* is less important OTA producers while *Aspergillus ochraceus* is considered a big producer of OTA but not found in this investigation while *Aspergillus niger* was the most frequent.

The aflatoxiginicity of isolated nine *A.flavus* from the examined fish feedstuffs showed that 66.67% of *A.flavus* isolates produced aflatoxins at rate of 50% above the permissible limit (6 isolates out of 9). This nearly agrees with (43) who reported that 55.5% of isolated *A. flavus* (10 isolates out of 18) from fish feed produced aflatoxins. It has been elucidated that a higher rate of aflatoxiginicity of *A. flavus* in fish feedstuffs produced by (39) that 83% (10 isolates out of 12) of isolated *A.flavus* were aflatoxigenic. Accordingly, PCR assay was performed in this study to detect the presence of aflatoxin genes produced by the isolated *A. flavus* from the examined fish feedstuff using specific primers indicating the aflatoxigenic ability.

Conclusion

All examined fish feedstuffs intended for fish farms in Kafr El Sheikh governorate showed the presence of fungal growth especially the mycotoxigenic fungi as well the incidence of carcinogenic mycotoxins AFB_1, AFB_2, AFG_1 and OTA were estimated. HPLC

and PCR techniques used for mycotoxins detection providing an accurate results. Either improper processing of fish feed ingredients concerning in their manufacturing or inadequate storage are catalyzers for growth of fungi so more adequate measures are required for manufacturing and storage. More reports are needed to provide a new vital insights into AFB_1 and OTA levels regarding to fish feeds and their bioaccumulation in fish flesh for human consumption. The relation between protein content of fish feedstuff and the mycotoxin incidence requires more investigations to limit mycotoxin production introduced by fungi.

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Original Research Article

ANTIBACTERIAL AND ANTI-OXIDANT ACTIVITIES OF LAUREL OIL AGAINST *Staphylococcus aureus* AND *Pseudomonas fluorescence* IN *Oreochromis niloticus* FILLETS

Ebeed A. Saleh[1]*, Ibrahim I. Al-Hawary[2], Mayada M.Elnajar[3]

[1]Department of Food Hygiene, Faculty of Veterinary Medicine, Damanhour University, Egypt, [2]Department of Fish Processing and Biotechnology, Faculty of Aquatic and Fisheries Science, Kafrelsheikh University, Egypt, [3]Department of Food Hygiene, Faculty of Veterinary Medicine, Damanhour University

*Corresponding author, E-mail: ebeed.saleh@yahoo.com

Abstract: *Laurus nobilis* L. commonly known as Bay belonging to the family *Lauraceae*, is one of the most useful plant used in foods as a spice in Mediterranean cookery. This study was designed to evaluate the antibacterial and anti-oxidant activities of different concentrations of laurel essential oil (0.5%, 1%, 1.5%) against two bacterial strains "*Staphylococcus aureus* as food poisoning bacteria and *Pseudomonas fluorescence* as food spoilage bacteria" in order to enhance fish safety and increase shelf life. A total of 80 samples of freshly dead *Oreochromis niloticus* fish were collected after experimental infection with the two bacterial strains followed by treatment with different concentrations of laurel essential at different storage time at chilling temperature. The results showed that laurel essential oil was efficient against the tested bacteria, but varied in its antibacterial activity as follows; the highest reduction percent of *S. aureus* and *P. fluorescence* at rate of 99.97% and 94.5% was detected 6 days after laurel oil treatment, respectively, and reached 100% 9 day after oil treatment at the same concentration. While the lowest value was obtained with 0.5% concentration. Laurel oil (1.5%) also showed a significant decrease in pH, TNB-N and TBA. Based on the obtained data, it could be concluded that, laurel essential oil has strong antibacterial and antioxidant activity against the tested bacteria, so that it can be used as natural food preservatives alternatives to other synthetic agents to keep quality and increase fish shelf life.

Key words: *Laurus nobilis*; essential oil; antibacterial activity; fish shelf-life; food safety

Introduction

Fish is an important source of protein to humans which rich of many sources of therapeutically important polyunsaturated fatty acids, vitamins and various other micro nutrients. Seafood has a limited shelf life due to it is highly perishable, microbial spoilage is the main cause of fish decomposition (1). It affects both the quality and the safety of fish and leads to food poisoning, so that the concepts of enhancing its shelf life and quality became needful in the recent years (2).

Due to the highly potential health hazard of synthetic preservatives and its toxicological effects, natural preservatives have been explored

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by the researchers for controlling the microbial and chemical mechanisms responsible for spoilage in fish (3).

Plant extracts are safe, effective and successful treatments with the potential to extend the shelf life of foods. Plant extracts and its essential oils have antimicrobial and anti-oxidant properties against food borne pathogens, today they have been considered as natural preservatives or food additives in food industry for raw and processed food preservation (4). Bay laurel (*Laurus nobilis* L.) is an evergreen shrub native to the Mediterranean region, being the only European representative of the *Lauraceae* family. Its dried leaves and essential oil are used in Italy France, Turkey, Algeria, Morocco, Spain, Portugal and Mexico as a valuable spice in the culinary and food industry (5).

Thus, the aim of this study was to evaluate the antibacterial and antioxidant activities of laurel essential oil against two bacterial strains "*Staphylococcus aureus* as food poisoning and *Pseudomonas fluorescence* as food spoilage bacteria " for enhancing shelf life of fish.

Materials and methods

Collection and preparation of samples

A total of 80 samples of freshly dead *Orechromis niloticus* fishes were collected from local market at Kafrelsheikh Governorates and transferred quickly to the Lab of "Animal Health Research Institute" for bacteriological and chemical examination. Then fishes were swabbed with ethyl alcohol 70% and scorched with flame, the fish flesh taken from the back muscle (6). Fillets cut with a sterile scalpel to (100 g), put under the UV light for 20 minutes to reduce number of the microorganisms attached to its surface.

Preparations of inocula

Staphylococcus aureus and *Pseudomonas fluorescence* strains were obtained from Food Analysis Center, Faculty of Veterinary Medicine, Benha University, Egypt. Bacteria were sub-cultured on Brain-Heart Infusion (BHI) broth and harvested by centrifugation (3000×g, 15 min), and resuspended with saline (NaCl, 0.85%, w.v) (7). For inoculation of the *Oreochromis niloticus* fillets, 1 ml of the dense suspension 3×10^6 /g for each strains was employed.

Essential oil extraction

Extraction of the plant material was applied by immersion in absolute methanol for three days using automatic shaker. Then twice filtrations were applied to remove solid and fine plant particles. The enriched extract was concentrated by evaporation with heating in water bath at 65 ° C until solid mass was obtained. Finally, spreading under shaded area till complete dryness and stored in the refrigerator until using. Each extract was used for preparation of 1% (w/v) solution (8), then was inoculated into *Oreochromis niloticus* fillets.

Bacteriological examination

To 25 g of the sample, 225 ml of sterile peptone water were added and mixed, for preparation tenfold serial dilutions for the further examinations (9). A volume of 0.1ml of this serial dilution was spread over Baired Parker agar plate, then the plate was incubated at 37°C for 48 h. *S.aureus* count /g was calculated (10). A volume of 0.1 ml of each sample homogenate was separately inoculated into duplicate petridishes of *Pseudomonas* selective agar medium and was evenly spread, then the developed colonies (greenish yellow colonies) were counted (11).

Chemical examination

Ten grams of sample were blended in 10 ml of neutralized distilled water. The pH value was determined by using an electrical pH meter (Bye model 6020, USA) (12). The technique applied for determination of total volatile nitrogen (TVN) was recommended by Food and Agriculture Organization "FAO" (1980) (13) **.** Determination of thiobarbituric acid number (TBA) (mg malonaldehyde/kg) was recommended by Pikul *et al.* (1989) (14).

Statistical Analysis

The obtained results were statistically evaluated by application of Analysis of Variance (ANOVA) test according to Feldman *et al.* (15).

Results

Antibacterial activity

S. aureus inoculated fillets showed a significant reduction following addition of different Laurel oil concentration at different storage time. The highest reduction percent was at 1.5% concentration of oil by 98.63%, 99.97% and 100% after 3, 6 and 9 day of treatment, while the lowest reduction showed at 0.5% concentration by 40%, 75.26% and 86.9% after 3,6 and 9 day of treatment (Table 1).

In the case of *P.fluorescence* inoculated fillets, it displayed a lower reduction percent compared to *S.aureus* inoculated fillets also, it showed significant reduction percent with different concentration, at 0.5% concentration the reduction was 21.33%, 62% and 69.13% after the 3, 6 and 9 day of treatment, at 1% concentration the reduction was 48.67%, 70.12% and 93.89% after 3,6, and 9 day of treatment, the higher reduction percent was at 1.5% concentration by 82.43%, 94.15% , 100% after 3, 6 and 9 day of treatment with laurel oil (Table 2).

Table 1: Antibacterial activity of essential oil of laurel on *S.aureus* inoculated into *Oreochromis niloticus* fillets by intensity of 3×10^6 /g (n=5)

Treatment	**Control**		**0.5 % Laurel oil**		**1 % Laurel oil**		**1.5 % Laurel oil**	
Storage time	**Count**	**R %***	**Count**	**R %**	**Count**	**R %**	**Count**	**R %**
Zero time	$3.0\times10^6\pm 0.2\times10^6$	------		------	$3.0\times10^6\pm 0.2\times10^6$	------	$3.0\times10^6\pm 0.2\times10^6$	------
3 days	$2.89\times10^6\pm 0.1\times10^6$	3.67	$3.0\times10^6\pm 0.2\times10^6$ $1.80\times10^6\pm 0.2\times10^6$	40	$9.64\times10^5\pm 2.0\times10^5$	67.86	$4.11\times10^4\pm 0.7\times10^4$	98.63
6 days	$2.66\times10^6\pm 0.3\times10^6$	11.33	$7.42\times10^5\pm 1.5\times10^5$ $3.93\times10^5\pm 1.1\times10^5$	75.26	$5.37\times10^4\pm 0.8\times10^4$	98.22	$9.0\times10^2\pm 1.6\times10^2$	99.97
9 days	$2.59\times10^6\pm 0.2\times10^6$	13.67		86.90	$2.50\times10^3\pm 0.4\times10^3$	99.91	ND	------

R %*= Reduction %; ND= Not detected; Reduction %= count before - count after / count before x100

Table 2: Antibacterial activity of essential oil of laurel on *P. fluorescence* inoculated into *Oreochromis niloticus* fillets by intensity of 3×10^6 /g (n=5)

Treatment	**Control**		**0.5 % Laurel oil**		**1 % Laurel oil**		**1.5 % Laurel oil**	
Storage time	**Count**	**R %***	**Count**	**R %**	**Count**	**R %**	**Count**	**R %**
Zero time	$3.0\times10^6\pm 0.2\times10^6$	------	$3.0\times10^6\pm 0.2\times10^6$	------	$3.0\times10^6\pm 0.2\times10^6$	------	$3.0\times10^6\pm 0.2\times10^6$	------
3 days	$2.95\times10^6\pm 0.24\times10^6$	1.67	$2.36\times10^6\pm 0.19\times10^6$	21.33	$1.54\times10^6\pm 0.12\times10^6$	48.67	$5.27\times10^5\pm 1.03\times10^5$	82.43
6 days	$2.87\times10^6\pm 0.17\times10^6$	4.33	$1.14\times10^6\pm 0.08\times10^6$	62	$8.97\times10^5\pm 1.65\times10^5$	70.12	$1.65\times10^4\pm 0.21\times10^4$	94.50
9 days	$2.84\times10^6\pm 0.22\times10^6$	5.33	$9.26\times10^5\pm 1.84\times10^5$	69.13	$1.81\times10^5\pm 0.25\times10^5$	93.89	ND	------

R %*= Reduction %; ND= Not detected

Different concentration of laurel oil showing significant reduction percent of *S.aureus* and *P.fluorescence* at different storage time; the highest reduction % was 1.5% laurel oil by 100% reduction after the 9 day of treatment.

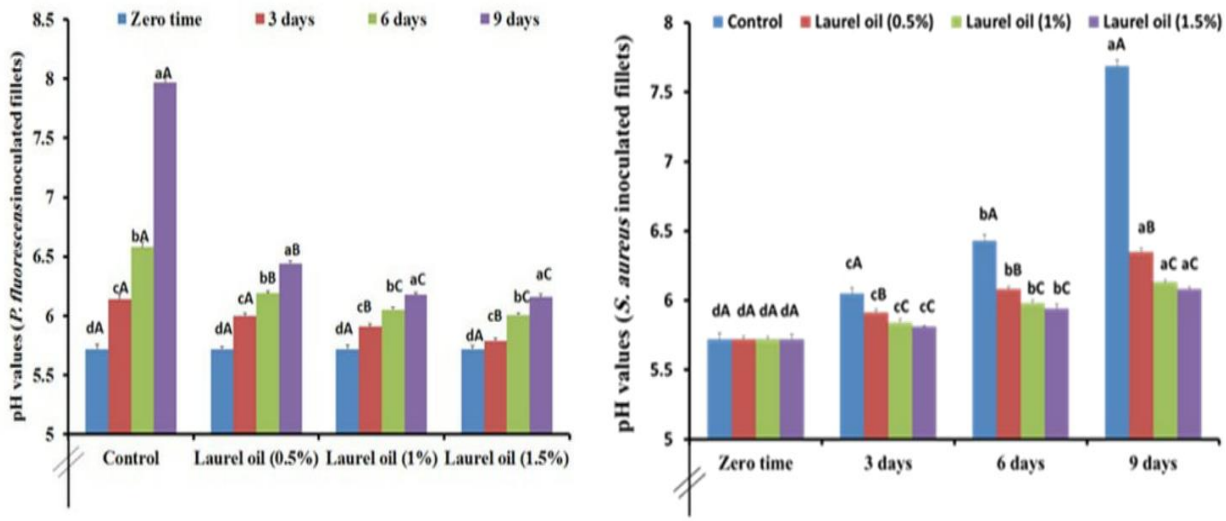

Figure 1: Showing pH values of *S.aureus* and *P.fluorescence* inoculated *Oreochromis niloticus* fillets for control and Laurel oil (0.5%, 1%, 1.5%) concentration at different storage time

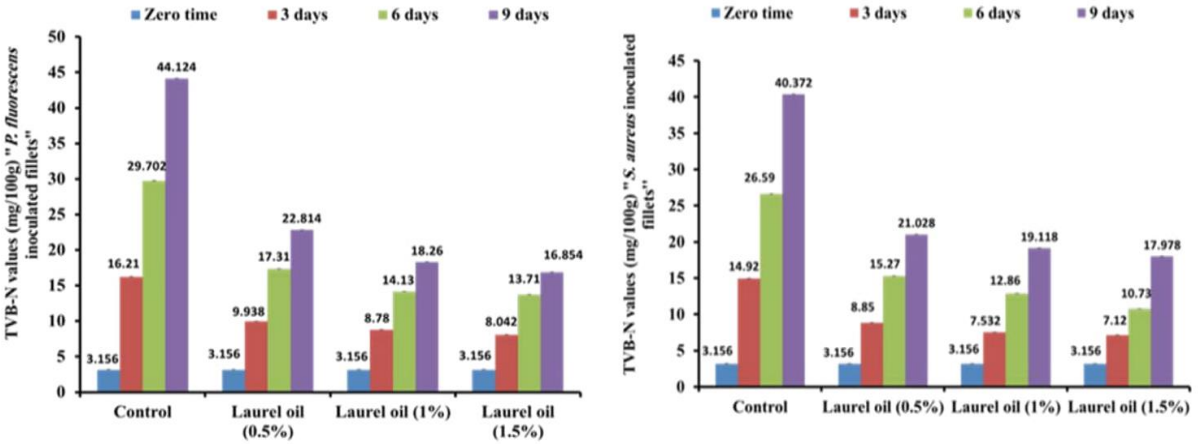

Figure 2: Showing TVB-N values of *S.aureus* and *P.fluorescence* inoculated *Oreochromis niloticus* fillets for control and Laurel oil (0.5%, 1%, 1.5%) concentration at different storage time

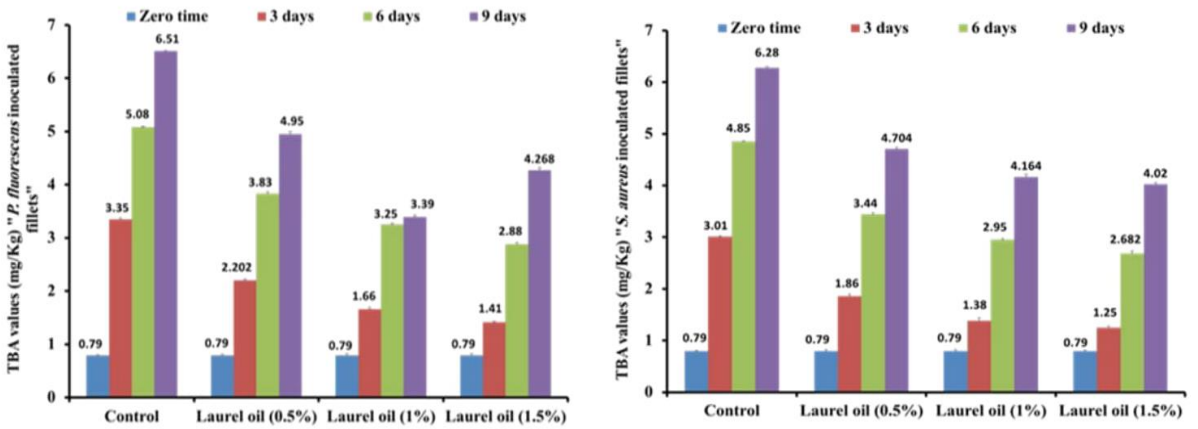

Figure 3: Showing TBA values of *S.aureus* and *P.fluorescence* inoculated *Oreochromis niloticus* fillets for control and Laurel oil (0.5%, 1%, 1.5%) concentration at different storage time

Chemical evaluation

The mean pH values of the examined *Oreochromis niloticus* fillets inoculated with *S. aureus* and *P.fluorescence* showed gradually decrease after addition of laurel oil in compared to the untreated group. Differences associated with the examined samples of the pH values between different concentration of oil and different storage time were highly significant ($P < 0.05$) (Fig.1).

Different concentration of laurel oil showed significant decrease in the mean TVB_N (mg %) value at different storage time; the most significant record was at 1.5% laurel oil after the 9 day of treatment by 17.99 ± 0.01 for *S. aureus* inoculated fillets and 16.85 ± 0.01 for *P.fluorescence*, compared to untreated fillets. Showed in (Fig. 2). Also, laurel oil showed significant decrease in the mean value of TBA (mg/Kg) at different storage time, but the highest significant value was at 1.5% concentration after the 9 day of treatment by 4.02 ± 0.01 and 4.27 ± 0.01 for *S. aureus* and *P. fluorescence* respectively compared to un treated fillets that was 6.28 ± 0.01 and 6.51 ± 0.01 after the 9 day (Fig. 3).

Data is presented as means ± SE. Mean values followed by different small (lowercase) letters indicates significant effect of the time factor with the same treatment. While, mean values followed by different uppercase letters indicates significant effect of time factor between the different treatments ($P < 0.05$).

Discussion

The present study reported that, treatment of *Oreochromis niloticus* fillets, experimentally infected with *S. aureus* by intensity $3x10^6$ \g with Laurel essential oil with different concentration (0.5%,1%,1.5%) at different storage time, it showed various reduction percent as follows; at 0.5% concentration it showed reduction by 40%, 75.26% and 86.9% after 3,6 and 9 day of treatment At 1% concentration the reduction percent was 67.86%, 98.22% and 99.91% after the 3 ,6 and 9 day of treatment, While the concentration 1.5% of Laurel oil showed the higher reduction percent was 98.63%, 99.97% at 3 and 6 day of treatment and 100% after the 9 day, as showed in (Table1).

While, fillets infected with *P.fluorescence* displayed different reduction percent with laurel oil , it was 21.33%, 62% and 69.13% after the 3, 6 and 9 day of treatment respectively ,at 0.5% concentration while at 1% concentration it showed 48.67%, 70.12% and 93.89% reduction percent after 3, 6 and 9 day of treatment, the higher reduction percent was at 1.5% concentration of oil by 82.43%, 94.15% and 100% after 3,6 and 9 day of treatment, as showed in (Table 2).

Another study has been found, that Eucalyptol that is the main component of laurel essential oil inhibit growth of S. aureus at the concentrations of 0.7%, 0.6% and 1% (16). while other, reported that *Pseudomonas* spp. were very sensitive to the essential oil of Laurel even very diluted (17). The present study concluded that, *S. aureus* and *P. fluorescence* are sensitive to different concentration of laurel oil due to the great number of different chemical compounds found in essential oil (18). The major constituents were eucalyptol (27.2%), alpha terpinenyl acetate (10.2%), linalool (8.4%), methyleugenol (5.4%), sabinene(4.0%) and carvacrol (3.2%) (5).

Eucalyptol is known to possess a strong antioxidant activity and antibacterial action against *Staphylococcus aureus*, Linalool is an aliphatic alcohol with strong to moderate antibacterial activity against several bacteria, such as *Shigella sonnei, S. flexneri , Pseudomonas* spp. and *L. monocytogenes (16). S. aureus* showed more reduction than *P. fluorescence*, this may be due to sensitivity of different *Pseudomonas* towards different antibiotics this sometimes paradoxical and it displays an intrinsic resistance to wide variety of essential oils (17).

Measuring of pH is used as indicator to evaluate the shelf life and quality of the fish fillets. Lipid and protein oxidation consider the main cause of reducing meat quality leading to off-flavor and rancidity, increasing value of TVN indicate incidence of spoilage of fish after different periods storage reaching 20mg/ 100g if

increased the meat decomposing occur , otherwise TBA number was related to the oxidation in meat causing rancidity (19).

The pH value was measured for control and different treatment of oil at different storage time, the results revealed that; the mean pH value of untreated groups showed marked change to alkalinity (7.69, 7.97) after the 9 day, as showed in (Fig.1). this indicated that the fish fillets was rapidly deteriorated, then after treatment with laurel oil the values of pH decreased gradually for maintaining the border line pH, also, ANOVA analysis indicated that there is low significance effect of the time factor within the same treatment, while there was a great significant of time factor for different oil treatment in compared with untreated group ($P < 0.05$). By measuring TVB-N it was found that, different concentration of laurel oil showed significant decrease in the (TVB_N) value at different storage time in compared to control; The most significant record of mean TVN was 1.5% laurel oil after the 9 day of treatment, followed by 1% concentration, followed by 0.5%, as showed in (Fig.2). ANOVA indicated that there was high significance (++) between different storage time of the same treatment group, and also, high significance of time factor between different treatment "control, 0.5%, 1%, 1.5%".

TBA number was measured for control and oil treated fillets and the results showed in (Fig.3), revealed that; different concentration of Laurel oil showed significant decrease in TBA value at different storage time in compared to control ; The most significant record of TBA was 1.5% Laurel oil after the 9 day of treatment, followed by 1% concentration, followed by 0.5%. The ANOVA indicated that there was a high significance (++) between the time factor within both the same treatment and between different treatment.

Laurel essential oil with different concentration (0.5%, 1%, 1.5%) maintain value of TBA that indicate decreasing of oxidative rancidity causing spoilage, thus Laurel oil suggested to has anti-oxidant properties. another matching study, reported that essential oil from laurel, eugenol and methyl eugenol may be considered the main mediators of antioxidant activity (20).

The antioxidant activity of eugenol has been reported in several studies and there are few reports regarding the radical scavenging activity of the constituents of bay laurel leaves. It was concluded from the present study that Laurel essential oil has strong antibacterial activity against *Staphylococcus aureus* and *Pseudomonas fluorescence* and it can inhibit growth of these bacteria with its different concentrations; also, treatment of *Oreochromis niloticus* fillets with laurel oil, help in keeping quality of fish fillets and prolong fillets shelf life, in addition to the anti-oxidant activity of laurel oil that maintain fillets freshness and prevent lipid peroxidation, so that enhance shelf life for a period of time

Conflict of interest statement

None of the authors have any conflict of interest to declare.

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Original Research Article

SEASONAL SCREENING OF THE MYCOTIC INFECTIONS OF CULTURED FRESHWATER FISHES IN KAFR EL-SHEIKH GOVERNORATE

Nadia B. Mahfouz[1], Eman M. Moustafa[1]*, Mohamed Kassab[2], Wesam H. Marzouk[3]

[1]Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt, [2]Department of of Cytology and Histology, Faculty of Veterinary Medicine, Kafrelsheikh University, [3]Food safety specialist in Directorate of Health in Gharbia, Egypt

*Corresponding author, E-mail: emantarek2002@yahoo.com

Abstract: The present study was carried out to screen the predominant mycotic infections among freshwater fishes (*Oreochromis niloticus* and *Clarias gariepinus*) in Kafrelsheikh fish farms; with special focus on the seasonal incidence; as well as, the histopathological changes induced by the detected fungi. 500 specimens of freshwater fishes (400 *O. niloticus* and 100 *C. gariepinus*) were investigated for seasonal incidence of mycotic diseases. Mycological examination revealed the isolation of 2148 fungal isolates from 375 diseased and 125 apparently healthy fish samples (1828 mould and 320 yeast isolates), of which 1258 were isolated from *O. niloticus* and 890 isolates from *C. gariepinus*. Saprolegnia was the predominant among diseased fishes with highest prevalence in late autumn (10.68%, 6.96%) and winter (6.81%, 7.87%) in *O. niloticus* and *C. gariepinus*, respectively. However, *Pencillium sp*. and *Aspergillus sp*. were the most predominant fungi isolated from apparently healthy fishes. The highest prevalence of *Pencillium sp.* were recorded in winter, whileas *Aspergillus* showed variations between species*; Aspergillus flavus*, *Aspergillus niger* were more prevalent in summer (25.44%, 23.22%) and (26.9%, 37.44%) and *Aspergillus terrus*, *Aspergillus fumigatus* were more prevalent in autumn (5.98%, 5.67%) and (7.69%, 8.23%) in *O. niloticus* and *C. gariepinus,* respectively. Moreover, the highest prevalence of *Fusarium species* was recorded in spring *(*11.8%, 5.91%) from O*. niloticus, C. gariepinus*, respectively. Mucor recorded the highest prevalence in autumn (20.09%) in *O. niloticus* and winter (29.21%) in *C. gariepinus*; whileas Rhizopus was highest in summer (7.89%, 5.21%) in O*. niloticus* and *C. gariepinus*, respectively. Four genera from yeast were isolated; *Candida sp.* (28.44%, 36.27%), *Rhodotorula sp.* (36.24%, 24.51%), *Cryptococcus sp.* (16.97%, 20.59%) and *Trichosporon Sp.* (18.35%, 18.63%) in *O. niloticus* and *C. gariepinus*, respectively. The histopathological findings revealed severe degenerative changes in skin and gills with presence of fungal hyphae and spores.

Key words: *Clarias gariepinus;* moulds; mycotic diseases; *Oreochromis niloticus*; yeast

Introduction

Fish serves as an important source of human dietary protein worldwide, especially in Afri-

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can countries (1). To compensate animal protein deficiency resulting from the increased interest for fish as human nourishment, fish farming is rapidly extending all over the world (2). In Egypt, the aquaculture industry provides about 77% of the total national fish production (3, 4).

Nile Tilapia (*O. niloticus*) is viewed as a standout amongst the most prevalent freshwater fishes in Egypt. It is widely cultured because of its palatability, cheap price, high growth rate, capacity to withstand pressure and infections, ability to spawn effectively and the minimal prerequisites with regard to management and energy inputs (5).

With expanding freshwater fish production movement around the world for farming, alongside enhanced ecological observing of fungal and fungal-like infections that are full degree of the effect of these pathogens on wild fish populations will soon rise as a noteworthy danger to freshwater biodiversity (6).

Serious aquaculture conditions can advance the transmission of fish diseases, particularly contagious fungal sicknesses, causing economic losses. Fungal infections are one of the primary drivers for mortalities and extraordinary financial misfortunes in cultured fishes. (7). The significance of fungal diseases in freshwater fish not halted just for frequency of mortalities but rather additionally as financial significance, such as decline growth rate, hatchability in choronic infection or by mycotoxins production by tainted organism in case of bad stockpiling feed. Fungal infections in fish are viewed as auxiliary to some other pathogen, water quality issues, poor conditions, injury (unpleasant taking care of or hostility), bacterial disease and/or parasites (8). Numerous fungi influencing fishes are considered opportunistic, assaulting the fishes only if they are stressed or immune-compromised as a result of troublesome natural conditions, or optional to bacterial or viral diseases, or when they have lost their bodily mucus protection due to trauma or excessive handling (9). Disregarding the fungal infections significance our insight about them is still poor for two fundamental reasons: difficult distinguishing proof of pathogenic fungi and the productive development of saprophytic fungi once the fish is dead (10).

Fungi is mostly attacked due to temperature change and bad water conditions which allow excessive zoospores to grow and the ammonia which is formed by rottening of fish waste wears away the mucus that protects the skin (11). Moreover, fungi can assault fishes of all the ages and it can also forestall fruitful hatching when it invades fish eggs (12). The most widely recognized fungal infection was saprolegniosis which is the real oceanic mycotic winter freshwater fish disease, frequently impacts wild and cultured fishes (13). *Saprolegnia sp*. taints the fishes because of sudden drop of water temperature and was regularly influencing fishes exhibiting fungal skin lesions which, unmistakable as cottony-white development on the epidermis of the influenced fishes (14). *Aspergillus sp.* causes systematic diseases with high death rates in fish, whereby the infections mostly occur through contamination of fish feed (15) and the pathogenesis of *Aspergillus fumigatus* and *Aspergillus niger* had been accounted in fresh water fishes by Chauhan (16).

The current study was carried out to screen the predominant mycotic infections with special focus on the seasonal incidence among freshwater fishes (*O. niloticus* and *C. gariepinus*) in Kafrelsheikh fish farms; as well as, detect the histopathological changes induced by the detected fungi.

Materials and methods

A- Materials

1. Fish

A total number of 500 examined cultured freshwater fishes; 400 *O. niloticus* and 100 *C. gariepinus*, were collected alive from different freshwater fish farms at Kafr El Sheikh Governorates along the four seasons of the year 2017. The samples were collected with an average body weight of (40±5 & 150±10 gm) for *O. niloticus* and *C. gariepinus*, respectively.

The alive collected fishes were transferred to the wet lab., Fish Diseases and Management Department, Faculty of Veterinary Medicine, Kafr El-Sheikh University, Egypt, held in well-

prepared glass aquaria supplied with sufficient amounts of dechlorinated water with continuous aeriation (17).

B- Methods

1. Clinical examination

The collected fish were examined clinically according to the methods described by McVicar (18) to detect any external changes or clinical abnormalities**.**

2. Postmortem examination

Postmortem examination of the internal organs was carried out on sacrificed and freshly dead fish according to Austin & Austin (19).

3. Mycological examination

A. *Isolation of the fungus from diseased fishes*

Mycological examination was done according to (18). Samples were taken from fish showing skin lesions using sterile dissecting needle from the skin, gills and internal organs (liver, kidney). Gathered specimens were inoculated into duplicate plates of SDA media with 500mg of cyclohexamid and 50 mg of chloramphenicol dissolved in 3 ml ethanol 95% were added to the media after autoclaving. The inoculated plates were incubated at 25℃– 30℃. For 3-5 days (20). Negative plates were not disposed before 2 weeks (21). All the positive moulds cultures examined for gross and micro morphological characteristics (22).

B. Identification of different fungi

B.1.Identification of moulds

Recognization of moulds was completed according to Refai (23). Preliminary recognization utilizing wet mount preparation of fish samples made in 10% KOH. The confirmatory test of identification was carried out using souletip technique (24). Slide culture technique was carried out on those isolates whose identification was inconclusive after staining with lactophenol cotton blue (25).

B. 2. Identification of yeast

Plates of suspected specimens were analyzed microscopically for the presence of chlamydospores, arthrospores and blastospores (20, 23) and the plan of recognizable proof of yeasts given by Terrence (26). Rice agar media was used for identification of yeasts especillay *Candida albicans* by production of characteristic chlamydospores (23). The confirmatory distinguishing proof was carried out by germ tube test (27). Biochemical reaction using urease test was also conducted (28).

4. Histopathological Examination

Tissue specimens from the skin, gills and kidney of the infected fish samples were taken for histopathological examination (29).

Results and Discussion

The present work was applied to investigate the seasonal incidence of mycotic diseases among some freshwater fishes (*O. niloticus* & *C. gariepinus*) in Kafrelsheikh Governorate.

Clinical examination

The external gross lesions of the examined *O. niloticus* revealed darkened skin, pale body coloration, scale detachment, fins erosion, and eye opacity as shown in **(**Plate 1: Fig. 1, 2**).** These results agree with (24, 30). However, in fish naturally infected with Saprolegnia, there was scattered grayish white cotton wool-like growth on various parts of the body as well as presence of ulcerative areas in some cases as shown in (Plate 1: Fig. 3, 4**);** with unilateral eye cloudiness or opacity **(**Plate 1: Fig. 5**)**; the result is in accordance with that of El-Atta, (31).

Fish death may be due to either blindness which consequently disable fish to feed or due to the fungal growth over gills causing suffocation. The ulcerative areas over the skin may be attributed to the lytic action of primary bacterial infection as all fungal infections are considered as secondary invader pathogen; these results agreed with many authors (8, 23, 32-36).

On the other side, the infected *C. gariepinus* showed skin ulceration and scattered hemorrhagic patches on the ventral abdomen and mouth **(**Plate 1: Fig. 6**)**. These symptoms may be attributed to the toxins secreted by moulds and yeasts causing severe symptomatic changes that appear on the fish in the form of haemorrhagic patches, ascitis and destruction and degeneration of the gills; the result agreed with (16, 24).

P.M. lesions

The main observed postmortem lesions were liver enlargement with moderate petechial hemorrhage **(**Plate 1: Fig. 6& 7**)**. This result may support that the saprolegnia is a secondary invader following systemic bacterial infection which is responsible mainly for this internal lesions due to toxins produced by fungi and yeasts that interfere with function of liver causing congestion in internal organs especially liver (2, 31).

Mycological examination

Mycological examination revealed the isolation of 2148 fungal isolates from 375 diseased and 125 apparently healthy freshwater fish samples; 1258 fungal isolates from *O. niloticus* and 890 isolates from *C. gariepinus* (from skin, gills, liver and kidney). Recognizable proof of fungi into mould and yeast revealed that the incidence of mould and yeast was marginally higher in *O. niloticus* (56.89%, 68.12%) in contrast with that in *C. gariepinus* (43.11%, 33.88%). The high frequency of mould isolates in *O. niloticus* agree with some authors (9, 24) and disagree with the incidence in *C. gariepinus*; as both of the two authors recorded high incidence of yeast isolates from catfish. This might be attributed to variable host susceptibility due to geographical distribution.

Morphological identification of isolated moulds

The colonies of *Saprolgnia sp*. appeared as white cotton-wool like growth on the petri dish (Plate 2: Fig. A) while, microscopically appeared as long, branched, un-septated hyphae (Plate 2: Fig. B, C). This result is in accordance with some reports (8, 31).

Pencillium sp. Colonies were white and fluffy then, turned into greenish blue in colour (Plate 2: Fig D**)**, while microscopically, there were septated hyphe with un branched condiophores possessing metule with flask- shaped strigmata forming brush appearance. (Plate 2: Fig E, F**).** Nonetheless, *Fusarium* sp. Colonies were cottony or wooly in texture, snow white, pink-violet or blushing red in shading, with dissemination of hued colors into the switch reverse surface of the medium (Plate 2: Fig G**)** and microscopically, they seemed long, extended and septated hyphae from which short conidiophores climbed and sometimes branched. Two kinds of conidia were watched, a huge banana shaped, septated macroconidia and a little, round, non septated microconidia. (Plate 2: Fig H**).** These results are in accordance with some authors (9, 37).

On the other side, *Aspergillus sp.* demonstrated few varieties within the same genus. *A. flavus* seemed smooth with various aerial growths; the shading changes from yellow to yellowish green by aging (Plate 2: Fig I**)**, and microscopically the conidiophores were long and thick, the vesicles were globose and the strigmata were biseriate and radiate (Plate 2: Fig J**)**. Colonies of *A. niger* had black color with radiated edges with wooly texture (Plate 2: Fig K), while microscopically had extremely long, smooth and the strigmata were biseriate, minimized and radiate and the conidia were globes and smooth (Plate 2: Fig L). Colonies of *A. terrus* were velvety cinnamon buff to dark brown (Plate 2: Fig M), and microscopically, little hemispherical vesicle with long and smooth conidiophore (Plate 2: Fig N). Colonies of *A. fumigatus* have unmistakable edge with a few shades of green, surface has a powder appearance with a white overskirt was seen at the edge in the zone of dynamic development (Plate 2: Fig O), and microscopically portrayed by hyaline and particularly septated hyphae, conidiophores were long with club-molded vesicle, round conidia were conceived from single row of strigmata (Plate 2: Fig P**)**. These results agree with some authors (9, 37).

Colonies of *Mucor sp.* appeared fast-growing, white-to-gray cotton candy, became dark with time and fills the petri dish with fluffy mycelium and microscopically, non-septate broad hyphae, Sporangiophores are long, might be expanded and end with bear terminal round sporangia. The spores scattered and no rhizoids are formed. *Rhizopus sp.* colonies were deeply cottony; white turned to gray-brown on surface with aging. Microscopically, broad hyphae

could be observed, Sporangiophores are unbranched and connect to each another by septated hyphae, large sac-like sporangia that contain sporangiospores. These results agree with some authors (9, 37).

Morphological identification of yeast isolates

The isolates were cultivated on Rice agar media after culturing on SDA. In the current study, four genera were identified (Candida, Rhodotorula, Trichosporon and Cryptococcus). All genera reacted positively with urease test except Candida. *Rhodotorula sp*. was identified on SDA by formation of carotenoid pigments; that vary from orange to red (light pink flat colonies). Microscopically, revealed budding of round, oval large cells when stained with Gram's stain. On Rice agar media, showed large round blasto-conidia with absence of pseudohyphae. *Cryptococcus sp.* appeared rapidly on SDA as flat or slightly heaped shiny moist mucoid colonies with smooth edges. Its color changed from creamy at first to brown later. Microscopically, the colonies were ovoid, spherical with thick wall and mostly showed capsule with budding. On Rice agar media, no pseudo-hyphae but appeared as budding cells. *Trichosporon sp.* appeared on SDA firstly as smooth flat, or wrinkled white to creamy colonies that turned waxy with central folds surrounded by wrinkled furrows. Microscopically, appearance of hyaline mycelium which is separated and fragmented into rectangular arthrospores. On Rice agar media, *Trichosporon sp.* appeared as septated hyphae, pseudohyphae and arthrospores. *Candida species* colonies on SDA appeared creamy colored pasty colonies within 48-72hrs. On Rice agar media, *C. albicans* showed terminal chlamydospores, blastoconidia and pseudo-hyphae. Other *Candida sp*. fails to produce pseudohyphae. This result is in accordance with some authors (9, 37- 39).

Incidences of moulds and yeast among different seasons and different organs

As shown in table (1 & 2), mycological examination of 400 *O. niloticus* and 100 *C. gariepinus* revealed an incidence of several moulds including *Saprolegnia sp*. (53, 32) isolates, *Penicillium sp*. (152, 131), *Fusarium sp*. (78, 38) *A. flavus* (233, 149), *A. niger* (247, 223), *A. terreus* (35,22), *A. fumigatus* (29, 15) *Mucor* (148, 136) and *Rhizopus* (65, 42) in *O. niloticus* and *C. gariepinus*, respectively.

Saprolegnia sp. showed the highest prevalence in late autumn (10.68%, 6.96%) and winter (6.8%, 7.8%) in *O. niloticus* and *C. gariepinus*, respectively. This may be attributed to that seasonal variation play an important role in spreading of the Saprolegnia infection among freshwater fishes where the water temperature was low. These results agree with some authors (9, 24, 37) where they mentioned that saprolegniasis occurred during the winter season and colder months of the year. The highest incidence within organs was observed to be from the skin and fins (77.4%, 65.63%) followed by the gills (22.6%, 34.4%) in *O. niloticus* and *C. gariepinus*, respectively but, not isolated from liver and/or kidney. The results are in accordance with those of many authors (8, 9, 14, 30, 31) as shown in table (2)**.**

Pencillium sp. was isolated with the highest prevalence in winter (18.98%& 23.22%) from *O. niloticus, C. gariepinus* respectively. The highest incidence within organs was observed to be from the skin and fins (36.2%, 47.3%) followed by gills **(**30.9%, 20.6%) in *O. niloticus* and *C. gariepinus*, respectively. These results are similar to those reported by Ali, (37). Besides, *Penicillum sp.* could be isolated also from liver (22.4%, 13.7%) and kidney (10.5%, 18.3%). These results agree with some authors (9, 30). Different species of Pencillium were isolated with high incidence from apparently healthy fishes rather than diseased one, therefore members of this genus can be considered as saprophytes (9, 37).

Fusarium sp. was isolated with the highest prevalence in spring *(*11.8%, 5.91%) from O. *niloticus, C. gariepinus* respectively. The highest incidence within organs was observed to be in gills (37.18%, 34.2%) in *O. niloticus* and *C. gariepinus*, respectively. This might be attributed to the high affinity of fungal spores to high oxygen tension (37). It could be isolated from skin, fins, liver and kidney as well.

Aspergillus sp. showed some variation according to species. *A. flavus* was recorded all over the year and more prevalent during hot weather with high incidence during summer (25.44%, 23.22%) followed by spring (16.24%, 22.08%) in *O. niloticus, C. gariepinus.* The result is similar to some previous papers (9, 37, 40). *A. niger* was more prevalent during hot weather with high incidence during summer (26.9%, 37.44%) in *O. niloticus, C. gariepinus*, respectively. These results are similar to some previous papers (9, 37, 40). *A. terrus* was more prevalent during autumn (5.98%, 5.69%) in *O. niloticus* and *C. gariepinus*, respectively. These results are similar to some previous papers (9, 37, 40). *A. fumigatus* was more prevalent during autumn (7.69%, 8.23%) in *O. niloticus, C. gariepinus.* These results are similar to some previous papers (9, 37, 40). In the current study, the highest incidence within organs was observed to be in liver in most of *Asperigillus sp.* This may support the fact that Asperigillosis is a systemic disease.

Zygomycetes (Mucor and Rhizopus) are the most common fungi isolated from apparently healthy fish and diseased *O. niloticus* and *C. gariepinus* with high incidence during autumn season and these results agree with Ali, (37). *Mucor species* were isolated with the highest prevalence in autumn (20.09%) from *O. niloticus* and winter (29.21%) from *C. gariepinus*, respectively. The highest incidence within organs was observed to be from the skin and fins (45.3%, 46.3%) followed by gills (29.7%, 27.9%) in *O. niloticus* and *C. gariepinus*, respectively. The result is similar to those reported by Ali (37). It could be isolated also from liver **(**15.5%, 16.9%) and kidney (9.46%, 8.8%) in *O. niloticus* and *C. gariepinus*, respectively. These results agree with many authors (9, 37).

On the other side, Yeast isolates revealed 4 genera; Candida (62, 37), Rhodotorula (79, 52), Trichosporon (40, 19) and Cryptococcus (37, 21) isolated from *O. niloticus* and *C. gariepinus*, respectively. Yeast was also isolated with high frequency from diseased fishes rather than apparently healthy; these results came in agreement with those recorded (9). *Candida sp*. accounted for (28.44%, 36.27%) of the isolates and *Rhodotorula sp*. (36.24, 24.51%) from *O. niloticus* and *C. gariepinus*. The current results came in agreement with those recorded by Tartor *et al.*, (39). Samples collected from skin, gills, liver, and kidney revealed that *C. albicans* and *Rhodotorula sp.* were the highest yeast isolates. These findings were supported by the view reported by (9). *Cryptococcus sp.* in the present study was isolated with prevalence of (16.97%, 20.59%) from *O. niloticus* and *C. gariepinus*, respectively; nearly similar results were recorded by (38) but disagree with Tartor *et al.*, (39). *Trichosporon sp.* was detected to be (18.35%, 18.63%) from *O. niloticus* and *C. gariepinus*, respectively; nearly similar results were recorded by (24, 38) but disagree with Tartor *et al.*, (39).

Histopathological findings

Histopatholgical examination of naturally infected *O. niloticus* with *Saprolrgnia sp*. revealed severe degenerative changes in the skin. Necrosis of dermis and hypodermis, the underling dermis was edematous with degenerative changes of muscle fibers containing fragments from the fungal hyphae (Plate 3: Fig. A). Ulceration, loss of epidermis and loss of texture of scales and sometimes ulcer can be observed (Plate 3: Fig. B, C). Gills showed severe hyperplasia and hypertrophy of the epithelial lining of secondary lamellae with congestion of branchial blood vessels (Plate 3: Fig. D, E). Kidney revealed necrosis in some tubules together with peritubular fibrosis (Plate 3: Fig. F). These results agreed with many authors (11, 31, 34).

Table 1: Seasonal Prevalence of Mould and yeast in *O. niloticus* and *C. gariepinus*

	Oreochromis niloticus (100 fish/season)								*Clarias garipinus* (25 fish/season)							
	Winter		Spring		Summer		Autumn		Winter		Spring		Summer		Autumn	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Mould																
Saprolegnia	28	6.81	0	0	0	0	25	10.68	21	7.87	0	0	0	0	11	6.96
Pencillium	78	18.98	11	4.06	39	11.40	24	10.26	62	23.22	29	11.42	23	10.90	17	10.76
Fusrium	17	4.14	32	11.81	20	5.85	9	3.85	8	2.99	15	5.91	10	4.74	5	3.16
Asp.flavus	65	15.82	44	16.24	87	25.44	37	15.81	10	3.75	56	22.05	49	23.22	34	21.52
Asp.Niger	83	20.19	50	18.45	92	26.90	22	9.40	37	13.86	82	32.28	79	37.44	25	15.82
Asp.Fumigtus	0	0	0	0	11	3.22	18	7.69	0	0	0	0	9	4.27	13	8.23
Asp.terrus	7	1.70	10	3.69	4	1.17	14	5.98	0	0	6	2.36	0	0	9	5.67
Mucor sp	62	15.09	12	4.43	27	7.89	47	20.09	78	29.21	27	10.63	6	2.84	25	15.82
Rhizopus sp	19	4.62	6	2.21	27	7.89	13	5.56	17	6.37	5	1.97	11	5.21	9	5.67
Yeast																
Rhodotorulla sp	26	6.33	30	11.07	6	1.75	17	7.26	7	2.62	9	3.54	5	2.37	4	2.53
Candida sp	10	2.43	25	9.23	19	5.56	8	3.42	13	4.87	8	3.15	12	5.69	6	3.80
Trichosporon sp	7	1.70	20	7.38	10	2.92	0	0	3	1.12	9	3.54	7	3.32	0	0
Cryptococcus sp	9	2.19	31	11.44	0	0	0	0	11	4.12	8	3.15	0	0	0	0
Total	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
	411	32.67	271	21.54	342	27.19	234	18.60	267	30	254	28.54	211	23.71	158	17.75
Total No. of isolates	1258								890							

Table 2: Incidence of Mould and yeast in organs of *O. niloticus* and *C. gariepinus*

	Oreochromis niloticus (100 fish/season)									*Clarias garipinus* (25 fish/season)								
		Skin&Fins		Gills		Liver		Kidney			Skin&Fins		Gills		Liver		Kidney	
	No.	No.	%	No.	%	No.	%	No.	%	No.	No.	%	No.	%	No.	%	No.	%
Mould																		
Saprolegnia	53	41	77.4	12	22.6	0	0	0	0	32	21	65.63	11	34.37	0	0	0	0
Pencillium	152	55	36.2	47	30.9	34	22.4	16	10.5	131	62	47.4	27	20.6	18	13.7	24	18.3
Fusrium	78	21	26.92	29	37.18	11	14.1	17	21.8	38	10	26.3	13	34.2	9	23.7	6	15.8
Asp.flavus	233	83	35.6	47	20.20	78	33.5	25	10.7	149	33	22.1	62	41.6	30	20.1	24	16.1
Asp.Niger	247	92	37.2	46	18.6	81	32.8	28	11.3	223	95	42.6	72	32.3	33	14.8	23	10.3
Asp.Fumigtus	29	10	34.48	12	41.40	3	10.34	4	13.8	15	10	66.7	5	33.3	0	0	0	0
Asp.terrus	35	13	37.1	7	20	10	28.6	5	14.3	22	9	40.9	5	22.7	7	31.8	1	4.5
Mucor sp	148	67	45.27	44	29.73	23	15.54	14	9.46	136	63	46.3	38	27.9	23	16.9	12	8.8
Rhizopus sp	65	42	64.6	23	35.4	0	0	0	0	42	30	71.4	12	28.6	0	0	0	0
Yeast																		
Rhodotorulla sp	79	25	31.6	40	50.6	4	5.1	10	12.7	25	7	28	2	8	12	48	4	16
Candida sp	62	18	29	20	32.3	16	25.8	8	12.9	37	7	18.9	4	10.8	17	45.9	9	24.3
Trichosporon sp	40	18	45	12	30	10	25	0	0	19	2	10.53	7	36.84	10	52.63	0	0
Cryptococcus sp	37	0	0	25	67.57	12	32.43	0	0	21	0	0	13	61.9	6	28.6	2	9.5
Total		No.	%	No.	%	No.	%	No.	%		No.	%	No.	%	No.	%	No.	%
		485	38.55	364	28.93	282	22.42	127	10.01		349	39.21	271	30.45	165	18.54	105	11.79
Total No. of isolates	1258									890								

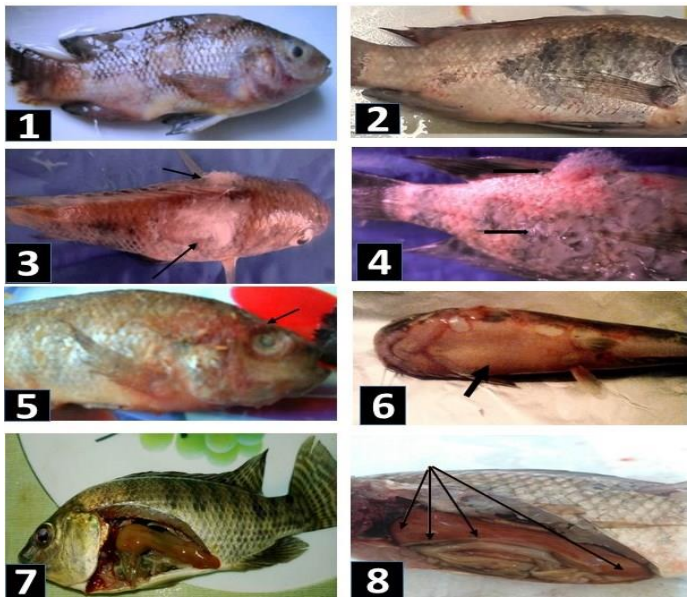

Plate 1: (1, 2) *O. species* showing skin darkening, scale detachment and erosion of membranous part of fins. **(3, 4)** *O. species* showing cotton wool-like growth on various parts of the body ,ulceration of skin. **(5)** *O.* species showing cloudy and opaque eye. **(6)** *C. gariepinus showing skin ulceration,* scattered hemorrhagic patches on the ventral abdomen and mouth. **(7)** Naturally diseased *O.niloticus* showing moderate petechial hemorrhage, dark liver enlargement **(8)** *A naturally examined O. niloticus showing threads of congestion along the surface of live*

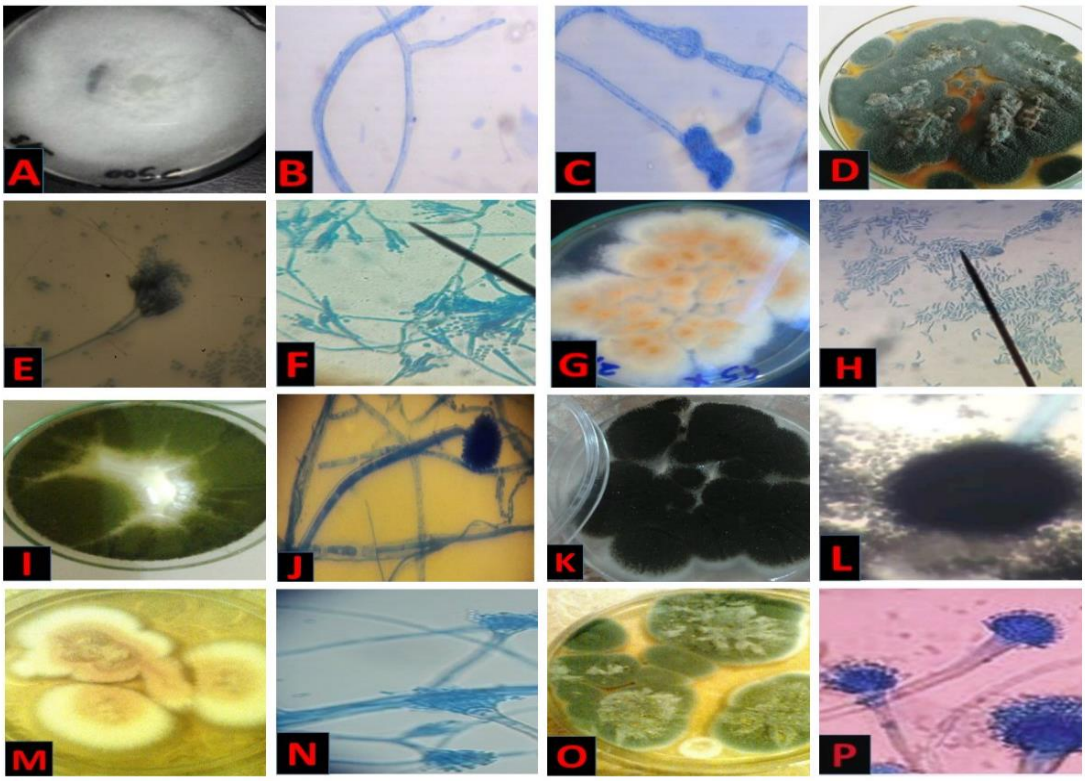

Plate 2: (A) *Saprolegnia species* with the characteristic cotton- wool like growth colony on SDA, **(B, C)** branched aseptic hyphe, **(D, E)** *Penicillium sp.* on SDA with different colour and texture, **(E, F)** *Penicillium sp.* showing brush- like arrangement, **(G)** a colony of *Fusarium sp.* on SDA with rose pigments on the center, **(H)** *Fusarium under light microscope* **(I)** *A. flavus* on SDA, **(J)** *A. flavous* showing characteristic typical head, **(K)** A colony of *A. niger* on SDA, **(L)** *Aspergillus niger* showing characteristic round head with black conidia, **(M)**, Colonies of *Aspergillus terreus* on SDA, **(N)** *A. terreus* with small hemispherical vesicle, **(O)** A colony of *A. fumigatus* on SDA, **(P)** *A. fumigatus* with columnar head

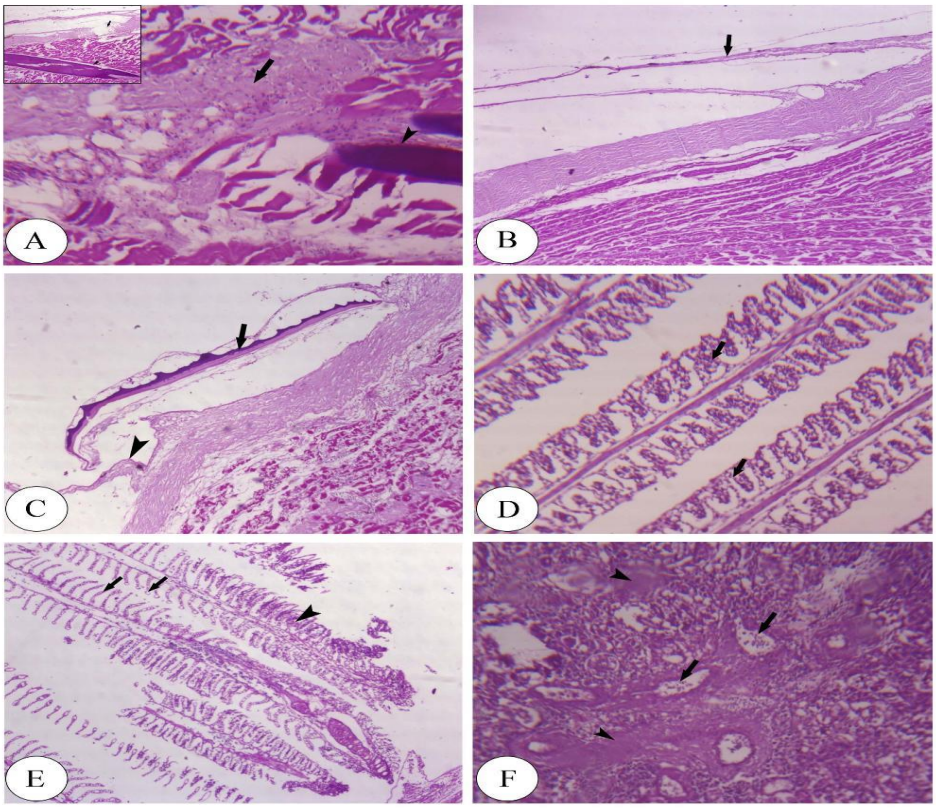

Plate 3: Photomicrograph of skin, gills and kidney of *Oreochromis niloticus* infected with *Saprolegnia sp*. **(A)** Necrosis of dermis, hypodermis, edema and degenerative changes of muscle fibers (Arrow) with presence of hyphae of saprolegnia (Arrow head) x 400. **(B)** Ulceration & loss of epidermis and loss of texture of scales (Arrow). **(C)** Normal scale (Arrow) and Ulcer in neighboring (Arrow head). **(D)** Gills showing sever Hyperplasia & Hypertrophy of the epithelial lining of secondary lamellae with fusion in neighboring (Arrow). **(E)** Infection of saprolegnia at the tip of primary lamellae with congestion of blood vessles and hyperplasia& hypertrophy (Arrow head) while other part filament is normal (Arrow). **(F)** Infected kidney with peritubular fibrosis(Arrow) and necrosis in some tubule (Arrow head).

Conclusion

From the present study, it could be concluded that Saprolegnia was the predominant among diseased fishes with highest prevalence in late autumn (10.68%, 6.96%) and winter (6.8%, 7.8%) in *O. niloticus* and *C. gariepinus*, respectively. However, *Pencillium sp.* and *Aspergillus sp*. were the most predominant fungi isolated from apparently healthy fishes. The highest prevalence of *Pencillium sp.* were recorded in winter, whileas *Aspergillus* showed variations between species*; A. flavus, A. niger* were more prevalent in summer and *A. terrus, A. fumigatus* were more prevalent in autumn in *O. niloticus* and *C. gariepinus,* respectively. Moreover, the highest prevalence of *Fusarium species* were recorded in spring from *O. niloticus* and *C. gariepinus*, respectively and Zygomycetes (Mucor and Rhizopus) recorded the highest prevalence in autumn in *O. niloticus* and winter in *C. gariepinus*. Four genera from yeast were isolated; *Candida sp., Rhodotorula sp., Cryptococcus sp.* and *Trichosporon Sp.* The histopathological findings revealed severe degenerative changes in skin and gills with presence of fungal hyphae and spores.

Conflict of interest

The authors declare that no conflict of interest.

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Original Research Article

QUALITY CHARACTERISTICS OF BEEF HAMBURGER ENRICHED WITH RED GRAPE POMACE POWDER DURING FREEZING STORAGE

Omima S. Abdelhakam, Essam M. Elsebaie*, Adel K. Ghazi, Mahmoud S. Gouda

Department of Food Technology, Faculty of Agricultural, Kafrelsheikh University, 33516, Kafrelsheikh, Egypt

*Corresponding author, E-mail: Essam.Ahmed@agr.kfs.edu.eg

Abstract: Integrating the consumption of agro-industrial residues with satisfactory consumers is an opposition that, may affect the financial dividend of the frozen meat manufacturing, aside from producing products with diversified nutritional values and satisfying technical characteristics. Thence, we aimed in this research to elaborate and appraise the physicochemical and microbiological characteristics of beef hamburger with the addendum 2 and 4 % of red grape pomace powder (RGPP) throughout its stockpiling at (-18 ± 2°C) over a period 12 weeks. A significant influence on pH value was observed at the beginning (zero time), however the control sample, 2% and 4% had pH values of 6.68, 6.41 and 6.22, respectively. Meantime, there was a significant influence of (RGPP) on thiobarbituric acid (TBA) evidenced on 4th week. The results showed that the microbial growth increased as a function to storage period for all and the addendum of RGPP in beef burger caused a reduction in total bacterial count in comparison with control. Thus, in general, it can be concluded that addendum of RGPP is an efficacious mean to improve burger quality and increasing its storage stability.

Key words: beef burger; red grape pomace; storage stability

Introduction

Meat products can be suffered from numerous negative changes throughout frozen and refrigerated storage as a result for the growth of microorganisms and oxidation of lipids, which result in quality shortage, meat deterioration, and economical damage (1). The growth of microorganisms in meat and its other products may be lead to slime forming, degrading structural constituents, reduction in water holding capability, bad odor, and alteration in appearance and texture which decrease nutritive value, quality, and shelf- life (2). Lipid oxidation is contingent on the composition of fatty acids, the concentration of vitamin E, and on oxidation-enhancing substances such as free iron in the muscles. Oxidation products can lead to lose color and nutritional value as a result to the breakdown of lipids, proteins, pigments, vitamins and carbohydrates (3). The oxidative rancidity may be cause a lot of alterations in meat and their products ranged from large changes in flavor, color loss and structural breakdown for proteins (freshness losing) that impede consumer's repeated purchase (4). Artificial anti-oxidative substances such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were utilized to hold up oxidation of li-

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pids in meat (5). Nonetheless, contemporary attention over its utilization has generated a necessity and induced research for alternate antioxidants, predominately from naturalistic origin. The usage of naturalistic preservatives with meat products increases the storage shelf life is a hopeful technology as numerous vegetative materials posse antimicrobial and antioxidant characteristics (6). Functional substances addendum into meat products might upgrade the health and nutritional attributes and extension their storage shelf-life (4).

Vegetation extracts which are affluent with polyphenolic components are perfect candidates, because of its extraction easiness from the naturalistic origins and their efficacy in the prevention of lipid oxidizing in food (6).

Grape seeds extract anti-oxidative efficacy has been assessed in a little number of meat sorts and was found to enhance the oxidative resistance in turkey patties (7), cooked beef (8) and nuggets (9).

Dietary fibers are considered to be a functional component of meat products. Incorporating fiber can get the texture, enhancing cooking performance because of its water binding and fat retention characteristic and dietary fibers can also produce technical characteristics that enhance physical, chemical and sensory attributes. Naturalistic antioxidants and dietary fibers are considered as dietetic agents that participate in enhancing human health. Natural products with high levels of phenolic antioxidants and dietary fibers are named antioxidant dietary fibers (ADF) (10).

The current investigation was intended to investigate the influence of red grape pomace powder (RGPP) integration in beef hamburgers on its physical, chemical, microbiological and sensory properties during frozen storage.

Materials and methods

Red grapes, variety Red Globe1, by products were obtained in August 2016 from Faragello factory for juice, Sixth of October City, Egypt. Red muscles beef, kidney fats and other components used for burger preparation were obtained from local market at Kafr El-Sheikh city, Egypt. The beef meat and fat tissue were transported to the food technology laboratory, faculty of agriculture, Kafrelshiekh University using an icebox.

All chemicals used in this study of HPLC grade (99.9% purity) were obtained from Sigma Company of chemicals and drugs, St. Louis, MO, USA.

Preparation of samples

The pomace (skin and seeds) was sorted by hand for removing stems and debris, and thereafter lyophilized, and milled up to pass through 20 mesh screen sieve. The yielded powder samples were mixed, and stored in polyethylene bags, then kept at room temperature until use (11) .

Burger preparation

Beef burger meat and kidney fat was grounded separately in a meat grinder by passing meat through a plate having 6 mm holes. The beef burger was formulated to contain the following ingredients 80% red muscles beef meat, 20% kidney fat, 18% (w/w) water (ice), 1.5% (w/w) salt, ground black pepper (0.3%), red pepper (0.2%) and cumin (0.2%) according to Aleson-Carbonell, et al.(12).The minced beef was treated with two concentrations of RGPP (2 and 4%). Control without RGPP was used in all assays. The beef burgers were frozen at -18 ºC until the start of analysis.

Thermal treatment

After the thawing at 4 °C for 12 hours, the beef burgers were stewed using an electrical grill (Genwex GW-066) at 220°C (the space between heat source and the samples was 4 cm) for 8 min (4 min for each side of beef burgers) (2)

Chemical analyses

Gross chemical composition of red grape pomace powder was analyzed according to A.O.A.C. (13). Where, total carbohydrates were calculated by difference. The amount of total polyphenols was estimated as mg Gallic Acid Equivalent/100gm using the method outlined by González-Centeno et al (11).

Identification of phenolic acids

For HPLC analysis, one gram of the red grape pomace powder was mixed with acidified methanol (10 ml)for 4 min and centrifuged at 10000 rpm for10 min and the supernatant was filtered through a 0.2 um Millipore membrane. The filtrates were collected in a vial for injection into HPLC Hewllet packared (series 1050) equipped with auto-sampling injector, solvents (methanol and acetonitril) were degassed and ultraviolet (UV) detector was set at 280 nm and quarter HP pump. The column temperature was maintained at 35◦C, where gradient separation was performed using the mobile phase (methanol and acetonitril) at flow rate of 1 ml/min following the method of Goupy, et al. (14).

Physical analyses of burger

The pH value of beef hamburgers was determined by using a pH meter (Jenwae model 3357, USA) according to the method described by (15)**.** Water holding capacity (WHC) was determined by filter press method as described by (15). Beef burgers were weighted before and after cooking to determine cook loss according to the method of [15] by using the following equations:

Cooking loss (%) = [(Raw weight - Cooking weight)/ Raw weight]*100

Chemical analyses of burger

Thiobarbituric acid reactive substances (TBARS) of beef hamburger samples were estimated as reported by (16).

Microbiological Analyses

Total count of aerobic microorganisms and fungi and yeasts were carried out in triplicate and using the methodologies mentioned by (17).

Statistical analysis

General linear model of SPSS (Ver. 16.0, 2007) was used to conduct ANOVA for determination of differences between means. The probability levels o f $P \leq 01$and $P \leq 0$ .05 were considered to be significant for statistical procedures. All measurements and trials were done in triplicate.

Results and discussion

Chemical composition of red grape pomace

There are many factors affecting the chemical composition of red grape pomace such as variation in organic compounds of the soil, varieties, fertilizers applied, climatic and environmental conditions (18)**.**

The data in Table (1) indicate that pomace is considered a good source of protein, crude fiber and total carbohydrates which are important from the nutritional point of view. The results revealed that the moisture, crude protein, ether extract, ash, crude fiber and total carbohydrate contents were 5.1, 12.5, 11.5, 1.8, 9.2 and 74.2%, respectively. These results were in harmony with those of (19)**.**

Identification of phenolic acids content of ethanolic red grape pomace powder extract

HPLC procedure was utilized for fractionation and identification of the phenolic components extricated from the tested sample. The results were tabulated in Table (2).

The results revealed that, red grape pomace ethanolic extract have 24 phenolic components. Pyrogallol was the highest compound of polyphenolic compounds found in ethanolic pomace extract (38.38%) followed by Catechol (8.72%) then Epicatechin (7.87%), where Cinnamic (0.09%) and Resveratrol (0.15 %) were the lowest ones. These results are in the same line with others obtained by (20).

Physical analyses of burger

Meat products physical characteristics such as pH and WHC are highly remarkable because of its influence on the quality attributes like cooking loss, juiciness and tenderness.

pH values

The results in Table (3) revealed that there was an increment in beef hamburger pH values as a function of the storage time which on the same trend with (21) who stated that the inconsiderable increment in pH values throughout the storage period might be back to protein liberation metabolites as a consequence for bacterial efficiency. The results also appeared that

the addendum of RGPP was performed to reduce beef hamburger pH values. Also, there was a decrement in pH values as a function for increasing the addendum levels of RGPP as compared to control treatment which has the highest pH values along storage period whereas beef hamburger containing 4 % RGPP treatment has the lowest pH values. This pH decrement might be lead to the low pH values of RGPP added (22). At the end of storage time, the pH value of the control sample was 6.93 which significantly different from the pH value of beef hamburger enriched with 4 % RGPP (6.51).

Water holding capacity (WHC)

The results given in Table 3 appeared that WHC (cm^2) of beef hamburger has been reduced along the storage period. This decrement may be back to proteins denaturation which causing a reduction in its ability to hold water leading to lower WHC. The addendum of RGPP into beef hamburger negatively influence on WHC which was reduced with increasing the addendum level of RGPP, Beef hamburger enriched with 4 % RGPP has achieved the lowest WHC values (the highest area of squeezed water) during storage period whereas control treatments were recorded the highest values which appear that RGPP had lower water holding capacity.

Table 1: Proximate chemical composition of red grape pomace (on dry weight basis)

Component	%
Moisture	5.1
Crude protein (N x 6.25)	12.5
Ether extract	11.5
Ash	1.8
Crude fiber	9.2
*Total carbohydrates	74.2
Total polyphenols (mg GAE/g)	4.92

*Total carbohydrates were calculated by difference.

Table 2: Phenolic compounds in ethanolic extract of red grape pomace powder

Compounds	% of the Total phenolics	Compounds	% of the Total phenolics
Gallic acid	3.64	Ferulic	0.62
4-Amino benzoic acid	0.20	Isoferulic	1.76
Protocatchuic	2.72	Resveratrol	0.15
Catechin	6.77	Ellagic	4.29
Chlorogenic	4.00	Alpha-Coumaric	1.18
Catechol	8.72	e-Vanillic	4.70
Epicatechin	7.87	Benzoic	7.58
Caffeine	0.68	3,4,5 Methoxycinnamic	0.38
Caffeic	1.14	Coumarin	0.31
Vanillic	1.96	Salicylic	1.56
β-coumaric acid	0.34	Cinnamic	0.09
Pyrogallol	38.38	P-OH-benzoic	0.92

Table 3: Changes in some physical characteristics of beef hamburger enriched with red grapes pomace powder throughout frozen storage

Storage period (months)	Beef hamburger treatments		
	Control	2% red grapes pomace	4% red grapes pomace
PH value			
0	6.68[a]	6.41[b]	6.22[c]
1	6.74 [a]	6.49 [b]	6.30 [c]
2	6.85 [a]	6.61 [b]	6.37 [c]
3	6.93 [a]	6.75 [b]	6.51 [c]
Water holding capacity (cm^2)			
0	3.80 [c]	3.96 [b]	4.10 [a]
1	3.86 [c]	4.10 [b]	4.21[a]
2	3.98 [c]	4.16 [b]	4.29[a]
3	4.20 [c]	4.33 [b]	4.42 [a]
Cooking loss (%)			
0	3.17 [a]	1.27 [b]	0.63 [c]
1	6.34 [a]	3.92 [b]	2.93 [c]
2	7.62 [a]	4.55 [b]	3.13 [c]
3	7.93 [a]	5.98 [b]	4.87 [c]

Values followed by the same letter in the same row are not significantly different at $P \leq 0.05$

Table 4: Changes in TBA values (mg malonaldehyde / kg) of beef hamburger enriched with red grapes pomace powder during frozen storage

Beef burger type treatments	Storage period per months			
	0	1	2	3
Control	0.367[a]	0.595[a]	0.709[a]	0.932[a]
2% red grapes pomace	0.352[b]	0.470[b]	0.501[b]	0.600[b]
4% red grapes pomace	0.338[c]	0.396[c]	0.412[c]	0.563[c]

Table 5: Changes in microbiological properties of beef hamburger incorporated with red grapes pomace powder during frozen storage

Storage period (months)	control	2% red grapes pomace	4% red grapes pomace
Plate count bacteria (log cfu/g)			
0	3.21	3.21	3.21
1	3.89	3.00	2.93
2	4.96	2.88	2.68
3	6.42	2.61	2.44
Yeast and molds count (log cfu/g)			
0	2.20	2.20	2.20
1	3.40	2.18	1.90
2	3.62	1.93	1.80
3	4.70	1.70	1.65

Cooking loss

According to data in Table (3), cooking loss percentage of beef hamburger is increased as a function of the storage period which on the line with the water holding capacity results. Also, the cooking loss of beef hamburger enriched with RGPP is decreased with increasing the addendum levels since beef hamburger enriched with RGPP had cooking loss values lower than control treatment along the storage period. Burger containing 4 % RGPP had the lowest cooking loss values at any storage period while, control treatment have the highest cooking loss value. This might be back to the fiber components of RGPP, which could influence on the cooking loss of the beef hamburger, since fibers could reduce the water loss during cooking by forming gels as reported by (22).

Changes in chemical properties of beef hamburger during frozen storage

Thiobarbituric acid (TBA) value

Lipid oxidation is one of the main limiting factors for the quality and acceptability of meat and meat products. Thiobarbituric acid (TBA) value (malonaldehyde / kg) is used as an index for measuring oxidative rancidity which takes place in meat products during storage.

Table (4) shows the effect of incorporation of RGPP on the oxidative stability of beef hamburger during frozen storage. The data show that the TBA value is increased as the storage time increased which in agree with (21). The TB A values of beef hamburger were decreased with increasing RGPP incorporation level. There are significant differences between control and the other red grapes pomace powder containing treatments exhibited lower TBA values than control treatments. These due to that polyphenols have been re-ported to have antioxidant activity in vitro (23).

At the end of frozen storage period TBA values of control was higher than the spoilage limit of Egyptian Standard Specifications (ESS) (24) (0.9 mg malonaldehyde / kg) since it recorded 0.932 mg malonaldehyde / kg. On the other hand beef hamburger treatments contain-ing 2 and 4 % RGPP were reached to 62 -66 % of the ESS spoilage limit. These results may be due to the polyphenol content of red grapes pomace powder which play important role in reducing the oxidative reaction of lipids which reflects in low TBA values. The data also show that at any storage time the lowest TBA value was observed for beef hamburger containing 4% RGPP.

Changes in microbiological load of beef hamburger incorporated with red grapes pomace powder during frozen storage

Table (5) shows the effect of incorporation of red grapes pomace powder (RGPP) on the microbial growth of beef hamburger during frozen storage. The results show that as the storage time increase the microbial growth increase for all treatments. The incorporation of red grapes pomace powder in beef hamburger results in lowering the total bacterial count as compared to control, since it was gradual decreasing as the RGPP incorporation level increased. This may be due to the higher phenolic compounds content in beef hamburger incorporated with RGPP. The results show that there are significant differences between all treatments. At any storage time the lowest total bacterial count was observed for beef burger incorporated with 4 % RGPP while the highest bacterial co unt was observed for control treatment which reached the spoilage limit of ESS (24) (6 log cfu/g) for total bacterial count at 90 d ays of frozen storage whereas treatments incorporated with RGPP reached to 30 – 35 % o f this limit. Also, the molds and yeasts counts were decreased as the incorporation level increased, the lowest counts were observed for treatment incorpor ated with 4 % RGPP at all sto rage times. At the end of storage period there were significant differences between control and treatments incorporated with RGPP.

Conclusion

The results of this study demonstrated that red grape pomace ethanolic extract contains 24 phenolic components. These compounds helped to improve pomace powder extract as antioxidant agent that can be used to prepare functional foods which provide the physical, chemical and microbiological properties of beef hamburger.

Conflict of interest

Authors declare that no conflict of interest.

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Original Research Article

LAYING PERFORMANCE, FAT DIGESTIBILITY AND LIVER CONDITION OF LAYING HENS SUPPLEMENTED WITH VITAMIN B_{12} OR BIOTIN AND/ OR BILE ACIDS IN DIET

Mohamed I. El-Katcha, Mosaad A. Soltan, Karima El-Naggar*, Set A. El-Shobokshy, Mohamed A. El-Erian

Department of Nutrition and Veterinary Clinical Nutrition, Faculty of Veterinary Medicine, Alexandria University, 22758, Egypt

*Corresponding author: karima.muhammad@alexu.edu.eg; ORCID ID: 0000-0002-7847-7613

Abstract: For 8 weeks feeding trial, two hundred and seventy, 53 weeks old laying hens were used to investigate the effects of dietary supplementation of vitamin B_{12} or biotin and/or bile acids on performance, egg quality, fat digestibility and liver composition and histopathology. Birds were randomly divided into 6 groups (three replicates) and fed the experimental diets; group1 (G1) fed on the basal diet without additives as control while G2 and G3 supplemented with 0.02 ppm vitamin B_{12} and 0.3 mg biotin/kg diet respectively, groups 4-6 fed as the previous detailed design of G1- G3 with the addition of 400 g of dried bile acid (DBA) /tone feed. Biotin supplementation significantly (P<0.05) increased body weight losses of laying hens, both vitamins significantly (P<0.05) decreased daily feed intake (FI) and improved FCR. DBA addition alone or with biotin reduced these weight losses along with significant (P<0.05) increase in daily FI. Vitamin B_{12} supplementation alone or with DBA increased average egg production (P<0.05) while was reduced with biotin supplementation. Fat digestibility was non-significantly improved (P≥0.05) with both vitamins supplementation without or with DBA. Biotin significantly (P<0.05) reduced the average yolk relative weight, which was increased when mixed with DBA, while significantly increased average egg albumin relative weight. Vitamin B_{12} or biotin addition without or with DBA non-significantly increased blood serum GOT and GPT activities, non-significantly reduced (P≥0.05) fat content of liver tissue (on dry matter or fresh basis) and serum lipid profile parameters except serum HDL concentration, was increased, with no histopathological changes in hepatic tissue. It could be summarized that vitamin B_{12} supplementation without or with DBA is recommended in layer diet as it gave the best performance, reduced serum lipid profile and improved fat digestibility and the hepatic health.

Key words: layers; egg quality; liver; vitamin B12; biotin; bile acids

Introduction

Recently, laying hens facing many challenges influencing egg production industry resulting in significant economic losses. Such

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challenges as environmental changes, imbalanced or poor-quality feed, frequent diseases affect the physiological condition of layers and bring out pathological burden to hen body especially to sensitive and important organs such as liver. Therefore, liver is in continuous overloaded condition, which disturbs its function. One of the common disorders influences liver in layers is the fatty liver hemorrhagic syndrome (FLHS), negatively affecting their performance. FLHS is a metabolic or nutritional disorder of layers characterized by excessive accumulation of fat depots in the hepatic tissue and abdominal cavity as liver become enlarged, pale and ends up with rupture and hemorrhage causing death of the affected birds (1).This syndrome occurs when the lipogenesis process exceeds the capacity of lipoprotein synthesis and secretion (2). The actual cause of FLHS has not been fully defined, but could be a combination of nutritional, genetic, environmental, and hormonal factors (3). Different studies have been done to follow up the actual factors inducing this problem in laying hens with the development of some managemental and nutritional approaches to reduce its occurrence. One of these strategies is dietary supplementation of some feed additives such as some lipotropic factors and antioxidants, which could control the lipid metabolism, reduce the production of free radicals and protect the liver from damage.

Methionine and choline as lipotropic factors are used to treat or prevent this syndrome as their inadequate dietary levels increase oleic acid absorption and decreased triacylglycerol secretion (4, 5). Other nutrients deficiency such as vitamin B_{12} and vitamin E increased hepatic triacylglycerol accumulation in laying hens (6, 7), increasing the incidence of occurrence. Earlier studies reported that biotin deficiency is an important dietary factor involved in inducing fatty liver in birds (8, 9).

Recently, the poultry production sector is continuously searching for new feed additives to improve feed efficiency and physiological condition of layers for optimal performance and egg production. Bile acids are natural components synthesized in human and animal body from cholesterol, which have a critical role in dietary fat digestion and absorption through the intestinal wall (10). Previous studies have been done investigating impacts of B-vitamins supplementation and emulsifiers as bile acids on broilers health and productivity (11-14), however, little literature is available regarding their effect on laying hens productive performance and egg quality parameters.

We hypothesized that dietary inclusion of these additives could help in the digestion of fat, maintain the healthy condition of the liver of laying hens. Therefore, the main objectives of the present study to investigate the effects of vitamin B_{12} or biotin supplementation without or with dried bile acids on productive performance, egg quality, fat digestibility, serum biochemistry and liver histopathology of laying hens.

Material and methods

Birds, experimental design and feeding program

Management procedures for all birds used during the course of experiment followed the guidelines approved by the Animal Care and Use Committee, Faculty of Veterinary Medicine, Alexandria University. A total of two hundred and seventy, 53 weeks old of *Isa brown* laying hens purchased from local company and used in this experiment. Hens were weighed separately, then randomly divided into 6 separate groups (45 bird /group) with three replicates each (15 bird/ replicate). Birds were kept in wire cages, and each cage was provided with feeder and water troughs. The control group (Group 1) was fed the basal diet (BD) without any additives, group 2 and 3 were fed the BD supplemented with 0.02 ppm vitamin B_{12} and 0.3 mg biotin/kg diet respectively, while group 4-6 were fed as the previous detailed design of the first three groups but, with the addition of 400 g bile acid/tone diet. BD were formulated to meet bird nutrient requirements according to NRC (15). BD composition and its chemical analysis is presented in table 1, which was analyzed according to AOAC (16). Additives used in this experiment included; vitamin B_{12} and biotin produced by Allgäu Vet. Co., China; the dried bile acids (DBA) (Shangdong Longchang

Animal Health Product Co., Ltd, Jinan, China). All the experimental groups were fed the designated diets with free access to fresh water for 8 weeks experimental period. Hens were weighed individually at the beginning and end of experiment and the live body weight (BW) changes were taken with recording of feed intake (FI). Feed conversion ratio (FCR**)** was calculated based on feed consumed (g) and produced egg mass (g).

Egg production and Egg quality

Eggs produced from various laying hens were collected daily for calculating the average daily egg production % (EP) and egg weight (g) for each group for 14 days period for four successive periods. Egg mass was calculated by multiplying egg weight by egg production. At the end of the experiment, 20 eggs from each group were collected to estimate the egg quality parameters (yolk weight and relative weight to total egg weight, yolk index, albumin weight, and its relative weight, albumen index, shell thickness and weight as well as its relative weight were recorded) according to Card and Nesheim (17). Six samples of egg yolk from each group were collected for egg yolk cholesterol determination according to Rotenberg and Christensen (18).

Fat digestibility

During the last week of the feeding trail, the excreta were quantitatively collected for 5 successive days in addition to recording the amount of feed consumed. The excreta then dried in hot air oven, ground and stored until chemical analysis of fat. Ether extract of feed, fecal and liver samples was determined according to AOAC (16)**.**

Serum biochemical parameters

Blood samples were collected (n= 6/group), then left to coagulate at room temperature. Separation of serum was done by centrifugation at 3000 rpm for 10 minutes. The clear serum samples were kept in freezer (-20̊ C) until analysis of the following blood serum indices; total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride (TG), glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT). All these parameters were determined by spectrophotometer using specific commercial kits produced by Biodiagnostic, Co. (Diagnostic and Research reagents).

Liver Histopathology

At the end of the experiment (61 week of hen age), three hens from each group (one hen from each replicate) were collected randomly and used for liver tissue collection. Samples were fixed in 10% formalin for at least 2 days then slides were prepared according to Bancroft et al. (19), and stained with Hematoxylin and Eosin (H&E) for general inspection.

Statistical analysis

Results were analyzed using analysis of variance (one-way ANOVA) using IBM SPSS Statistics 22 statistical package (SPSS Inc., Chicago, IL, USA) to measure the significant differences between the means of different variables. Differences among experimental groups were considered significant at (P<0.05).

Results and discussion

Layer Performance

Biotin supplementation significantly (P< 0.05) increased BW losses of laying hens. Both vitamins significantly (P<0.05) decreased average daily FI, but, improved FCR compared with control. On the other hand, DBA addition alone or with biotin reduced these losses in BW along with significant (P<0.05) increase in daily FI compared with laying hens without DBA addition (table 2). Moreover, FCR was significantly improved with DBA inclusion with vitamin B_{12} and biotin compared with birds fed the same diet without DBA or control group.

Unlike the obtained results, earlier studies examined the effects of vitamin B_{12} (20, 21), concluded that it had no clear effect on FI of commercial laying hens, while increased FI with Halle and Ebrahem (22). Improvement of FCR with vitamin B_{12} supplementation in the present study is in line with Halle and Ebrahem (22), while inconsistent with Kato et al. (20). Additionally, Abdel-Mageed and Shabaan (23) documented another different result as similar

FI was obtained in laying hens groups fed biotin-supplemented diets while, improved FCR with increasing its level from 162.5 to 325.5 µg/kg. Additionally, Whitehead (24), found that biotin supplementation during lay did not have any beneficial effect upon FI and FCR of laying hens.

Bile acids are the principal constituents of bile, playing a significant role in emulsification, digestion, and absorption of fat and lipid-soluble vitamins (25). Best FCR was obtained with birds fed BD contained DBA plus vitamin B_{12} (2.08), while the worst value with birds fed BD supplemented with DBA (2.49). Previous reports documented improvements of broiler performance (weight gain, higher FI and FCR) with the dietary inclusion of bile acids (13, 26-28), improved FCR in quail laying hens (29). The variation in response may be related to different breeds considered in these trials.

Regarding EP (table 2), vitamin B_{12} supplementation alone or with DBA increased average EP ($P<0.05$) with the highest EP (86.34%) in birds supplemented with the 2 additives suggesting that they could have a synergistic effect. On the other hand, biotin supplementation showed dissimilar result as it reduced EP (72.71%), even when added with DBA (78.22%), compared with control birds. Unlike the obtained result, Kato et al. (20) found that vitamin B_{12} supplementation had no effect on EP, attributing it to short experimental period. Additionally, Bunchasak and Kachana (30) reported that supplemental vitamin B_{12} didn't alter egg production or FI. However, Daryabari et al. (31) reported that 0.3g biotin/L water supplementation improved EP of breeding hens, as well as Abdel-Mageed and Shabaan (23) who found that biotin inclusion in diet enhanced it. Biotin concentration in the blood has been reported to be lower in young broiler breeder hens and increases linearly with age (32), therefore, the lower or unaffected EP in the biotin supplemented groups in the present study could be attributed to the higher level of biotin in birds. Response due to bile acids addition is inconsistent with the finding obtained with (29) as 1.2% supplemental ox bile addition in quail laying hens diet significantly improved EP %.

Both vitamins supplementation non-significantly ($P\geq0.05$) reduced average egg weight (table 2). The same response was obtained when diet was supplemented with DBA alone or combined with vitamin B_{12} while, when DBA mixed with biotin showed slightly higher egg weight compared with birds fed on the same diet without DBA. On contrary, Kato et al. (20) found increased egg weight of laying hens with vitamin B_{12} supplementation.

Fat digestibility

Fat digestibility showed non-significant improvement ($P\geq0.05$) with vitamin B_{12} or biotin compared with control (table 2). The same result was obtained when DBA was supplemented alone or with both vitamins. Fat digestibility improvement especially in laying hens supplemented with DBA being the highest was supported by Alzawqari et al. (28), who found that supplemental increased levels of bile in diet linearly increased fat digestibility in broiler chicks. This improvement with the addition of DBA might be attributed to inadequate amount of bile salts secreted by bird, so more emulsification of fats occur by the exogenous bile acids or for replacement of the active catabolism of bile salts occur by gut microflora (26). Moreover, Lai et al. (12) indicated that supplementation with 60 and 80 mg/kg bile acids significantly increased the activity of intestinal lipase and lipoprotein lipase on day 21 and 42. The present study revealed a close relationship between dietary DBA supplementation and fat digestibility. Most of the previous studies reported, were concerned with investigation of the effects of these exogenous emulsifiers in broiler diet especially at younger age of chicks due to the physiological limitation of the digestive tract to produce endogenous emulsifiers. However, literature on their application in layers diets is scarce.

Egg quality (External and internal quality)

Average egg shell relative weight was non-significantly increased ($P\geq0.05$) with vitamin B_{12} or biotin compared with the reference group (table 3). DBA addition single or combined with vitamin B_{12} increased ($P\geq0.05$) egg shell

relative weight, while, was reduced ($P \geq 0.05$) when biotin was added to diet. Regarding egg shell thickness, no changes were found among different treatments. In support, Keshavarz (33) concluded that vitamin B_{12} supplementation without or with methionine improved egg shell quality, Rajalekshmy (34) reported that shell weight was decreased at higher levels of folic acid (4 ppm) when vitamin B_{12} was added (0.01 ppm). However, Kato et al. (20) found another different result as egg shell thickness and weight per unit of surface area were higher ($p<0.01$) for birds fed diet without vitamin B_{12} supplementation.

In terms of internal egg quality (table 3), biotin supplementation significantly reduced ($P<0.05$) average egg yolk relative weight, but this effect was reversed when DBA was added with biotin. On the other hand, average egg albumin relative weight was significantly increased with biotin supplementation, though the lowest relative weight was recorded with birds received diet supplemented with DBA only. Regarding average yolk index, it was significantly reduced in birds supplemented with biotin and DBA, with no significant difference between the other experimental groups. The present data are in line with El-Husseiny et al. (35), who found that dietary supplementation with 0.01-0.02 ppm vitamin B_{12} did not have any significant effect on egg shell thickness, shell percentage, Haugh units and yolk index. Moreover, Whitehead (24), reported that albumen height was slightly improved with biotin supplementation. Additionally, Abdel-Mageed and Shabaan (23), showed that biotin inclusion at 325.5 μg/kg diet gave the highest increase in egg shape index, yolk index and Haugh unit. Furthermore, Soltan (29) concluded that ox bile addition in quail laying hens had no significant influence on internal and external egg quality.

Egg yolk triglyceride and cholesterol content were non-significantly ($P \geq 0.05$) reduced with BDA supplementation compared with birds fed the same diets without DBA addition. These finding could support that DBA has an important role in fat metabolism through enhancing its digestibility and utilization. The egg is a highly nutritious food for human consumption, however, its higher content of cholesterol associated with cardiovascular diseases restricts its consumption. So, previous studies were concerned with the production of egg with low cholesterol content through genetic selection; dietary interventions or using some drugs such as bile acids sequestrants in the diet of laying hens (36, 37).

Serum biochemical parameters

Blood serum lipid profile (table 4) was non-significantly altered with additives used. However, serum total cholesterol, TG, LDL, and VLDL concentrations were non-significantly ($P \geq 0.05$) reduced with DBA addition single or in combination with vitamins used except for serum HDL concentration which was increased. The higher reduction was observed in birds supplemented with DBA.

Serum TG, LDL, and HDL concentrations considered an important diagnostic indicators of lipid metabolism. The synthesis of adipose tissue, fat deposition, and formation of yolk in poultry is dependent on available serum TG. Most of fatty acids are produced in the liver and transported via LDL or chylomicrons for storage in adipose tissue as TG (2). On the other hand, HDL enhances the uptake and transport of cholesterol from extra hepatic tissues to hepatic tissue for catabolism (38), which was confirmed in the current study as it was non significantly increased especially with DBA addition. Therefore, these findings suggesting that the used feed additives (vitamin B_{12}, biotin and DBA) could have a hypolipidemic effect by affecting lipid metabolism. On contrary, Alzawqari et al. (13), reported that dietary inclusion of DBA significantly increased serum components of cholesterol, TG, HDL and LDL concentrations at days 21 and 42 of age, however, serum cholesterol concentration was numerically decreased (28). Furthermore, Lai et al. (12) documented that bile acid inclusion in broiler diets showed no differences in serum TG, HDL, and LDL concentrations among all groups at days 21 and 42. Inconsistency in results between experiments maybe related to differences in breed, age of birds and feeding experimental design.

Vitamin B12 or biotin supplementation without or with DBA non-significantly increased blood serum GOT and GPT activities compared with their reference birds (table 4). Serum GOT and GPT activity are traditional biochemical indices of liver function and are used clinically for diagnosis of liver injury. Yousefi et al. (5), documented that AST enzyme activity (GOT) could be used as diagnostic indicator for FLHS in laying hens. All liver function related parameters in the present study were within normal range (39)**,** which could confirm that these supplemental additives in laying hen diet had no adverse effects on liver function.

Liver composition and histopathology

As presented in table 4, both vitamin supplementation without DBA addition non-significantly decreased liver moisture % while significantly increased ($P<0.05$) it when combined with DBA compared with their reference birds. On the other hand, the fat content of the liver tissues (on dry matter or fresh basis), was non-significantly reduced ($P\geq0.05$) with both vitamins supplementation without or with DBA. Fatty liver is associated with the reductions in egg production with poor egg shell quality (40), which is particularly a serious problem during the late period of laying. With the long-term accumulation of fat and various toxins in the hepatic tissue, hepatocytes are injured with impaired function, reducing absorption and utilization of fat-soluble vitamins which consequently affecting the assimilation and deposition of calcium resulting in poor egg shell quality. So, liver health is closely associated with egg production and egg shell quality. Dietary inclusion of bile acids enhances the clearance function of very low-density lipoproteins, which transports fat from liver to systemic tissues, and reduces fat deposition in hepatocytes. Additionally, it stimulates the thyroid hormone activity (41), increases energy expenditure, reducing excessive accumulation of fat inside the liver and other body tissues of laying hens.

The examined liver of laying hens fed on the BD without any supplemental additives showed centrilobular vacuolation represented with clear vacuoles with round border consistent with fatty changes (Fig.1A). Most of the hepatic sinusoids were congested in addition to multifocal lymphocytic cells aggregation. Vitamin B_{12} or biotin supplementation markedly decreased these pathological vacuolations with nearly normal hepatocytes (Fig.2 B and C). DBA inclusion in the diet without or with vitamin B_{12} or biotin supplementation showed mild vacuolation of hepatocytes or even normal hepatocytes (Fig.1D, E, and F). The present findings are in line with Lai et al. (42), who concluded that liver of broiler chickens supplemented with bile acids was normal and no histological changes. Therefore, these findings revealed that these feed additives especially bile acids could help in maintaining liver health by reducing fat deposition in the hepatic tissue.

Table 1: Ingredients composition and chemical analysis of experimental diet

Ingredients	%
Yellow corn ground	56.83
Soybean meal (44% CP)	20.0
Corn gluten meal	5.0
Wheat bran	5.0
Vegetable oils (sunflower oil)	1.25
Ground lime stone[1]	9.80
Monocalcium phosphate[2]	1.30
Common salt	0.25
Vitamin premix [3]	0.15
Mineral premix [4]	0.15
Lysine[5]	0.02
Methionine[6]	0.10
Choline[7]	0.10
Mycotoxin adsorbent	0.05
Chemical analysis (%)	
Moisture	11.65
Crude protein	17.07
Ether extract	4.87
Ash	12.76
Crude fiber	4.43
NFE[8]	49.22
Calcium	3.72
Phosphorus	0.63
ME (Kcal/kg)[9]	2746.88

[1]Lime stone contains 37 % calcium and locally produced. [2]Mono calcium phosphate contains 21% phosphorus and 17 % calcium. [3]Vitamin premix: Each 1.5 kg contains: Vit. A (12000000 IU), vit. D (2000000 IU), vit. E (10 g), vit. K3 (2 g), vit. B1 (1 g), vit. B2 (5 g), vit. B6 (1.5 g), vit. B12 (10 g), nicotinic acid (30 g), pantothenic acid (10 g), folic acid (1g), biotin (50 mg), produced by Archar Daniels Midland Co., LL., USA. [4]Mineral Premix: Each 1kg contain, Manganese 100000 mg, Zinc 600000 mg, Iron 30000mg, Copper 10000 mg, Iodine 1000 mg, Selenium 200 mg, Cobalt 100 mg. [5]Lysine: 98% lysine hydrochloride, Shandong Longue Co., China. [6]DL-methionine, Evonik Co. (guaranteed analysis 99.5% DL- methionine).[7]Choline: choline chloride 60 % with vegetable carrier (corn powder) produced by Shandong pharmaceutical Co., China. [8]Nitrogen free extract was calculated by difference. [9]ME were calculated according to NRC (1994).

Table 2: Effect of dietary vitamin B_{12} or biotin supplementation without or with dried bile acids on performance and fat digestibility of laying hens

Parameters	Supplementation					
	Control	Vitamin B_{12}	Biotin	Control	Vitamin B_{12}	Biotin
	Without DBA[1]			With DBA		
Initial body weight (g/hen)	1851.82 ±21.07^{a}	1826.55 ±19.19^{a}	1815.66 ±23.77^{a}	1798.47 ±22.20^{a}	1844.27 ±19.63^{a}	1829.60 ±24.50^{a}
Final body weight (g/hen)	1827.74 ±35.02^{a}	1802.24 ±29.49^{ab}	1731.11 ±31.14^{b}	1794.15 ±30.66^{ab}	1815.27 ±28.93^{ab}	1795.38 ±26.79^{ab}
Weight changes (g/hen)	-24.08 ±20.02^{b}	-24.31 ±16.06^{b}	-84.55 ±19.26^{a}	-4.32 ±17.40^{c}	-29.00 ±17.35^{b}	-34.22 ±10.38^{b}
Average Feed Intake (g/hen/day)	113.41 ±0.07^{b}	109.99 ±0.19^{d}	96.90 ±0.13^{f}	120.21 ±0.06^{a}	111.35 ±0.12^{c}	107.34 ±0.16^{e}
Average FCR	2.35 ±0.03^{b}	2.14 ±0.03^{c}	2.14 ±0.06^{c}	2.49 ±0.03^{a}	2.10 ±0.03^{d}	2.19 ±0.03^{c}
Average egg production (%) 53 – 61	76.65 ±0.69^{bc}	81.56 ±0.74^{ab}	72.17 ±1.37^{c}	78.60 ±0.79^{b}	86.34 ±4.27^{a}	78.22 ±0.76^{b}
Average egg wt. (g)	62.83 ±0.22^{a}	62.74 ±0.15^{a}	62.48 ±0.15^{a}	61.38 ±0.16^{a}	61.75 ±0.14^{b}	62.65 ±0.14^{a}
Average egg mass (g/hen)	48.17 ±0.46^{bc}	51.21 ±0.50^{ab}	45.20 ±0.89^{c}	48.21 ±0.48^{bc}	53.33 ±2.63^{a}	49.02 ±0.50^{b}
Fat digestibility %	75.07 ±2.84^{a}	81.67 ±2.40^{a}	78.97 ±4.65^{a}	84.40 ±0.60^{a}	82.30 ±1.95^{a}	79.20 ±6.45^{a}

Values are means ± standard error. Means within the same row of different litters are significantly different at ($P < 0.05$).[1] DBA: Dried bile acids

Table 3: Effect of dietary vitamin B_{12} or biotin supplementation without or with dried bile acids on egg quality parameters

Parameters	Supplementation					
	Control	Vitamin B_{12}	Biotin	Control	Vitamin B_{12}	Biotin
	Without DBA[1]			With DBA		
	External egg quality					
Average eggshell relative weight	12.86 ±0.49^{a}	13.25 ±0.20^{a}	12.90 ±0.39^{a}	13.43 ±0.34^{a}	12.97 ±0.13^{a}	12.23 ±0.29^{a}
Average eggshell thickness (mm)	38.78 ±0.36^{a}	37.56 ±0.47^{a}	37.04 ±0.70^{a}	37.19 ±0.64^{a}	37.94 ±1.42^{a}	37.33 ±0.94^{a}
	Internal egg quality					
Average yolk relative wt.	29.99 ±0.89^{ab}	29.82 ±0.50^{ab}	29.22 ±0.66^{b}	30.06 ±0.58^{ab}	30.74 ±0.69^{ab}	31.47 ±0.76^{a}
Average yolk index	0.43 ±0.01^{a}	0.41 ±0.01^{ab}	0.43 ±0.00^{a}	0.43 ±0.00^{a}	0.41 ±0.01^{ab}	0.40 ±0.01^{b}
Average albumin relative wt.	57.15 ±0.60^{ab}	57.17 ±0.39^{ab}	58.24 ±0.64^{a}	54.84 ±2.02^{b}	56.51 ±0.50^{ab}	55.17 ±0.82^{ab}
Average albumin index	8.89 ±0.24^{a}	8.71 ±0.09^{a}	8.78 ±0.23^{a}	8.68 ±0.20^{a}	8.81 ±0.16^{a}	8.84 ±0.15^{a}
	Yolk cholesterol					
(mg/g yolk)	9.27 ±0.41^{a}	8.93 ±0.89^{a}	10.11 ±1.44^{a}	8.85 ±0.25^{a}	8.85 ±0.33^{a}	8.89 ±0.34^{a}
(mg/whole yolk)	181.48 ±3.65^{a}	165.58 ±20.61^{a}	181.54 ±35.65^{a}	170.35 ±6.36^{a}	161.63 ±11.76^{a}	162.76 ±6.47^{a}
	Yolk triglycerides					
(mg/g yolk)	10.11 ±0.49^{a}	10.31 ±1.55^{a}	9.74 ±0.13^{a}	9.75 ±0.52^{a}	9.95 ±0.66^{a}	9.56 ±0.48^{a}
(mg/whole yolk)	197.86 ±3.73^{a}	189.29 ±23.47^{a}	176.66 ±11.79^{a}	187.14 ±4.53^{a}	181.00 ±11.40^{a}	174.69 ±5.72^{a}

Values are means ± standard error. Means within the same row of different litters are significantly different at ($P < 0.05$). [1] DBA: Dried bile acids

Table 4: Effect of dietary vitamin B_{12} or biotin supplementation without or with dried bile acids on blood serum parameters of laying hens

	Supplementation					
	Control	Vitamin B_{12}	Biotin	Control	Vitamin B_{12}	Biotin
	Without DBA[6]			With DBA		
Blood serum lipid profile parameters						
Cholesterol (mg/dl)	198.15 ±0.70 [a]	191.23 ±6.12[a]	196.30 ±2.37[a]	195.53 ±3.43[a]	190.69 ±2.31[a]	190.77 ±2.75[a]
Triglyceride (mg/dl)	205.14 ±1.14[a]	205.86 ±3.62[a]	206.91 ±1.70[a]	203.61 ±3.06[a]	201.08 ±2.29[a]	204.90 ±0.58[a]
HDL[1] (mg/dl)	47.17 ±2.06[a]	48.43 ±2.10[a]	47.23 ±1.57[a]	49.80 ±1.42[a]	50.80 ±2.60[a]	51.63 ±1.49[a]
LDL[2] (mg/dl)	109.95 ±1.13[a]	101.62 ±5.46[a]	107.69 ±3.61[a]	105.01 ±3.24[a]	99.67 ±0.75[a]	98.15 ±3.06[a]
VLDL[3] (mg/dl)	41.03 ±0.23[a]	41.17 ±0.72[a]	41.38 ±0.34[a]	40.72 ±0.61[a]	40.22 ±0.46[a]	40.98 ±0.12[a]
Blood serum parameters related to liver functions						
GOT[4] (u/L)	65.33 ±17.9[a]	96.33 ±3.18[a]	71.33 ±16.51[a]	94.00 ±2.08[a]	82.50 ±12.50[a]	77.00 ±10.50[a]
GPT [5](u/L)	20.90 ±3.51[a]	24.67 ±1.45[a]	21.50 ±0.50[a]	21.67 ±1.45[a]	22.50 ±0.50[a]	24.67 ±1.67[a]
Liver tissue composition (%)						
Moisture	70.37 ±0.53[ab]	70.23 ±0.43[ab]	69.99 ±2.54[ab]	69.08 ±1.59[b]	73.03 ±0.71[a]	71.13 ±0.93[ab]
Fat (DM basis)	25.43 ±3.30[a]	22.78 ±2.47[a]	19.62 ±3.50[a]	22.86 ±4.18[a]	21.51 ±1.67[a]	23.61 ±0.99[a]
Fat (fresh basis)	7.55 ±1.05[a]	6.08 ±0.81[a]	5.96 ±1.27[a]	7.06 ±1.72[a]	5.82 ±0.59[a]	6.80 ±0.12[a]

Values are means ± standard error. Means within the same row of different litters are significantly different at ($P<0.05$). [1]HDL: high-density lipoprotein, [2]LDL: low-density lipoprotein (LDL), [3]VLDL: very low-density lipoprotein, [4] GOT: glutamic oxaloacetic transaminase, [5]GPT: glutamic pyruvic transaminase. [6] DBA: Dried bile acids

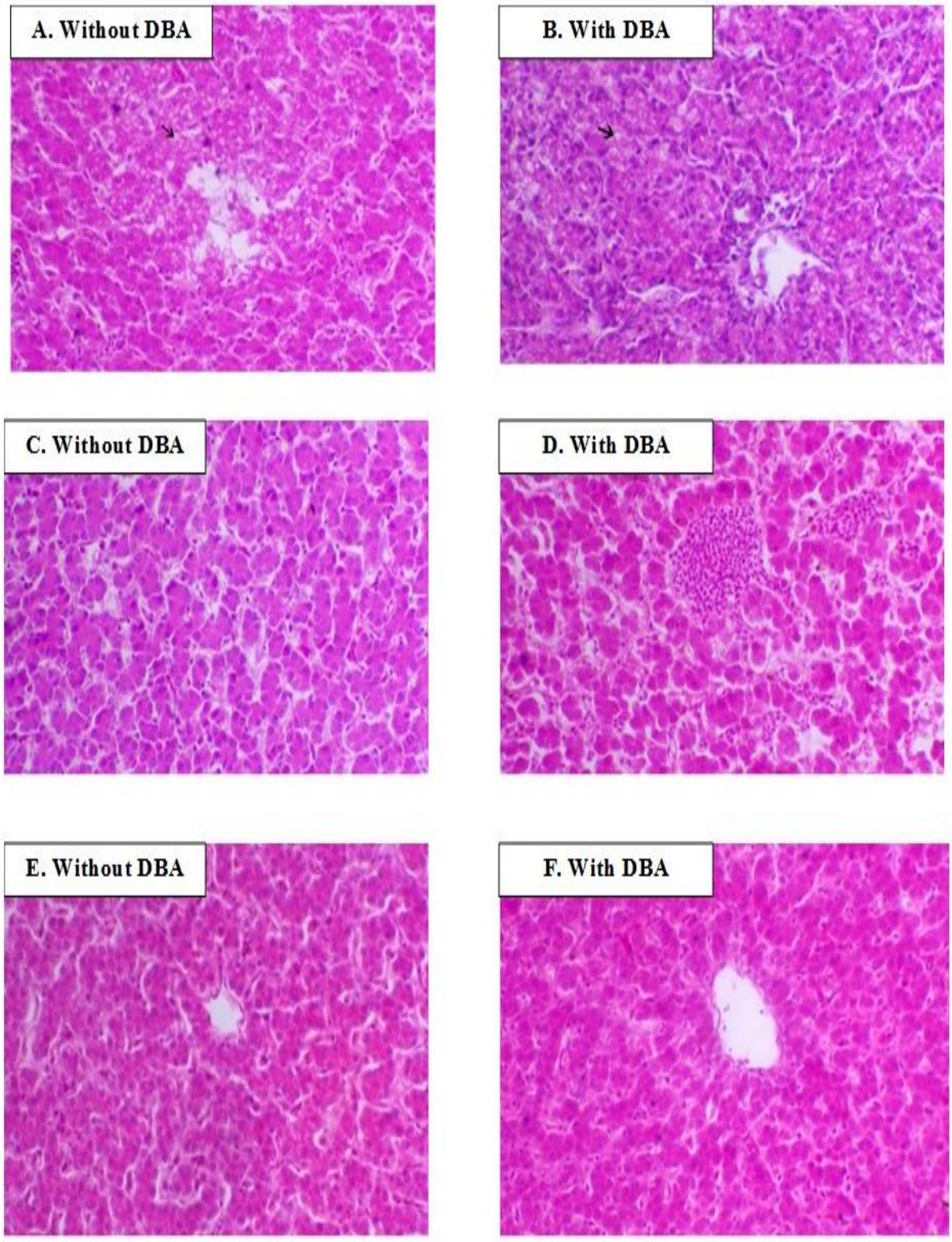

Figure 1: Histopathological changes of hepatic tissue of laying hens fed on control (without DBA) showing centrilobular hepatic vacuolation with fatty changes (arrow) (A); or with DBA mild degree of hepatic vacuolation consistent with fatty changes (arrow) (B); hepatic tissues of birds supplemented with vitamin B_{12} supplementation and without DBA or with DBA addition showing normal hepatocytes (C and D); Birds supplemented with biotin without or with DBA addition showing normal hepatocytes (E and F)

Conclusion

Under the conditions of the present study, vitamin B_{12} supplementation single or combined with dried bile acids is recommended in laying hen diet as it gave the best productive performance and improved fat digestibility, hepatic health and reduced the serum lipid profile. On the other hand, biotin supplementation is not recommended for laying hens except if combined with DBA.

Conflict of interest

All authors declared that no conflicts of interest, as they are responsible for the content and writing this article.

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Original Research Article

INFLUENCE OF VITAMIN E AND SELENIUM SUPPLEMENTATION ON THE PERFORMANCE, REPRODUCTIVE INDICES AND METABOLIC STATUS OF OSSIMI EWES

Sherief M. Abdel-Raheem[1]*, G. B Mahmoud[2], Waleed Senosy[3], Taymour M. El-Sherry[4]

[1]Department of Animal Nutrition and Clinical Nutrition, Faculty of Veterinary Medicine, Assiut University, 71526, Egypt, [2]Department of Animal production, Faculty of Agriculture, Assiut University, [3]Department of Theriogenology, Faculty of Veterinary Medicine, New Valley University, 51714, Egypt, [4]Department of Theriogenology, Faculty of Veterinary Medicine, Assiut University

*Corresponding author, E-mail: sherief.diab@aun.edu.eg

Abstract: The objective of this study was to investigate the effects of pre and post-lambing intramuscular (i.m.) injections of vitamin E (VE) and selenium (Se) on the performance, reproductive indices and metabolic status of Ossimi ewes. A total of 20 pregnant ewes were divided randomly into two equal groups of ten animals. The control group (CG) was administered 3 ml of normal saline. The treated group (TG) was administered a combination of 5 mg of sodium selenite and 450 mg of VE twice weekly for 2 weeks pre and post lambing. The performance, reproductive indices, colostrum and milk mineral profiles, blood metabolites and antioxidant enzyme biomarkers were investigated in this experiment. VE and Se supplementation had a significant effect on ewe, lamb performance, colostrum and milk mineral profiles. The treated ewes lost less weight (0.7 kg) compared with the ewes in the control group (3.68 kg) on 30th day postpartum (P<0.001). Lambs born to treated ewes were heavier than those born to control ewes (4.47 kg vs 3.99 kg, respectively), gained more daily weight quickly (268.66 vs. 226.00 g, respectively, P<0.001) and weighed more on 30th day post-lambing (12.53 vs. 10.77 kg, respectively, P<0.001). Treated ewes had higher concentrations of blood glucose, total protein, total cholesterol, antioxidant enzyme biomarkers, and lower concentrations of serum non-esterified fatty acids (NEFA). VE and Se supplementation had a significant effect (P<0.05) on postpartum reproductive performance of ewes in terms of fast uterine involution, earlier resumption of ovarian function and ovulation with large size ovulatory follicles, and greater numbers of small, medium, and large-sized follicles. In conclusion, VE and Se supplementation during late gestation and early lactation could be considered an effective strategy for minimizing the weight loss of ewes, enhancing lamb performance, the antioxidant and metabolic status, which improves the reproductive performance of Ossimi ewes.

Key words: vitamin E; selenium; lamb performance; reproductive performance; antioxidant status; Ossimi ewes

Introduction

Over the last decade, ewe fertility has been progressively deteriorating around the world.

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Improving productivity by improving the peripartum period has become an important problem. Ewes are often deficient in certain trace elements and vitamins important for lambing because they graze on dry pastures of poor-quality during pregnancy, which can influence lamb survival, birth weight, subsequent poor animal production and welfare (1). Vitamins and minerals play an important role in the growth, physiology, and reproductive performance of animals. Se deficiency is linked to several reproductive disorders of cattle including abortion, retained placenta, neonatal weakness, metritis, poor uterine involution, cystic ovaries, impaired fertilization of ova, impaired fertility, and repression of immunity (2, 3). Selenium (Se) and vitamin E (VE) both prevent oxidative degeneration of biological membranes. Vitamin E and the Se-containing enzyme glutathione peroxidase (GSHpx) are critical components of the antioxidant system that functions in all cells (4). Vitamin E and Se are integral parts of the antioxidant defense system and participate in critical enzymatic reactions, which directly influence growth performance, function of the immune system, and reproductive performance of animals (5, 6). Newborns are likely to experience VE deficiency; due to the insignificant amount of VE transmitted to the fetus in the uterus, it is important that colostrum must supply enough VE to the lamb (7). Ewes that were supplemented with VE and Se during late gestation gave birth to heavier lambs with reduced mortality as compared to lambs born to non-supplemented ewes (8, 9). Although fresh grass contains high amount of VE, the amount of VE decreases during storage and conservation and depends on forage species and time of harvest (7). The previous National Research Council (NRC, 1985) (10) recommendation of 15 IU of VE/kg dry matter (DM) was increased by approximately 17-times in the NRC's most recent set of recommendations (2007) (11) to increase the immunocompetence of animals. However, it has not been fully documented that VE and Se supplementation of ewes has a beneficial synergistic effect on reproductive and productive lamb performance. Therefore, the objective of this study was to investigate the effect of pre and post lambing intramuscular (i.m.) injections of VE and Se on the performance, reproductive indices and metabolic status of Ossimi ewes

Material and methods

The study was performed according to the regulations and procedures approved by the Ethics Committee on Animal Experimentation of Assiut University, Faculty of Veterinary Medicine and the Guide for the Care and Use of Laboratory Animals (National Institute of Health publication no. 8023, revised 1978).

Experimental site

The study period was the winter breeding season from December to February. The ambient temperature ranged from 21.6-26 °C while the relative humidity was ranged from 50-54%. Ewes were kept indoors at the Research and Production Farm, Department of Animal Production, Faculty of Agriculture, Assiut University (latitude 28 ° 07′N and 30° 33′E).

Animals, management, and treatments

A total of 20 pregnant Ossimi ewes (identified by ultrasonography) were used of nearly similar age (4-5 years), with 1-2 parity and their average weight of 53.86 kg. Two weeks prior to anticipated lambing, healthy ewes clinically free from external and internal parasites were selected for the present study. Each ewe had free access to food and water and was maintained in an individual pen. Ewes were fed a late pregnancy diet (Table 1) until a parturition lactation diet was offered. Ewes were divided into two equal groups of 10 animals. The control group (CG) received no supplementation and administered 3 ml of normal saline. The treated group (TG) received an injection of VE and Se (3 ml i.m. from Viteselen 15®, Adwia Company, Egypt). Each ewe in the TG was injected with 5 mg of sodium selenite and 450 mg VE twice weekly for two weeks pre and post lambing; lambing occurred over a two week period. Food was mixed daily and ewes were fed two times a day. All nutrients met or exceeded the requirements for 60 kg ewe at the last four weeks of gestation and ewes at the first 6-8 weeks of lactation, according to guidelines

from the National Research Council for sheep (NRC, 1985). The late pregnancy diet consisted of 70 % Berseem hay and 30 % concentrate mixture. The lactation diet consisted of 40 % Berseem hay and 60 % concentrate mixture along with wheat straw offered ad libitum (Table 1). For both groups, the experimental diet contained approximately 0.38 ppm Se and 23 mg VE/kg diet. Two meals of equally allotted feed were given to all ewes at 08:00 and 17:00 and leftover feed was removed and weighed. The amount of feed consumed daily was recorded and representative samples were subjected to chemical analysis.

Ovarian and uterine examinations

Sonographic examinations were performed on postpartum days 2, 4, 6, 8, 11, 14, 17, and 25 using a real-time B-mode ultrasound scanner (Pie Medical, 100 LC, Holland) with a line array transducer of 5.0 MHz. Ovarian examination was carried out by ultrasonography to monitor follicular growth, ovulation, and corpus luteum formation. Uterine involution was investigated by measuring the maximal transversal cross-sectional diameter and the lumen diameter of both the right and left uterine horns. Furthermore, the presence and echogenicity of uterine cavity contents were included in this investigation. Entire follicles of 3 mm in diameter or greater were recorded. The term wave was defined as one or more antral follicles growing 3 to $\geq$ 5 mm in diameter before regression.

Blood sampling and estimation of biochemical parameters and steroid hormones

Blood samples were collected from the jugular vein parallel to sonographic examination. Duplicate blood samples were collected per animal, which were subjected to centrifugation at 3000 rpm for 20 min. Following centrifugation, plasma was harvested and stored at -20 ºC prior to testing. A colorimetric method was used to evaluate the blood metabolites (glucose, total protein, triglycerides, total cholesterol, AST, and ALT) using kits from Spinreact ® Company (Spain). A direct ELISA was used to determine concentrations of progesterone (P4) (Diagnostic System Laboratory Co. (DSL), USA, catalogue no. 3900). The intra- and inter-assay coefficient of variation for estrogen and progesterone was 3.6% and 12.43%, respectively. The sensitivity of the assay for P4 was 0.12 ng.

Determination of oxidant and antioxidant levels

The levels of the oxidant (malondialdehyde (MDA); catalog no. MAK085, Sigma-Aldrich, USA), the antioxidant (reduced glutathione (GSH); catalog no. RS505, Randox Laboratories Ltd, UK) and total antioxidant capacity (TAC) (catalog no. NX2332, Randox Laboratories Ltd, UK) were determined by a colorimetric method using kits according to the instructions provided by the manufacturer's (Sigma-Aldrich, USA and Randox Laboratories Ltd, UK).

Plasma and milk concentrations of trace elements

A flame atomic absorption spectrophotometer (Shimadzu, AA-7000, Japan model) was used to measure Cu and Zn levels in colostrum and milk according to the procedure described by El-Bahr and Abdelghany (12). A graphite furnace (GFA-EX7) atomic absorption spectrophotometer (Shimadzu, Koyoto, Japan) was used to measure Se. Sample digestion was performed according to Official Methods of Analysis of AOAC (13). The graphite furnace program for measuring Se by GFA-EX7 and instrument settings were similar to those previously described by Meligy (14).

Statistical analysis

SPSS software (SPSS analytical program for windows version 16; SPSS) (15) was used to conduct the statistical analyses. Independent t-test was used to detect the significant differences in the treatment means of estrus onset, estrus duration, time of ovulation, emergence of ovulatory follicles, maximum size of ovulatory follicles, and blood metabolites. The effect of time on each variable in each group was evaluated using analysis of variance with repeated measurements of the general linear model using the Bonferroni multiple comparison test. Prob-

ability values less than 0.05 (P <0.05) were considered significant. Results are presented as mean ± SE.

Results

Lamb and ewe performance

There was a significant improvement in lamb performance of TG in terms of heavier birth weight and higher weight gain at 30 days post-partum (Table 2). Similarly, there was a significant improvement in ewe body weight of TG at the time of lambing and 30 days post-partum.

Postpartum uterine involution

The rate of uterine involution was significantly faster (P<0.05) in the TG compared with the control group at 6 and 11 days post-lambing (Figure 2). Uterine diameter was smaller in the TG than that of the CG on days 4, 6, 8, and 11 postpartum while there were no differences between both groups on day 14, 17, 20, and 25 postpartum (Figure 2).

Postpartum ovarian resumption

The first ovulation and ovarian resumption occur earlier in TG compared with CG. As confirmed by the recorded first elevation in progesterone (P4) postpartum that was coincided with ultrasonic lutein tissue on the ovarian surface was at 16.7 ± 1.0 days in treated ewes, which was significantly earlier (p<0.01) compared with the CG (25.7 ± 1.0 days). This result indicates that the first ovulation and ovarian resumption occurred earlier in the treated group compared with CG. The size of the first ovulatory follicle was larger (P<0.05) in the TG (6.6 ± 0.02 mm) in comparison with the CG (5.8 ± 0.03 mm). Moreover, progesterone concentration at that time was 1.4 ± 0.08 ng/ml and 1.1 ± 0.07 ng/ml for the TG and CG, respectively. The number of small follicles was higher (P<0.05) in the TG at different times during the postpartum period (Fig. 2, A). The overall number of small follicles during the study period was higher (P<0.001) in the TG (Fig. 2, D). The number of medium sized follicles fluctuated during the study period but was higher in the TG (Fig 2, B). In addition, the overall number of medium-sized follicles was high (P<0.01) in treated ewes compared with the CG (Fig. 2, D). The number of large follicles was higher in the TG (P<0.05) during different intervals of the postpartum period (Fig. 2, C). However, the overall number of large follicles did not differ between both groups during the study period (Figure 2, D).

Metabolic and biochemical profiles

The concentration of glucose in blood (mg/l) was higher in the treated ewes (P <0.05) in comparison with the control during the early postpartum period until day 20 postpartum (Figure 3, A). Similarly, total protein (g/l) was higher in the TG (P<0.05) on day 2, 4, 8, 14, 17, and 20 postpartum. Furthermore, the concentration of total cholesterol (mg/dl) was higher in the treated ewes during the postpartum period of the study. The concentration of non-esterified fatty acids (NEFA; mmol/l) significantly elevated (P<0.05) in the CG during the postpartum period except at day 6 postpartum as compared to the CG. The concentration of triglycerides (mg/dl) was higher (P<0.05) at day 2 and day 6 postpartum than control while it lowered during days 17, 20 and 25 postpartum in the treated ewes.

Antioxidant enzyme biomarkers and selenium concentrations

The MDA concentration (mmol/l) was higher (P<0.05) in control ewes compared with the treated ones during the postpartum period (Table 3). The blood concentration of TAC was significantly higher (P< 0.05) in the TG at days 2, 4 and numerically not statistically on days 14, 17, 20, and 25 compared to the CG. In addition, plasma levels of reduced GSH (U/g Hb) were significantly higher on days 4, 14, 17, 20, and 25 in the TG compared with the CG. The concentration of Se (µmol/L) in serum was significantly higher in the TG on days 2, 4, 14, 17, 20, and 25 compared with the CG.

Trace element profile in colostrum and milk

The data exhibited in table 4 revealed that the concentrations (ppm) of copper and Se in colostrum and milk were significantly (p<0.05) higher in the TG. However, the concentration

of zinc (ppm) in the colostrum and milk harvested from treated ewes was decreased significantly (P<0.05) than those of CG.

Table 1: Ingredients and analyzed chemical composition of experimental diets during pregnancy and early lactation (as fed basis)

Item	Late pregnancy diet	Early lactation diet
Ingredient, %		
Concentrate mixture	30	60
Berseem hay	70	40
Wheat straw	*Ad libitum*	*Ad libitum*
Total	100	100
Concentrate mixtures, %		
Ground yellow corn	75.7	75.7
Soybean meal (44 %)	3	15
Wheat bran	19	7
Vitamin and mineral Premix*	0.3	0.3
Limestone	1.5	1.5
Salt	0.5	0.5
Total	100	100
Chemical composition (%, unless stated)		
Dry matter	87.67	87.42
ME Mcal/head/day**	3.67	5.47
Crude protein	13.00	14.00
Crude fiber	20.11	12.81
Crude Fat	2.46	2.38
Nitrogen free extract	41.02	49.74
Ash	11.08	8.49
Organic matter	76.59	78.93
Calcium	1.00	0.84
Phosphorus	0.30	0.30
Selenium, mg/kg	0.38	0.38
Vitamin E, mg/kg	23.00	23.00

*Vitamin and mineral premix each 3 kg contain: 1250000 IU Vit. A; 2500000 IU Vit. D3; 1000 mg Vit E; 80000 mg Mn; 60000 mg Zn; 50000 iron, 20000 copper, 5000 iodine, 250 Se, 1000 Co mg tell 3 kg $caco_3$.
**ME, metabolizable energy calculation based on NRC tables for sheep (1985).

Table 2: Lamb and ewe performance of control and treatment groups (means ± SE)

Item	CG	TG	P
Lamb performance			
Number of lambs/ewe	1.5 ± 0.17	1.6 ± 0.16	0.67
Lamb birth weight, kg	3.99 ± 0.05^{b}	4.47 ± 0.03^{a}	<0.001
Lamb weight, kg (30 day post-lambing)	10.77 ± 0.04^{b}	12.53 ± 0.06^{a}	<0.001
Total weight gain, kg	6.78 ± 0.06^{b}	8.06 ± 0.07^{a}	<0.001
Daily weight gain, g	226.00 ± 1.84^{b}	268.66 ± 2.28^{a}	<0.001
Ewe performance			
DMI g/day (late pregnancy)	1699.92 ± 3.68	1695.85 ± 3.88	0.46
Ewe BW, kg (d 130 pregnancy)	53.86 ± 0.11	54.02 ± 0.09	0.29
Ewe BW, kg (d 140 pregnancy)	54.86 ± 0.12	55.02 ± 0.1	0.30
DMI g/day (early lactation)	2296.74 ± 6.02	2303.38 ± 5.60	0.43
BW after lambing (d 1)	44.86 ± 0.14^{b}	46.02 ± 0.15^{a}	<0.001
BW after lambing (d 30)	41.18 ± 0.25^{b}	45.32 ± 0.13^{a}	<0.001
Weight loss, kg	-3.68 ± 0.23	-0.70 ± 0.05	<0.001

*Means bearing different superscript letters in the same raw differ significantly (P < 0.05).
BW, body weight; DMI, dry matter intake; d, day

Table 3: Activities of antioxidant enzymes and selenium concentrations of treated and control groups (means ± SE)

	Group	TAC, mmol/L	GSH U/g Hb	MDA, µmol/L	Selenium, umol/L
Day 2	CG	42.33±3.28[b]	206±1.73[b]	15.44±0.18 [a]	0.96±0.04[b]
	TG	73.81±3.26[a]	312.24±9.8[a]	14.27±0.09 [b]	1.74±0.05 [a]
	P	0.03	0.13	0.004	<0.001
Day4	CG	39.00 ± 3.6[b]	204.00±0.58[b]	15.4±0.12[a]	0.91±0.04[b]
	TG	70.63 ± 3.5[a]	592.33±1.45[a]	14.63±0.09	1.71±0.02 [a]
	P	0.03	<0.001	0.006	0.001
Day 14	CG	70.91±5.75	205.67±1.2[b]	13.17±0.12[a]	0.97±0.07 [b]
	TG	72.49±11.65	593.00±1.53[a]	11.90±0.26[b]	1.72±0.04[b]
	P	0.9	<0.001	0.01	0.001
Day 17	CG	70.91±5.75	201.33±1.76[b]	10.8±0.17[a]	1.01±0.03[b]
	TG	72.49±11.65	594.21±8.17[a]	9.53±0.22[b]	1.75±0.05[a]
	P	0.9	0.001	0.01	<0.001
Day 20	CG	70.91±5.75	204.00±1.15[b]	8.33±0.09 [a]	1.00±0.06[b]
	TG	72.49±11.65	595.33±0.88[a]	7.7±0.06[b]	1.84±0.02[a]
	P	0.9	<0.001	0.004	<0.001
Day 25	CG	70.91±5.75	196.0±1.73[b]	7.4±0.15[a]	1.19±0.07[b]
	TG	72.49±11.65	563.33±1.76[a]	6.73±0.09[b]	3.48±0.26[a]
	P	0.9	<0.001	0.02	0.001

*Means with different superscripts (a, b) in the same column (for each day separately) differ significantly (P<0.05). TAC, Total antioxidant capacity; MDA, Malondialdehyde; GSH, Glutathione peroxidase

Table 4: Mineral profile (ppm) of colostrum and milk of control and treatment groups (means ± SE)

Item	CG	TG	P
Colostrum			
Cu,	0.51 ± 0.03[b]	0.94±0.09 [a]	0.01
Se	0.08 ± 0.003[a]	0.17±0.001[a]	<0.001
Zn	13.94±0.07[a]	10.59 ± 0.06[b]	0.02
Milk			
Cu	0.49 ± 0.05 [b]	1.14±0.22 [a]	0.04
Se	0.06 ± 0.003[b]	0.13±0.02 [a]	0.02
Zn	4.98 ± 0.24[a]	3.64±0.27 [b]	0.01

*Means bearing different superscript letters in the same raw differ significantly (P < 0.05)

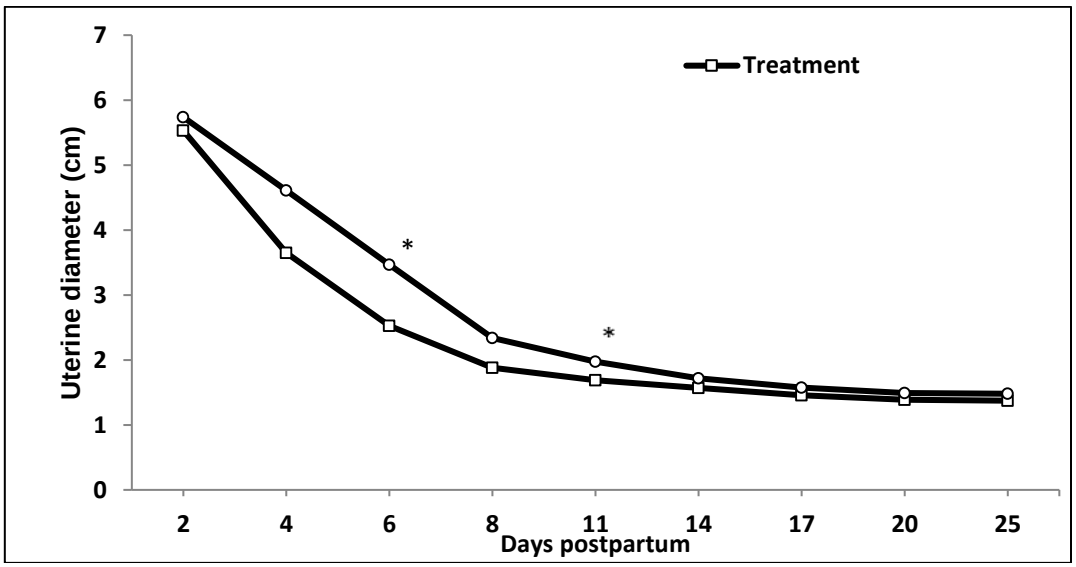

Figure 1: Pattern of uterine involution in treated and control group during postpartum period

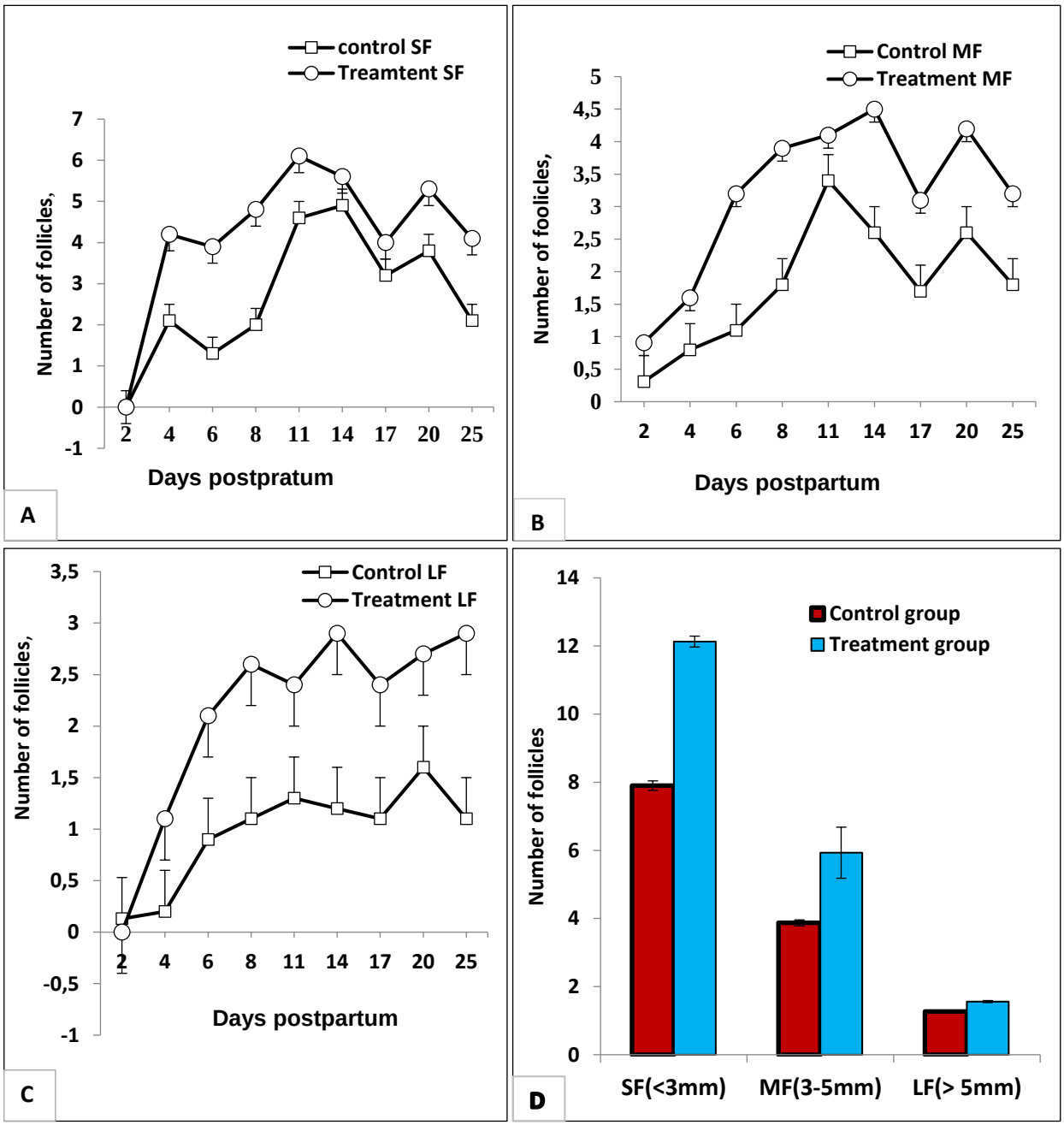

Figure 2: A, Number of small follicle (SF); B, medium follicle (MF); C, large follicle (LF); D, Overall number of small, medium and large follicles in treated and control ewes during postpartum period. Means are significantly different between groups in all parameters in comparison ($P < 0.05$)

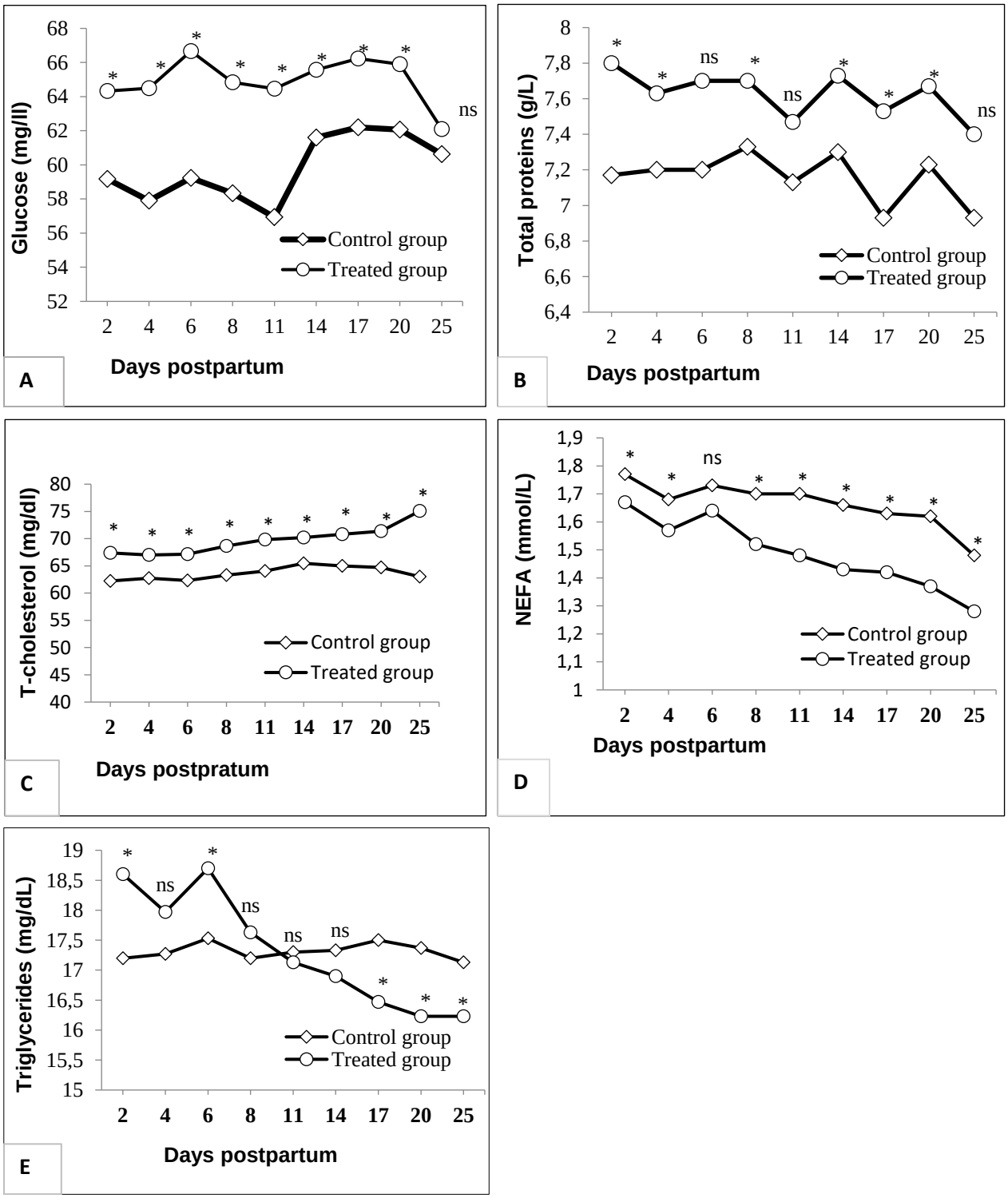

Figure 3: Concentration of blood glucose (A), total proteins (B), total cholesterol (C), non-esterified fatty acids, NEFA (D) and triglycerides (E) in control and treated group during postpartum period ($P < 0.05$).

Discussion

Previous studies have suggested that supplementation with VE and Se can improve the function of the immune system, reproductive performance, and growth performance of goats and can increase the survival of Merino lambs (1, 5, 16). The improved productivity of Ossimi ewes was consistent with the previous studies in terms of higher lamb numbers, heavier birth

weight, and higher body weight gain on day 30 in the TG (9, 17). These studies showed that the supplementation or inclusion of VE and Se in the diet of the mother can improve the lamb performance. Lambs reared by ewes supplemented by VE and Se in the late gestation period showed better weight gain and heavier weaning weight. An explanation for that, VE and selenium supplementation increased their concentrations in colostrum and milk, which improve the immune function, feed efficiency and daily weight gain of suckling lambs (18, 19). The rate of uterine involution was significantly faster ($P<0.05$) in the TG in comparison to control ewes. This result is in agreement with the previous observations of Kiracofe (20) who reported that the diameter of uterine horns returned to their non-pregnant size by 21 days postpartum in ewes and that the uterus was similar in size to the non-pregnant uterus by the day 25. The earlier first ovulation with large-sized ovulatory follicles with large numbers of small, medium, and large-sized follicles in the treated ewes indicated the earlier resumption of ovarian activity and agreed with the results of previous investigations (9, 21). The Role of VE in the development of follicles and ovarian activity is unknown, but ovulation-induced oxidative damage to the ovarian epithelium of ewes can be prevented by supplementation of VE (22). Selenium is important for formation of the selenoprotein glutathione peroxidase 1(GPx1) which plays an important role in female reproductive function; i.e., involved in determining the follicle growth, maturation, and dominance in both cows and women (23). Potential role of antioxidant like vitamin E and Se in follicle dominance are protecting the dominant follicle from increasing levels of reactive oxygen species (ROS) (23).

The significant increase of Se in serum and selenium and copper in colostrum, and milk is in agreement with earlier studies (24, 25). These previous studies indicated that VE and Se supplementation resulted in increasing the levels of VE and Se in the blood and consequently in the colostrum and milk of ewes during lactation.

On the other hand, significant differences ($P<0.05$) in blood biochemical parameters were observed between the TG and the CG. Ewes showed a significant increase ($P<0.05$) in the levels of plasma total protein, glucose, total cholesterol, and a low level of NEFA after VE plus Se supplementation during the late gestation and suckling periods. These results consistent with previous studies (26, 27). A higher level of glucose (relative to control ewes) following the supplementation of Se and VE was observed in previous studies (28, 29). Although, Se and VE directly affect the metabolism of carbohydrates, it is not clear whether increased glucose level directly or indirectly through increasing thyroxin and triiodothyronine hormones in supplemented animals as reported in previous studies (26). In the pregnant ewes, the increased concentrations of total protein and albumin following VE and Se supplementation were reported by previous studies (28, 29). The exact mechanism that leads to a higher total protein in the serum of the TG was not elucidated in these studies but increased concentrations of γ globulin could be a part of the mechanism. Another supporting research to our findings reported that the concentration of blood metabolites and the total antioxidant status in plasma were elevated in sheep received Se and VE supplements (27, 30). In this study, significant increase of GSH-Px and TAC was noticed after VE and Se supplementation compared with the control. This indicates that the maternal supplementation of Se and VE during late pregnancy and early lactation can improve the antioxidant status of Ossimi ewes.

The increased levels of NEFA in CG were nearly similar with the findings observed by Avci and Kizil (31) in transition cows and are indicative of the increasing depletion of lipid reserves existed because of the increased energy demand. Also, the increased NEFA in the CG indicates the utilization of fat storage reserves to meet the rapid and dramatic energy responses in the ewes of this group. It can therefore be assumed that VE and Se supplementation may improve the energy balance in lactating ewes and decrease the weight loss during lactation.

Conclusion

Supplementation of pregnant ewes with VE and selenium (two weeks pre and post-lambing) had a significant effect ($P<0.05$) on the postpartum reproductive performance of ewes in terms of higher uterine involution, earlier ovarian resumption and first ovulation with large size ovulatory follicle, higher numbers of small, medium and large sized follicles. Furthermore, VE and Se supplementation had improved ewe and lamb performance, colostrum and milk mineral profiles, the metabolic and antioxidant status of Ossimi ewes. In conclusion, during the periods of late gestation and early lactation, VE and Se supplementation could be considered an effective strategy for minimizing the weight loss of ewes, enhancing lamb performance, the antioxidant and metabolic status, which improves the reproductive performance of Ossimi ewes.

Conflict of interest

The authors declare that they have no conflict of interest

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Author contributions

S. M. A. designed the experiment, shared in running the experiment, sample collection and analysis, carry out statistical analysis of the data and wrote the manuscript. G.B. M. shared in running the experiment, sample collection and analysis and shared in revising the manuscript. W.S. shared in running the experiment, writing and revising the manuscript. T. M. E shared in sample collection, performed ultrasound measurements and revised the manuscript. All authors read and approved the final manuscript.

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Original Research Article

COMPARATIVE THREE DIMENSIONAL COMPUTED TOMOGRAPHY (CT) SCANS AND ANATOMICAL INVESTIGATION OF RABBIT (*Oryctolagus cuniculus*) AND CAT (*Felis domestica*) SKULL

Attia A. A. Moselhy*, Eman A. A. Mahdy

Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Zagazig University, Egypt

*Corresponding author, E-mail: atiaanatomy@gmail.com

Abstract: This study was conducted to elucidate the comparative anatomical features of the skull of rabbits and cats using CT scans. Adult healthy New Zealand rabbits and Domestic Baladi cats of both sexes, were prepared for X-ray and CT scan of the heads at different positions. The heads were also processed for bone preparation and were photographed at various views. Some measurements were taken to exhibit the main differences between the two animal species. There was a cranial pharyngeal canal in the basisphenoid bone of rabbit, which was absent in cat. The retroarticular process situated caudal to the mandibular fossa in cat and absent in rabbit. The carotid foramen was large and situated only in rabbit ventromedial to the bulla tympanica. The supraorbital process of rabbit extended anteriorly and posteriorly forming rostral and caudal supraorbital fissures. The orbit was located laterally in rabbit rostrally in cat. The facial surface of the maxilla was perforated by several formina in rabbit. A large retroalveolar foramen was found only in the mandible of rabbit. The condyloid process of the mandible was large and present longitudinally in rabbit and transversally in cat. Also, the hyoid bones and the paranasal sinuses were compared at both animals. The hyoid bone located in the mandibular space in rabbit and caudal to this space in cat. The lingual process of the hyoid bone was absent in cat. The middle and great cornuae of the hyoid bone were absent in rabbit. The paranasal sinuses of rabbit were maxillary and ethmoidal sinuses. While in cat, there were frontal, sphenoidal and maxillary sinuses. The combination between the traditional gross morphology of the skulls, X-ray and scan aimed to clarify and confirm all the points of comparison between the two animal species used, which was not achieved by using only one method of them.

Key words: rabbit; cat; skull; computed tomography; anatomy

Introduction

The skull is a highly constructed and integrated part of the axial skeleton. All breeds of domestic rabbits descend from European rabbit (Oryctolagus cuniculus), which is a member of the family Leporidae (rabbits and hares) (1). The skull is divided into two primary units, neurocranium and basicranium. The brain case provides protection for the brain and opening for cranial nerve connections, the bone of the face provide a location and protection for the organs of special senses.

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The domestic cat (Felis catus), a common buddy animal, is only one of the species in the family Felidae (2). The rabbit is utilized for numerous purposes, including biomedical research, meat and fur production. Rabbits and cats are often used as human substitutes in olfaction and inhalation tests, both of which require the knowledge of their anatomy (3, 4).

There are many papers about the morphometric features of the head of cat and rabbit and the gross anatomical studies were required. (5, 6).The cat as carnivorous and the rabbit which is herbivores animal, they show certain anatomical peculiarities specially their skeleton. The cat has large brain case and anterior located orbit and the rabbit skull long, narrow and laterally situated orbit.

The current study was conducted to elucidate the comparative anatomical features of the skull of a rabbit and cat as educational tools in veterinary studies.

Materials and methods

Animals

The handling of animals in this study was followed the guidelines of the Institutional Animal Care and the Research Ethics Committee of the Zagazig University, with an ethical approval number of (ZU-IACUC/2/F /92/2018).

Adult healthy of each New Zealand rabbits and domestic cats (n = 10 for each) of both sexes weighed about 2-4 kg, were used. Rabbits were obtained from a laboratory farm in Faculty of Agriculture, Zagazig University. The age of rabbit was 10 months and cat was 13 months. The cats were purchased from a pet animal's clinic in Zagazig city, Sharkia Governorate, Egypt. Rabbits were injected through the ear vein with Xylazine (3 mg/kg) followed by injection of Ketamine (3 mg/kg) (7). The dose in cats was 1 mg/kg of xylazine followed by 5 mg/kg of I.M ketamine for sedation and anesthesia (7).

Radiography

For radiography, the animal heads were photographed in dorsal and lateral position using Mobile Fischer X-ray machine H.G. Fischer, inc. Franklin Park, Illinois, USA, in Department of Surgery, Faculty of Veterinary Medicine, Zagazig University.

Computed tomography

For (CT), both species were used, at AL-Bayan Center of radiology and CT in Belbes, Sharkia Governorate, Egypt. CT images were taken without contrast medium using multislices CT system, which was capable of acquiring up to 32 slices per second with fast whole-body scan time of 0.5 seconds, 50 kW X-Ray Generator, Multiple kV and mA techniques and 5.0 MHU X-Ray Tube. TOSHIBA 600HQ (third generation) Japan (8).

Anatomical dissection of heads

The heads of both species were carefully separated and cleaned from all attached tissues. Also, the intact hyoid bones were carefully dissected. The skulls were prepared following the method of (9). The measurements were taken with a caliper to demonstrate the main differences between the two animal species. The obtained skulls were photographed using a Sony digital camera, Dsc W810 20.1 MP. The weight of animals obtained by using a digital scale. The nomenclatures were taken as a basis for the denomination on the skulls (10). The cranial and facial portions of the skull were described the most characteristic points of differences of both animals. Also, the hyoid bones and the paranasal sinuses were compared at both animals.

Statistical analysis

All statistical procedures were performed using the SAS statistical system Package V9.2 (SAS, 2009) (11). Differences between means due to different anatomical parameters were tested by student's t-test. Data were reported as mean ± SEM, and the differences were considered significant at $P \leq 0.05$

Results

he apparent point of comparison between two species skull in the current study that, rabbit skull as a whole was long, narrow and compressed. But, that of a cat was short, broad and heavy in weight table 1.

The skull is composed of two main portions; cranial and facial.

I- Bones of the cranium

The occipital bone

The occipital bone (Fig.1 A, B, C and D) was subdivided into three parts; supra-occipital, lateral part and basioccipital. In rabbit, the occipital bone, shared in the formation of the roof of the cranial cavity and the supra-occipital part was fenestrated and depressed dorsally. The external sagittal crest was sharper, higher and longer in cat than in rabbit. The nuchal crest of rabbit was low, curved and located caudolaterally. However, in a cat, the latter crest was sharp and long. The external occipital protuberance appeared as a sharp elevated ridge in the rabbit and reached to the foramen magnum. While in cat, it was small and separated from the foramen magnum by the external occipital crest. The occipital condyles in a cat were larger and broader than that of the rabbit. The jugular processes were longer in rabbit and closely attached to bulla tympanica in cat. The hypoglossal foramen was double in rabbit and single in cat. The foramen magnum is diamond in shape and notched dorsally in rabbit and rounded in cat. The basilar part of rabbit was longer, wider and increased in thickness rostrally, although in the cat, it had the same width along its whole length.

The interparietal bone

The interparietal bone was larger and longer in cat than in rabbit (Fig.1A and B). There was an intracranial extension of the interparietal bone termed osseous tentorium cerebeli, which was very large in cat and small in rabbit.

The sphenoid bone

The basisphenoid in rabbit was wedge shaped and directed rostodorsally with its narrow rostral part was lodged between the ptergyoid bones. In cat, the bone was directed rostrally and quadrate in shape (Figs.1E and F and 2A, B, and C). The basisphenoid bone was grooved dorsally in rabbit at its middle; there was a cranial pharyngeal canal which was absent in cat. The wing of the sphenoid bone in cat contained four foramina; rotundum, orbital, optic and ethmoidal foramen. In rabbit, the optic foramen was sited.

The presphenoid

The presphenoid bone (Fig. 1 E and F) in a cat was large, wedge shape separated from the pterygoid bone by the openings of the pterygoid canal. While in rabbit, this bone was located anterior to basisphenoid and anterio- ventral to the optic foramen.

The temporal bone

The temporal bone (Figs.1 A, B, E and F, 2 C and 3 A and B) composed of two parts; Pars petrosa and Pars squamosa. In rabbit, the temporal fossa was small, short, shallow (nearly flat) and it participated in the formation of the posterior part of the orbit. In cat, the fossa was longer, larger and deeper (concave) and located caudal to the orbit. The mandibular fossa articulated with the mandible forming temporomandibular joint. It was located more dorsally in rabbit than in cat (Figs. 2C and 3C and D). The retroarticular process situated caudal to the mandibular fossa in cat and absent in rabbit. The bulla tympanica of rabbit was smaller than in cat and its shape was seashell like but resembling an egg in cat. The external acoustic process was located at high level in rabbit and absent in cat. The external acoustic meatus positioned dorsal to the level of bulla tympanica in rabbit, and it placed rostodorsal to bulla tympanica in cat. The jugular and lacerum anterior foramina were larger in cat than that of rabbit. The carotid foramen was large and situated only in rabbit ventromedial to bulla tympanica (Fig. 1F). The internal acoustic meatus was larger and deeper in rabbit than in cat.

The parietal bone

The parietal bones participated in the formation of the roof of the cranial cavity in rabbit. But in cat, they were large and shared in the lateral boundary in addition to the roof of the cranial cavity (Fig. 1A and B).

The frontal bone

The main parts of the frontal bone were Squama, nasalis and Pars orbitalis. In rabbit, the frontal bone (Figs. 1A, B, 3A, B, C and D and 3A, B, E and F) was large, narrow, depressed dorsally and tapered anteriorly, forming inverted V-shape between the nasal bones. In cat, it was broad, short and lodged rostrally between the nasal bones and maxilla. The supraorbital process of rabbit was large, prominent. It extended anteriorly and posteriorly forming rostral and caudal supraorbital processes. It constituted the dorsal margin of the orbit. In cat, the supraorbital process was one part, long and it formed the caudal half of the orbital boundary.

The orbit

The orbit of rabbit was located laterally, slightly oval in shape and it contained one large optic foramen in the interorbital septum (Fig. 3A and C). The ethmoidal foramen was present just craniodorsal to the latter foramen. The orbit of cat was situated rostrally, rounded in shape and incomplete caudally, but closed by the orbital ligament (Figs. 3B and D and 3G and H).

The ethmoid bone

The ethmoid bone (Fig. 4 A and B) was consisted of cribriform plate and perpendicular plate in both species. The olfactory fossa of the cribriform plate was deep and small in rabbit but, long dorsovental and shallow in cat. The perpendicular plate in cat was longer, larger and situated in the caudal two third of the short nasal cavity, it was lodged in the sulcus vomeris ventrally. In rabbit, the forementioned plate was small, it did not reach to the level of vomer bone and it was restricted to the most caudal part of the long nasal cavity. So, the bony nasal septum was longer in cat than in rabbit which it was mostly cartilaginous.

II- Bones of the face

The lacrimal bone

The lacrimal bones (Figs. 1 B and 3 A and B) can be seen from lateral view of the skull and formed the rostromedial part of the orbit. In cat, it was smaller than that of rabbit. In both species, the bone had an opening for the lacrimal canal, which it located between this bone and the maxilla in rabbit.

The nasal bone

The nasal bones (Figs. 1 A and B and 3) were larger and longer in rabbit than that of cat. In rabbit, they had the same width along its whole length with notched rostral end. The nasal bones of cat were short, broad rostrally and narrow caudally with the rostral end was curved C -shaped. In both animals, the caudal end was fitted between the frontal bones. The nasal bone of rabbit was separated from the maxilla by the nasal process of the premaxilla. But in cat, the caudal two third of the bone was related to the maxilla and only the rostral third correlated to the nasal process of the premaxilla. The nasal surface of the nasal bone had a dorsal turbinate crest for attachment of dorsal turbinate bone. In rabbit, the latter crest was long and present in the middle of this surface of the bone, while in cat, the crest was short and ventrally situated.

The premaxilla

The premaxilla (Figs. 1E and F, 2, A, B, C, and D 3 and4 A and B) of rabbit was large, long and it constituted the rostral part of the lateral wall of the nasal cavity. The premaxilla of cat was short, small and it represented the lateral and ventral boundary of the rostral osseus nasal aperture. In rabbit, the body of the premaxilla had two pairs of upper incisors teeth, the interdental space was very long and the interincisive canal was absent. In cat, it had three pairs of upper incisors teeth and the interdental space was very short or absent. The nasal process of premaxilla of rabbit was long, narrow and it extended caudodorsally until it reached to the frontal bone and making deep nasoincisive notch. While in cat, it was short and extended slightly between the nasal bone and maxilla, forming a shallow nasoincisive notch. In rabbit, the palatine process of premaxilla was long, extended caudally and formed the rostral part of the basis of the hard palate. It united with its followed and surrounded its lateral and caudal sides by long palatine fissures. Meanwhile, the palatine process of premaxilla of cat was very

short, joined the other side and the palatine fissures were small and short. The premaxilla formed most the basis of the hard palate in rabbit while in cat it formed only one quarter.

The maxilla

The maxilla consisted of body and two processes; zygomatic and palatine. The body of the maxilla (Figs. 1E and F, 2 and 3) formed the lateral wall of the nasal cavity. In rabbit, the facial surface of the bone was perforated by several formina and had a facial tuberosity laterally and alveolar bulla appeared from the rostroventral part of the orbit. Three foramina were present medial to the maxillary tuberosity; maxillary, sphenopalatine and posterior palatine foramen. In cat, there were no facial nor maxillary tuberosities. The infraorbital canal was very short in cat and extended dorsoventrally but, it was long and extended in a craniocaudal manner in rabbit. The facial tuberosity prolonged caudally into zygomatic process and reached to the malar bone. The dorsal border of the maxilla related to the nasal process of premaxilla in rabbit and to the nasal bone in cat. The alveolar border of the maxilla carried two premolar, three molar and a small molar tooth present caudally in rabbit. However, in cat, this border had one pair of canine, three pairs of premolar and one molar teeth. The zygomatic process of maxilla directed upward and backward joined the zygomatic bone. The palatine process of maxilla of cat was larger and broader than that of rabbit. This process of rabbit had a pointed rostral end and it formed the caudal boundary of the palatine fissure. In both species, the nasal surface of the maxilla contained maxillary sinus and ventral turbinate crest. In rabbit, the bony lacrimal canal was long and crossed the whole length of latter surface of the maxilla at its middle part. In cat, the bony lacrimal canal was very short and situated ventrally in the caudal part of the nasal surface of the maxilla.

The turbinate bones

In rabbit, the ethmoturbinate bone was narrower than cat and it was restricted to the caudal half of the nasal cavity. In both species, it was situated below the dorsal turbinate bone and caudal to the ventral turbinate bone. The dorsal turbinate bone in rabbit was long, narrow and it had a middle bulged part while in cat; it was broad, short and placed dorsal to the ethmoturbinate. The ventral turbinate bone in rabbit was larger than cat and situated rostral to the ethmoturbinate and ventral to the dorsal turbinate bone. While in cat, it was very small and positioned ventral to the ethmoturbinate (Fig. 4A and B).

The cavities of the skull

The cranial cavity in general was larger in cat than rabbit. The rostral end of this cavity in cat was wide and separated from the frontal bone by the frontal sinus. But in rabbit, it was narrow and located in higher level just below the frontal bone due to the absence of the frontal sinus. The nasal cavity was longer in rabbit than cat. In cat, it was about half the length of the cavity in rabbit (Fig. 4 A and B).

The palatine bone

It consisted of Lamina horizontalis and perpendicularis (Fig. 1E and F). In rabbit, the horizontal part of the palatine bone represented eighth the bony part of the hard palate. In cat, it symbolized half of the latter. The bone contained the rostral palatine foramen at the level of the 3rd upper premolar tooth in rabbit and located between the level of the 2nd and 3rd upper premolar teeth in cat. The perpendicular part of the palatine bone was narrow in rabbit and broad in cat. In both species the perpendicular part formed the lateral boundary of the posterior nares.

The pterygoid bone

In rabbit, the pterygoid bone (Figs. 1E and F, 2A and 2C) was larger than that of cat. It was very thin, transparent bone. Also, it had two pterygoid processes, each of them enclosed a triangular cavity termed pterygoid fossa. At the base of the latter fossa, there was alar foramen. In cat, the pterygoid bone was small connected rostrally with the perpendicular plate of the palatine bone by pterygopalatine suture. Its ventral border carried long pointed hamulus pterygoideus. There were two foramina between the

pterygoid bone and the body of the sphenoid bone (foramina of pterygoid canal).

The zygomatic bone

In rabbit, the zygomatic bone (Figs. 1A and B, 2B, C and D and 3A and B) was smaller, shorter and narrower than that of a cat. It formed a small ventral part of the rabbit orbit and all the ventral boundary of the cat orbit. The zygomatic process of this bone in rabbit extended more caudally than the level of the same process of the temporal bone. In cat, the caudal end of this bone was bifid into dorsal frontal and ventral zygomatic processes. The zygomatic process was longer, narrower and joined the zygomatic process of the temporal bone.

The vomer bone

The vomer bone (Figs. 1E, 3G and H and 4A and B) was short, straight and located more rostrally in cat. It was long and curved rostroventrally in rabbit. The bone was broader in rabbit than cat while, the sulcus vomeris was larger in cat. The vomer bone ended at the level of the rostral end of the nasal bone in cat. However, in rabbit it was parallel to the caudal two third of the nasal bone. In rabbit, the bone can be seen from the large palatine fissure. While in cat, it is not seen from the short palatine fissure. In both species, the vomer bone not divided the posterior nares.

The mandible

The mandible (Figs. 4 C and D and 5A) composed of body and two rami. In rabbit, the body was longer, narrower and represented by two parts; incisive and molar. While in cat, the body was short, broad and thick. The lower jaw in rabbit carried one pair of lower incisor, two premolar and three molar teeth. But in cat, there were two pair of incisor, one pair of canine, two premolar and one molar tooth. The rabbit had no canine teeth so, there was long inter-dental space (diastema). The latter was very short or nearly absent in cat. In rabbit, Symphysis mandibulae was long and clear ventrally than dorsally. However, in cat it was short and well distinct dorsally and ventrally. The lateral surface of the body of the mandible of rabbit was perforated by several formina resemble that of the maxilla. The mental foramen of rabbit was only one and present laterally on the body, away from the incisor teeth, just in front the first premolar tooth. In cat, the latter foramen was two in number; rostral and caudal. These foramina located more forward than in the rabbit. A large retroalveolar foramen was found only in rabbit, just caudal to the last molar tooth. The ramus of the mandible in rabbit was thin, transparent plate of bone its lateral surface had sallow massetric fossa and the medial one contained deep pterygiod fossa and fovea pterygoidea. At the cranial margin of the latter fossa, the mandibular foramen was located. The ramus of the mandible of cat was thick and compact. It had deep massetric fossa and the mandibular foramen present caudally than in the rabbit. The angle of the mandible and the vascular notch were clear in rabbit. The condyloid process was large and present longitudinally in rabbit and transverslly in cat. The mandibular notch in rabbit was shallower than in cat and it is located rostral to the condyloid process. In rabbit, the coronoid process was thin and it bent medially above a deep groove on the rostral border of the ramus. While in cat, the coronoid process was thick and elevated dorsally. Distal to the condyloid process, positioned Collum mandibulae in rabbit and angular process in both species. The madibular space was narrow in rabbit and wide in cat.

The hyoid bone

In rabbit, the hyoid bone (Figs. 2A and C, 3D and 5B, C, D and E) located in the mandibular space. While in cat, it situated caudal to the mandibular space. The hyoid bone of rabbit was smaller than that of a cat. It consisted of four parts; body (basihyoideum), small cornua (ceratohyoideum), somewhat long thyrohyoideum and short lingual process. In cat, the bone formed of five parts. The lingual process was absent in cat. In addition to the parts of the bone in rabbit present middle (epihyoideum) and great (stylohyoideum) cornuae. Basihyoideum of rabbit was large and quadrilateral in shape. While in cat, it was narrow and small plate of bone (Fig. 5A, B, C, D and E). There was no articulation between the hyoid bone and the skull of rabbit, but in cat, the great cornua

had tympanohyoideum which articulate with the mastoid process of the temporal bone (Figs. 3D and 5C).

Paranasal sinuses:

The paranasal sinuses of rabbit were two in number; maxillary and ethmoidal sinuses. While in cat, there were three paranasal sinuses; frontal, sphenoidal and maxillary sinuses (Figs. 4A and B and 5F, G, H and I).

Table 1: Anatomical measurements of the skull and mandible between rabbits and cats

Anatomical measurements	Rabbits	Cats	P-Values
Skull length (cm)	8.98±0.18	10.05±0.13	0.003
Skull width (at the middle of the orbit)	4.02±0.11	6.47±0.12	0.000
Cranium length	4.27±0.17	4.52±0.11	0.281
Nasal cavity length	4.07±0.11	3.05±0.16	0.002
Nasal bone length	3.97±0.13	1.20±0.10	0.000
Orbit Height	1.47±0.08	2.07±0.11	0.005
Breadth	2.25±0.11	2.97±0.10	0.004
Base of the skull (length)	1.05±0.11	2.05±0.13	0.001
Foramen magnum			
Height	0.40±0.4	0.50±0.04	0.157
Breadth	0.62±0.03	0.70±0.01	0.111
External acoustic meatus			
Height	0.39±0.13	0.49±0.16	0.004
Breadth	0.39±0.02	0.78±0.16	0.000
Zygomatic arch length	4.10±0.12	5.52±0.28	0.008
Mandible length	7.02±0.16	6.57±0.13	0.081
Ramus of the mandible height	4.00±0.15	3.02±0.11	0.002
Mandibular space	3.05±0.17	4.97±0.17	0.000
Mandibular symphysis (length)	2.22±0.12	1.45±0.11	0.008
Weight of the skull (without mandible) (gm)	15.07±0.11	40.12±0.41	0.000
Weight of mandible (two rami) (gm)	8.02±0.13	10.00±0.12	0.000

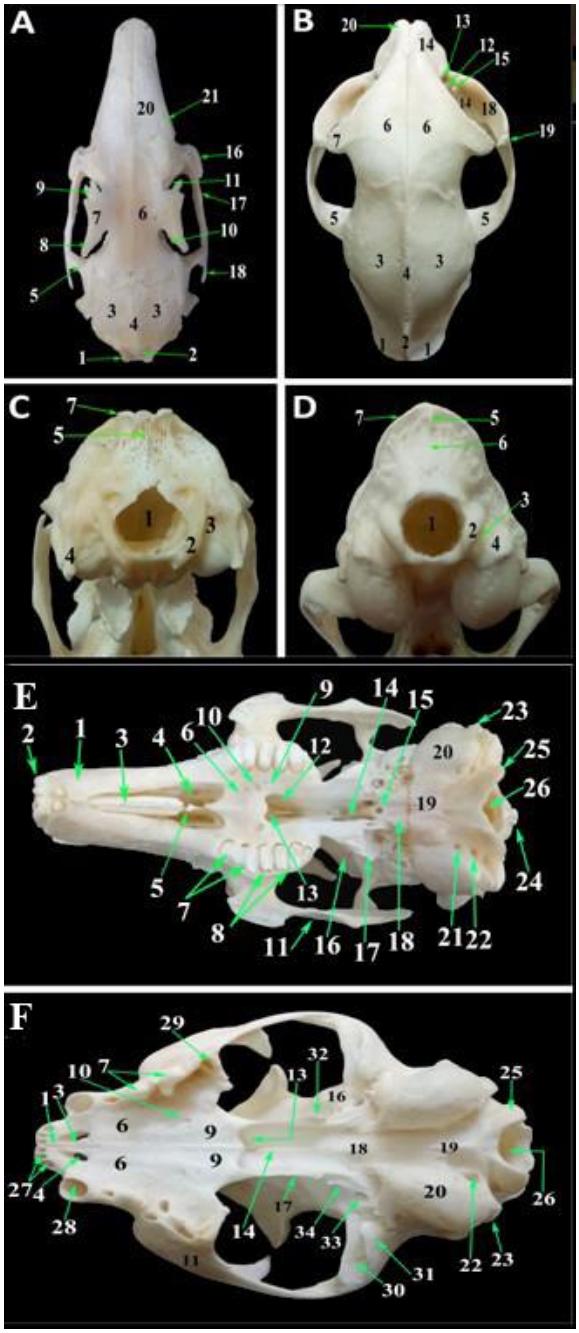

Figure 1: Photomacrographs of the dorsal aspect of the rabbit skull (A) and cat (B): 1- Squama occipitalis 2- Os interparietale 3-Os parietale 4- Crista sagittalis externa 5- Processus zygomaticus os temporal 6- Os frontale 7- Processus supraorbitalis 8- caudal branch of (7) 9- rostral branch of (7) 10- Incisura supraorbitalis caudalis 11- Incisura supraorbitalis rostralis 12- Os lacrimale 13- Foramen lacrimale 14- Maxilla 15- Foramen maxillare 16- Tuber faciale 17- Processus zygomaticus Maxilla 18- Os zygomaticum 19- Processus frontalis os zygomaticum 20- Os nasale 21- Processus nasalis os incisivum. Photomacrographs of the caudal aspect of the skull rabbit (C) and cat (D): 1- Foramen magnum 2- Condylus occipitalis 3- Fossa condylaris 4-Processus jugularis 5- Protuberantia occipitalis externa 6- Crista occipitalis externa 7- Crista nuchae. Photomacrographs of the ventral aspect of the skull of rabbit (E) and cat (F) 1- Corpus ossis incisivi 2- Dentes incisivi 3- Processus palatinus os incisivum 4- Fissura palatines 5- Vomer 6- Processus palatines Maxilla 7- Dentes premolars 8- Dentes molars 9- Lamina horizontalis os palatinum 10-Foramen palatinum majus 11- Os zygomaticum 12- Lamina perpendicularis os palatinum 13- Choanae 14- Os presphenoidale 15- Canalis craniopharyngeus 16- Ala presphenoidale 17- Os pterygoideum 18- Os basisphenoidale 19- Pars basilaris os occipital 20- Bulla tympanica 21- Canalis caroticus 22-Foramen jugulare 23- Processus jugularis 24- Protuberantia occipitalis externa 25- Condylus occipitalis 26- Foramen magnum 27- Alveoli incisivi 28- Alveoli caninus 29- Alveoli molars 30- Fossa mandibulae 31- Processus retroarticularis 32- Hamulus pterygoideus 33- Foramen alare caudalis 34- Foramen alare cranialis

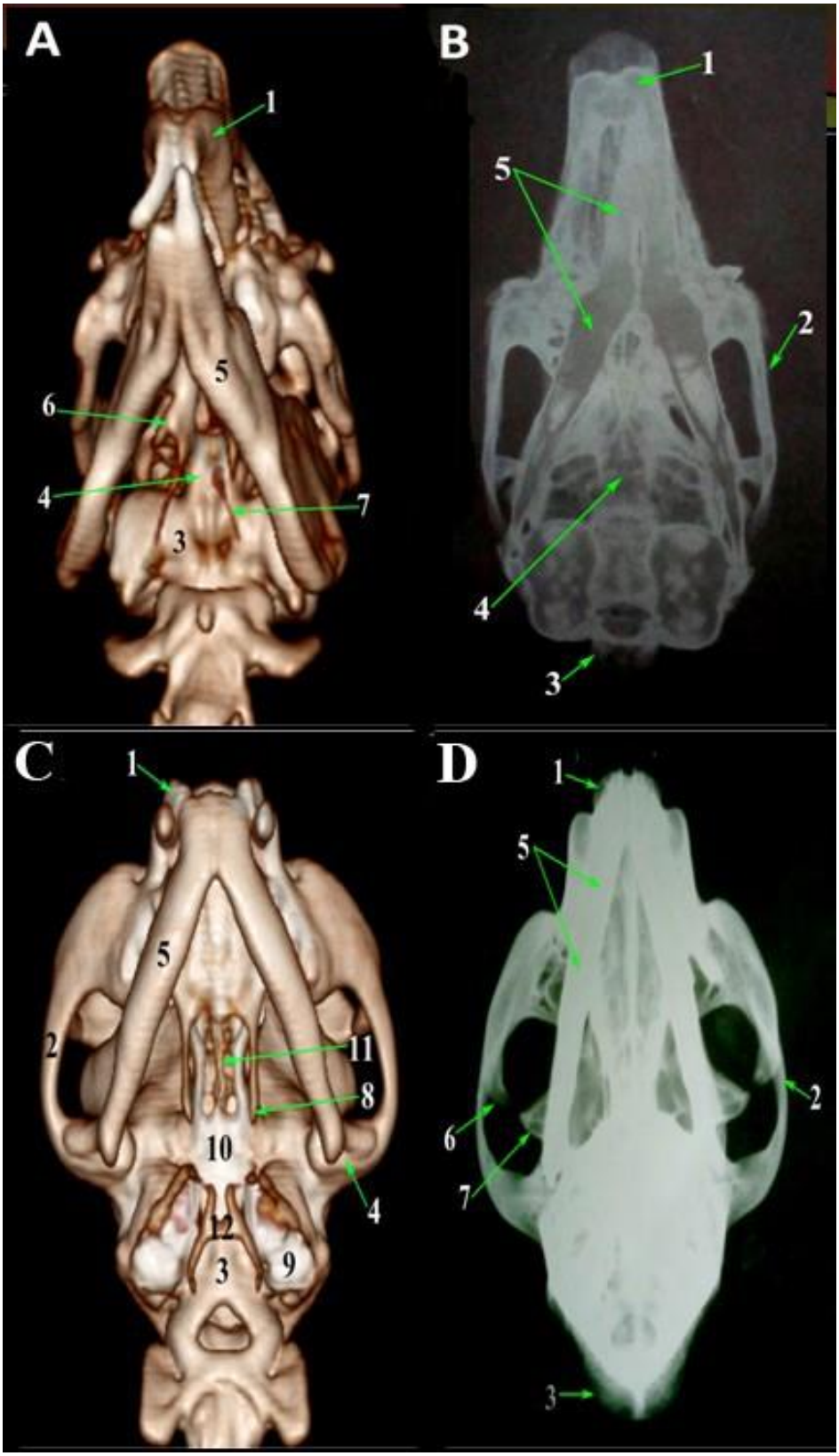

Figure 2: CT scan (A) and X- ray (B) images of the rabbit head (ventral view): 1- Os incisivum 2- Os zygomaticum 3- Os occipitale 4- Os basisphenoidale 5- Mandibula 6- Processus pterygoideus 7- Os hyoideum. CT (C) and X- ray (D) images of the cat head (ventral view): 1- Os incisivum 2- Os zygomaticum 3- Os occipital 4- Articulatio temporo-mandibularis 5- Mandibula 6- Processus frontalis os zygomaticum 7- Processus zygomaticus os frontale 8- Hamulus pterygoideus 9- Bulla tympanica. 10-Os basisphenoidale 11- Os presphenoidale 12- Os hyoideum

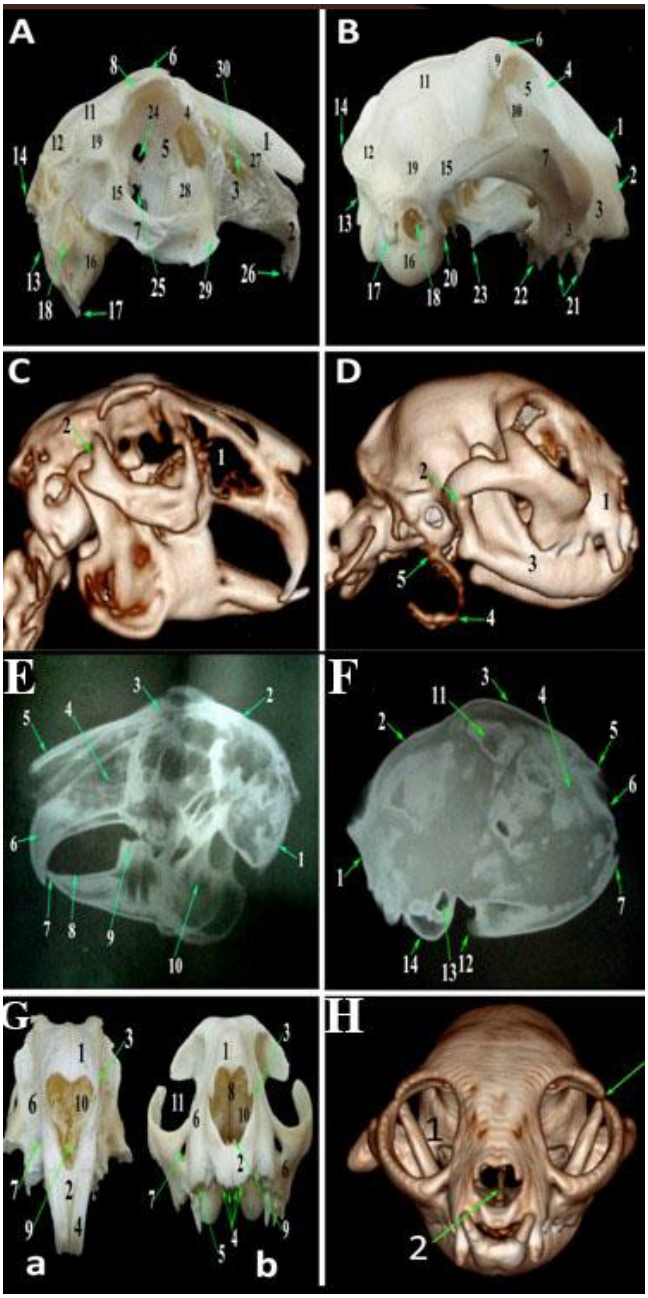

Figure 3: Photomacrographs of the lateral aspect of the rabbit skull (A) and cat (B): 1-Os nasale 2-Os incisivum 3-Maxilla 4-Os lacrimale 5-Orbita 6-Os frontale 7-Os zygomaticum 8-Processus supraorbitalis 9- Processus zygomaticus os frontale 10-Processus frontalis os zygomaticum 11-Os parietale 12-Os interparietale 13-Os occipitale 14-Crista nuchae 15-Processus zygomaticus os temporale 16-Bulla tympanica 17-Processus jugularis 18-Meatus acusticus externus 19- Pars squamosa os temporale 20-Processus retroarticularis 21- Dentes premolares 22- Dentes molars 23-Hamulus pterygoideus 24- Canalis opticus 25- Fissura orbitalis 26- Dentes incisivi 27- Processus nasalis os incisivum 28- Tuber maxillae29- (Tuber faciale 30- Sinus maxillaries. CT images of the rabbit (C) and cat (D) heads (lateral view) : 1- Maxilla (fenestrated in rabbit) 2- Articulatio temporomandibularis 3- Mandibula (fenestrated in rabbit) 4-Os hyoideum 5- Site of attachment of Os hyoideum with the skull. X- ray images of rabbit (E) and cat (F) heads (lateral view) demonstrating: 1- Os occipitale 2- Os parietale 3- Os frontale 4- Maxilla 5- Os nasale 6- Os incisivum 7- Dentes incisivi (Alveoli incisivi) 8- Margo interalveolaris 9- Dentes premolares (Alveoli premolars) 10- Fossa masseterica 11- Sinus frontalis 12- Processus angularis 13- Meatus acusticus externus 14-Bulla tympanica. Photomacrographs of the rostral aspect of the skull of a rabbit (G-a) and cat (G-b) showing: 1- Os nasale 2- Os incisivum 3-Processus nasalis os incisivum 4- Dentes incisivi (Alveoli incisivi) 5- Alveoli caninus 6- Maxilla 7- Foramen infraorbitale 8- Lamina perpendicularis os ethmoidale (Septum nasi osseum) 9- Vomer 10- Os conchae nasalis 11- Orbita. CT image of the cat head (H) (rostral view) demonstrating the orbit (1) which closed with orbital ligament (arrow) and vomer bone (2)

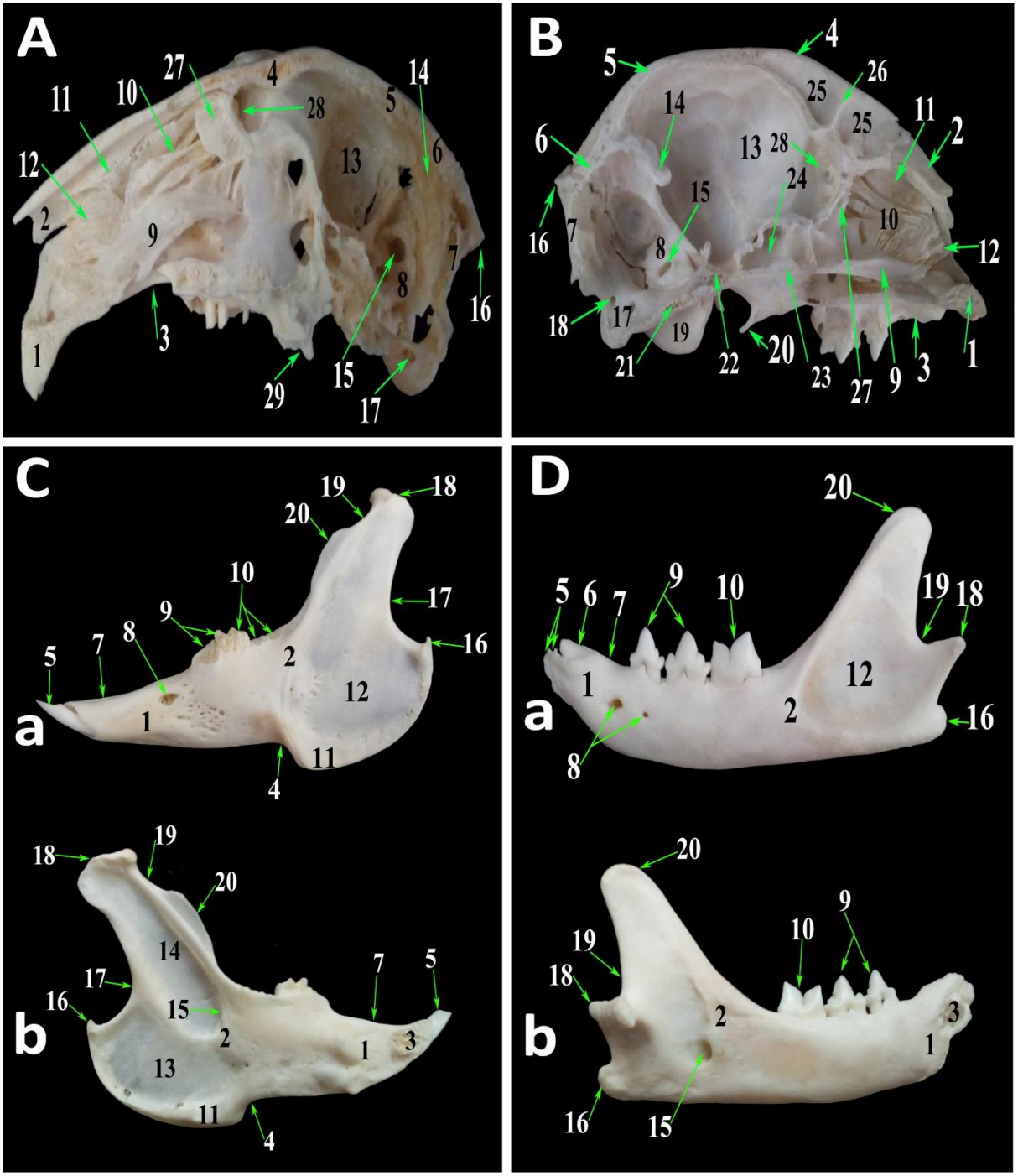

Figure 4: Photomacrographs of the sagittal section of the skull of rabbit (A) and cat (the perpendicular part of the ethmoid bone removed) (B): 1- Os incisivum 2- Os nasale 3- Maxilla 4- Os frontale 5- Os parietale 6- Os interparietale 7- Os occipital 8- Os temporal 9- Vomer 10- Ethmoturbinalia 11- Os conchae nasalis dorsalis 12- Os conchae nasalis ventralis 13- Cvaum crania 14-Tentorium cerebelli osseum 15- Meatus acusticus internus 16- Crista nuchae 17- Processus jugularis 18- Canalis hypoglossi 19- Bulla tympanica 20- Hamulus pterygoideus 21- Pars basilaris os occipital 22- Os basisphenoidale 23- Os presphenoidale 24- Sinus sphenoidalis 25- Sinus frontalis 26- Septa sinuum frontalium 27- Lamina perpendicularis os ethmoidale 28- Lamina cribrosa os ethmoidale 29- Processus pterygoideus. Photomacrographs of the mandible of rabbit (C) and cat (D) lateral (a) and medial (b) surfaces: 1- Corpus mandibulae 2- Ramus mandibulae 3- Symphysis mandibulae 4- Incisura vasorum facialium 5- Dentes incisivi 6- Alveoli caninus 7- Margo interalveolaris 8- Foramina mentalia 9- Dentes premolares 10- Dentes molars 11- Angulus mandibulae 12- Fossa masseterica 13- Fossa pterygoidea 14- Fovea pterygoidea 15- Foramen mandibulae 16- Processus angularis 17- Collum mandibulae 18- Processus condylaris 19- Incisura mandibulae 20- Processus coronoideus

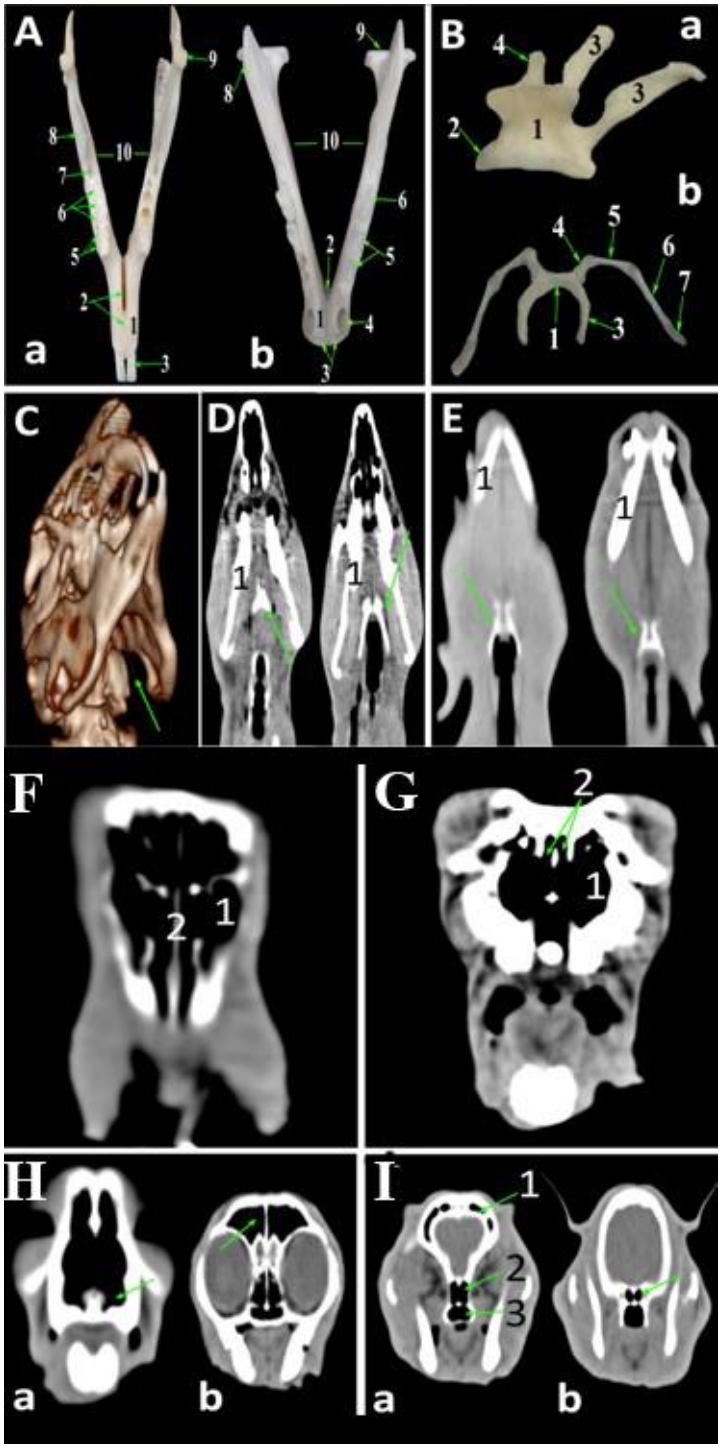

Figure 5: Photomacrographs of the mandible (rostral view) of rabbit (A-a) and cat (A-b) 1- Corpus mandibulae 2- Symphysis mandibulae 3- Dentes incisivi 4- Alveoli caninus 5- Dentes premolares 6- Dentes molars 7- Foramen retroalveolaris 8- Processus coronoideus 9- Processus condylaris 10- Mandibular space. Photomacrographs of the hyoid bone of rabbit (B-a) and cat (B-b) 1- Basihyoideum 2- Processua lingualis 3- Thyrohyoideum 4- Ceratohyoideum 5- Epihyoideum 6- Stylohyoideum 7- Tympanohyoideum. CT image of the rabbit head (D) (ventrolateral view) demonstrating the absence of articulation between the hyoid bone and the skull (arrow). CT images of the rabbit (D) and cat (E) heads (ventral view) demonstrating the hyoid bone at different levels (arrows) and Mandibula (1). CT image of the rabbit head (F) sinus maxillaries at anterior level (1) and septum nasi (2). CT image of the rabbit head (G) sinus maxillaries at posterior level (1) and sinus ethmoidales (2). CT images of the cat head (H) sinus maxillaries (a-arrow) and sinus frontalis at anterior level (b- arrow). CT images of the cat head (I) sinus frontalis at posterior level (a-1) which divided by septa sinuum frontalium, Sinus sphenoidalis (a-2), nasopharynx (a-3) and Sinus sphenoidalis at posterior level (b- arrow) which was divided by a median longitudinal partition

Discussion

The cranium was much longer than the facial region in leopard cat. The orbit and the cranial cavity were large in the domestic cat, which agreed with the same result in leopard cat (12). The head of Persian cats was characterized by a short face and open orbits (13, 14) which was in the same line with the current study. The shape of the skull was differed in the same species as quadrate in domestic rabbits and elongated flat in wild rabbits (15). In agreement with (16), the parietal bones in cat were large and they cover the most of the roof of the cranial cavity.

The features of the occipital bone in rabbit were confirmed by (17). The caudal aspect of the skull of cat was formed by the occipital bone, which it was triangular in shape, this finding correlated with (16) in Jungle cat. The jugular process was short and small and the foramen magnum was rounded in cat, this finding agreed with (18). The paracondylar process was sharper in leopard cat than in domestic cat (12). The supraorbital process of rabbit extended anteriorly and posteriorly forming rostral and caudal parts, which was similar to that observed by (17, 19, 20). The middle depression of the frontal bone in domestic cat was not observed in Jungle cat (16).

In the present study, the bulla tympanica of the rabbit was smaller than in the cat and its shape was a seashell like but an egg like in the cat. On the other hand (17, 20) described the tympanic bulla of the rabbit as a rounded structure. (21) added that the tympanic bulla of dog and cat extended in a lower level than the occipital bone. The mastoid process was short in cat and the jugular processes were longer in rabbit. The external acoustic process in rabbit appeared as a long tube, but in cat it was nearly absent or took the shape of a bony ring that came in accordance with (21) in cat. The bulla tympanica of cat was very large and was lodged caudolaterally which was similar to the leopard cat (12) meanwhile, the external acoustic meatus found dorsally but more rostrally in the present work and at the dorsal border of bulla tympanica in leopard cat.

The zygomatic process of the zygomatic bone in rabbit extended more caudally than the level of the same process of the temporal bone in addition to the absence of the retroarticular process that made a difference in the temperomandibular joint from that of dog and cat that agreed with the results of (17,19). There were two optic canals in cat and single optic foramen in rabbit, similar observation recorded by (17, 19). The incisive bones were large, long in the rabbit and characterized by very long nasal process, had two pairs of upper incisor teeth, long interdental space and absence of the interincisive canal and the canine tooth. This observation was characteristic to the skull of the rabbit as an herbivorous animal and simulated the results of (17, 19). In rabbit, the palatine processes and fissure of the premaxilla were long, so the former composed most the basis of the hard palate. On the other hand, the palatine processes in cat were very short formed only one quarter of the basis of the hard palate and the palatine fissure was extremely short and small. Similar descriptions were evidenced by (17). In contrast to the current study, (15) in cat demonstrated that, the horizontal part of the palatine bone showed two elevations on either side of the median plane.

The infra-orbital foramen in rabbit was situated at the level of the first premolar tooth, similar result was recorded by (22, 23). The facial crest was absent in domestic cat and the infraorbital canal was very short as in dog and cat (24, 25) in contrary to the other domestic animal, this crest was clear (18). The basisphenoid bone of rabbit was characterized by the presence of cranial pharyngeal canal, this foundation agreed with (17). This canal had different names in the previous studies, foramen cavernosum (19). In the present study, the mandible of the rabbit was characterized by lack of canine teeth, long diastema and symphysis mandibulae, perforated lateral surface, presence of retroalveolar foramen and peculiar condyloid and coronoid processes. This result was similar to that recorded by (17, 26). The lateral surface of the mandibular ramus located had masseric fossa and there were several mental foramina at the rostrolateral aspect of the mandible in, it

correlated (16). The hyoid bone of rabbit lacked epihyoideum and stylohyoideum as reported by (17, 27, and 28). So, there was no articulation between the hyoid bone and the skull of rabbit. The latter authors found muscular attachment between the bone and the skull. On the contrary, (27) in rabbit referred to the articulation between the ceratohyoideum and the occipital bone.

The paranasal sinuses of rabbit were maxillary and ethmoidal sinuses, meanwhile, (29, 30) stated that maxillary and dorsal conchal sinuses were existed. On the contrary, (31, 32, 33) recorded only the maxillary paranasal sinus. The ethmoidal sinus of the rabbit in the present work was confirmed by the observations of (34, 35, 36). The paranasal sinuses of the cat were frontal, sphenoidal and maxillary sinuses, this finding was agreed with that of (36). This study can be applied in a veterinary clinic of cat and rabbit such as regional anesthesia during the treatment of head injury and dental extraction.

Conclusion

There were great differences between rabbit and cat in the bones forming the cranial cavity, nasal cavity, orbit, hard palate and jaws. Also, numerous variations recorded in the hyoid bone of the two animals; in its position, parts and articulation between this bone and the skull. The paranasal sinuses varied in rabbit than cat; in its number and bones contained them. So, the skull of each animal gave it complete adaptation with the nature of rabbit as herbivores and cat as carnivorous animals.

Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

IMPACT OF ALTERING DIETARY OMEG 6 TO OMEGA 3 FATTY ACIDS RATIO ON GROWTH PERFORMANCE, CARCASS COMPOSITION, HEMATO-BIOCHEMICAL PARAMETERS AND ABSORPTIVE CAPACITY OF THE INTESTINE OF NILE TILAPIA

Eldsokey Nassef[1]*, Elsayed Hegazi[1], Ahmed Sherif[2], Mohamed Elshamy[2], Abdelnasser Bakr[1], Tarek k. Abouzed[3]

[1]Nutrition and Clinical Nutrition Department, Faculty of Veterinary Medicine, Kafrelsheikh University, Kafrelsheikh, Egypt, [2]Agriculture Research Center, Animal Health research Institute, Kafrelsheikh, Egypt, [3]Biochemistry Department, Faculty of Veterinary Medicine, Kafrelsheikh University, Kafrelsheikh, Egypt.

*Corresponding author, E-mail: dsokeynassef@yahoo.com

Abstract: The present study investigated the influence of altering omega 6 to omega 3 fatty acids (FA) ratio on growth, hemato- biochemical parameters and absorptive capacity of Nile tilapia. One hundred and fifty fingerlings (25.1±0.3 g), were assigned into 3 groups (5 replicates each) and fed on iso-caloric and iso-nitrogenous diets. Diets were supplemented with fish and soy oil to create 3 different ratio of omega 6 to omega 3 FA, namely, 0.91, 2.85 and 9.3 parts of omega 6 to 1 part of omega 3 (R1, R3 and R9, respectively). Increasing the ratio from R1 to R9 significantly decreased average daily gain and increased feed conversion ratio. Also this alteration decreased ash content in the carcass on the expense of ether extract. Number of erythrocytes, packed cell volume, hemoglobin concentration were significantly increased in fish fed R1. Feeding R9 increased heterophils and decreased lymphocytes as compared with R1 and R3. Fish fed R1 significantly had high serum protein and serum antibacterial activity (47.6%) than R9 (43.5%) and R3 (44.9%). Feeding R1 significantly decreased serum triglycerides, cholesterol and creatinine. Feeding R3 and R1 enhanced absorptive capacity of jejunum villi more than the group fed R9. Increasing the ratio of omega 6 to omega 3 FA from R1 to R9 decreased growth performance through decreasing absorptive capacity of jejunum villi. However, Feeding R3 had a positive effect on growth performance and absorptive capacity of the intestine of Nile tilapia.

Key words: fish oil; growth performance; intestinal villi; Nile tilapia; soybean oil

Introduction

Fish oil is the main lipid source used in the formulation of commercial aqua feeds. The continuous little global production of fish oil into 2030 (1), the highly variable cost have forced intensive research activities to evaluate alternative lipid sources (2). Vegetable oils are important lipid sources with low cost and wide availability. To our knowledge, there was scarce information on the assessment impact of altering omega 6 to omega 3 fatty acids (FA)

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ratio on antibacterial activity and absorptive capacity of the intestine of Nile tilapia.

Tilapia is the most common type of fish used in aquaculture where its production reaches to 66.8% of total aquaculture production (3). Fish meals and oils are essential part of diet composition in tilapia as a source of protein and lipid (2). Dietary lipids are important for regular growth and modulating immune responses in mammals, fish, sea cucumber, and shrimps (4). Because the expansion of aquaculture, it is expected that the requirements of fish meals and oils by the aquaculture sector will increase, but there are troubles of the high cost and limiting availability of fish meals and fish oils (1; 4).

We hypothesized that vegetable oils could replace fish oil due to its low cost and wide availability. However, the difference in the ratio of omega 6 to omega 3 FA can impact fish health and disease resistance. Lipids affect on the immune system by influencing the phospholipids of immune cell membranes, membrane-associated signaling molecules (eicosanoids) and receptor sites (5). Many previous studies used fish meal as a protein supplement when evaluating vegetable oils which supplied a certain amount of long chain poly unsaturated fatty acids. This study aimed to investigate the influence of altering omega 6 to omega 3 FA on growth performance, hematological and biochemical parameters, carcass composition and antibacterial activity of Nile tilapia and assessment of the absorptive capacity of duodenum, jejunum and ilium.

Materials and methods

Fish and experimental design

One hundred and eighty fingerlings of Nile Tilapia (25.1±0.3 g) were purchased from commercial farm in Kafrelsheikh governorate and were acclimatized to tank conditions for 2 weeks. One hundred and fifty apparently healthy fingerlings of homogenous size were selected and randomly distributed into 3 groups (5 replicates each). Each replicate contained 10 fish per tank of 50-L which was equipped with continuous aeriation, inlet and outlet. The procedures have been approved by the Institutional Aquatic Animal Care and Use Committee, Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University.

Three experimental diets were supplemented with fish and soy oils to create 3 different ratio of omega 6 to omega 3 FA. The first ratio (R1) was of 0.91 parts of omega 6 to 1 part of omega 3 FA. The second ratio (R3) was of 2.85 parts of omega 6 to 1 part of omega 3 FA. The final ratio (R9) was of 9.3 parts of omega 6 to 1 part of omega 3 FA. The diets were nearly similar in protein and energy (Table 1). The ingredients were ground to pass through a sieve of 1 mm and mixed for 20 min. the oil was added with the continuous mixing. Distilled water was added to the diets till forming soft dough. The diets were pelleted in a laboratory pellet mill through 2 mm diameter die. Pellets were dried in an oven at 60 °C for 2 h and freshly used. The experimental period lasted for 8 weeks. The fish were fed twice daily at 8.00 and 15.00 h at a level of 4% of body weight for 2 weeks; 3.5% from 3 to 4 weeks then 3% from 5-8 weeks. Feed refusal was recovered after feeding and dried using oven at 60 °C for 2 h then subtracted from the offered feed.

Growth trial

Growth parameters were determined according to the following equations:

Body weight gain (BWG, g $fish^{-1}$) = Final BWT - Initial BWT;

Average daily gain (ADG, g) = BWG/duration of the experiment (56 days);

Feed intake (FI, g $fish^{-1}$, 56 days) = (offered feed–feed refusal recovered and dried)/no of fish

Feed conversion ratio (FCR) = FI (g)/ BWG (g);

Protein efficiency ratio (PER) = BWG (g)/dry protein intake (g);

Protein retention (PR, %) = (protein gain, g/protein intake, g) ×100

Energy retention (ER, %) = (energy gain, kcal)/energy intake, kcal) ×100

Chemical analysis of feed and fish

Representative feed samples were ground through 1mm screen (Cyclotec, Foss Sweden). The ground samples were analyzed for crude

protein based on Kjeldahl method and ether extract (Ankom Technology method), according to (6). NFE was calculated according to the following formula: NFE= 100- (moisture + crude protein + ether extract + ash + crude fiber). Lysine, methionine, calcium and available phosphorus were calculated based on feed composition tables in (2).

At the end of the experiment, 3 fish were randomly collected from each replicate; dried in oven at 60 °C for 48 h. Dry matter and moisture were determined. The dried samples were analyzed for crude protein, ash and ether extract according to (6).

Blood analysis and immune response assay

At the end of the experiment, pooled blood sample was collected from 5 fish per tank via the caudal vein into heparinized disposal syringe for complete blood count (CBC). Further blood sample without anticoagulant was collected, centrifuged at 3000 rpm for 15 min to obtain the serum and stored at -20 °C for the biochemical analysis. Blood film was prepared according to (7). Differential leukocyte count was calculated according to (8). Hematocrit, erythrocytes, white blood cell (WBCs) and hemoglobin (HB) were examined according to (9). Serum protein and albumin were calorimetrically measure based on (10, 11) respectively. Serum globulin was determined by subtracting the concentration of albumin from protein. The activity of alanine amino transaminase (ALT) and aspartate amino transaminase (AST) was determined as previously described (12).

Histopathological analysis

Intestinal tissue specimen were obtained at the end of experiment to examine absorptive capacity of villi. The samples were immersed in formalin 10%, embedded in paraffin, stained with haematoxylin and eosin (H & E) and documented photographically with a digital camera (DCM 130E/1.3 megapixels, CMOS Software Scopephoto, China) connected to a light microscope (Leica).

Serum antibacterial test

Serum bactericidal activity was done following the procedure of (13). Equal volumes (100µl) of serum and A. hydrophila bacterial suspension $2x10^8$ (CFU) were mixed and incubated at 25 ^oC for 1 h. Blank control was made by replacing serum with sterile phosphate buffer saline (PBS). The mixture was then diluted with sterile PBS at a ratio of 1:10. The diluted mixture (100µl) was plated on blood agar and incubated at 37 ^oC for 24 h. The number of viable bacteria was determined by counting the colonies grown on the agar plates.

Statistical analysis

Analysis of variance (ANOVA) was done for all data using the SPSS program (14). Duncan's multiple range was used to determine the significant difference among means at $P<0.05$.

Results

Growth performance

As shown in Table 2, the final weight, BWG and ADG were significantly higher in fish fed R1 than those fed R9. But, fish fed R3 had nearly similar growth parameters to fish fed R1. In sum, ADG of fish fed R1 was the greatest followed by R3 then R9, There was no significant difference in feed intake of fish fed either R1 or R3 or R9. But, FCR was significantly improved in fish fed R1 followed by R3 then R9. PER and PR were greater in fish fed R1 than those fed the other diets. In contrast, ER was significantly greater in fish fed R9 followed by R3 then R1.

Body chemical composition

As illustrated in Table 3, there is no significant differences between different groups in dry matter, moisture and crude protein. Feeding omega 3 FA (R1) significantly increased mineral density in the skeleton as compared to R9. In contrast, feeding R1 decreased fat deposition in the whole body of fish. Whereas, Feeding R3 still had the same effect of R1 on ash and ether extract concentrations.

Hematological parameters

As illustrated in Table 4, feeding R1 increased RBCs count, Hb concentration and PCV as compared to R3 or R9. Feeding R1, R3 increased WBCs than R9. However feeding R9 increased heterophil but decreased lymphocyte when compared to the other groups, while monocytes, eosinophil and basophil still unchanged among the dietary treatments.

Biochemical analysis

As shown in Table 5, although the ALT and AST were significantly lower in fish fed R1. The values of further groups were within the acceptable range. The same trend was recorded in creatinine analysis. Serum globulin was markedly high in fish fed R1. Subsequently, it elevated serum protein because the serum albumin was nearly similar among all treatment groups. Serum triglyceride and cholesterol were significantly lesser in fish fed R1 as compared to R9 or R3 groups. No significant difference in cholesterol concentration between R9 and R3 groups was detected. Feeding R1 increased HDL and decreased LDL than the other two groups. On the other hand, fish fed R3 had a higher HDL and a lower LDL than R9 group.

Antibacterial activity

As shown in Table 6, the result indicated a significant enhancement of the immune status of group fed R1 but differences in other groups were in acceptable range which showed no threat on fish life.

Table 1: Physical and chemical composition (%) of the diets for Nile tilapia.

Items	Dietary treatments [a]		
	R1	R3	R9
Corn gluten meal	10	10	10
Soybean oil	0	2.5	5
Fish oil	5	2.5	0
Corn grain	30.0	30.0	30.0
Dehulled soybean meal	51.6	51.6	51.6
Monocalcium phosphate	1.57	1.57	1.57
Limestone	0.68	0.68	0.68
Salt	0.35	0.35	0.35
Methionine, DL	0.2	0.2	0.2
Premix b	0.1	0.1	0.1
Antimycotoxin	0.2	0.2	0.2
Vitamin C	0.1	0.1	0.1
Binder	0.2	0.2	0.2
Chemical composition of dried pelleted diets			
Digestible energy, Kcal/kg c	3325	3313	3302
Crude protein %	32.23	32.23	32.23
Lysine %	1.73	1.73	1.73
Methionine %	0.75	0.75	0.75
Crude fat %	6.93	6.93	6.93
Calcium %	0.7	0.7	0.7
Available phosphorus %	0.45	0.45	0.45

[a] Treatments represent the ratio between omega 6 to omega 3 FA in the diet. R1 is a ratio of 0.91 parts of omega 6 to 1 part of omega 3; R3 is a ratio of 2.85 parts of omega 6 to 1 part of omega 3; R9 is a ratio of 9.3 parts of omega 6 to 1 part of omega 3. [b] Premix: Magnesium 40mg ; Manganese 10mg ; Zinc150mg ; Iron 30mg ; Copper 5mg ; Iodine 5mg ; Cobalt 0.005mg ;Selenium 0.1mg; vit A 5500 UI; vit D3 1000UI; vit E 50mg ; vit B1 20mg ; vit B2 20mg ; vit B6 20mg ; vit B12 0.02 mg ; niacin 100mg; vit K3 10mg ; biotin 0.1mg ;folic acid 5mg. [c] Calculated according to (2).

Table 2: Impact of altering omega 6 to omega 3 FA ratio on growth performance and feed utilization

	Dietary treatments [1]		
Items	R1	R3	R9
Initial weight	25.23±0.18	25.17±0.03	25.40±0.10
Final weight	54.96±0.6 [a]	51.12±2.84 [ab]	50.27±3.1 [b]
Body weight gain	29.3±0.90 [a]	25.96±2.87 [ab]	24.87±1.1 [b]
Average daily gain	0.52±0.02 [a]	0.47±0.05 [ab]	0.44±0.06 [b]
Feed intake	57.16±0.72	55.2±1.41	54.4±1.10
Feed conversion ratio	1.95±0.04 [a]	2.1±0.17 [ab]	2.24±0.21[b]
Protein efficiency ratio	1.59± 0.01[a]	1.45± 0.12 [b]	1.42b± 0.13
Protein retention	25.8± 1.5 [a]	23.55± 0.1 [b]	23.19± 1.05 [b]
Energy retention	45.6± 2.28 [c]	48.78± 0.16 [b]	50.05± 0.6 [a]

Means ± SE with different letter within the same raw are significantly different at P<0.05. [1] Treatments represent the ratio between omega 6 to omega 3 FA in the diet. R1 is a ratio of 0.91 parts of omega 6 to 1 part of omega 3; R3 is a ratio of 2.85 parts of omega 6 to 1 part of omega 3; R9 is a ratio of 9.3 parts of omega 6 to 1 part of omega 3.

Table 3: Impact of altering omega 6 to omega 3 FA ratio on body chemical composition (on dry matter basis)

	Dietary treatments [1]		
Items	R1	R3	R9
Dry matter	28.37±0.56	29.01±0.29	28.97±0.88
Moisture	71.63±0.58	71.0±0.29	71.03±0.88
Crude protein	55.73±0.15	55.7±1.7	55.9±0.11
Ether extract	21.8±1.25 [b]	23.1±1.1 [a]	23.8±0.4 [a]
Ash	19.6±1.98 [a]	17.98±1.9 [b]	16.46±0.78 [c]

Means ± SE with different letter within the same raw are significantly different at P<0.05. [1] Treatments represent the ratio between omega 6 to omega 3 FA in the diet. R1 is a ratio of 0.91 parts of omega 6 to 1 part of omega 3; R3 is a ratio of 2.85 parts of omega 6 to 1 part of omega 3; R9 is a ratio of 9.3 parts of omega 6 to 1 part of omega 3.

Table 4: Impact of altering omega 6 to omega 3 FA ratio on hematological parameters

	Dietary treatments [1]		
Items	R1	R3	R9
RBCS	2.57±0.09 [a]	2.01±0.08 [b]	1.88±0.1 [b]
WBCS	121.9±0.3 [a]	119±0.7 [a]	116.3±1.4 [b]
Heterophil	25±0.6 [b]	26.3±0.6 [b]	30±0.6 [a]
Monocyte	4.7±0.3	3.7±0.9	5.3±0.7
Lymphocyte	66.7±0.3 [a]	65.7±1.2 [a]	60.7±0.7 [b]
Eosinophil	3.7±0.3	4.3±0.6	4±0.6
Basophil	0.3±0.3	0.3±0.3	0.7±0.3
Hb	8.7±0.26 [a]	7.14±0.14 [b]	6.66±0.1 [b]
PCV	26.1±0.8 [a]	22.1±0.4 [b]	21±0.03 [b]

Means ± SE with different letter within the same raw are significantly different at P<0.05. [1] Treatments represent the ratio between omega 6 to omega 3 FA in the diet. R1 is a ratio of 0.91 parts of omega 6 to 1 part of omega 3; R3 is a ratio of 2.85 parts of omega 6 to 1 part of omega 3; R9 is a ratio of 9.3 parts of omega 6 to 1 part of omega 3.

Table 5: Impact of altering omega 6 to omega 3 FA ratio on biochemical parameters and antibacterial activity

	Dietary treatments 1		
Items	R1	R3	R9
ALT (µ/l)	34.3±1.3 [c]	41.2±0.8 [b]	47.3±0.9 [a]
AST (µ/l)	106.3± 2.96 [b]	112.5±1.3 [b]	130±6.4 [a]
Creatinine (mg/dl)	0.89±0.05 [c]	1.47±0.09 [b]	1.86±0.14 [a]
Total protein (g/dl)	6.6±0.15 [a]	5.7±0.2 [b]	5.3±0.09 [b]
Albumin (g/dl)	4.1±0.06	4.07±0.02	4±0.03
Globulin (g/dl)	2.5±0.15 [a]	1.6±0.3 [b]	1.2±0.1 [b]
Triglycerides (mg/dl)	149.5±1.9 [c]	295.5±1.45 [b]	303.3±2 [a]
Cholesterol (mg/dl)	98.5±1.6 [b]	118.2±0.7 [a]	123.5±2.4 [a]
HDL (mg/dl)	56.2±0.7 [a]	51.3±0.9 [b]	48.6±0.6 [c]
LDL (mg/dl)	2.87±0.3 [c]	11.5±3.3 [b]	20±0.8 [a]
Serum antibacterial (%)	47.6 ±0.6 [a]	44.9 ±0.4 [b]	43.5 ±0.8 [b]

Means ± SE with different letter within the same raw are significantly different at P<0.05. [1]Treatments represent the ratio between omega 6 to omega 3 FA in the diet. R1 is a ratio of 0.91 parts of omega 6 to 1 part of omega 3; R3 is a ratio of 2.85 parts of omega 6 to 1 part of omega 3; R9 is a ratio of 9.3 parts of omega 6 to 1 part of omega 3

Table 6: Impact of altering omega 6 to omega 3 FA ratio on mucosal and villi parameters of the intestine

Dietary treatments	Duodenum		Jejunum		Ilium	
1	length	width	length	width	length	width
R1	541±33	141±13	938±35 [a]	115±4 [b]	570±37 [a]	149±23
R3	510±30	139±13	769±27 [b]	113±6 [b]	187±23 [b]	133±12
R9	539±31	150±10	901±37 [a]	142±10 [a]	405±23 [c]	140±10

Means ± SE with different letter within the same column are significantly different at P<0.05. [1]Treatments represent the ratio between omega 6 to omega 3 FA in the diet. R1 is a ratio of 0.91 parts of omega 6 to 1 part of omega 3; R3 is a ratio of 2.85 parts of omega 6 to 1 part of omega 3; R9 is a ratio of 9.3 parts of omega 6 to 1 part of omega 3

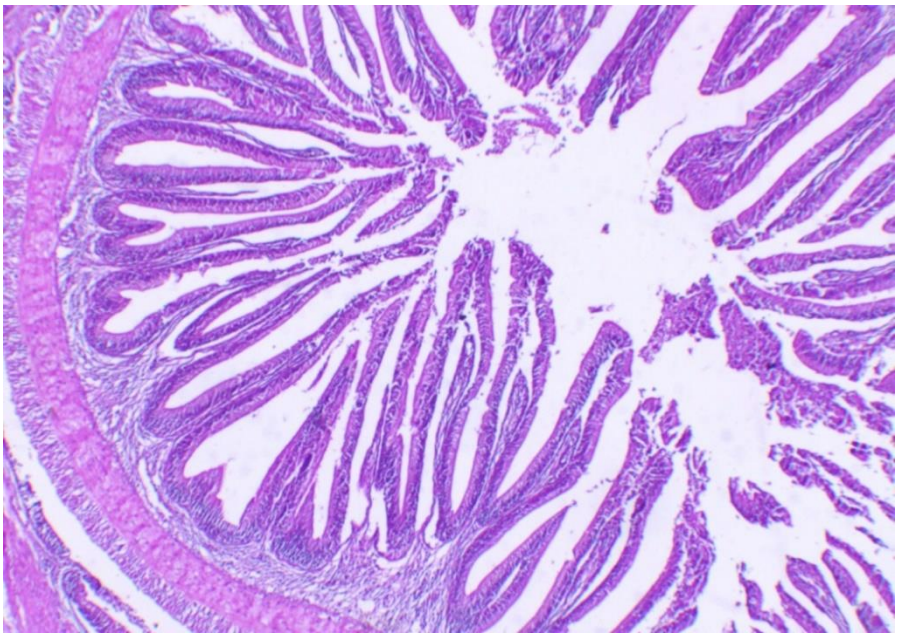

Figure 1: Jejunum of fish supplemented with diet containing fish oil showing normal long villi lined with normal epithelium, (H&E, X200)

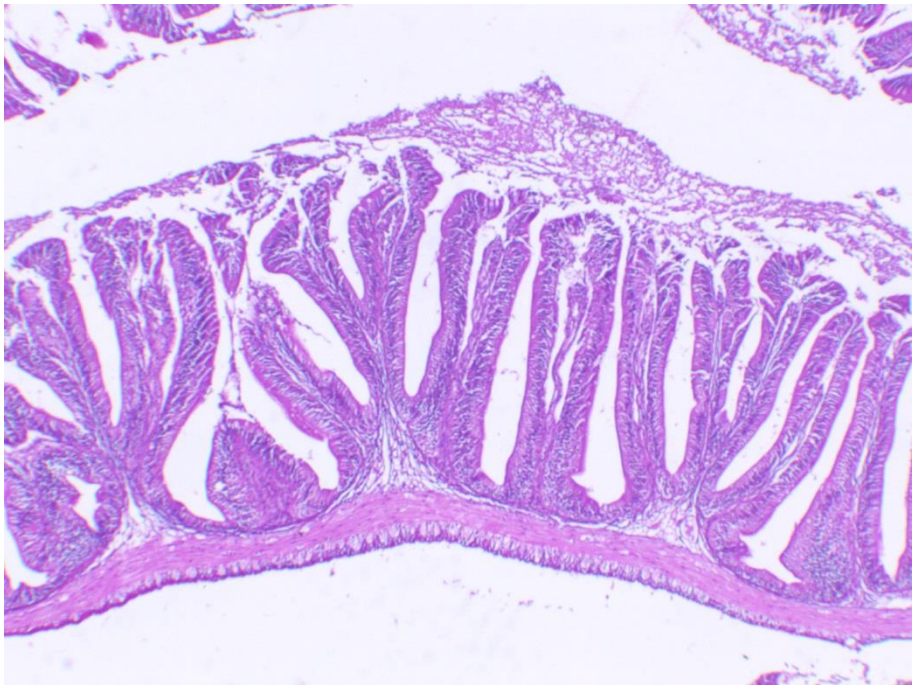

Figure 2: Jejunum of fish supplemented with diet containing vegetable oil showing normal villi, (H&E, X200)

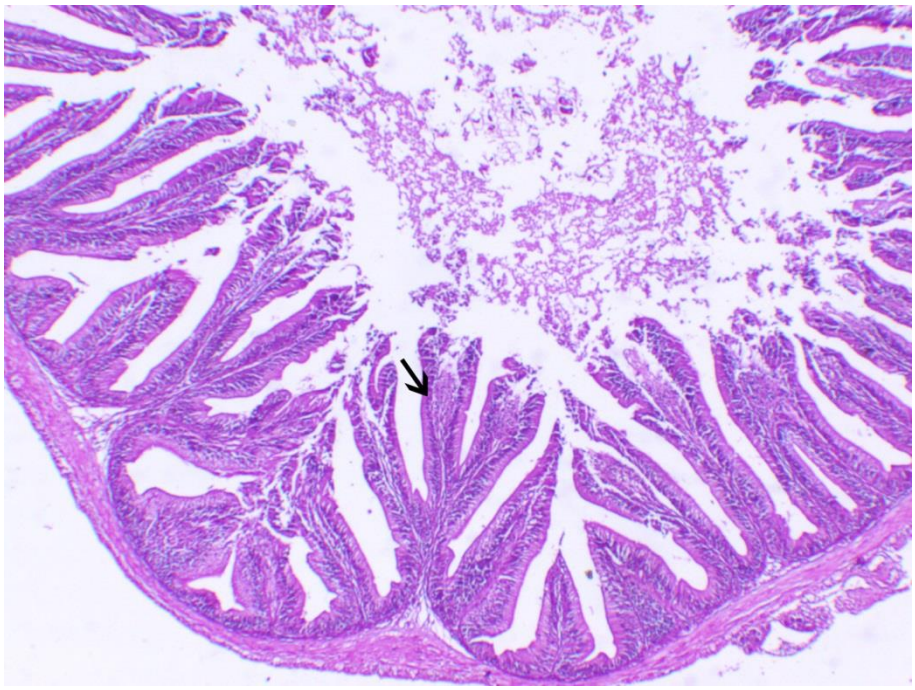

Figure 3: Jejunum of fish supplemented with diet containing a mix of fish and soybean oils showing most of villi length was similar to group 1 with increase branches (arrow), (H&E,X200)

Histopathological analysis

Increasing ratio of omega 6 to omega 3 FA from R1 to R9 decreased length of jejunum and ilium villi. Feeding R3 improved the absorptive capacity of intestinal villi as similar to the group fed R1. Jejunum of fish fed R1 showing normal long villi lined with normal epithelium (Fig. 1). Jejunum of fish fed R9 showing normal villi but

was shorter than the other groups (Fig. 2). Jejunum of fish fed R3 showing most of villi length was similar to fish fed R1 with increase branches and width (Fig. 3). The effect of dietary treatments on duodenum histopathology was not significant (Table 6).

Discussion

The result of the present study revealed that the fish fed a diet containing a blend of omega 6 and omega 3 FA (R3) had similar BWG to the group fed more omega 3 FA (R1) but higher than that fed omega 6 FA (R9). Similarly, (15) found that the final weight was lower when increasing dietary omega 6 FA by using 6% soy oil in fish diet for 6 weeks. Also, a recent study denoted that increasing omega 6 to omega 3 FA ratio for 8 weeks depressed growth of tilapia (16). (17) who reported that a moderate increase of omega 6 FA by using 4.5% SO in replacement of FO in hybrid tilapia diets for 10 weeks, found that all groups had similar final weight. Also, (18) investigated a moderate omega 6 to omega 3 ratio in red hybrid tilapia diets for 5 months and detected no significant difference in either the growth performance or feed efficiency. Some studies have designated that both omega-3 and omega-6 FAs are indispensable for tilapia (2). These results implied that the presence of moderate dietary levels of omega 6 FA may spare a part of the requirement for omega 3 FA series.

This study revealed that feeding R1 reduced body fat content. This might be due to the high phospholipid content of fish oil than the soy oil. Lipid rich in omega 3 PUFA is reported to impede the fat synthesis and to reduce the deposition of these FAs in the liver and whole body of fish (19). With regard to phospholipids, (20) found that it decreased total lipids of whole body of rainbow trout as compared to a mixture of olive and linseed oils, also, (21) stated that omega 3 FA decreased lipid concentration in rainbow trout than the omega 6 FA. On the other hand, feeding R1 increased mineral deposition in the skeleton. The relation between type of FA and mineral metabolism in fish is less investigated. In some studies on European sea bass, (22) demonstrated that EPA and DHA of marine origin supported vertebral and cephalic growth with less deformities. In terrestrial animals, phospholipid facilitated cartilage mineralization and stimulated insulin like growth factor production which activate bone formation and matrix production. On the other hand, prostaglandins E2 derived from arachidonic acid is a powerful stimulator of bone resorption (23).

These results clarify that feeding R1 improved general health condition of Nile tilapia as indicated by increasing HB concentration, PCV and RBCs count which positively affected the growth performance. Blood cells reflect dietary changes due to their fast renovation (24). Feeding omega 3 FA increases DHA incorporation in cell membrane of erythrocytes which maintains osmotic pressure and nutrient transport across the cell membrane. On the other hand, feeding omega 6 FA to channel catfish increased C18 PUFA and decreased LC-PUFA which appeared to increase erythrocyte fragility (25). In another study on salmon, vegetable oils (blend of rapeseed and linseed oils), which are rich in omega 6 FA, decreased erythrocyte counts and hematocrit (26).

Fish fed more omega 3 FA or a mixture of omega 6 and omega 3 FA (R3) had high lymphocyte and low heterophil count than those fed omega 6 FA (R9). However, total count of WBCs was high in fish fed R1 than the other groups. These results differed from the previous reports which documented that omega 3 PUFA inhibit lymphocyte proliferation (27).

The current results revealed that feeding R1 or R3 enhanced liver function as indicated by lowering serum ALT and AST levels than fish fed R9. This may be due to the associative influence of dietary lipid types on the histological structure of the liver (28). Increasing omega 6 FA in gilt head sea bream diets increased fat accumulation in hepatocyte (29) which subsequently reduced hepatocyte activity leading to metabolic imbalance. Omega 6 FA had a lipogenic effect and lowered oxidation capacity of fish (30). More certainly, feeding omega 6 FA to Atlantic salmon increased molecular expression of adipophilin in the liver (31) which is a marker for lipid accumulation.

Altering the ratio of omega 6 to omega 3 FA modified immune status (32). Our result of serum antibacterial test indicated that the group fed R1 had significant enhancement of antibacterial activity of fish over the other groups. The use of a moderate omega 6 to omega 3 FA ratio did not alter the health conditions (33).

Results of the histopathology confirmed the beneficial effect of partial replacement of omega 3 with omega 6 FA on absorptive capacity of jejunum villi. Increasing ratio of omega 6 to omega 3 FA from R1 to R9 had a harmful effect on the absorptive capacity of the intestine, subsequently it decreased the growth performance of Nile tilapia fish. To our knowledge, there was scarce information on the effect of altering omega 6 to omega 3 FA ratio on the absorptive capacity of the intestine. Fish oil is rich in omega 3 FA which maintain integrity of cell membrane of the enterocytes which might show the higher length of the jejunum villi than soy oil.

Conclusion

Increasing the ratio of omega 6 to omega 3 FA from R1 to R9 decreased growth performance and feed utilization of the Nile tilapia. Also, it cleared that feeding more omega 3 and less omega 6 FA was very essential for blood and liver cells. Feeding R9 weakened immune response against bacterial infection and decreased absorptive capacity of jejunum villi. However, it could feed R3 without adverse effect on growth performance, feed utilization and absorptive capacity of the intestine.

Acknowledgments

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Conflict of interest

There is no conflict of interest.

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Original Research Article

MONITORING OF PATHOLOGICAL LESIONS IN NON-GRAVID OVINE UTERUS

Karam H. Al-Mallah[1]*, Mariem M. Hussien[2]

[1]Department of pathology and poultry diseases, college of Veterinary Medicine, University of Mosul, Iraq, [2]Veterinary hospital, Ninavah directorate of agriculture, Iraq

*Corresponding author, E-mail: karamalmallah@yahoo.com

Abstract: A total of 108 non-gravid ovine uteri were collected from slaughtered ewes at Mosul slaughter house and private butcheries to monitor and identify the uterine pathological lesions. The uteri were grossly examined for observation of gross pathological lesions. Tissues samples were collected for histopathological studies. The incidence of different pathological lesions was calculated. Diffuse or nodular endometrial hyperplasia, endometrial degeneration and desquamation and endometrial coagulative necrosis at ratios 36.11%, 20.37% and 16.66% respectively. Endometritis and subendometrial fibrosis were noticed in 7.40% and 3.70% of the examined samples. Also vascular hyperemia and vascular wall thickening were observed in 12.96% and 5.55% of total examined samples. It is concluded that most of the detected lesions in nongravid uteri appears to be related with previous infections or pregnancies.

Key words: Nongraviduteri; lesions; ewes

Introduction

Uterus is the largest part of female genital tract which is formed from perimetrium, myometrium and the endometrium. Endometrium is essential for fetal implantation and placentation. The uterine glands in the endometrium secrets uterine milk necessary for nutrition of the conceptus during the preimplantation period. Also the uterine glands secrete PGF2 α necessary for luteolysis of the CL periodicum with subsequent initiation of a new oestrous cycle (1). The uteri in farm animals including ewes can be affected with a wide range of pathogens. Hyperemia, hemorrhage and hydrometra were recorded as circulatory disturbances in the uterus (2, 3) .Uterine atrophy may arise due to ovarian dysfunction or nutritional deficiency (4). Physical affections or trauma may result in uterine torsion, uterine prolapse and uterine rupture especially when occur near the time of parturition (2). Metritis and endometritis are common uterine affections in farm animals which may be acute or chronic resulting in infertility (5).They may develop more complicated sequels including pyometra and mucometra (2). Parasitic infestation like Hydatid cysts, *Echinococcusgranulosus*, was recorded in the uterus (6). Uterine tumors including leiomyoma and lymphosarcoma(2) squamous cell carcinoma (7) and hemangioma (8) were also recorded in cattle and sheep.

The present study aimed to monitor and identify the types and incidence of uterine lesion in the ovine uterus in the Mousl region.

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Materials and methods

Directly after slaughtering 108 non-pregnant uteri were collected from ewes at Mosul abattoir and several private butcheries in the city for a period extended from November 2012 to May 2013. The uteri were identified and transported in ice tank to the Department of pathology and poultry disease, College of Veterinary Medicine, University of Mosul.

The uteri were macroscopically examined for presence of gross pathological lesions. These lesions were recorded and some of them were photographed. Tissues samples were collected from two uterine horns (apex, middle part and base of each horn as well as from uterine body.

The samples were preserved in 10% neutral buffered formalin for two days. The samples were prepared for histopathological examination and stained with H&E according to Khodakarm-Tafti and Davari (4). The prepared slides were examined under light microscope, Kruss, Germany, and photographed using digital camera (SONY Japan)**.** The pathological changes were recorded and classified as has been mentioned in table, 1. Also the incidence of each specific pathological lesion was calculated in relation to the total number of the examined uteri.

Results

Mild to moderate cellular adaptations lesions as being manifested by endometrial hyperplasia and endometrial atrophy were recorded at the percentages of 36.11% and 1.85% respectively (Table, 1 and Figs. 1, 2).

Disturbances in cell metabolism as being represented by endometrial degeneration and desquamation (20.37%) and endometrial coagulative necrosis (16.66%) were recorded (Table, 1 and Figures, 3, 4).Inflammations and repair were recorded as endometritis (7.4%), sub endometrial fibrosis (3.7%), placental retention and adhesions (0.93%), pyometra (1.85%). Also endometrial scarification, endometrial ulceration, myometrial abscesses and granuloma were recorded at the rate of 0.93% for each (Table1 and Figs. 5-8).

The circulatory disturbances including hyperemia, hemorrhage and vascular wall thickening were recorded at the rates of 12.96%, 2.77% and 5.55% respectively (Table1 and Fig. 9). Disturbances of pigmentation were observed as hemosiderosis and jaundice at the rates of 1.85% and 0.93% respectively. A single case of uterine tumor was diagnosed as metastatic squamous cell carcinoma in the uterus at a rate of 0.93% (Figs. 10, 11, Table 1).

Discussion

The aim of the present study was to screen the incidence and the types of pathological lesions in the ovine uterus in the Mousl region. The incidence of endometrial hyperplasia (36.11%) is higher than that (2.3%) recorded in Basarha region (10). This may be attributed to the variations in the environmental condition and the in the type and incidence of diseases producing such conditions between the two regions. The endometrial hyperplesia may arise as result of oestrogen level. Reynolds et al, (2009) (9) observed s similar conditions in overectomized ewes infected with higher doses of oestrogen. Also endometrial hyperplasic condition was recorded in ewes fed on estrogen rich clover (11).

Endometrial atrophy was recorded in the bovine uterus in Al-qisysia province (12). It was mainly detected in the uteri affected with chronic endometritis (4, 13). Disturbances in cell metabolism appeared as endometrial degeneration and necrosis. The degenerative changes may be confused with physiological apoptosis normally occurring during estrus cycle (14). However the pathological endometrial degenerations and necrosis usually accompany abortions, endometritis and prolonged dystocia as mentioned by (5). Endometrial necrosis and desquamation were diagnosed in case of chlamydial infections. Also stated endometrial degenerations and fibrinonecrotic endometritis were induced in pregnant ewes after experimental intravenous injection of *Trypanosomaevansi* (16). Melo et al, (2017) (17) found that feeding of pregnant goats on green leaves of the plant *Multiglandulosa terapterys* resulted in abortion, focal placentitis and coagulative necrosis of endometrium.

Table 1: The incidence of pathological lesions in the non-pregnant uteri of ewes collected from Al-Mousl region

Type of lesion Classification of the lesion	Incidence Rate	Percentage
Cellular adaptations		
Endometrial hyperplasia (Nodular or diffused)	39/108	36.11%
Endometrial atrophy	2/108	1.85%
Disturbances in cell metabolism		
Endometrial degeneration and desquamation.	22/108	20.37%
Endometrial coagulative necrosis	18/108	16.66%
Inflammation and repair		
Endometritis	8/108	7.4%
Sub endometrial fibrosis	4108	3.7%
Placental retention	1/108	0.93%
Pyometra	2/108	1.85%
Endometrial scarification	1/108	0.93%
Ulceration	1/108	0.93%
Myometrial abscess	1/108	0.93%
Granuloma	1/108	0.93%
Circulatory disturbances		
Hyperemia	14/108	12.96%
Hemorrhage	3/108	2.77%
Vascular wall thickening	6/108	5.55%
Disturbances of pigmentation		
Hemosiderosis	6/108	1.85%
Jaundice	1/108	0.93%
Tumors		
Squamous cell carcinoma	1/108	0.93%

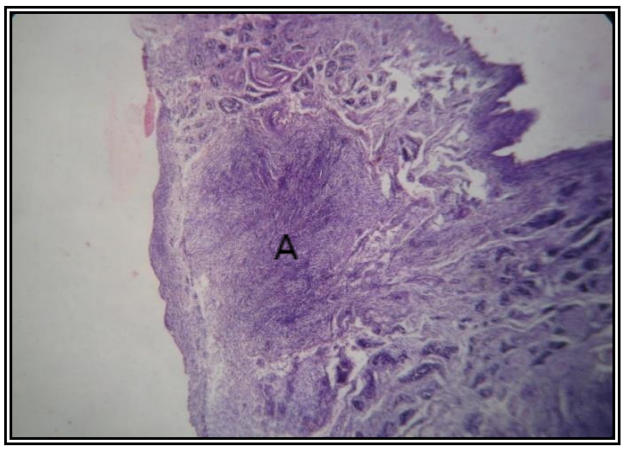

Figure 1: Photomicrograph of a section in ovine uterus showing nodular hyperplasia in endometrium (A). H&E, 40X

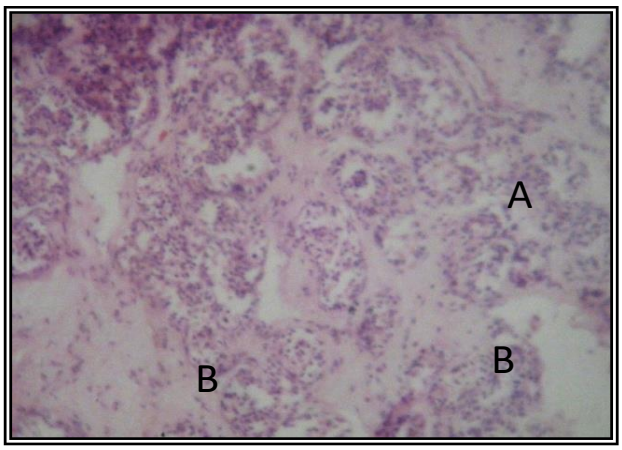

Figure 2: Photomicrograph of a section in ovine uterus showing endometrial atrophy manifested by shrinking and reduction in the size of the uterine glands (A) and subendometrial. H&E, 115 X

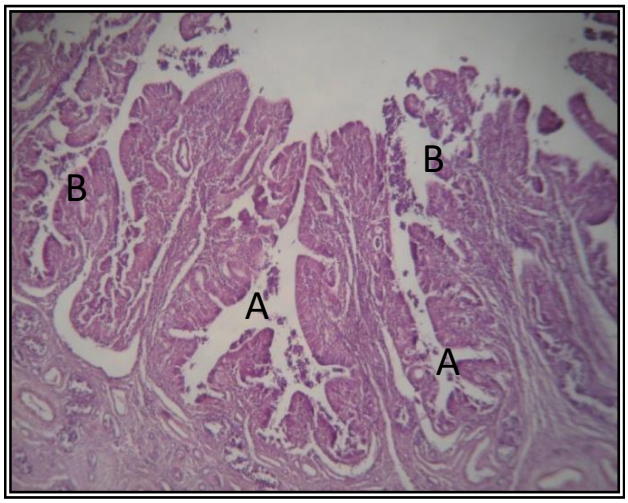

Figure 3: Photomicrograph of a section in ovine uterus showing vacuolar degeneration in endometrial cells (A) Sloughing of endometrium (B). H&E, 40X

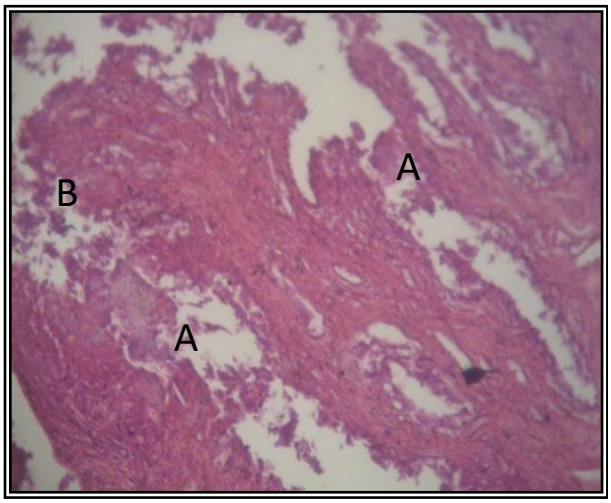

Figure 4: Photomicrograph of a section in ovine uterus showing coagulative necrosis in endometrial cells (A) Sloughing of endometrium (B). H&E, 115X

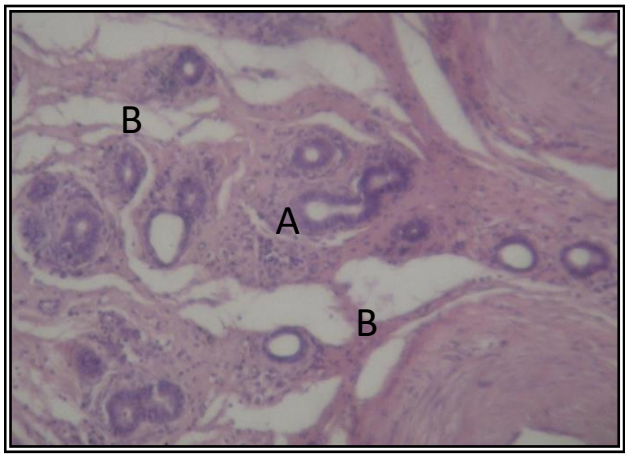

Figure 5: Photomicrograph of a section in ovine uterus showing endometritis manifested by infiltration of inflammatory cells (A) and edema (B). H&E, 165 X.

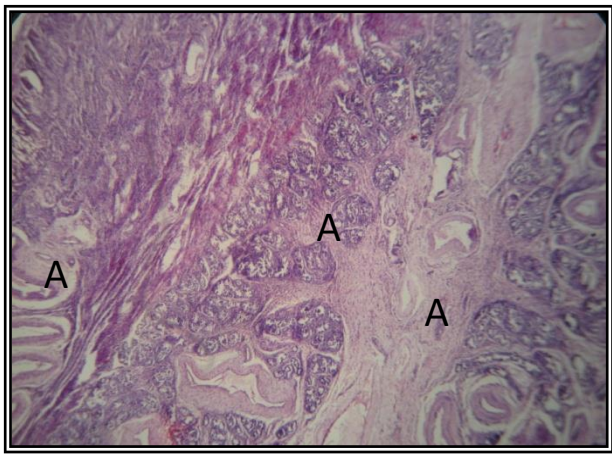

Figure 6: Photomicrograph of a section in ovine uterus showing subendometrial fibrosis (A). H&E, 40X.

Figure 7: Photomicrograph of a section in ovine uterus showing scarification being obvious by fibrous tissue deposition (A) extending through endometrium and myometrium. H&E, 68X

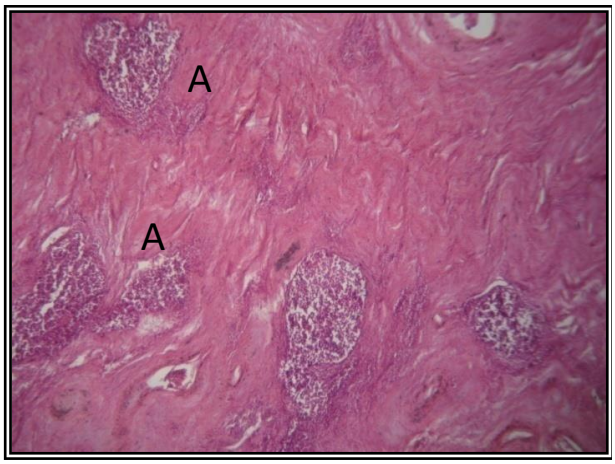

Figure 8: Photomicrograph of a section in ovine uterus showing microabscesses (A) within myometrium. H&E, 40X

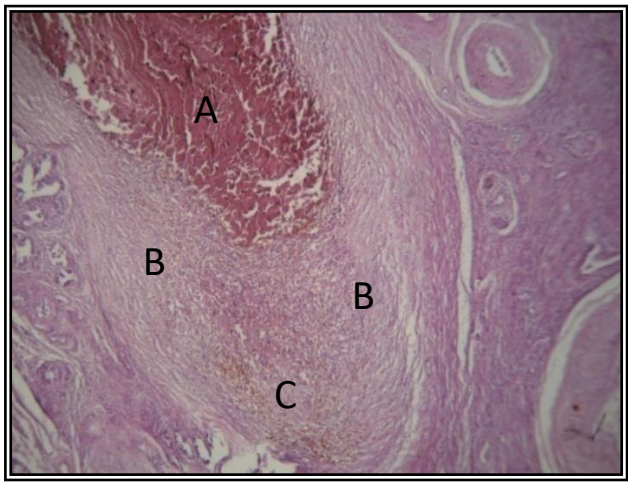

Figure 9: Photomicrograph of a section in ovine uterus showing hyperemic artery (A) thickening of vascular wall (B) hemosiderosis of vascular musculature (C). H&E, 68X

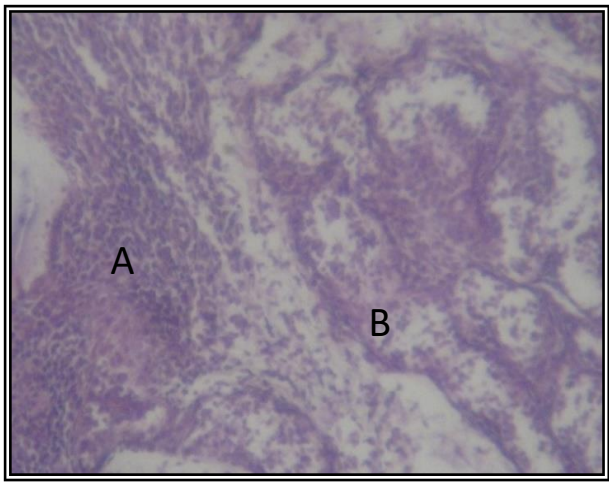

Figure 10: Photomicrograph of ovine uterus showing sequamous cell carcinoma appeared as actively mitotic cell sheathes (A) and hyperchromatic multi mitotic figure cells invades uterine glands (B). H&E, 256 X

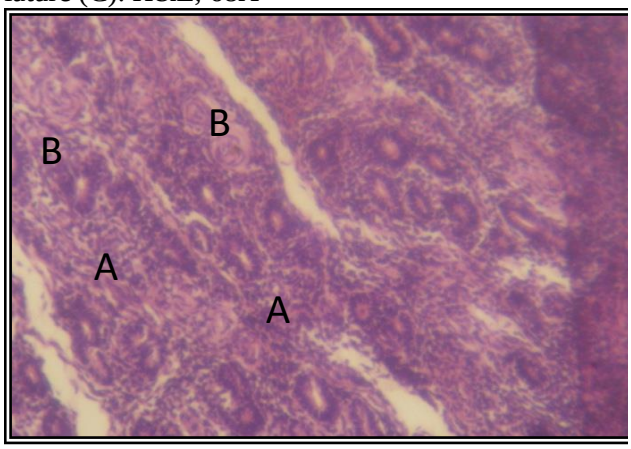

Figure 11: Photomicrograph of a section in ovine uterus showing sequamous cell carcinoma appeared as actively mitotic sequamous cell sheathes (A) and keratin nest structures (B). H&E, 200 X

The incidence of the endometritis in the current study (7.4%) was higher than those recorded in Ireland (2.3%) (18), Iran (2.3%) (4) and India (24.8%) (20) and lower than that (24.8%) in Al-Basraha Iraq (19).

The variations in rate may be attributed to the differences in environment and animal administration systems but still common affections worldwide. Many pathogens causing endometritis such as *Brucella abortus* (21), *Salmonella, Klebsiella , E.coli , Staphylococcus aureous* and *Proteus*, were isolated from genital tract (22) suffering from endometritis . Viral endometritis accompanies diseases like FMD (23).

Subendometrial fibrosis indicates chronic metritis which is usually associated with extensive deposition of collagen fibers. Charlotta et al, (200) (24) stated that fibrosis was observed in the uteri of mares with the advancement of age. Retained placental tissue was recorded at lower rate (0.93%) in the current study. The etiology may be mechanical, nutritional also bacterial, viral and mycotic infections (25).

Pyometra was recorded at rate of 1.85% of examined uteri. It was higher than that (0.15%) recorded by Khodakarm (2013) (4) and comparable to that recorded by Sharma et al, (2014) (20) in ewes. It was realized that pyometra may be related with cystic ovaries in affected animals (26). Endometrial scarification (0.93%), as indicated by scar granulation tissue extending through endometrium and myometrium can be explained as previous healing process from previous injuries of parturition (27). This lesion was recorded in 3 out of 30 ewes experimentally infected with *Mycoplasma capricolum capripneumoniae* (28).

The presence of Microabscesses in myometrium resembles the results of (29) in goats who confirmed the lesion , also (30) suggested that systemic pyogenic diseases like tuberculosis may induce that form of lesions in the genital system .granulomatous reaction was identified at 0.93% of samples , it may be expressed by bacterial , viral , fungal or parasitic infection of

uterus (31), Also (8) recorded cases of granulomatous metritis in cattle and classified as military tuberculosis and (29) stated that granulomatous endometritis in goat was recorded at 1.29% and explained as brucellosis .

Circulatory disturbances manifested by hyperemia at 12.96% of samples mostly accompanied physiological changes as increasing estrogen level. Ali and Madboli (2013) (32) stated that increasing alpha and /or beta oestrogen receptors and decreasing norepinephrine in uterus causes hyperemia in guinea pig. Hemorrhage in myometrium and sub endometrium was noticed in three samples at rate of 2.77%. It has been thought that it accompanies sever constrictions of the myometrium during delivery or abortion which is called postpartum atonic haemorrhage (33). Thickening of the vascular walls of uterus was described by (34) as morphological changes in the myometrial arteries resulting from accumulation of collagen and mucopolysaccharides in subendothelium with focal fibrotic changes in smooth muscle layers. It is usually seen in women after multiple pregnancies. .Hemosiderosis is a precipitation of hemosiderin pigment in tissue and it is reported by Beena et al, (2015) (29) in the caprine uteri in India at 25.32% of the examined samples. Jaundice stains body organs and tissues yellow by bilirubin and not specific to genital system. Squamous cell carcinoma was diagnosed in only one uterus sample at ratio 0.93%. It may arise outside the uterus and metastasized to the uterus because this type of tumors is not specific to the genital system. This suggestion may be supported by the observations of (35, 36) who recorded this type of tumors in human uteri

Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

COMPARATIVE HISTOPATHOLOGICAL CHANGES OF LIVER, KIDNEY AND APPENDIX OF RABBITS TREATED WITH INORGANIC NANO CHROMIUM TO AMELIORATE HEAT STRESS EFFECT

Sahar H. El-Nagar[1]*, Mohamed A. Helal[1], Shawky Mahmoud[2] , Leanne Dillard[3]

[1]Department of Animal Wealth Development, Faculty of Veterinary Medicine, Kafrelsheikh University, 33516, Egypt, [2]Department of Physiology, Faculty of Veterinary Medicine, Kafrelsheikh University, [3]Department of Animal Sciences, Auburn University, Auburn, 36849 USA

*Corresponding author, E-mail: sahr_hamdy@vet.kfs.edu.eg

Abstract: The objective of the current experiment was to describe the effects of nano-chromium chloride ($CrCl_3$) on chronic hyperthermia (32.8 ± 1.5 °C) on rabbit liver, kidney, and appendix in comparison with rabbits at room temperature (24.5 ± 1.3 °C) and treated with the same concentration of nano-chromium chloride. For this study, 108 rabbits of two different breeds (New Zealand White and Rex) were used and randomly allocated into 12 groups. The study was conducted as a completely randomized 2 × 2 × 3 factorial (n = 9) design. Treatments were temperature, breed, and concentration of nano-chromium chloride (0, 1, or 2 mg/L) the results showed that heat stress caused granular hepatic vacuolation, severe congestion of the central vein, and sinusoids in the liver. As well as degenerative changes within the epithelial lining of tubules in the kidney and lymphoid depletion in the appendix. The liver tissue of the New Zealand rabbits was affected more by heat stress than Rex Rabbits, but no difference was observed in the kidney or appendix tissues. The addition of 2 mg/L nano-chromium was more effective than 1 mg/L on the heat stressed rabbit tissues, but it caused hepatic vacuolation with glycogen infiltration in liver tissue and mild vacuolation in the renal tubular epithelium.

Key words: rabbit; nano-chromium; heat stress; histopathology

Introduction

The thermal comfort zone for rabbits is 21^0C. Any elevation from this temperature is considered heat stress (1). Heat stress is divided into two types acute and chronic heat stress. This division depends on the period of exposure and the presence of tissue damage including: pyknosis, apoptosis, necrotic areas, and an increase in melanomacrophage centers in liver tissues through**,** DNA digestion and cell membrane destruction (2, 3). Previous studies (4-6) observed vacuolated hepatic degeneration with dilation and congestion of sinusoid, widespread necrosis, and infiltration of leukocyte in some parts of the liver and kidney in heat stressed rabbits. The authors also reported damage to the glomeruli of the kidneys. As a consequence, Ondruska et al. (7) reported high animal mortality rates, leading to economic losses for rabbit producers during summer season.

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More recent studies have tried to reduce heat stress through different management and nutritional methods. Nano-chromium is one of the most important nutritional supplements that can be used to decrease and possibly eliminate heat stress damage. However, low doses must be used as higher doses may lead to adverse animal effects such as degenerative changes and necrosis in liver as well as hyaline casts in kidney and tubules and glomeruli (8).

Chromium is an essential trace mineral required for most vital metabolic processes. It is used in carbohydrate, protein, and lipid metabolism (9); therefore it has anabolic effects during periods of stress, including heat stress. Research has shown that nano-chromium has an anabolic effect when used in low concentrations and enhances the nucleic acid synthesis in the liver of the mouse (10). The objective of the current study was to determine what dosage of nano-chromium chloride is needed to ameliorate the degenerative changes in the vital tissues of heat stressed rabbits.

Materials and methods

This study was carried out at the Department of Animal Wealth Development in the Faculty of Veterinary Medicine at Kafrelsheikh University in Kafrelsheikh, Egypt. The experiment was approved by the guiding of committee on Animal Welfare and Ethics of the Faculty of Veterinary Science, Kafrelsheikh University, in accordance with Egyptian national laws regarding animal welfare. One hundred eight weaning aged rabbits of two different breeds (Rex and New Zealand White; 35 ± 3 and 33 ± 2 d weaning age, respectively) were used to evaluate the effect of nano-chromium chlordie on liver, kidney, and appendix tissues.

Experimental design

Each breed had an initial body weight of 602 ± 9.5 and 531 ± 6.5 g for Rex and New Zealand Breed, respectively. Animals of each bred were divided equally into 6 groups with 9 rabbits in each group. Three groups of each breed were subjected to severe heat stress (32.8 ± 1.5 °C; HT) and the other three groups were reared under room temperature conditions (24.5 ± 1.3 °C; RT) (11). Each group contained 3 replicates with 3 rabbits in each replicate. Each replicated group was reared on wire cages 50 × 50 ×50 cm and fed commercial rabbit pellets (18% crude protein) ad libitum.

The nano-chromium particles were prepared by chemical precipitation method of chromium chloride salt (12), and then characterized by transmission electron microscope (TEM) at the Nanotechnology Institution of Kafrelsheikh University, Egypt. The resulting particles were 40 – 60 nm. These particles were then added to the drinking water at 3 different concentrations (0, 1 or 2 mg/L; C, 1, and 2, respectively) for the duration of the 8 week study. At the end of the experiment rabbits were slaughtered for tissue collection. The liver, kidney and appendix tissues were collected in 10% formalin, sectioned, and stained with H&E stain for microscopic examination (13).

Results

Liver

The liver of the control groups from both breeds and untreated with nano-chromium showed the normal arrangement of the hepatocytes in cords around the central vein and normal sinusoids (Figure 1- A and C). However, the liver of the groups that were exposed to heat stress but untreated with nano-chromium showed that the New Zealand breed was more susceptible to heat stress than the Rex breed. This is due to the appearance of severe congestion of the central vein and sinusoids, as well as granular hepatic vacuolation (Figure 1-B and D). Mild hepatic valuation in New Zealand rabbits treated with 1 mg/L nano-chromium at room temperature was observed (Figure 2). This is consistent with excessive glycogen storage in the liver. This was also seen when New Zealand rabbits received the same concentration of nano-chromium, but that were also exposed to heat stress (Figure 2-C). However, when Rex Rabbits were treated with 1 mg/L of nano-chromium under room temperature conditions, they appeared to have

normal hepatocyte and liver tissue as seen in Figure 2-B. When exposed to heat stress with the same concentration of nano-chromium, the Rex rabbits responded the same as the New Zealand rabbits, and also appeared to have congestion of the sinusoids. The addition of 2 mg/L nano-chromium caused hepatic vacuolation with glycogen infiltration that was severe in New Zealand breeds (Figure 3-A) and mild in Rex Rabbits (Figure 3-B). While in animals exposed to heat stress, the same effect was observed in both breeds which appears as marked decrease in hepatic vacuolation.

Kidney

There was no observable difference in kidney tissues between breeds under the same temperature conditions. Under room temperature conditions, the two breeds showed normal renal glomeruli and tubules (Figure 4-A and C). However under heat stress, they showed degenerative changes within the lining epithelium of the renal tubules (Figure 4-B and D). In regards to renal tissues, there was no observable difference in the response between the breeds when treated with 1 mg/L nano-chromium and under the same temperature (Figure 5-A and C). The tissue appeared to be normal under room temperature conditions. Mild degenerative changes in the lining of the epithelium of the renal tubules appeared in heat stressed animals (Figure 5-B and D). In rabbits treated with 2 mg/L nano-chromium, there was no observable difference in response between two breeds under room temperature. Rex rabbits showed normal renal glomeruli and tubules (Figure 6-A). The New Zealand rabbits showed normal renal glomeruli, but showed mild vacuolation of the renal tubular epithelium (Figure 6-B). There was also no difference between the two breeds under heat stress compared with New Zealand under room temperature.

Appendix

Under room temperature conditions and treated with 0 mg/L nano-chromium, the two breeds showed normal lymphoid follicles (Figure 7-A and C), but heat stress caused a mild lymphoid depletion in the germinal center of the follicle of Rex rabbits and on the basal follicle of the New Zealand rabbits (Figure 7-B and D). When rabbits were treated with 1mg/L nano-chromium under room temperature conditions, they were not different from the untreated rabbits under the same temperature (Figure 8-A and C). However under heat stress, the addition of 1mg/L of nano-chromium ameliorated the lymphoid depletion that resulted from the heat stress in untreated rabbits (Figure 8-B and C). Figure 9 from A to D showed that there was no difference between breeds treated with 2 mg/L nano-chromium under different temperature treatment.

Discussion

Liver

The granular hepatic vacuolations resulting from exposure to heat stress observed in Figure 1 may be due to the activation of mitochondrial reactive oxygen species (ROS; 14) which can lead to cytotoxicity, apoptotic cell death, and necrosis (15). This degenerative condition appears as vacuoles in hepatocytes. However, the severe congestion of the central vein was likely due to the fact that the liver has the densest concentration of mitochondria, which are overloaded during stress. This can lead to increased blood supply to this organ as compensatory mechanism (16). Figure 2-A and B represent the liver tissue unexposed to heat stress and treated with 1mg/L nano-chromium. The New Zealand breed showed a better response to nano-chromium treatments than the Rex breed. This is evident in the mild hepatic vacuolation that is consistent with excessive glycogen storage. This is in agreement with Huskisson et al. (17) that illustrated that Cr III enhanced glucose uptake by the liver cells. On the other hand, Muthulingam et al. (18) observed that Cr lead to decreased glycogen in the gills, liver, and kidneys of fish. This was observed in Figure 3, where the degenerative changes and congestion of the liver caused by heat stress disappeared and were replaced by marked hepatic vacuolation due to the increased dose of nano-chromium.

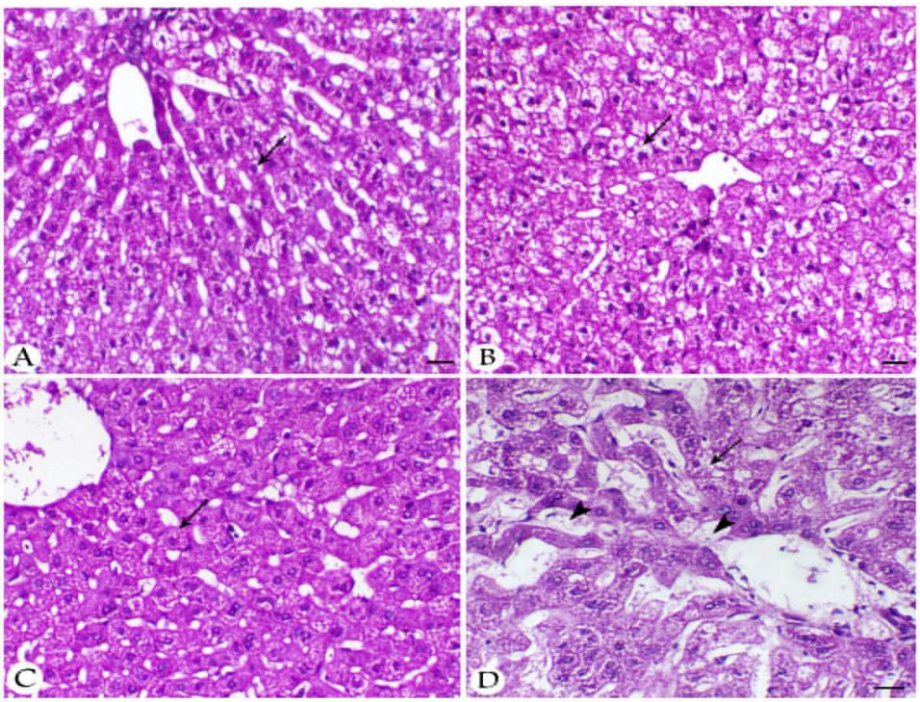

Figure 1: A) Liver of room temperature (R) Rex rabbits showing normal hepatocyte arranged in cords around the central vein (arrow), H&E, bar = 40 µm. **B)** Liver of heat stressed (HS) Rex breed showing granular hepatic vacuolation (arrow), H&E, bar = 40 µm. **C)** Liver of New Zealand-R rabbits showing normal hepatocytes arranged in cords around the central vein (arrow), H&E, bar = 40 µm. **D)** Liver of HS-New Zealand rabbits showing severe congestion of the central vein and sinusoids (arrowheads) and granular hepatic vacuolation (arrow), H&E, bar = 40 µm

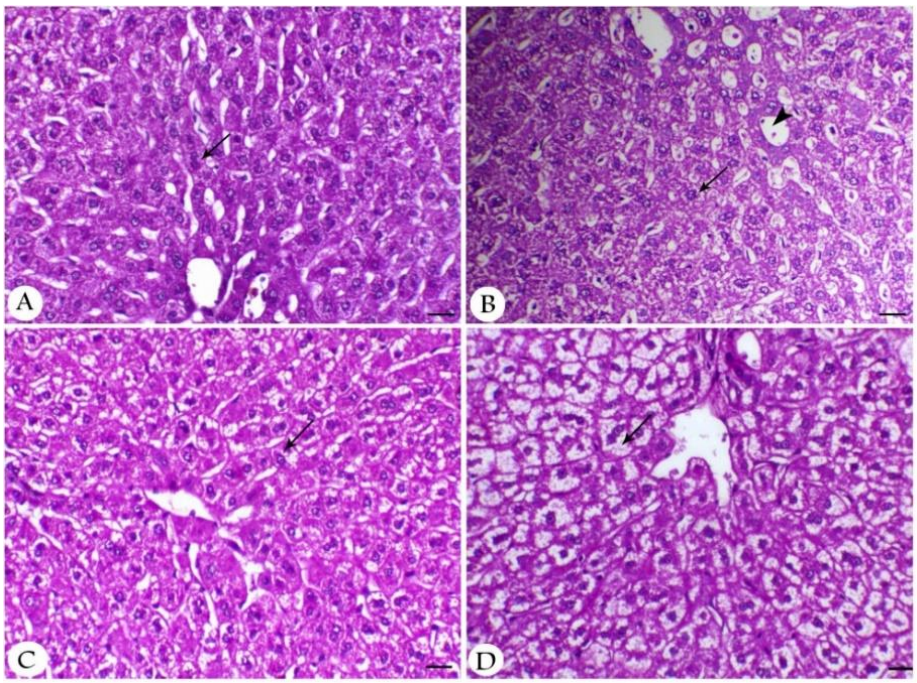

Figure 2:A) Liver of room temperature (R) Rex treated with 1 mg/L nano-chromium (T1) showing normal hepatocytes (arrow), H&E, bar = 40 µm. **B)** Liver of HS Rex rabbits T1 showing sinusoidal congestion (arrowhead) and mild hepatic vacuolation consistent with hydropic changes (arrow), H&E, bar = 40 µm. **C)** Liver of R -New Zealand rabbits T1 showing mild hepatic vacuolation consistent with over glycogen storage (arrow), H&E, bar= 40 µm. **D)** Liver of heat stressed (HS) New Zealand rabbits T1 showing hepatic vacuolation consistent with over glycogen infiltration (arrow), H&E, bar = 40 µm

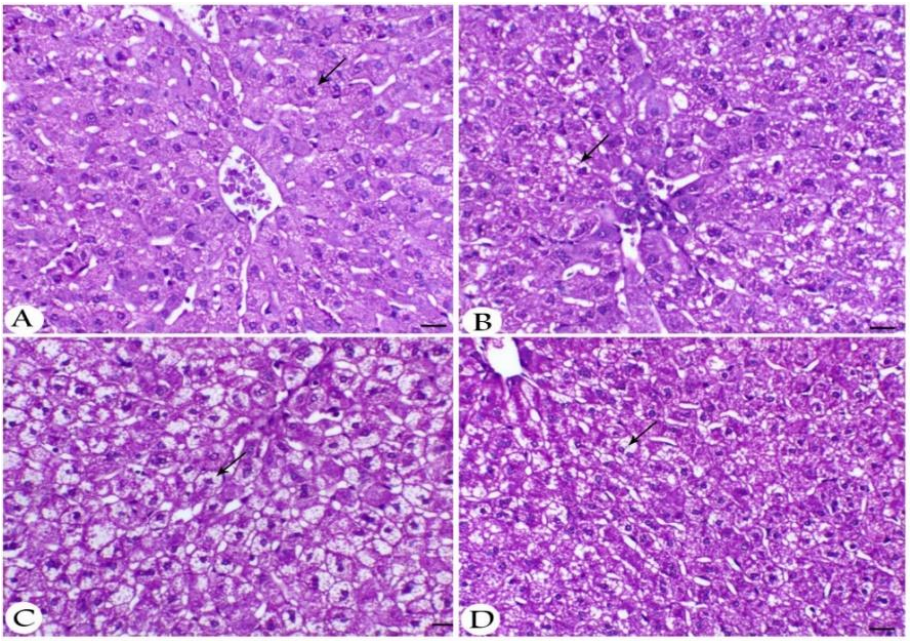

Figure 3:A) Liver of room temperature (R) Rex rabbits treated with 2mg/L nano-chromium (T2) showing mild hepatic vacuolation consistent with glycogen storage (arrow), H&E, bar= 200 µm. **B)** Liver of Rex heat stressed (HS) rabbits T2 showing marked decrease of hepatic vacuolation (arrow), H&E, bar = 40 µm. **C)** Liver of R New Zealand rabbits T2 showing marked hepatic vacuolation consistent with glycogen infiltration (arrow), H&E, bar= 200 µm. **D)** Liver of HS New Zealand rabbits T2 showing marked decrease of hepatic vacuolation (arrow), H&E, bar= 40 µm

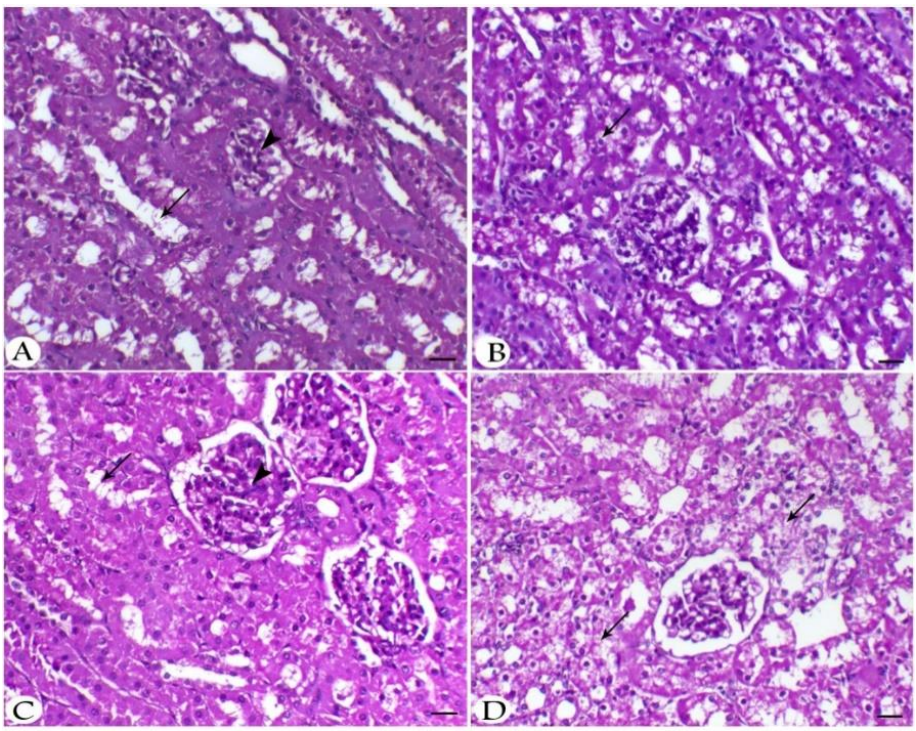

Figure 4:A) Kidney of room temperature (R) Rex rabbits showing normal renal glomeruli and tubules (arrowhead and arrow respectively), H&E, bar = 40 µm. **B)** Kidney of heat stressed (HS) Rex rabbits showing marked degenerative changes within the lining epithelium of the tubules (arrows), H&E, bar= 40 µm. **C)** Kidney of R New Zealand rabbits showing normal renal glomeruli and tubules (arrowhead and arrow respectively), H&E, bar= 40 µm. **D)** Kidney of HS New Zealand rabbits showing marked degenerative changes within the lining epithelium of the tubules (arrows), H&E, bar= 40 µm

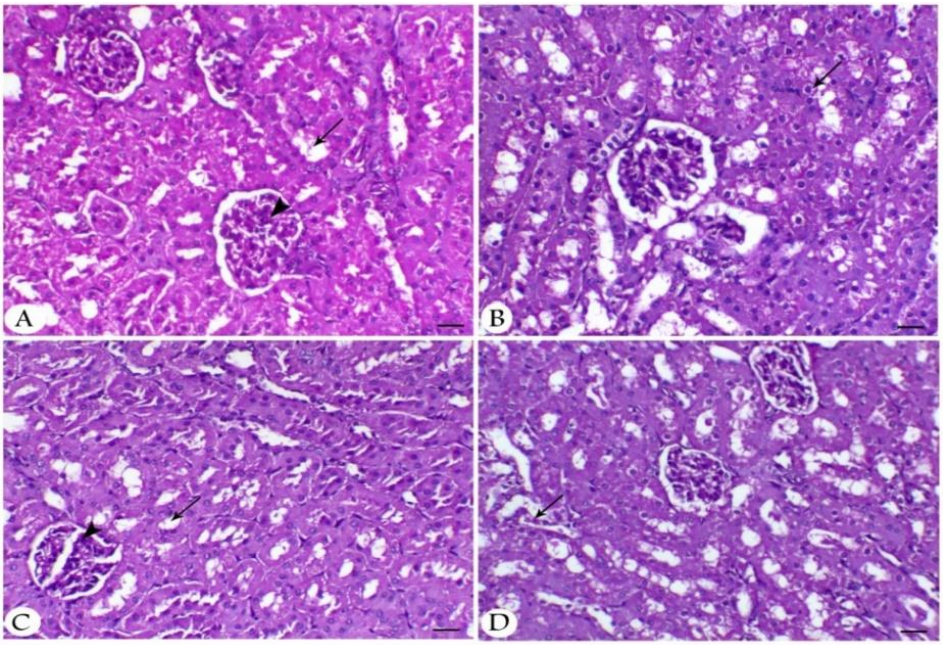

Figure 5:A) Kidney of room temperature (R) Rex rabbits with 1 mg/L nano-chromium (T1) showing normal renal glomeruli and tubules (arrowhead and arrow respectively), H&E, bar = 40 µm. **B)** Kidney of HS Rex rabbits T1 showing mild degenerative changes within the lining epithelium of the renal tubules (arrow), H&E, bar = 40 µm. **C)** Kidney of R New Zealand rabbits T1 showing normal renal glomeruli and tubules (arrowhead and arrow respectively), H&E, bar= 40 µm. **D)** Kidney of HS New Zealand rabbits T1 showing mild degenerative changes within the lining epithelium of the renal tubules (arrow), H&E, bar = 40 µm

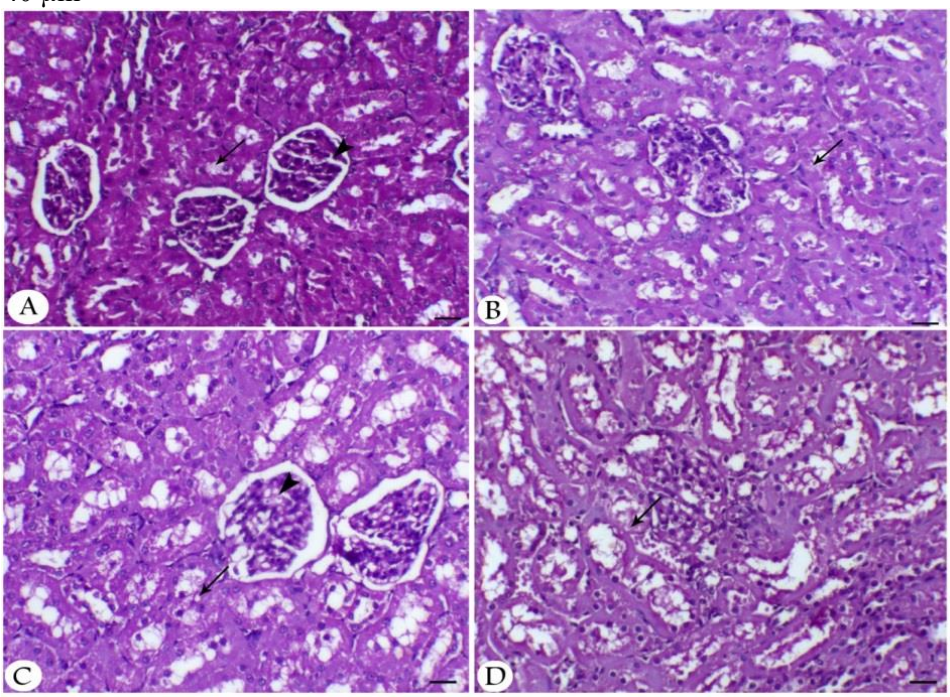

Figure 6:A) Kidney of room temperature (R) Rex rabbits with 2 mg/L nano-chromium (T2) showing normal renal glomeruli and tubules (arrowhead and arrow respectively), H&E, bar= 40 µm. **B)** Kidney of heat stressed (HS) Rex rabbits T2 showing normal renal glomeruli and mild vacuolation of the renal tubular epithelium (arrow), H&E, bar = 40 µm. **C)** Kidney of R-T2 New Zealand rabbits showing normal renal glomeruli (arrowhead) and mild vacuolation of the renal tubular epithelium (arrow), H&E, bar = 40 µm. **D)** Kidney of HS-T2 New Zealand rabbits showing normal renal glomeruli (arrowhead) and mild vacuolation of the renal tubular epithelium (arrow), H&E, bar = 40 µm

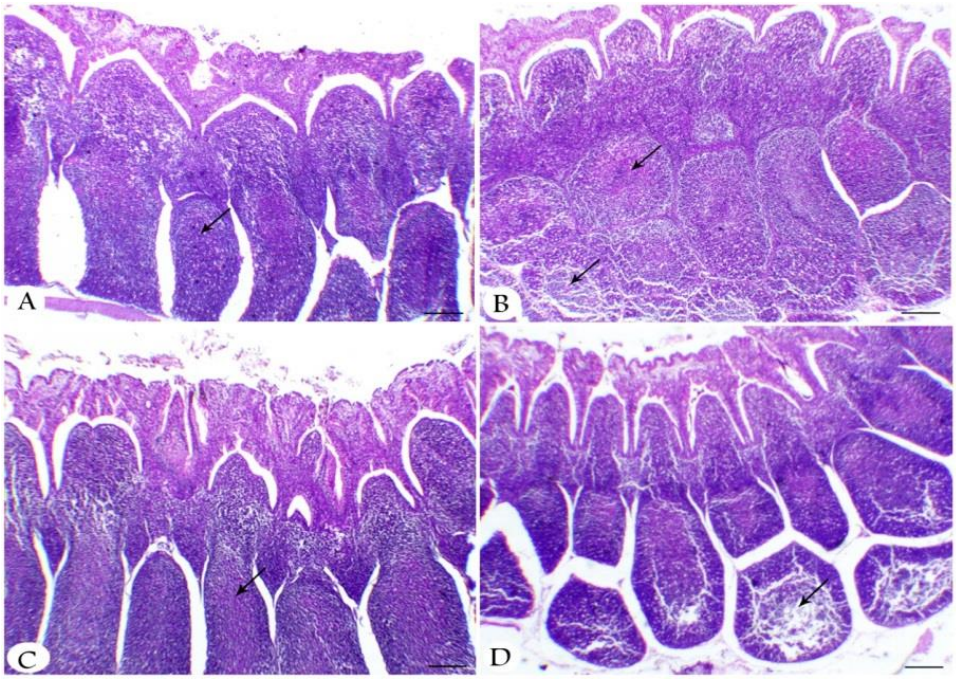

Figure 7: A) Appendix of room temperature (R) Rex rabbits showing normal lymphoid follicles covered with epithelial covering (arrow), H&E, bar = 200 µm. **B)** Appendix of heat stressed (HS) Rex rabbits showing mild degree of lymphoid depletion of the germinal center of the follicles (arrow), H&E, bar= 200 µm. **C)** Appendix of R New Zealand rabbits showing normal lymphoid follicles covered with epithelial covering (arrow), H&E, bar = 200 µm. **D)** Appendix of HS New Zealand rabbits showing lymphoid depletion of the germinal center of the basal follicles (arrow), H&E, bar= 200 µm

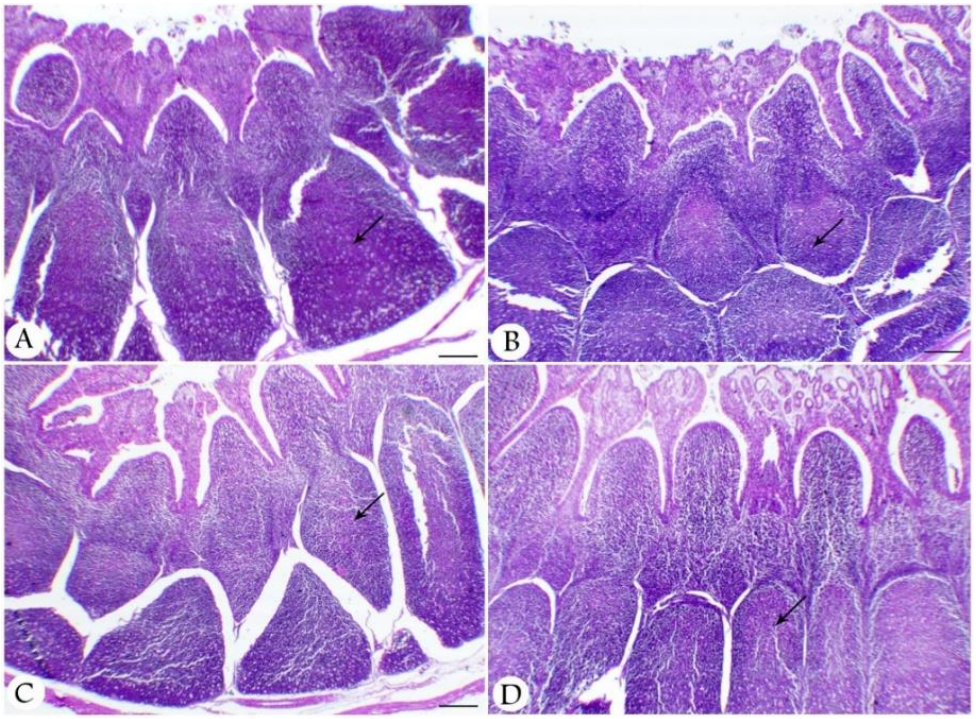

Figure 8: A) Appendix of room temperature (R) rabbits treated with 1 mg/L nano-chromium (T1) showing normal lymphoid follicles (arrow), H&E, bar = 200 µm. **B)** Appendix of heat stressed (HS)-T1 Rex rabbits showing normal lymphoid follicles (arrow), H&E, bar = 200 µm. **C)** Appendix of R-T1 New Zealand rabbits showing normal lymphoid follicles (arrow), H&E, bar = 200 µm. **D)** Appendix of HS-T1 New Zealand rabbits showing normal lymphoid follicles (arrow), H&E, bar = 200 µm.

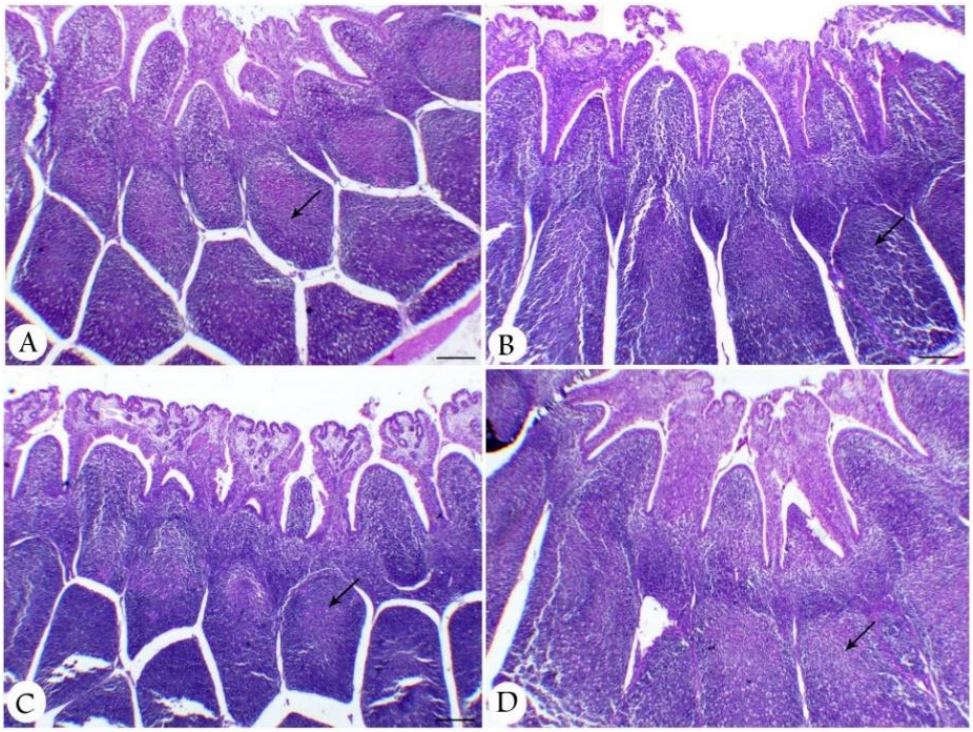

Figure 9: A) Appendix of room temperature (R) rabbits treated with 2 mg/L nano-chromium (T2) showing normal lymphoid follicles (arrow), H&E, bar = 200 µm. **B)** Appendix of heat stressed (HS) rabbits T2 showing normal lymphoid follicles (arrow), H&E, bar = 200 µm. **C)** Appendix of New Zealand R-T2 rabbits showing normal lymphoid follicles (arrow), H&E, bar = 200 µm. **D)** Appendix of HS-T2 New Zealand rabbits showing normal lymphoid follicles (arrow), H&E, bar = 200 µm

Kidney

When both breeds were exposed to room temperature conditions and untreated with nano-chromium they showed normal glomeruli and tubules (Figure 4-A and C). Kidneys of animals exposed to heat stress, but also untreated with nano-chromium particles showed degenerative changes within the lining of the epithelium of the renal tubules. This is likely due to the cytolytic response of the tissues by the heat followed by endotoximia that enhanced the release of inflammatory cytokines and consequently caused vascular endothelium injury (19). This appeared as degenerative changes in tubular lining epithelium in this study.

Figure 5-A and C showed normal renal and glomerular structures when 1mg/L of nano-chromium was administered. This is interrupted to mean that this concentration of nano-chromium is safe and does not alter the renal structure in rabbits housed in room temperature conditions. This level of nano-chromium also decreased, but did not remove, the degenerative changes resulting from heat stress in both breeds. While the addition of 2mg/L nano-chromium did ameliorate the degenerative changes in the heat stressed rabbits, this was replaced by mild vacuolation in lining epithelium. This may be due to glucose uptake by the cell increased by addition of this concentration (17) rather than toxicity.

Appendix

Animals of both breeds that were not treated with nano-chromium and kept under room temperature conditions appeared to have normal lymphoid follicles, as they were covered with epithelium. This indicates that this temperature was comfortable for the immune system of the rabbits. On the other hand, the groups that were exposed to heat stress and untreated with nano-chromium appeared to have a lymphoid depletion of the germinal center of the follicle (Figure 7-B and D). This may be due to the heat stress enhanced the production of ROS (20) that lead to death of

most of cells (21), including immune cells and resulted in lymphoid depletion.

The addition of both 1 and 2mg/L of nano-chromium had the same effect on appendix tissues during heat stress. The addition of nano-chromium prevented the lymphoid depletion resulting from this stress (Figure 8-B and D; Figure 9-B and D). These results may be due to the catalytic effect of Cr III on the H_2O_2 inactivating enzyme, consequently decreasing the cell apoptosis (22). The structure of the appendix was not altered in groups treated with 2mg/L of nano-chromium, but not exposed to heat stress. This in the agreement with Yucesoy et al. (23), that illustrated that Cr salts do not have an effect on the immune system cells.

Conclusion

In conclusion, addition of 2 mg/L nano-chromuim particles to the drinking water of the heat stressed rabbits could be used to alleviate the granular degenerative changes and the sinusoidal congestion of the rabbit liver. As well as to ameliorate the effects of the previous concentration on the degenerative changes in the epithelium lining renal tubules. It is also able to reduce the lymphoid depletion in the lymphoid follicles of the rabbit appendix.

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Original Research Article

EFFECTIVENESS OF EXOGENOUS DIGESTIVE ENZYMES SUPPLEMENTATION ON THE PERFORMANCE OF RABBITFISH (*Siganus rivulatus*)

Mahmoud A. O. Dawood[1]*, Mohamed Mohsen[1], Ashraf El-dakar[2], Elsaid Abdelraouf[1], Eman M. Moustafa[3], Hamada A. Ahmed[4]

[1]Animal Production Department, Faculty of Agriculture, Kafrelsheikh University, 33516 Kafrelsheikh, Egypt, [2]Aquaculture Department, Faculty of Fish Resources, El-Arish, Suez University, Egypt, [3]Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Kafrelsheikh University, 33516, Kafrelsheikh, Egypt, [4]Department of Nutrition and Veterinary Clinical Nutrition, Faculty of Veterinary Medicine, Damanhour University, Damanhour, Egypt

*Corresponding author, E-mail: mahmouddawood55@gmail.com

Abstract: The current study was established to test the possibility of using exogenous digestive enzymes (EDE) in rabbitfish (*Siganus rivulatus*) diets. Five experimental diets containing 34% soybean meal were prepared and supplemented with EDE at 0, 1, 2, 3, and 4 g kg^{-1} diet. Fish with average initial weight (± 1.1 g) were fed the test diets for 74 days. Rabbitfish offered the diets with EDE supplements exhibited significantly (P<0.05) higher growth performance, feed intake, protein efficiency ratio, protein gain, and protein retention over the basal diet. But, no significant (P>0.05) differences were observed in carcass composition and somatic parameters index with EDE supplementation. The physiological condition of fish fed diets with EDE showed that the hematocrit (%), total plasma protein and total plasma globulin were significantly (P<0.05) higher in fish fed EDE than the control group. It can be concluded that the addition of EDE in rabbitfish diets improved the growth performance, feed efficiency and health condition.

Key words: blood parameters; exogenous digestive enzymes; feed efficiency; growth; rabbitfish

Introduction

Aquaculture is one of the most economically important food sectors, representing the cheapest animal protein source for the increased population around the globe (1). Aquafeeds is contributing by around 70 to 80% from the total cost of the fish aquaculture (2). Thus, high quality feeds with balanced composition should be formulated to present the nutritional requirement and welfare for aquatic animals (3).

Fish meal has been served as the major protein source in aquafeed of carnivorous fish which tend to possess higher dietary protein requirements than omnivorous and herbivorous due to its balanced amino acid profile and palatability (4-7). However, the resource shortages and rising price of fish meal seriously restrict the use of fish meal in aquaculture. In

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this light, much efforts have been conducted to seek the sustainable supplies of protein sources to substitute fish meal. The plant protein sources have been limited used due to their relative low protein content and palatability, the presence of anti-nutritional factors and unbalanced amino acids profiles (4, 8, 10, 11). Digestive enzymes are helping in the food digestion process by transforming the non-digestible ingredients into more absorbable nutrients (9). Using of digestive enzymes is also recommended to facilitate the digestion process of fibrous ingredients existed in plant proteins such as: starch, cellulose and protein into simpler substances (3). Amylases, lipases, cellulases, xylanases, phytases and proteases are different forms of exogenous digestive enzymes which has been applied successfully in animal nutrition and aquafeed (10, 11). Since specific enzyme preparations have become valuable tools for economically manipulating digestive processes in animals (12), there are also considerable interests in using enzymes as supplements in fish feeds (3,9,13).

Rabbitfish species have long been identified as appropriate candidates for aquaculture in the Indo-Pacific and the Middle East area (14). Although they are herbivorous and could potentially be fed soy bean meal-based diets, non-fish meal feeds might reduce the digestibility and assimilation of this species. Under the circumstances, fish diets should be supplemented by using digestive enzymes to further utilize the prepared feeds efficiently (13, 15, 16).

The present study was performed to examine the possibility of using exogenous digestive enzymes "cellulases, xylanases, α-amylase and proteases mixture" on growth, feed efficiency, carcass composition and health condition of rabbitfish (*Siganus rivulatus*).

Materials and methods

Experimental system

Rabbitfish (*S. rivulatus*) juveniles were obtained from the coast of Mediterranean Sea, El-Arish, Egypt. The collected fish were carried carefully in well prepared tanks to the "Mariculture Research Center, Faculty of Environmental Agricultural Sciences, Suez Canal University, El-Arish, Egypt", then acclimatized for 1 week before starting the trial. A total of 150 fish were distributed in 15 glass aquaria (50 l) where each aquarium was stocked with 10 fish with similar size (initial weight 1.1 g). The trial was conducted in triplicates. Each aquarium was provided with submerged air diffusers for aeration and about 30-35% of water was changed daily. The water quality parameters were measured daily and remained at 34 g kg^{-1}, 25±1°C, 8.9 and 7 mg kg^{-1}, for salinity, temperature, pH and DO, respectively.

Diet preparation

The basal diet (32.2% crude protein and 8.5% lipids) was supplemented with exogenous digestive enzymes (EDE) (ZADO®, patent No. 22155 of Egypt) at 0, 1, 2, 3 and 4 g kg^{-1}. The basal diet was prepared to contain fish meal (65%) and soybean meal (45%) as protein sources and other ingredients were added by following El-Dakar et al. (13) (Table 1). The pellets size was 1.5-mm die. Pellets were air dried and stored at -20°C. Each diet was offered to three randomly chosen aquaria at 2-3% of biomass daily at 8.00, 12.00 and 16.00 h for 74 days. Fish were weighed at 2-week intervals and ration was adjusted according to new fish weight per cage.

Sample collection and analysis

After 74 days, all fish in each aquarium were weighed, counted and total length was measured to calculate the specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), protein gain (PG), protein retention (PR), survival and condition factor (CF) using the following formulae:

SGR = 100 *(ln FBW -ln IBW)/t, FCR = FI (g)/WG (g), PER = WG/protein intake, PG = {(FBW (g) ×final whole-body protein content (%)/100) − (IBW (g) ×initial whole-body protein content (%)/100)}/ (WG (g)) ×1000, PR= (protein gain (g kg WG^{-1}) ×100)/ protein intake (g kg WG^{-1}), CF = 100 *[body weight (g)/$length^3$ (cm)] and survival= (final number/initial number)*100. Where FBW is final body weight (g), IBW is initial body

weight (g), WG is weight gain, FI is feed intake (g) and t is 74 days.

Fish for initial carcass composition and diet samples were taken at the beginning of the trial and stored at -20 °C until analysis. 3 fish were randomly taken from each aquarium and killed, then the liver was dissected and weighed to calculate the hepatosomatic index (HSI) where HSI = 100 *(liver weight/body weight).

Three fish from each aquarium were collected, pooled and stored at -20 °C for carcass analysis. Using heparinized syringes, blood was collected from the caudal vein of 3 fish in each replicate aquarium and pooled. A small fraction of the heparinized blood was used to analyze the hematocrit and hemoglobin levels. Plasma samples were obtained by centrifugation at 3000×g for 15 min using a high-speed refrigerated micro centrifuge and kept at -20°C. Hematocrit was determined by using micro hematocrit-heparinized capillary tubes and a micro hematocrit centrifuge (10,000 g for 10 min.) (17). Hemoglobin concentrations were determined by the cyan hemoglobin method, at 540 nm. Total plasma protein and albumin were determined using commercial kits produced by Pasteur labs (Egypt). However, the total plasma globulin was calculated by subtracting the total plasma protein from total plasma albumin according to Coles (18). Chemical analysis of feed and fish were performed according to the methods described in AOAC (19).

Statistical analysis

Data were analyzed using one-way analysis of variance (Package Super-ANOVA 1.11, Abacus Concepts, Berkeley, California, USA). Significance differences between means were evaluated using Duncan's new multiple range test at a probability level of 0.05 when t-test was significant.

Results

Growth, survival and feed efficiency

Survival rate of all fish groups fed varied levels of EDE ranged between 90 to 100 %. Using of EDE resulted in improved FBW, WG and SGR significantly (P<0.05) than the control. Group 4 which supplemented with 3 g kg^{-1} EDE recorded the highest FBW and WG significantly (P<0.05) than other groups. Fish gained 2025%,2181, 2307, 2453 and 2212 % of their initial weight for the control, 1, 2, 3 and 4 g kg^{-1} groups than their weights at the start of the experiment (Table 2).

Feed efficiency (FI, PG, and PR) of fish fed EDE is presented in Table 2. Results showed that rabbitfish fed diet contains different levels of EDE gave higher values significantly (P<0.05) than EDE free group. Moreover, those fed 3g kg^{-1} EDE gave significantly the higher values than the other groups (P<0.05). Although, FCR recorded no significant differences (P>0.05) among all experimental groups, rabbitfish fed 3g kg^{-1} recorded the lowest value numerically comparing the other experimental groups. PER of fish fed diets supplemented with EDE at 1, 2, and 3 g kg^{-1} was significantly (P<0.05) higher than the non-supplemented group.

Carcass composition

No significant differences (P>0.05) were observed in the analyzed carcass compositions for fish collected at the end of the trial in comparison with the values before the feeding trial (Table 3). Further, no difference was detected in CF and HSI values of fish fed different test diets (Table 3).

Blood parameters

Hematological parameters were presented in Figure 1A; hematocrit of rabbitfish was significantly (P<0.05) improved in fish fed either 3 or 4 g kg^{-1} EDE than the other experimental diets. Although, hemoglobin value was not affected by EDE supplementation among tested groups, diets supplemented with 3 and 4 g kg^{-1} EDE recorded higher hemoglobin value significantly (P<0.05) than the EDE free group (Fig. 1B).

Blood chemical parameters in rabbitfish were presented in Figure 2. Fish fed diets with EDE had significantly (P<0.05) higher levels of total plasma protein compared to fish fed EDE free diet (Fig. 2A). Total plasma albumin recorded the lowest value significantly

(P<0.05) in fish fed 2 g kg^{-1} EDE among the other experimental groups (Fig. 2B). Fish fed EDE at 2, 3, and 4 g kg^{-1} recorded higher total plasma globulin significantly (P<0.05) than other experimental groups (Fig. 2C).

Table 1: Formulation and chemical proximate composition of the experimental diets (g kg^{-1} dry weight basis).

Ingredients	EDE supplementation level (g kg^{-1})				
	0	1	2	3	4
Fish meal	120	120	120	120	120
Soybean meal	340	340	340	340	340
Wheat bran	100	100	100	100	100
Wheat milling by product	170	169	168	167	166
Corn starch	50	50	50	50	50
Rice bran	150	150	150	150	150
Oil mixture[1]	50	50	50	50	50
Vitamin and mineral premix [2]	20	20	20	20	20
Digestive enzyme mixture [3]	0	1	2	3	4
Total	1000	1000	1000	1000	1000
Proximate composition and energy content (% dry matter basis)					
Crude protein	32.9	31.5	31.8	32.5	32.2
Crude lipid	9.7	7.3	7.5	8.3	8.8
Crude fiber	2.5	2.5	2.3	2.4	2.4
Crude ash	9.8	8.5	9	9	8.6
Nitrogen free extract	45.1	50.2	49.4	47.8	48
GE (kJ g^{-1} diet)[4]	19.34	19.00	19.01	19.19	19.35
DE (kJ g^{-1} diet)[5]	16.69	16.41	16.40	16.55	16.72
P/E ratio (mg CP kJ^{-1} DE)	19.71	19.20	19.39	19.64	19.26

[1]Mixture of Sunflower oil and linseed oil with a ratio of 1:1.

[2]Commercial Vitamin (Super Vit, Arab veterinary Industrial Co., Jordan). 15.000 IU vitamin A, 0.7 g vitamin C (Stay C®, 35% active), 15.000 IU vitamin D_3, 2 mg vitamin E, 2.5 mg vitamin B_2, 2 mg vitamin K_3, 10 mg nicotineamide, 3 mg vitamin B_6, 5 mg vitamin B_{12}, 2 mg vitamin B_1, 2 mg folic acid, 5.5 mg Ca-D-pantothenate, and mineral premix (Eco Vit, Egyptian veterinary produced and feed a additives Co., Demyatta, Egypt). 200 g calcium, 90 g phosphate, 40 g sodium, 2.5 g copper, 48 g magnesium, 3.6 g manganese, 23.5 g zinc, 8 g iron, 450 mg cobalt, 200 mg iodine and 20 mg selenium.

[3]Exogenous digestive enzymes (EDE), ZADO® (enzymic preparation containing cellulases, xylanases, α–amylase and proteases from anaerobic bacterium, patent No. 22155 of Egypt).

[4]Gross energy was calculated by using factors of 23.62, 39.5 and 17.56 kJ g^{-1} for protein, lipid and carbohydrate, respectively (20).

[5]Digestible energy content was estimated as 16.72, 16.72 and 37.62 kJ g^{-1} for protein, carbohydrate and lipid, respectively (21).

Table 2: Growth performance, survival and feed efficiency utilization of rabbitfish fed diets supplemented with EDE for 74 days*

Parameters	EDE supplementation level (g kg^{-1})				
	0	1	2	3	4
INW	1.13±0.02	1.14±0.01	1.09±0.01	1.1±0.03	1.14±0.03
FBW	24.01±0.07^{a}	26.01±0.46^{b}	26.24±0.17^{b}	28.07±0.19^{c}	26.41±0.35^{b}
WG (g)	22.88±0.07^{a}	24.87±0.46^{b}	25.15±0.18^{b}	26.97±0.21^{c}	25.26±0.36^{b}
WG (%)	2025.42±31^{a}	2181.12±33.6^{b}	2307.29±26.6^{bc}	2453.55±56^{c}	2212.88±71. 7^{b}
SGR (%)	4.13±0.02^{a}	4.23±0.02^{b}	4.3±0.02^{bc}	4.38±0.03^{c}	4.24±0.04^{b}
Survival (%)	100	95	90	100	95
FI	55.01±0.29^{a}	59.25±0.14^{b}	59.38±0.28^{b}	62.26±0.09^{c}	59.76±0.32^{b}
FCR	2.4±0.01	2.38±0.05	2.36±0.02	2.31±0.02	2.37±0.04
PER	1.26±0.01^{a}	1.34±0.03^{b}	1.33±0.01^{b}	1.33±0.1^{b}	1.31±0.02^{ab}
PG	142.18±0.00^{a}	146.41±0.01^{b}	147.04±0.04^{c}	147.52±0.01^{d}	146.45±0.08^{b}
PR	25.73±0.14^{a}	27.33±0.07^{b}	27.76±0.13^{c}	29.85±0.05^{e}	28.18±0.17^{d}

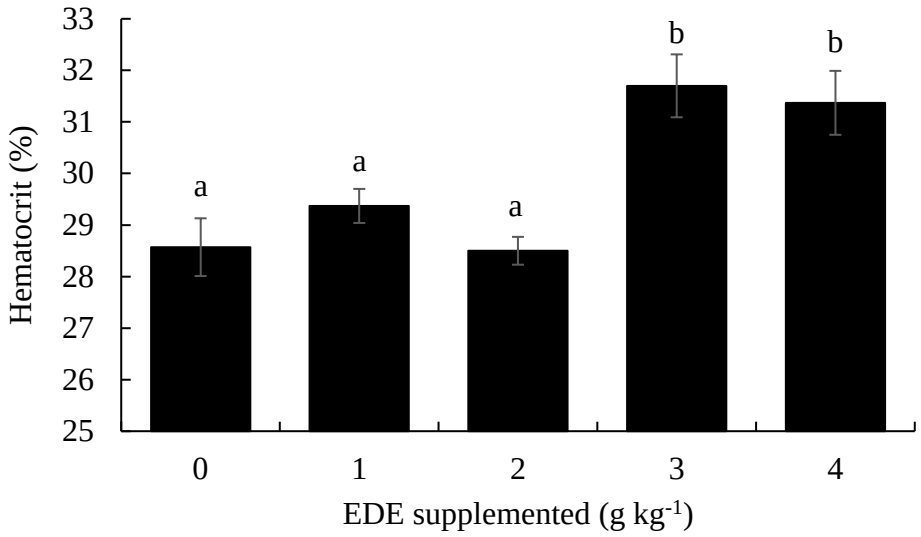
*Values are means of triplicate groups' ±S.E.M. Within a row, means with different alphabet are significantly different (P<0.05), means with the same letters are not significantly different (P>0.05). Absence of letters indicates no significant difference between treatments.

Table 3: Whole body proximate analysis (%) and somatic parameters of rabbitfish fed diets supplemented with EDE for 74 days*

Items		EDE supplementation level (g kg^{-1})				
	Initial fish	0	1	2	3	4
Moisture	70.89±0.65	69±0.4	69.6±0.32	69.3±0.24	69.6±0.06	69.15±0.06
Crude protein	14.18±0.15	14.22±0.29	14.62±0.21	14.68±0.15	14.73±0.1	14.64±0.1
Crude lipid	10.18±0.16	11.06±0.22	11.79±0.45	11.81±0.47	11.79±0.45	11.46±0.75
Crude ash	4.58±0.15	4.62±0.15	4.43±0.33	4.22±0.34	4.27±0.09	4.67±0.09
CF [3]	-	1.98±0.04	1.86±0.01	1.82±0.07	1.76±0.11	1.93±0.03
HSI[4]	-	1.39±0.09	1.45±0.18	1.65±0.09	1.64±0.09	1.54±0.07

*Values are means of triplicate groups ±S.E.M. Within a row, Absence of letters indicates no significant difference between treatments, Initial values are not included in the statistical analysis.

(A)



(B)

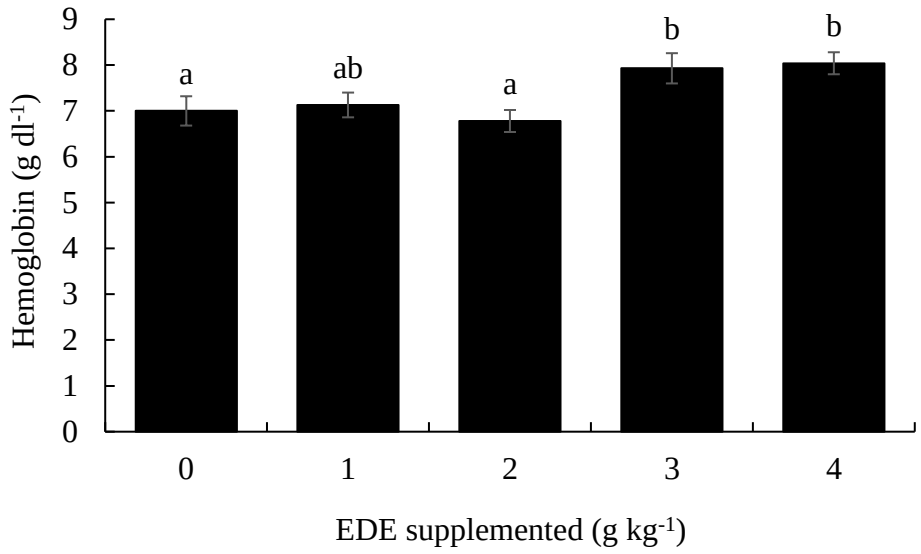
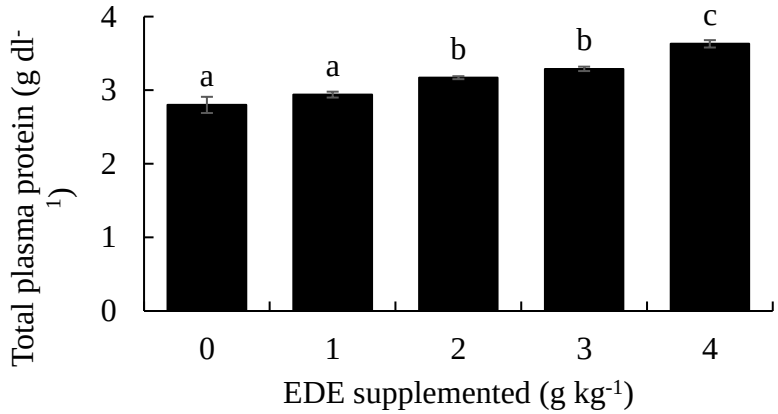
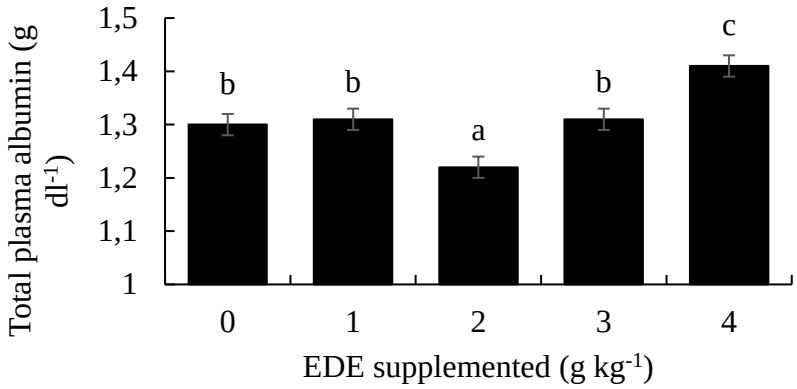


Figure 1: Average percentage of hematocrit (A) and hemoglobin values (B) in the blood of rabbitfish fed diets supplemented with different levels of EDE for 74 days. Values are means ± pooled SEM from triplicate groups. Means with different alphabet are significantly different (P<0.05)

(A)



(B)



(C)

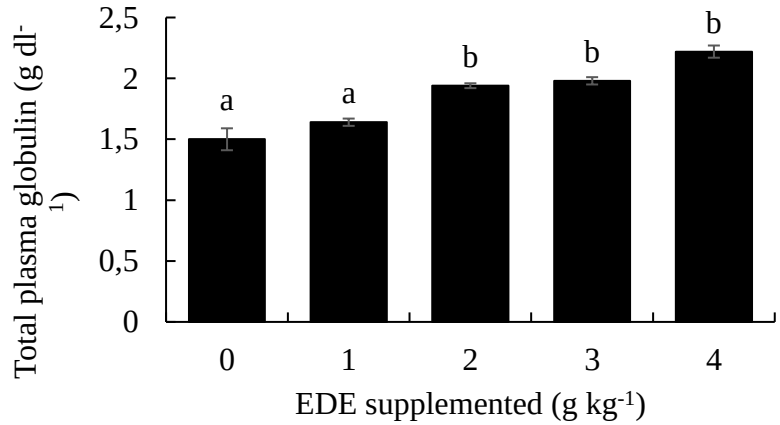


Figure 2: Average percentage of total plasma protein (A), total plasma albumin (B) and total plasma globulin (C) for rabbitfish fed diets supplemented with different levels of EDE for 74 days. Values are means ± pooled SEM from triplicate groups. Means with different alphabet are significantly different (P<0.05)

Discussion

Nowadays, EDE are extensively used all over the world as additives in fish feeds to improve the nutritional value of diets, especially with the increase of plant proteins (22). In this study, FBW, survival, WG and SGR of fish fed diet containing 3 g kg^{-1} EDE were significantly improved. It means that the enzymatic treatment with EDE improved the digestion process to be more suitable for increasing anaerobic bacteria and exogenous enzymes activity. EDE was used as an exogenous enzyme extracted from anaerobic bacteria, would provide cellulases, xylanases, α-amylase and proteases in fish feed. Diets containing EDE at level of 3 g kg^{-1} showed significantly higher SGR than other experimental diets. Similarly, the effects of dietary supplementation of exogenous enzymes increased the growth performance in several fish species including, Channel catfish (23, 24), Clarias catfish (25), Nile tilapia (26-28), Rainbow trout (29), and Common carp (30), respectively. However, Yigit and Keser (9) reported that, using of digestive enzymes in canola-based diets had no positive effect on the growth of Rainbow trout. The discrepancy with the present study was probably due to the differences in species and of plant protein meal used.

In the present study, EDE supplementation in rabbitfish feeds improved feed intake (FI). Fish fed diet containing 3 g kg^{-1} EDE showed increased FI (62.26 g per fish) compared to 55.01 g per fish in the control diet. These findings might be due to the action of EDE, which may be caused by better utilization of some dietary fibre. As a result, the growth rates improved when fish fed the diets containing dietary EDE, reflecting that the lowest FCR value was detected in case of presence EDE in diets at level of 3 g kg^{-1} EDE. In this connection, McAllister et al. (31) reported that proposed modes of action for direct-fed enzymes include "solubilisation of dietary fibre before ingestion, provision of readily fermentable substrate for micro-organisms and/or enhancement of microbial enzyme activity in the gut". Shalaby et al. (32) reported that rabbitfish could utilize efficiently carbohydrates in their feeds.

The results indicated that the diets with EDE improved PER, PG, and PR values. This may be ascribed to stimulating the digestion of the fibrous components by increasing the rate of fibre digestion. Singh et al. (30) reported that the protease enzyme can hydrolyses proteins to short peptides in food which in turn increased the protein digestibility in the gastrointestinal tract and accordingly enhance the feed efficiency and growth performance of fish (33). The enzyme efficiency can be affected by the level of inclusion and species of organism (31). Chakrabarti et al. (34) demonstrated that the amylase activity was enhanced when the formulation contains 13-25% corn starch. Rabbitfish showed a strong activity of β-glucuronidase which is responsible about the digestion of carbohydrate (35, 36). Rainbow trout also exhibited increased levels of absorbed protein as a result of increasing the activity of amylase (37). The amylase activity is affected by several factors including: feeding habit, structural of the carbohydrate, temperature and season of rearing (20, 38). The activity of amylase enzyme is higher in herbivorous and omnivorous fish than the carnivores (36). However, use of EDE in fish feeds will need to be more investigated on the basis of feeding habits, fish size and dietary composition. It is suggested that smaller fish may give better results in growth rate when received EDE containing diets than bigger ones.

Body compositions of rabbitfish were not affected by dietary supplementations of EDE. The present work confirmed the results obtained by Lara-Flores et al. (39). They found that no differences were observed in the whole-body moisture, protein, lipid, and ash contents. On the other hand, Goda et al. (3) reported that Nile tilapia carcass protein, lipid, and ash contents were affected by EDE addition.

Results showed that the somatic indices (CF and HSI) were not affected by the inclusion of EDE in rabbitfish diets. Similar results were obtained by El-Dakar et al. (13) who reported that the HSI was not affected by EDE feeding.

The CF of the fish fed EDE was lower than that of control, which gave a CF similar to fish fed EDE at 4 g kg^{-1}. These results reflect the small differences in weight together with length of the harvested fish.

The general health status of fish can be evaluated by measuring the blood indices which reflect the physiological response of fish towards different feeding strategies (40-42). In this study, high values of hematocrit were observed in fish fed EDE. However, EDE-free group exhibited lower hematocrit levels, which may trigger anaemia in fish (3). Results of the present study also revealed that all fish fed EDE gave higher levels of total plasma protein and total plasma globulin. This may be attributed to fast metabolism which in turn resulted in better total plasma protein and globulin contents.

Conclusion

In conclusion, our results showed that using of EDE for rabbitfish is recommended to enhance the growth, nutrient utilization and health condition. Improved performances were observed in fish fed diets supplemented with 3 g kg^{-1} EDE for 74 days.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

HEMATO-BIOCHEMICAL AND MOLECULAR MARKERS (IS900) OF CATTLE INFECTED WITH JOHNE'S DISEASE IN EGYPT

Ahmed M. Abdelaal[1], Magdy M. Elgioushy[2]*, Shaimaa M.Gouda[1], Mohamed M. El-Adl[3], Emad A. Hashish[4], Shimaa A. Elgaml[4], Mayar O. Ali[5], Attia A El-Gedawy[6]

[1]Department of Animal Medicine, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44511, Egypt,[2]Department of Animal Medicine, Faculty of Veterinary Medicine, Aswan University, Aswan 37916, Egypt, [3]Department of Biochemistry and Chemistry of Nutrition, Faculty of Veterinary Medicine, Mansoura University, Mansoura 35516, Egypt, [4]Department of Clinical Pathology, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44511, Egypt, [5]Department of Animal welfare and Husbandry, Faculty of Veterinary Medicine, Mansoura University, Mansoura 35516, Egypt, [6]Department of Bacteriology, Animal Research Institute, Egypt

*Corresponding author, E-mail: magdy.gioushy@aswu.edu.eg

Abstract: In Egypt, limited studies were performed to investigate Jhon's disease (JD) caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). This study was concerned with diagnosis of MAP in cattle dairy farms using indirect-ELISA technique and amplification of IS900 using PCR technique for confirmation of infection and investigated adequate diagnostic markers for MAP through determining hemato-biochemical profile in cattle dairy farms. Screening of MAP was done by indirect ELISA between the periods from January 2017 to June 2018. A total of 200 cows suffered from persistent diarrhea (n=17) and apparently healthy cows in contact with them (n=183) were used in this study. Positive JD was reported in 62 cases (31%) and negative JD was reported in 138 cases (69%). Out of the positive JD, 17 cases were clinically affected by persistent diarrhea and weight loss (clinical JD), where 45 cases were apparently healthy (sub-clinical JD). Typical colonies of MAP were isolated from the fecal samples of clinically affected cows with positive staining of MAP bacilli. Amplification of IS900 using PCR technique confirmed the presence of MAP infection. Phylogenetic analysis revealed identical similarities with MAP strains in India and Egypt. Hemato-biochemical investigations revealed significant decrease in RBCs, PCV, total protein, globulin and glucose with non-significant change in oxidative stress markers in clinically affected cows. This study is considered one of the limited researches that highlight the role of MAP infection in dairy farms in Egypt.

Key words: dairy cattle; ELISA; IS900; Johne's disease; MAP

Introduction

Johne's disease is a chronic infectious enteric disease of ruminant where MAP is the causative agent of the disease. It is a slow growing, gram-positive and acid-fast bacterium that requires several months of incubation (1). Severe economic losses in the cattle industry were reported in farms infected with MAP (2). Ingestion of colostrum, water and feed contaminated with fecal matters via the fecal oral route can predispose to JD infection (3).

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The disease has a characteristic cross infection between species without restrictions, thus it is hard to be controlled (4). Vaccination may be effective in controlling infection and reduction shedding of MAP in the fecal matter with no complete elimination of infection (5). During the clinical stage of JD, the infection causes excessive diarrhea and wasting of the affected animal. Sub-clinically affected animals may, additionally, shed the bacterium with colostrum, milk and feces, particularly in the last stage of the disease (6). Furthermore, JD has zoonotic importance as MAP existed in the pathogenesis of Crohn's disease (CD) (7).

Several methods might be used for diagnosis of JD. Identification of MAP through fecal subculture consumes a long time and therefore it is not considered an appropriate method of diagnosis. Additionally, ELISA can be used for the detection of the immune reaction of the microorganism (8). Polymerase chain reaction (PCR) can be used in amplification of certain genes in MAP (9). Several specific genetic sequences for MAP have been identified, such as IS900, F57element, and hsp X gene. The IS900 is considered the target of choice and the most widely used for MAP detection (10).

The aim of the current study was to determine hemato-biochemical profile of cattle herds infected with MAP with the aid of indirect-ELISA technique and amplification of IS900 using PCR technique for confirmation of infection as well as to construct a phylogenetic analysis for determining the genetic difference between current infection and other infected strains in other localities.

Materials and methods

Animals and clinical examination

Two hundred dairy cows were selected from three dairy farms in Sharqia and Ismailia Governorates in Egypt during the period from January 2017 to June 2018. The selection of cows was based on the existence of persistent diarrhea in addition to all cows over 3 years of age in the same farms were also selected. Cows were classified into three categories based on the ELISA screening test. Clinically affected cows were classified based on positive ELISA results in association with persistent diarrhea (n=17). The sub-clinically infected cows with Johne's disease was classified based on positive ELISA results without any clinical signs (n=45). Out of the remaining negative cows (n=138), 15 cows were randomly selected as negative control cows. All cows enrolled in this study received a thorough clinical examination via methods that have been previously described in cattle (11).

Blood and fecal samples collection

Blood samples were collected from the jugular vein of two hundred selected cows for the initial ELISA screening test. Seventy seven blood samples were collected from the clinically, sub-clinically and control cows for hemato-biochemical profile. Fecal samples were collected from clinically affected cows from the rectum for the bacteriological isolation, identification and amplification of IS900 using PCR technique.

MAP antibodies titration

Serum from all selected cows was examined for anti-MAP antibodies in ELISAs. To titrate anti-MAP antibodies, we used *Mycobacterium paratuberculosis* (MAP) test Kit for dairy cattle PARACHEK2 (Prionics, Switzerland) according to (12).

Examination of fecal samples

Fecal samples were collected from 17 clinically affected cows which were positive by ELISA and associated with persistent diarrhea. Two grams of fecal sample were collected from each animal and kept into a sterile mortar and decontamination of the fecal sample was done using 30 ml of 0.9% Hexadecylpyridinium chloride solution (HPC), to 3g of aliquot for 24 h through vortexing and shaking (13). After 30 min, at room temperature, 20 ml of the supernatant was transferred into another sterile tube and placed for 24 h at room temperature in a dark place in a vertical position, and then the tubes were centrifuged for 10 min by 3000 rpm (14). Three hundred µl of the decontaminated pellet was used after discarding of the supernatant and inoculated with Herrold's egg yolk medium (HEYM) slants and supplementation with

mycobactin J. One week incubation of slants was done in a horizontal position at 37ºC, and then the tubes were kept in a vertical position for 8-16 weeks incubation period. Later the tubes were being checked for the growth of typical MAP colonies. Then smears were taken, from the suspected samples for microscopic examination.

Ziehl-Neelsen staining of fecal smears

Prepared fecal smears were air dried for 10 minutes, followed by a heat fixation (60-70ºC), for 2 h. The smears were dipped in carbol fuchsin 1%, and then gently heated until a small portion of steam rises without reaching the boiling point. All slides were kept for 5-10 minutes, and then rinsed with tap water followed by addition of acid alcohol (3% Hcl in 70% methyl alcohol) for 1-2 minutes then thoroughly rinsed with tap water again. Slides were immersed in Methylene blue 5%, and left for 1-2 minutes, then rinsed with tap water and dried in air. Finally, the slides were examined under oil immersion lens.

Molecular detection of MAP using IS900

Extraction of DNA from isolated MAP was carried out, using QIAamp DNA Mini kit (Qiagen, Germany), according to manufacturer's instruction. Amplification of IS900 MAP was performed on Bio-Rad T100 Thermal cycler (Hercules, CA, USA) with a 60 ng of DNA template, 12.5 µl of Emerald master mix (Takara, Japan) and 2 µl of forward and reverse primer for MAP IS900 marker (F: CCG CTA ATT GAG AGA TGC GAT TGG; R: AAT CAA CTC CAG CAG CGC GGC CTC G) (15). PCR was started with an initial denaturation step at 94ºc for 10 minutes, then followed by 35 cycles of denaturation for 60 seconds at 94 ºc, annealing for 90 seconds at 61 ºC and extension for 60 seconds at 72 ºC to amplify the fragment of DNA of 298 bp for IS900. PCR products were then run on gel electrophoresis (2%) for visualization of the amplified PCR product. The required product size was optimized using a 100bp DNA ladder (Jena bioscience, Germany) and was cut with a clean scalpel for purification of DNA fragment with Gene JET Gel extraction kit (Thermo Scientific, Lithuania). The purified DNA products were then sent for sequencing in 3130 X DNA Sequencer (Genetic Analyzer, Applied biosystem, Japan) with both forward and reverse primer used in PCR.

Phylogenetic analysis

The obtained nucleotide sequence was aligned, trimmed with the aid of ClustalW that was integrated with MEGA 6 software (16). The trimmed DNA samples were checked on Genbank for determining similarity index between other species of MAP on Genbank**.**

Hematological analysis

Two ml of blood samples were collected into EDTA tubes for the investigation of hematological parameters. Total RBCs count was manually done by using Neubauer-ruled hemocytometer. Hemoglobin (Hb) concentration was determined using the method of cyanomethemoglobin (J.T. Baker, London, England). Packed cell volume (PCV) was determined by microhematocrit centrifugation. The value of PCV was determined by using a built-in reader. The erythrocytic indices, include mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC), were calculated from the value of total RBCs count, Hb concentration and PCV by using the standard formulas (17).

Biochemical analysis

Five ml of blood was collected into plain test tubes without anticoagulant. It was used for the separation of serum samples for the biochemical investigations. Blood left at room temperature for 2 h to be clotted, and then centrifuged at 3000 rpm for 10 min. All biochemical parameters were analyzed colorimetrically by commercial kits (BioMérieux, Marcy, L'Etoile, France). The serum glucose (18), serum cholesterol (19), triglycerides (20), serum total protein (21) and albumin (22) were measured. Globulin was calculated based on subtracting serum albumin from serum total protein. Liver transaminases (ALT & AST) (23) and alkaline phosphatase (24) were determined. Creatinine (25) and urea (26) were also measured.

Antioxidant status and oxidative stress markers

Several parameters were evaluated in the serum samples to measure the antioxidant status and stress markers. The total antioxidant capacity (TAC) was calculated (27), serum glutathione–S-transferase (GST) activity was estimated using Chloro dinitro benzene derivative (28). Reduced glutathione (GSH) concentration was colorimetrically determined using dithio dinitrobenzene derivatives (29). Hydrogen peroxide (H_2O_2), malondialdehyde (MDA) and nitric oxide (NO) concentrations were determined colorimetrically according to (30-32), respectively.

Statistical analysis

Differences between subclinical, clinical JD and control cows were analyzed using the Kruskal-Wallis with post hoc Dunn's multiple comparison tests because of the non-normal distribution of markers. Results were presented as medians ± standard errors (S.E.). The significance was determined at $P< 0.05$. All statistical analysis was performed using the statistical software (SPSS Inc., Chicago, IL, USA).

Results

Clinical signs

Signs of clinically affected cows with JD were recorded, (Table 1). Clinically affected cows showed normal systemic reactions (body temperature, respiratory rate and heart rate). Persistent diarrhea, chronic loss of body weight, pronounced decrease in milk yield and dehydration (sunken eyes) were the most pronounced clinical signs observed in 100% of cases. Reduced ruminal sound, right ping sound and sub-mandibular edema were observed in 29.4% of clinically affected cows. Rectal palpation revealed corrugated rectal mucosa in 70.6% of cases. Diarrhea was watery, contains air bubbles. Marked reduction was observed in milk production in clinically affected cows when compared with sub-clinically infected cows and control (average milk production was 10±2.4 kg/day in clinically affected cows, 18±3.1 kg/day in sub-clinically infected cows and 21± 5.3 kg/day in control cows).

Detection of MAP antibodies

The MAP antibodies were detected in the serum samples of clinically affected and sub-clinically infected dairy cows of two hundred investigated dairy cows for the presence of MAP IgG antibodies for in serum of cattle. Specific anti-MAP IgG antibodies were detected from the serum samples of the clinically infected cows 8.5% and sub-clinically affected cows 22.5%, but no IgG antibodies were detected from the serum samples of the control cows.

Isolation and microscopic examination of MAP

MAP was usefully isolated from 4 out of 17 fecal samples of clinically affected cows which diagnosed by indirect ELISA. After 16 weeks of incubation on Herrold's media containing egg yolk slope, typical colonies of MAP were observed and appeared very small, convex (hemispherical), soft, non-mucoid and initially colorless and translucent. Staining of direct fecal smears revealed 3 of 17 fecal samples were positive. Microscopic appearance of MAP showed red acid alcohol-fast Ziehl-Neelsen staining bacilli.

The amplification of MAP DNA samples was sequenced, matched and deposited on Genbank with accession number of MH663496 and named as *Mycobacterium avium* subsp. *paratuberculosis* Egy1. The aligned MAP species are KY587112, KT075353, KT075351, KT075350, MH663496, KJ173784, JQ837281, EU130943, EF514831, EF536056, EF536046, S74401, AF416985, AF305073, AJ250018, AJ011838, AB052552, AJ250023, AJ251434, AJ250022, X16293, EF536047, AY974347, KT275243, EF536041, FJ775182, AY974346, JN983503, EU232747, EF514825, AF455252, EF536048, EU714039, EF514828, KJ173781, EU232756, EU714041, EU232748, HQ830160, EU057153, EU057170, EU714040, EU232755, KJ882903, HM172613, GQ144322. It showed a 100% identity with MAP strain KVAFSU_S196. Phylogenetic tree (Fig. 1) was divided into two clades (clade A and B) where our isolated species of MAP was located in subclade B among other related species of M.avium (MAP strain

KVAFSU_S196, MAP strain 25, MAP strain steroyl ACP desaturase, MAP strain B42, MAP strain 25C) that was isolated from India. Moreover, pairwise distance showed 0.0009 with our isolated strain. The Indian strain and our Egyptian strain were shown a close genetic distance with high node support. The highest pairwise difference was observed in JQ837281 (1.3 EU130943 (1.4), S74401 (1.3), AJ011838 (1.3) and KT275243 (1.4). Those species were located in different localities in Australia, India, and United Kingdom and in Egypt.

Hematological analysis

Clinically affected cows showed a significant *(P<0.05)* decrease in the RBCs, PCV and the level of MCV with a significant *(P< 0.05)* increase in the level of MCHC, meanwhile the Hb concentration revealed a non-significant changes when compared with the control and sub-clinically infected group. The blood indices in this group reflect microcytic hyperchromic anemia. There was a non-significant change in the RBCs, Hb, PCV, MCV, and MCHC level in sub-clinically infected cows when compared with the control ones. The blood indices were close to the level of normal in this group, (Table 2).

Biochemical parameters

Clinically affected cows showed a non-significant change in the levels of cholesterol, triglycerides, albumin, serum hepatic marker enzymes (ALT, AST, ALP) and the markers of renal damage (urea and creatinine), meanwhile the levels of serum total protein, globulin and glucose were significantly *(P< 0.05)* decreased when compared with the control and sub-clinically infected group. All these parameters revealed a non-significant change in the sub-clinically infected cows when compared with the control, (Table 3).

Antioxidant status and oxidative stress markers

Clinically affected cows showed non-significant changes in total antioxidant capacity (TAC), glutathione S-transferase (GST), glutathione (GSH), Hydrogen peroxide (H_2O_2), malondialdehyde (MDA) and nitric oxide (NO) when compared with the control and sub-clinically infected group. All these oxidative and lipid peroxide markers revealed a non-significant change in sub-clinically infected cows, meanwhile There was a significant *(P< 0.05)* decrease in the level of Hydrogen peroxide (H_2O_2) when compared with the control, (Table 4).

Table 1: Clinical picture of 17 cows affected by Johne's disease

Clinical signs	Clinically affected cows by Johne's (n=17)	
	Number of cows	%
Normal vital parameters*	17	100
Normal ruminal motility**	12	70.6
Hypomotile rumen	5	29.4
Right ping sound	5	29.4
Chronic weight loss	17	100
Persistent diarrhea	17	100
Dehydration (sunken eye)	17	100
Decrease in milk production	17	100
Submandibular edema	5	29.4
Corrugated rectal mucosa by rectal palpation	12	70.6

*Normal vital parameters mean normal reference range of temperature (38.2-39.2° C), heart rate (70- 90/ minute) and respiratory rate (20- 30/minute) (11).

** Normal ruminal motility (3-5 ruminal contraction/2minute) (11).

Table 2: Hematological picture in control, sub-clinically and clinically affected cows

Parameter	Control (n=15)	Sub-clinical Johne's (n=45)	Clinical Johne's (n=17)
RBCs 10^{12}/L	5.8±0.19 ^{a}	5.85 ±0 .16^{a}	3.97±0.18^{b}
Hb g/dl	6.6±0.45^{a}	6.9±0.27^{a}	7±0.29^{a}
PCV %	38±1.17^{a}	35.1±0.85^{a}	21 ± 1.5^{b}
MCV fl	65.32±0.9^{a}	60.4±0.32^{a}	53.03±0.8^{b}
MCH pg/ cell	12.1±0.77^{b}	11.8 ±0.55^{b}	17.64 ± 1^{a}
MCHC %	17.37±.02^{b}	19.64±0.12^{b}	33.33 ±.01^{a}

Medians with different superscripts indicate significant difference at P <0.05

Table 3: Biochemical picture in control, sub-clinically and clinically affected cows

Parameter	Control (n=15)	Sub-clinical Johne's (n=45)	Clinical Johne's (n=17)
Glucose mg/dl	48±4.5^{a}	45.45±1.9^{a}	38±4.1^{b}
Cholesterol mg/dl	157±11.2^{a}	165±10.6^{a}	169±21.2^{a}
Triglycerides mg/dl	49±3.17^{a}	55±2.55 ^{a}	58±4.7^{a}
Total protein g/dl	8±0.3^{a}	8.64±0.28^{a}	5.15 ± 0.48^{b}
Albumin g/dl	3.52±0.28^{a}	3.8±0.15 ^{a}	3.15±0.31^{a}
Globulin g/dl	4.48±0.38^{a}	4.84 ± 0.27^{a}	2 ± 0.37^{b}
ALT u/l	10±0.4^{a}	13±0.66^{a}	11±0.62 ^{a}
AST u/l	19±0.81^{a}	16±0.85^{a}	18±0.77^{a}
ALP u/l	112±30.4^{a}	89±33.7^{a}	99±24.67^{a}
Urea mg/dl	25.06±2. 83^{a}	20.14± 2.97^{a}	17.17±2.76^{a}
Creatinine mg/dl	0.87±0.068^{a}	0.8±0.041^{a}	0.6±0.067^{a}

Medians with different superscripts indicate significant difference at P <0.05

Table 4: Oxidative stress biomarkers in control, sub-clinically and clinically affected cows

Parameter	Control (n=15)	Sub-clinical Johne's (n=45)	Clinical Johne's (n=17)
GST u/L	163.7±11.76^{a}	160.7±54.1^{a}	168.7±37.8^{a}
GSH mg/d	9.73±1.04^{a}	11.7±0.5^{a}	11±1.17^{a}
TAC mM/L	4.1±2.28^{a}	3.8±2.3^{a}	3.9±0.21^{a}
H2O2 mM/L	0.3±0.012^{a}	0.26 ± 0. 01^{b}	0.28±.01^{ab}
MDA nmol/ml	6.6±1.3^{a}	6.2±1.2^{a}	5.6±0.9^{a}
NO umol/L	18±3.6^{a}	23±4.2^{a}	29± 6.06^{a}

Medians with different superscripts indicate significant difference at P <0.05

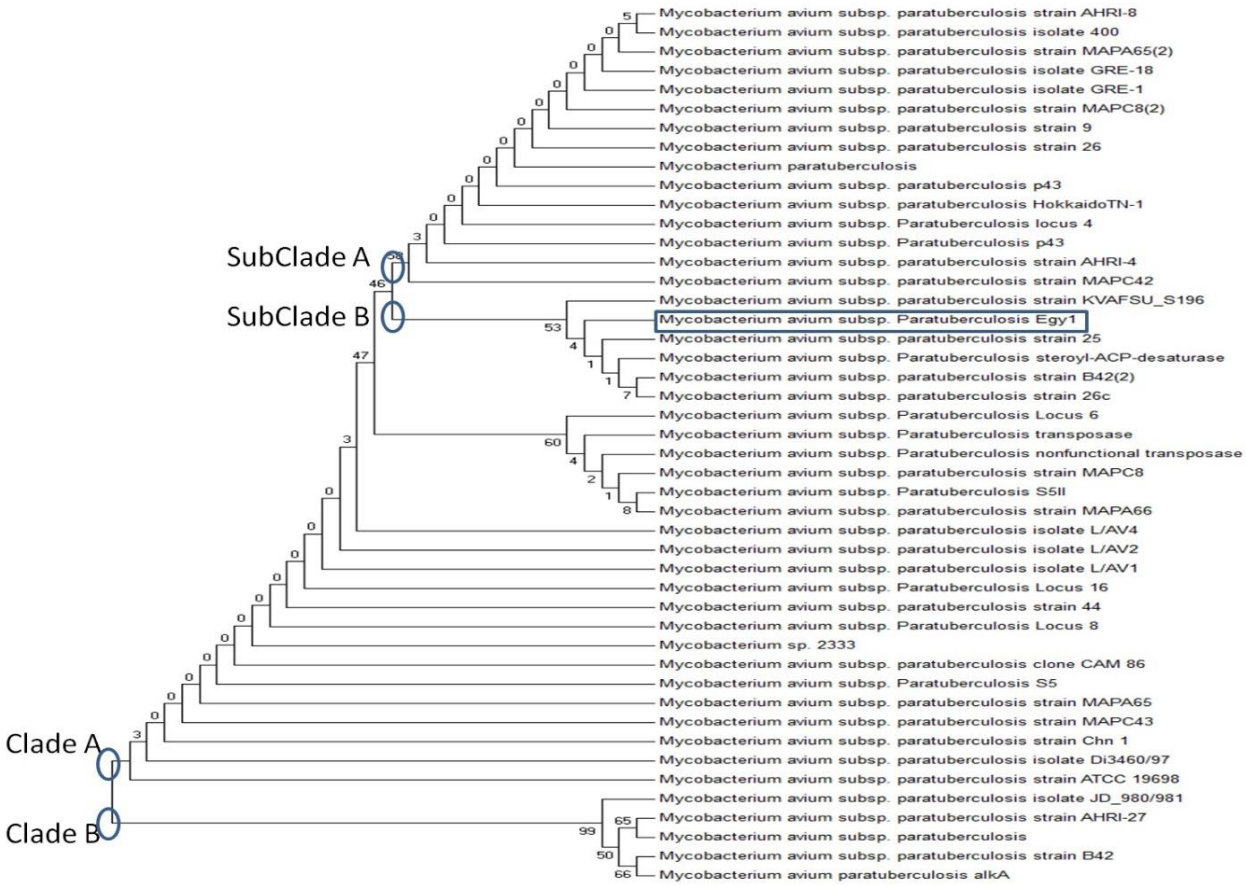

Figure 1: Phylogenetic analysis of different strains of MAP infection with our isolated strain"blue rectangle". phylogenetic tree was divided into two clades; clade A and clade B. Clade A was subdivided into two sub clades A and B where our isolated sequence (Mycobacterium avium subsp paratuberculosis EGY1) was located (sub-clade B)

Discussion

MAP has severe hazard to animal health. It causes significant economic losses to livestock animals worldwide. Limited studies were carried out to investigate MAP infection in Egypt, which directs our attention to this disease. Identification of MAP requires either culturing organisms in specific media or detecting the antibodies in sera of infected animals (33). The use of diagnostic markers to study the response of the host toward MAP infection is crucial for diagnosis of Johne's disease. Generally, ELISA is considered the most widely used test that assigned to detect MAP infection in animals. ELISA has been used in this study for the differentiation of the clinically affected cows from the sub-clinically infected cows. This based on the immune reaction of the microorganism in correlation to the presence or absence of the clinical signs in animals.

Alterations in the general health condition were observed. The heart rate, respiratory rate, and temperature remain normal which coincided with the previously reported data (4). The most common clinical signs reported in clinically affected cows were persistent diarrhea, chronic loss of body weight and dehydration which were nearly similar to those previously reported(34). The associated diarrhea may be due to the release of histamine as a result of antigen antibody reactions in the intestine (35). The characteristic thickened corrugated intestine by rectal examination might be attributed to the associated inflammatory process which involves the lymph node draining the intestine (36). The chronic loss of body weight might be

due to the inflammation which resulted in thickening of the intestinal wall, leading to poor nutrient absorption in affected animals (4). Submandibular odema was observed in some cows, it might attribute to protein loss from the blood stream into the digestive tract (37).

Generally, the use of IS900 had been used extensively for studying MAP in domestic animals (38, 39). PCR positive result of MAP for IS900 can be used correctly in the identification of the causative agent for Johne's disease (40). Different sequences for IS900 were deposited in Genbank, besides the original sequence isolated from United Kingdom (X16293) (33) with a product size of around 1500 bp which showed a change in a nucleotide sequence of our obtained sequence with a pair wise genetic difference of 0.009.

Our obtained sequence of IS900 was identical to a sequence that was isolated from India (KY587112), while it was showing a higher difference when compared with other strains from Egypt (KJ173784 and KJ173784), although they were isolated from the fecal matter of cattle. In the two strains from Egypt, our isolated strain of MAP showed 98% similarity index, but was named as transposase sequence where it is responsible for transposition of gene in prokaryotes. Insertion sequence regulates gene transposition (41). On the contrary, another isolated sample from Egypt (JN983503) was isolated from fecal matter of bovine species would find to share higher than 99% similarity index with our obtained sequence.

Johne's disease is reported to induce some hematological alterations. Significant decrease in the RBCs count and the PCV was reported in clinically affected cows when compared with sub-clinically infected and control ones. The significant decrease in the RBCs count is similar to that observed in JD clinically affected camels (42). Similar results of a significant decrease in PCV were also reported (43). These findings indicated the presence of anemia among the clinically affected cows, which is similarly reported before (44). Anemia is an important common extra intestinal complication associated with Crohn's disease in human. The microcytic hyperchromic anemia was reported in our study, where Crohn's disease show the same type of anemia which characterized by microcytic hyperchromic cells (45).

The use of serum biochemical parameters can be used as a diagnostic marker for certain diseases(46). Changes in the serum biochemical parameters were noticed. Significant decrease in the total protein in clinically affected cows was reported compared with sub-clinically infected and control cows. This was previously reported in the serum samples of positively infected cows with MAP (47). The hypoproteinemia might attributed to protein-losing enteropathy which might be due to the impaired integrity of the mucosa of the gastrointestinal tract (48) or due to the increase in the gut motility with a decreased in the absorption results in an increase in the intestinal loss of protein (43). Most of the cows suffer from clinical Johne's disease showed a low level of albumin with a normal level of globulin (49). Significant decrease in globulin was reported in our study. Hypoglobulinemia was previously reported in Crohn's disease which attributed to the decrease in globulin synthesis or increased catabolism of immunoglobulins (50). Hypoglycemia was reported in clinically affected cows when compared with sub-clinically infected and control cows. The presented findings matched with other researchers who reported hypoglycemia in response to gastrointestinal inflammatory disorders resulted from infection (51, 52). While serum biochemical parameters (cholesterol, triglycerides, ALT, AST, ALP, urea and creatinine) were reported to be non-significant changed. The non- significant change in the serum creatinine was similar to that observed in camel infected with JD (43).

With concern to oxidative and antioxidative markers, the non-significant changes in antioxidative markers (GST, GSH and TAC) in clinical and subclinical groups compared to the control group is nearly similar (53) who noted non-significant changes in serum superoxide dismutase and glutathione perioxidase activities of *M. paratuberculosis* positive cows. Non-significant changes in oxidative markers (H_2O_2, NO

and MDA) in clinically affected cows are indicative they are not associated with oxidative stress.

It could be concluded that the infection with the clinical form has a serious significant impact on the health of cows and the production of Egyptian dairy farms. A more continuous exact evaluation of its economic impact might provide a greater impact with an insight into the cost efficiency of detection and screening for subclinical infections, which could help in prevention the new infections and also improve the health and productivity of the herd. The parameters related with hemato-biochemical parameters such as glucose, total protein and globulin level in the present study may be considered in the differential diagnosis of clinical and subclinical *M. paratuberculosis* infection in dairy farms and waiting for further confirmation of the JD by definitive tests as culture, ELISA and PCR. Still more deep investigation on a large scale is needed to identify the number of cases affected with JD in different localities in Egypt.

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Original Research Article

EFFECT OF FEEDING DIFFERENT CONCENTRATE ROUGHAGE RATIO ON GROWTH, REPRODUCTIVE PERFORMANCE AND BEHAVIOR OF SHEEP

Anaam E. Omar [1]*, Heba S. Gharib[2], Enas N. Said[2]

[1]Department of Nutrition and Clinical Nutrition, Faculty of Veterinary Medicine, Zagazig University, 44511, Egypt, [2]Department of Veterinary Public Health, Faculty of Veterinary Medicine, Zagazig University, Egypt

*Corresponding author, E-mail: dr.anaamomar85@gmail.com, madamreda72@yahoo.com

Abstract: The goal of this study was to examine the influence of feeding different concentrate roughage ratios on growth, reproductive parameters and behavior of sheep. The study design involved the use of a 30 sexually mature ewes assigned to three groups consisting of three ratios of concentrate (C) to roughage (R), Group1. 80%C: 20%R, Group2. 70%C: 30%R and Group3. 60%C: 40%R. The variables of the studies were live weight changes and Body condition score (BCS) of ewes during pregnancy and postpartum till weaning and behavior of ewes in addition to the birth weight and average daily gain of neonatal lambs and subsequent changes till weaning. The conception rate of ewes and their overall reproductive performance are under investigation. The results reveled that, different ratios of concentrate and roughage feeding had no significant effect on body weight, BCS and average dry matter intake of ewes during pregnancy and postpartum till weaning. Lambs birth weight and subsequent weights till weaning and average daily gain were significantly (P≤0.05) increased by feeding on ration contain 80%C: 20% R, followed by those feeding on 70% C: 30% R and lowest in those feeding on 60% C: 40%R. Rumination was significantly increased in Group3 lambs than other groups. Higher concentrate ratios had positive effect on reproductive performance of ewes and body weight, daily body weight gain and neonatal behavior of lambs till weaning.

Key words: concentrate; roughage; ewe; lamb; growth performance; behavior

Introduction

There are about 5.6 and 4.13 million heads of sheep and goats in Egypt (1). Sheep and goat production constitute a major part of Bedouins income but in extensive system of production, quality and quantity of pastures affected by weather and environmental changes which would affect feeds availability and quality for sheep (2). These changes had an effect on the nutrition and performance of lambs and their dams. Above pasture conditions would have an impact on birth weight, on quantity of milk production and on weaning weight of lambs. Behavioral understanding is essential for the performance enhancement of sheep on grazing, mainly due to intense climate changes which influence animal physiology. However, it is important to enhance the utilization of pastures

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and applying strategies for nutritional management achieving the system highly productive (3).A study in India by (4) also reported significant effect of raising lambs on ADG under intensive system where lambs feed on complete rations compared with extensive system.

Feed quality and quantity are the main factors affecting ruminant productivity under tropical conditions, providing large quantities of concentrated feeds in the diet increase energy density of the diet that may improve feed efficiency and animal performance (5) however, feeding on high concentrate diet for long time may be result in decreased ruminal pH due to accumulation of volatile fatty acids and lactic acid and cause chronic digestive disorder (6). Therefore, feeding on proper concentrate level is one of the most important factors to ensure the growth and reproduction of ewes and their lambs. Nutrition play important role on reproduction performance of ruminant animals (7) also affect the onset of postpartum ovarian cyclicity of ruminant animals (8) but there is lack of information regarding the effects of diet on the lambing rate and overall reproductive efficiency of the fat-tailed sheep. In a study, (9) showed that high concentrated feed caused higher ovulation rate and less ova viability. The survival of a neonatal is mainly depending on vigor (the ability to stand up and suck milk during a few hours after birth), maternal behavior and colostrum production. These factors are influenced by the ewe's level of nutrition during late pregnancy (10, 11).

The objectives of our study are to define appropriate concentrate roughage ratio that ensure high growth and reproduction performance and behavior of ewes and their lambs.

Materials and methods

This study was conducted at private farm belonging to Sharkia governorate, Egypt.

Animals, housing, diets and experimental design

A total of thirty Baladi ewes were purchased locally and selected on the basis that they had lambed at least once before. Their body weights ranged between 40 to 50 Kg and their ages varied between 2.5 to 3 years. They were identified by ear tags, vaccinated against pasteurellosis, sheep pox, colostridial, pestedespetits ruminant (PPR) and enterotoxaemia potential infections. The ewes were exposed to the feedlot housing management and feeding as confined animals (zero grazing). Ewes were randomly divided considering age and body weight in to 3 experimental groups (10 ewes/ group) with 10 replicates of 1 ewe in each (Group1. 80%C: 20%R, Group2. 70%C: 30%R and Group3. 60%C: 40%R) in different stages of the reproduction of ewes. Daily observation was carried out on animals for any health disorders. No mortalities were occurred among different groups during the whole experimental period. The sheep sheds were respectively cleaned thoroughly and disinfected to eliminate any possible infection that transferred from previous sheep flocks. The water and feeding troughs were also cleaned. Individual housing for each animal in individual pens with feed and water troughs. All animals were reared under the same management condition. Ewes lambed indoor and new born lambs live together with their dams in individual pens. Lamb weight was calculated at birth and fortnightly till weaning.

The experimental diets were formulated according to specific requirements of ewes (12) as shown in Table 1. Experimental feeds were analyzed for (DM, Moisture, CP, CF, EE and Ash) according to (13). The animals were fed individually twice daily, equally dividing concentrate and roughage components, once in the morning between 6.00 and 7.00 AM, and again between 5.00 and 6.00 PM. The left-over feed was collected on a daily basis in the morning. Actual intake of the concentrate mixture and roughages were recorded.

Growth performance of ewes and lambs

The ewes were individually weighed at beginning of experiment to obtain the average initial body weight then the body weight was

recorded monthly during pregnancy period and biweekly after lambing till weaning of lambs to calculate the average body weight of the animals in each group. Feed intake of each ewe was recorded as the difference between weight of the feed offered and residues left. The birth weight of new born lambs was taken within one hour after birth. The subsequent weight of lambs was recorded fortnightly in the morning and before feeding up to weaning. The lambs weight gain was measured as the difference between final body weight at the intended period and primary body weight at same period.

Body Condition Scoring Procedures

The BCS evaluates the body fat reserve of the sheep and was determined by examining the tail head and the loin areas according to (14). The BCS of sheep was measured by feeling the backbone and the transverse processes of vertebrae in the loin region by the finger.

Reproductive performance

The ewes were treated with progestogen-impregnated (30 mg) intravaginal sponges for 14 days. After 14 d, the sponges were withdrawn and 400 I.U. of Pregnant Mare Serum Gonadotropin (PMSG) was injected intramuscularly. The ewes were then introduced to rams for natural mating. The number of conceived ewes and number of lambs born a live and number of abortion and still birth were recorded. Litter size was calculated as total number of lambs born / total number of ewes. The live weight of lambs was measured within one hour after lambing.

Behavior measurement

Focal sampling technique was used to record the time spent by ewes with each behavior. The behavioral activities were recorded through 10 hours (from 7.00 am till 17.00 pm) per week at 10 minutes interval. Behavioral traits recorded are eating, drinking, rumination, standing, walking, lying, urination and defecation. Eating: the mean time (min) spent in eating ration per 10h observation, Drinking: The mean time (min) spent in drinking water per 10 hours observation, Rumination: the mean time (min) spent in rumination /10h observation. Standing: The time (min.) spent in standing without doing any activities during 10hr, observation, Lying: time (min) spent in lying on the ground during 10hr observation, Defecation: when ewe makes voiding of food and water that are not retained in the body for metabolic processes as a solid material from the anus (faeces), Urination: when ewe makes voiding of water that are not retained in the body for metabolic processes as a liquid. While the data for neonatal lambs were collected for the first 2 hours following lambing. The observer recorded the duration in minutes that the neonate spent to be able to stand up without help, to reach udder (approach udder and nudge the inguinal region), time to suck (holding the teat with its mouth and appears to be sucking for more than 5 seconds) and the suckling attempts as described by (15,16). Behavior was evaluated by an observer stood up 2 meter from the pen using an observation sheet, a stop watch and a photograph during the observation period.

Statistical analysis

The collected data were subjected to analysis of variance using one-way ANOVA using SPSS 24.0 for Windows (SPSS Inc., Chicago, IL, USA) and the differences between experimental groups were calculated by Duncan's multiple-range test at $(P \leq 0.05)$(17).

Results and discussion

In this study three ratio of concentrate and roughage diets were considered and hypothesized as main contributing factors affecting the productive and reproductive performances of ewes under the feedlot (zero grazing) management system.

The effect of C: R ratios assigned to 80%C: 20%R, 70%C: 30%R and 60%C: 40%R on the live weight and BCS of ewes during pregnancy and after parturition till weaning are presented in Table 2. No significant ($P \geq 0.05$) influence of C: R ratios on live weight and BCS of ewes are observed. Our data disagree with results of previous studies where the final live weight and total live weight gain of does increased with higher level of concentrate supplementation (18,19,20), moreover (21) reported that ewes

under sub-feeding level (0.500, 0.750 and 1.00 kg concentrate /ewe) during first 15 weeks of pregnancy, last 6 weeks of pregnancy and 60 days from parturition till weaning, respectively had lower final weight and BCS than those fed on normal (0.750, 1.00 and 1.25 kg/ewe) and high concentrate level (1.00,1.25 and1.5 kg /ewe). The lack of our effect on growth performance of ewes might be related to the management practice, genetic factor, composition of basal diets (diets are isonitrogenous) and constant feed intake (22).

Average daily DM intake of ewes for the entire duration of feeding periods covering the pregnancy and lactation periods was shown in Table 3. The results showed that different ratio of concentrate and roughage had no effect on average dry matter intake/ewe through advanced weeks of experiment from 1-28 weeks. The lack of effect on DM intake may be due to the diets were isonitrogenous (22). Our results were consistent with previous researches in goats were DM intake was not affected with increasing concentrate percentage in diet from 30% to 70% (23) and (24) in Holstein cow were increasing forage concentrate ratios (47:53, 54:46, 61:39 and 68:32) had no effect on dry matter intake. In contrast feeding high level of concentrate increase dry matter intake of goat (18) moreover, (25) found that increasing concentrate percentage in diet from 30% to 60% increasing dry matter intake of dairy goats. Similarly, cow fed on 30% forage: 70% concentrate diet had significantly higher dry matter intake than those fed on 50% forage: 50% concentrate diet (26).

New born lamb performance

It is important that farmers feed their animals cheap, effective feed ingredients to remain gainful in the face of increasing feed prices. How quickly and efficiently the lambs grow is most important agents affecting economic capability in livestock production. High growing rate of lambs reduce the time needed to reach market weight, which in turn reduces the employments and the feed costs associated with raising of lambs. Composition and quantity of milk are essential for newborn lambs as the milk supplies the main nutrients needed (27), so growth rate of suckling lambs increase if the quantity or nutritive composition of the milk increase.There are also non-genetic factors can affect the lamb growth rate, one of them is system of production, the results showed that the feed lot rations were related with improved lamb growth rates, bigger carcass fatness and better dressing percentage (28).

The birth weight and biweekly changes of live weight of new born lambs till weaning are shown in Table 4. The mean lambs birth weight was significantly ($P \leq 0.05$) increased by greater concentrate level feeding. The average BW and ADG at week 2 were significantly ($P \leq 0.05$) increased in group1 in comparison with group 2&3. The average body weight and ADG at week 4 were significant ($P \leq 0.05$) the highest in lambs fed on 80% C: 20% R followed by lambs fed on 70% C: 30% R and lowest in lambs fed on 60% C: 40 % R. The lambs fed on 80%C: 20%R had ($P \leq 0.05$) significant higher average BW and ADG at week 6 and 8 followed by lambs fed on 70%C: 30%R while lambs fed on 60%C: 40%R had the lowest. Overall ADG was significantly ($P \leq 0.05$) increased by feeding higher levels of concentrate and it is the highest in group 1 and lowest in group 3. Our results of increasing ADG of lambs with increasing concentrate level in diets are consistent with results of (29,30,31). Also, (32,33) indicated that the ADG and FCR of lambs were better in high concentrate diets than low C diets under intensive system of production. Similar types of observations were also recorded by (34) in lambs, (18,35) in kids. However, our data disagree with result of (21) who observed no significant effect on lamb birth weight by feeding different levels of concentrate (0.750, 1.00 and 1.25 kg/ewe). Growth rate of kids feeding different levels of concentrate did not significant different (20). There was no effect of increasing levels of concentrate on feed intake, final live weight, ADG, FCR in lambs (36).

Effect of concentrate: roughage ratio on behavior of pregnant and lactating ewes

Data in the Table 5 revealed that the concentrate percent in ration had no significant impact on most of maintenance behavior of pregnant and lactating ewes. Regarding ingestive behavior, it is observed that ewe fed on Group 3(60%C: 40%R) spent longer eating time than other rations (Group 1and Group 2). These results may be explained to the fact that rapid consumption of concentrates by animals. Our results are in accordance with (22) who found that the eating time was decreased linearly with increasing the concentrate level. The time spent ruminating was significantly higher in pregnant and lactating ewes of group fed on 60%C: 40%R (12.26±0.38 and 11.26±0.4 min/h respectively) than other groups. This may explained to more time needed to ruminate the forage. In a reverse trend, (22) noted that the level of concentrates had no effect on time spent in rumination. Diets rich with concentrate prefered time spent in ruminating during the day while time spent in resting through the night (37).

Effect of concentrate: roughage percent on neonate lamb behavior

The impact of different ratio of concentrate and roughage during pregnancy on some neonatal lamb behavior are listed in Table 6 generally Group 1, Group 2 and Group 3 lambs showed significant behavioural differences. Lambs' response time to stand was shorter in Group 1 lambs, search for the udder and also response times to first suckling were significantly shorter at Group 1 lambs. Supplementation of high concentrate in peripartum period has an impact on lamb vigour. Lambs' response time to stand up without help, search for the udder and also response time to first suckling were significantly shorter in Group 1 neonates in comparison with other groups. These results were comparable with the results described in other sheep breeds (38). Beginning of suckling could achieve after a longer time because Group 2 and Group 3 neonates tend to tumble more times comparing to Group 1 neonates. Postpartum Group 1 ewes moved and cleaned their neonates more frequently, and depending on their maternal experience which can stimulate lamb activity (39). Feeding supplies at birth had an impact on lamb strength. Generally, latency to standing and suckling was slower in low-birth weight lambs than heavier lambs. Lamb vigor is affected by amount of adipose tissue and locomotor ability. Brown adipose tissue provides the neonate lamb with energy needed for thermoregulation and nutrition from birth till colostrum intake (40). Development of CNS of lamb, and subsequently locomotor activity, may be impacted by the nutrition of dam through pregnancy (41).

Reproductive performance of Ewes

In sheep production operations, the producers are interested in the number of ewes becoming pregnant, lamb born alive and lambs survive till weaning and then finishing for the markets. These important variables were summarized showing dietary influences in Table 7. About 80%, 50% and 40% of ewes fed groups 1, 2 and 3 respectively conceived producing a total of 8, 5 and 4 lambs from 10 ewes joined. No abortion or still birth has been reported. All births were for single lamb. The conception rate for ewes was higher in ewes fed on 80%C: 20%R followed by ewes fed on 70%C: 30% R then ewes fed on 60%C: 40%R. No twin birth was observed. The average litter size was higher in group 1 compared to groups 2 and 3. Group 1 was found to have more lambs born/ewe than other two groups. Reproductive performance of the sheep was known to be one of the major factors determining the efficiency of their production (42). Seasons of production, nutrition, breed, age, management and environment are some factors that affect reproductive activities on sheep (43, 44). Our results are consistent with results of (19) where the concentrate supplementation had positive effect on litter size and birth weight of kids moreover, concentration feeding had positive effect of reproductive traits of goat (20). (21) reported no effect of feeding different levels of concentrate on lambing, fecundity or prolificacy rates. In earlier work, (45) observed that the level of feeding or energy in the diet is

a major influencing factor on the birth weight of the lambs. The ewes receiving high energy diet during the last trimester of pregnancy gained more live weight than those receiving low energy diets. (46) found that the ovulation rate of induced estrus increased with improved nutritional regime of ewes, therefore the conception rate and litter size were significantly and positively influenced by the interaction of nutrition and induced estrus. . However, (47) reported no effect of nutrition on estrus and ovulation rate of ewes.

Table 1: Proximate and chemical composition of Experimental diets

Ingredients	**Group 1 (80% C: 20% R)**	**Group 2 (70% C: 30% R)**	**Group3 (60% C : 40% R)**
A. Concentrates			
Wheat Bran, kg	32.4	23	9.00
Corn, kg	34.5	33	35.5
Soyabean Meal (48%),kg	11	12	13.5
*Vit. & Min. Premix, kg	0.3	0.3	0.3
Limestone, kg	100	0.6	0.2
Calcium dibasic Phosphate, kg	0.3	0.6	1.00
Salt, kg	0.5	0.5	0.5
Total A, kg	80.00	70.00	60.00
B. Roughages			
Alfalfa Hay, kg	10.00	15.00	20.00
Wheat Straw, kg	10.00	15.00	20.00
Total B, kg	20.00	30.00	40.00
Total A + B, kg	100	100	100
Chemical composition			
DE, Kcal/Kg	2545	2529.2	2554.1
CP, %	14.07	14.09	14.05
EE, %	2.23	2.03	2.00
CF, %	9.31	9.24	10.44
Ca, %	0.66	0.67	0.69
Available phosphorus %	0.53	0.52	0.51

* Vitamins and minerals premix: Phosphorus 5.00%, Calcium 18 %, Sodium 5 %, Magnesium 5 %, Manganese 500 mg/kg, Cobalt 100 mg/kg, Zinc 2000 mg/kg, Iodine 125 mg/kg, Selenium 10 mg/kg, Vitamin A 400000 IU/kg, and Vitamin E (Alpha-Tocopherol) 400IU/kg. C: Concentrate, R: Roughage, DE: Digestible energy, CP: Crude protein, EE: Ether extract, CF: Crude fiber, Ca: Calcium.

Table 2: Effect of feeding different concentrate roughage ratio on live weight (kg) and body condition score (BCS) of ewes during pregnancy and after parturition till weaning.

Period	Variable	Group 1 (80%C:20%R)	Group 2 (70%C:30%R)	Group3 (60%C:40%R)	*P*-value
During pregnancy					
First month	Weight	46.30 ± 3.16	44.71 ± 4.16	44.29 ± 4.28	0.498
	BCS	3.00 ± 0.00 [a]	3.00 ± 0.00 [a]	2.78 ± 0.26 [b]	0.006
Second month	Weight	48.43 ± 2.10	47.27 ± 3.96	46.46 ± 5.04	0.566
	BCS	3.00 ± 0.00	3.00 ± 0.00	2.89 ± 0.22	0.123
Third month	Weight	49.10 ± 3.82	48.96 ± 3.96	48.68 ± 4.76	0.976
	BCS	3.10 ± 0.21	3.06 ± 0.18	3.06 ± 0.17	0.857
Fourth month	Weight	51.50 ± 3.11	51.21 ± 6.28	50.57 ± 3.98	0.903
	BCS	3.40 ± 0.21	3.25 ± 0.27	3.17 ± 0.25	0.123
Fifth month	Weight	57.01 ± 5.78	56.13 ± 2.25	55.68 ± 3.78	0.801
	BCS	3.65 ± 0.24	3.56 ± 0.32	3.44 ± 0.18	0.227
Postpartum to Weaning					
At Birth	Weight	51.86 ± 2.76	55.44 ± 7.36	49.50 ± 2.38	0.226
	BCS	3.50 ± 0.35	3.58 ± 0.38	3.25 ± 0.29	0.355
At week 2	Weight	49.86 ± 3.56	53.42 ± 7.91	47.70 ± 1.99	0.297
	BCS	3.30 ± 0.27[ab]	3.58 ± 0.20[a]	3.12 ± 0.25[b]	0.032
At Week 4	Weight	50.16 ± 4.25	52.25 ± 7.67	48.10 ± 0.96	0.599
	BCS	3.30 ± 0.27	3.33 ± 0.41	3.00 ± 0.00	0.345
At Week 6	Weight	48.44 ± 3.45	49.67 ± 5.33	48.33 ± 1.15	0.857
	BCS	3.00 ± 0.00	3.17 ± 0.26	3.00 ± 0.00	0.251
At Week 8	Weight	46.84 ± 4.07	49.67 ± 4.56	48.67 ± 1.26	0.519
	BCS	3.10 ± 0.22	3.17 ± 0.26	3.17 ± 0.29	0.895

[ab] Means within the same row carrying different superscripts were significantly different at ($P \leq 0.05$).
C: Concentrate R: Roughage BCS: Body condition score

Table 3: Effect of feeding different concentrate(C) roughage (R) ratio on daily DM intake of ewes.

Advancing weeks (pregnancy and lactation)	Group 1 (80%C:20%R)	Group 2 (70%C:30%R)	Group 3 (60%C:40%R)	*P*-value
1	993.25 ± 8.16	985.17 ± 11.86	991.38 ± 9.36	0.181
2	998.57 ± 1.21	997.71 ± 2.49	999.14 ± 1.00	0.187
3	1095.00 ± 4.54	1097.36 ± 6.60	1099.00 ± 3.16	0.214
4	1097.86 ± 5.63	1097.64 ± 4.30	1100.00 ± 0.00	0.376
5	1094.50 ± 9.83	1097.78 ± 5.48	1100.00 ± 0.00	0.182
6	1100.00 ± 0.00	1099.57 ± 1.35	1100.00 ± 0.00	0.381
7	1100.00 ± 0.00	1100.00 ± 0.00	1100.00 ± 0.00	---
8	1144.88 ± 10.02 [b]	1156.07 ± 9.24 [a]	1151.67 ± 10.27 [ab]	0.053
9	1233.18 ± 17.10	1221.50 ± 25.09	1225.86 ± 17.91	0.442
10	1224.53 ± 17.83	1228.71 ± 14.02	1229.50 ± 17.60	0.773
11	1240.27 ± 12.95	1239.01 ± 9.53	1238.54 ± 8.67	0.931
12	1232.56 ± 20.15	1241.86 ± 6.23	1240.72 ± 9.11	0.249
13	1244.43 ± 6.21	1243.86 ± 6.84	1239.64 ± 6.03	0.204
14	1244.50 ± 5.55	1245.64 ± 3.82	1245.14 ± 5.77	0.883
15	1244.78 ± 6.94	1245.93 ± 3.91	1245.07 ± 5.56	0.894
16	1245.50 ± 5.31	1244.50 ± 4.69	1244.78 ± 4.49	0.894
17	1246.57 ± 5.21	1245.14 ± 3.94	1243.07 ± 7.29	0.392
18	1246.00 ± 4.94	1245.07 ± 4.72	1244.86 ± 6.25	0.879
19	1244.21 ± 6.86	1244.86 ± 5.63	1242.71 ± 8.42	0.787
20	1245.07 ± 6.28	1243.07 ± 7.89	1242.14 ± 8.89	0.693
21	1243.07 ± 10.16	1240.14 ± 15.33	1245.00 ± 5.17	0.617
22	1245.71 ± 6.60	1244.50 ± 7.65	1244.86 ± 6.26	0.921
23	1247.00 ± 3.65	1243.00 ± 9.17	1245.43 ± 6.73	0.437
24	1246.71 ± 3.61	1245.43 ± 6.86	1245.50 ± 4.97	0.832
25	1247.71 ± 2.95	1244.00 ± 5.94	1245.86 ± 4.06	0.200
26	1245.64 ± 3.76	1244.00 ± 6.67	1247.36 ± 2.18	0.280
27	1243.07 ± 10.16	1240.14 ± 15.33	1245.00 ± 5.17	0.617
28	1245.71 ± 6.60	1244.50 ± 7.65	1247.36 ± 2.18	0.280

[ab] Means within the same row carrying different superscripts were significantly different at ($P \leq 0.05$).

Table 4: Effect of feeding different concentrate roughage ratio on body weight and body weight gain of newborn lambs till weaning (Mean ±SD)

Variable	Group 1 (80%C:20%R)	Group 2 (70%C:30%R)	Group 3 (60%C:40%R)	*P*- value
BW at Birth (kg).	5.133 ± 0.37 [a]	4.817 ± 0.73 [ab]	4.337 ± 0.50 [b]	0.027
BW at week 2 (kg).	9.017 ± 0.80 [a]	8.06 ± 0.83 [b]	7.519 ± 0.46 [b]	0.002
BW at week 4 (kg).	13.650 ± 1.06 [a]	12.250 ± 0.50 [b]	10.887 ± 0.64 [c]	0.000
BW at week 6 (kg).	17.893 ± 1.29 [a]	16.125 ± 0.25 [b]	14.625 ± 0.69 [c]	0.000
BW at week 8 weaning (kg).	21.87 ± 1.50 [a]	19.63 ± 0.25 [b]	17.63 ± 1.22 [c]	0.000
ADG From birth to week 2 (g).	277.45 ± 38.60 [a]	231.79 ± 21.42 [b]	227.23 ± 36.77 [b]	0.033
ADG From week 2 to week 4 (g).	330.92 ± 33.58 [a]	299.11 ± 26.79 [a]	240.63 ± 43.74 [b]	0.001
ADG From week 4 to week 6 (g).	303.06 ± 17.91 [a]	276.79 ± 17.86 [ab]	266.94 ± 26.43 [b]	0.019
ADG From week 6 to week 8 weaning (g).	284.18 ± 40.82 [a]	250.00 ± 29.16 [ab]	214.29 ± 50.51 [b]	0.023
Overall ADG from birth till week 8 (kg)	16.739 ± 1.23 [a]	14.807 ± 0.75 [b]	13.288 ± 1.22 [c]	0.000

BW: Body weight; ADG: Average daily gain.
[abc] Means within the same row carrying different superscripts were significantly different at ($P \leq 0.05$).

Table 5: Means (±SE) of time of some maintenance behavior of pregnant and lactating ewes in relation to different ratio of roughage and concentrate feeding (min/h)

Behavior	Pregnant ewes				Lactating ewes			
	Group 1	Group 2	Group 3	*P*-value	Group 1	Group 2	Group 3	*P*-value
Eating	19.0±4.8	15.1±5.93	27.0±2.05	0.217	19.6±4.01	16.9±5.56	25.8±1.8	0.264
Drink	6.4±1.2	5.2±1.39	5.4±1.12	0.771	7.0±0.83	5.60±1.28	7.2±0.80	0.489
Rumination	6.4±1.77	6.88±1.54	12.26±0.38	0.012	6.4±1.63	7.28±1.16	11.26±0.4	0.009
Lying	12.86±3.6	18.40±7.4	14.2±6.7	0.807	13.26±3.51	21.8±6.43	16.4±6.51	0.574
Standing	5.2±0.95	7.06±1.06	2.54±0.5	0.011	5.4±0.91	7.6±1.33	2.02±0.72	0.007
Walking	3.7±0.24	3.1±1.32	0.90±0.40	0.072	8.8±1.06	3.9±1.33	1.10±0.33	0.386
Urination	0.62±0.19	0.44±0.20	0.41±0.17	0.717	0.82±0.21	0.52±0.18	0.41±0.17	0.333
Defecation	2.02±0.6	1.16±0.47	0.61±0.24	0.138	2.42±0.5	1.48±0.36	1.08±0.0	0.058

Table 6: Effect of feeding different concentrate roughage ratio during gestation period on some neonatal behavior

	Group 1 (80%C:20%R)	Group 2 (70%C:30%R)	Group 3 (60%C:40%R)	*P*-value
Birth to stand(min)	19.75±0.64	21.20±0.58	23.25±0.85	0.014
Birth to udder(min)	23.75±0.75	27.40±0.74	28.5±1.70	0.009
Birth to suck (min)	27.00±0.70	32.40±0.92	32.75±0.85	0.000
Suckling attempt	4.25±0.36	5.20±0.37	6.75±0.47	0.003

Table 7: Effect of feeding different concentrate roughage ratio on reproductive performance of ewes

Variables	Group 1 (80%C:20%R)	Group 2 (70%C:30%R)	Group 3 (60%C:40%R)
No. of ewe mated	10	10	10
No. conceived	8	5	4
Abortions/stillbirths	0	0	0
No. of lambs born alive	8	5	4
No. of lambs aborted/stillborn	0	0	0
Total number of Lambs produced	8	5	4
Conception rate%	80	50	40
Lambing rate % (Lambs produced/ewe lamb mated)	80	50	40
Average litter size	0.8	0.5	0.4

C: Concentrate R: Roughage

Conclusion

It could be concluded that feeding higher concentrate roughage ratio (80C%: 20%R) and (70%C: 30%R) improved body weight, body weight gain and vigour of newborn lambs and reproductive performance of ewes.

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Original Research Article

IMMUNOLOGICAL RESPONSE OF A NEW TRIVALENT CAPRIPOX-VIRUS VACCINE IN PREGNANT EWES AND DOES

Eman B. Abd-Elfatah[1], Mamdouh F. El-Mekkawi[1], Emad A. Aboul-Soud[2], Elshaima M. Fawzi[1]*, Sherif A. El-Soally[3]

[1]Department of Animal Medicine, Division of Infectious Diseases, Faculty of Veterinary Medicine, Zagazig University, Sharkia , 44511, Egypt, [2]Veterinary Serum and Vaccines Research Institute, Department of Pox, Abbasia, Cairo, Egypt, [3] Military Veterinary Hospital, Cairo, Egypt

*Corresponding author, E-mail: elshaimafawzi@yahoo.es, a.fawzy@zu.edu.eg

Abstract: This study was conducted as a field trial of using a new candidate trivalent Capripoxvirus (CaPVs) vaccine in pregnant ewes and does to detect safety degree and level of immunity, in comparison with commercial Romanian *sheep pox viral* (RSPPV) vaccine. Monitoring the post-vaccinal reaction and measurement of cellular and humoral immunity by evaluating the lymphocyte proliferation assay (LPA) and serum neutralization test (SNT), respectively, in addition to detect the acquired maternal immunity in new-born lambs and kids .The post-vaccine reaction in animals vaccinated with trivalent CaPVs vaccine was more noticeable than that vaccinated with RSPPV vaccine without significant difference (P-Value>0.05). In vitro, the evaluation of cellular and humoral immunity revealed that the animals vaccinated with trivalent vaccine can significantly induce lymphocyte proliferation response in addition to of high level of antibodies (Abs) in comparison to that vaccinated with RSPPV vaccine (P-Value <0.05) with peak titre of lymphocyte proliferation and Abs titre at 10^{th} and 28^{th}, respectively. Furthermore, the evaluation of maternal immunity in new-born lambs and kids from previously vaccinated dams with trivalent CaPVs and RSPPV vaccines by SNT was shown that the peak level of receiving immunity was appeared at 1st week after birth with increasing its level in lambs and kids of dams vaccinated with trivalent CaPVs vaccine than those of dams vaccinated with RSPPV vaccine with a significant difference in between (P-Value <0.05). The result of this study provide a good profile about a high immune response with a long level of maternal immunity provided with trivalent CaPVs vaccine in comparison to the RSPPV vaccine so toward about the preferable use of new vaccine to control infection with sheep and goat pox diseases. Trivalent CaPVs vaccine could be candidate to be used against lumpy skin disease (LSD) due to antigenic relationship between genus CaPVs strains.

Key words: does; ewes; monovalent RSPPV; safety; trivalent CaPVs

Introduction

Sheep and goat pox (SGP) diseases are one of the contagious viral diseases affecting small ruminants caused by *sheep pox virus* (SPPV) and *goat pox virus* (GTPV), belonged to genus *Capripoxvirus* (CaPVs) along with *lumpy skin disease virus* (LSDV) of cattle, within family

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Poxviridae (1). CaPVs strains are antigenically and serologically identical but can be differentiated at the genetic level (2). In adequate management, bad hygiene and a route of CaPVs diseases transmission corroborate the diseases spreading particular in the poor farmers and this adversely affects on central economy that relay on the production of livestock (3).

SPPV and GTPV were considered to be host specific viruses but some strains as Yemen, Kenya and Oman isolates infect both goats and sheep while most SPPV and GTPV isolates show distinct host preferences with more virulence in one of the two species (1). Cross protection between SPPV and GTPV or vice versa is partial so a homologous vaccine was recommended for an optimum protection (4, 5). In the most countries at least two different vaccines containing the isolates of either GTPV or SPPV are necessary to protect small ruminants against both viruses (6, 7).

In endemic countries with CaPVs, control of the diseases depend mainly on a laboratory diagnosis that followed by a quarantine application, in addition to a vaccination is considered the most easy and effective protection way against all CaPVs diseases (8, 9,10). Live attenuated tissue culture vaccines in a lyophilized form provided a good protection and able to control an outbreaks of CaPVs diseases (11).

CaPVs strains share a major neutralizing site; consequently it is possible to protect cattle against LSD using strains of CaPVs derived from either sheep or goats as used in Egypt by using Romanian sheep pox (RSPPV) vaccine. Furthermore SPPV and GTPV do not occur in Southern Africa so only attenuated LSD vaccine are used, whereas in northern, central Africa and in the Middle East where the distribution of SPP, GTP and LSD viruses overlap, an attenuated SPP vaccines such KSGP O-240, RM65 and Romanian SPPV strains have been used against LSD (12,13).

Kenyan SGP O108 vaccine used to immunize both small ruminant and cattle against CaPVs with remarkable success (11, 14, 15). In Egypt, it was reported that the live attenuated RSPPV vaccine did not provide cattle with complete protection against LSD and reoccurrence of an outbreaks in vaccinated animals (16-18), goats vaccinated with RSPPV vaccine are fully unsuccessful (6,19).

In Egypt, three CaPVs (SPPV, GTPV, LSDV) were endemic and appeared in sporadic and an outbreak cases, trying to use a trivalent vaccine of SPPV strains (Romanian, Kenyan 0180) and GTPV vaccine with an equal volume and nearly equal titre used as abroad-spectrum vaccine candidates against all CaPVs diseases is a good trail with a remarkable high immunity compared with commercial RSPPV vaccine alone (20-22). SPPV and GTPV vaccines do not replicate in small animals models to evaluate its efficacy, an evaluation of the vaccine in its preferable and specific host is the first step to detect the efficacy of the vaccine experimentally and in field application.

This study was done to evaluate the safety and efficacy of trivalent CaPVs vaccine in pregnant animals by evaluating both cell-mediated immunity (CMI) and humoral immunity, in comparison with positive control group of vaccinated pregnant small ruminants with commercial RSPPV vaccine. In addition to evaluate the passive maternal immunity in new-born lambs and kids to determine the suitable time to start vaccination in both types of vaccines.

Materials and methods

The experimental protocol was approved by Zagazig University Institutional Animal Care and Use Committee (ZU-IACUC/2/F/98/2018)

Animals

Pregnant small ruminants (ewes, does)

Eighteen ewes and seventeen does at last third of pregnancy and free from antibodies (Abs) against SGPV in which 5 of these animals (3ewes and 2 does) were applied to check the safety test of new candidate trivalent CaPVs vaccine, other thirty ewes (15 ones) and does (15 ones) were divided into 3 groups: Group 1 (G1) composed of 12 ewes and does, divided equally, were vaccinated with trivalent vaccine; (G2) composed of 12 ewes and does, divided equally, were vaccinated with RSPPV vaccine

(control positive) and (G3) composed of 6 un-vaccinated animals (control negative).

New-born lambs and kids

Thirteen lambs and thirteen kids with good healthy, well-body condition and have adequate amount of colostrums directly after parturition were chosen in this experiment. The new-born lambs and kids were divided into 3 groups: (G4) 10 lambs and kids from dams vaccinated with trivalent vaccine, (G5) 10 lambs and kids from dams vaccinated with RSPPV vaccine (control positive), (G6) 6 from unvaccinated dams (control negative).

Vaccines and animals vaccination program

Experimentally trivalent vaccine composed of three strains (KSPP O108, RSSP and Held GTP) with a titer ($10^{4.75}$ TCID50) and monovalent RSPPV vaccine ($10^{5.25}$TCID 50) that was obtained kindly from the veterinary serum and vaccine research institute (VSVRI), Egypt. The titer of each virus vaccine was titrated according to Reed and Muench (23). Reconstituted vaccines with sterile phosphate-buffered saline were kept on ice, protected from direct sunlight and used within 1hour (h). Pregnant dams were vaccinated at last 2 months of pregnancy with 1 ml /intradermal or/and subcutaneous according to manufacture instructions of the reconstituted vaccines contain ($10^{2.5}$TCID50/ml) according to the manufacturer's instructions, while the control group kept un-vaccinated.

Safety test

The new candidate trivalent CaPVs was tested to check its safety by inoculation 5 pregnant small ruminants (3 ewes and 2 does) with 20x field dose I/D (14).

Samples

Peripheral blood mononuclear cells (PBMCs)

Whole blood collected from both vaccinated and control animals regularly at 0, 2, 5, 10, 20, 30 days post-vaccination from jugular vein, PBMCs separated by collection 3 ml of peripheral blood from each animal on Na heparin, blood was overlaid on the surface of lymphocyte separation medium by lymph prep (1:1) in sterile centrifuge tubes then centrifuged at 2500 rpm for 30 minutes, PBMCs aspirated by pipette from interphase layer and washed 3 times by heparinized PBS after centrifuged at 2000 rpm for 10 min (24), cells re-suspended in RPMI-1640 with antibiotic and 10% FCS.

Serum

Serum samples were collected from pregnant sheep and goats to monitoring the immune response Abs for 3 months at day 0 before vaccination then every week during 1st month post vaccination then every 2 weeks during 2nd and 3rd months post vaccination (0, 1, 2, 3, 4, 6, 8, weeks…etc). Good healthy new-born lambs and kids were selected for assessment of maternal immunity which were clinically examined for well-weight and good general body condition, serum samples were collected weekly at 1st month then every two weeks at 2nd and at 3rd months of age (1, 2, 3, 4, 6, 8, weeks…etc), to determine the level maternal Abs against the two types of vaccines which transferred from vaccinated dams to their new-lambs and kids and the best time of immunization, all the serum samples were tested for the presence of Abs by using SNT.

Lymphocyte proliferation assay (LPA)

LPA was done in 96-well flat-bottom plates using XTT assay according to (25, 26), the assay was carried on PBMCs according to the instructions KitX6493 purchased from thermofisher scientific company, briefly PBMCs cells grown in a 96-well plate at a density of 10^4–10^5 cells/well in 100 µL of culture medium, the plate was incubated in a CO_2 incubator for 24–48 h, 10 µL of the prepared PMS solution in the prepared 4 mL of XTT solution created immediately before labeling cells, 25 µL of XTT/PMS solution directly to each well then the plate incubate for 2 h at 37°C in a CO_2 incubator and read absorbance at 450 nm.

Serum neutralization test (SNT)

The test was applied to detect Abs level before and after vaccination of pregnant ewes and does and passive maternal Abs in new- born

lambs and kids to detect the best time to start vaccination. SNT was applied according to the standard protocol of VSVRI following the OIE manual (14), the neutralization index (NI) was calculated according to Reed and Muench (23).

Statistical analysis

In the present study, the obtained data was analyzed by two-way repeated measures ANOVA procedures of the Statistical Package for Social Sciences version 21.0 (SPSS for Windows 21.0, Inc, Chicago, IL, USA). Results were documented as means ± standard error (SE), P-value< 0.05 was used to indicate statistical significance and the difference among the means were determined by Duncan multiple range test (DMRT).

Results

Safety test and the post-vaccine reaction

The safety test of new trivalent CaPVs vaccines was checked before field administration of the vaccine, the vaccine was safe without abortion case recorded, after that the trivalent CaPVs vaccine was applied in the field trial in comparison with RSPPV. Both vaccinated groups (G1, G2) were put under a closed observation for 14 days to detect the post-vaccine reaction particularly measuring of rectal temperatures. Clinical examination reveal that there is no clinical signs of CaPVs infection were appeared except rise of temperature was observed in G1 after 24-72h post- vaccination while in G2 slight rise of temperature was appeared after 48- 72h post-vaccination (Table 1) without significant difference between both group (P-value>0.05), this rise of temperature was accompanied by slight loss of appetite and slight increase of respiratory rate without any abortion. Moreover all animals were still in complete alert state with good body condition. Mild local reaction at the inoculation site in both vaccinated groups was appeared in the form of slight redness and swelling as shown in (Fig. 1) that disappeared within 4-5 days from vaccination.

Evaluation of cellular immunity by lymphocyte proliferation response

Lymphocyte response was calculated as optical density (O.D) by using sensitive XTT substance that varies from time to time and from animal to animal in each group. PBMCs of vaccinated groups were shown higher lymphocytes proliferation than un-vaccinated group with significant difference (P-value <0.05). Mean lymphocytes proliferation of G1 (trivalent vaccinated group) were higher than G2 (monovalent vaccinated group) with significant difference (P-value <0.05) at all time of PBMCs samples collection, along the time of experiment O.D of lymphocytes proliferation in sheep was higher than goats in response to vaccines. Peak level of lymphocytes proliferation in both G1 and G2 was appeared at 10^{th} DPV then decreased thereafter (Fig. 2).

Evaluation of humoral immunity by SNT

Serum samples were collected to detect Abs titer using SNT in vaccinated and un-vaccinated groups, the mean protective neutralization index (NI) was detectable after 2^{st} WPV in sheep and goat vaccinated with trivalent CaPVs vaccine (1.750±0.102) then increased gradually till reached to the peak level at 4^{th} WPV with average means (2.6250±0.143, 2.1563±0.105) in G1 and G2 respectively with significant difference (P<0.05) in between. Sheep and goats in G1 were shown higher mean NI than sheep and goats in G2 with significant difference (P<0.05) between weeks of samples collection (Fig. 3).

Evaluation of maternal immunity in newborn lambs and kids using SNT

Peak protective (NI>1.5) was recorded at 1^{st} week in lambs and kids of G4 and G5 compared with non-protective NI in G6 with significant difference (P<0.05) between groups, average means of NI in each group differ between weeks of serum samples collection with significant difference (P<0.05). In G4 (lambs and kids from trivalent vaccinated dams) the level of NI of Abs remain till 10^{th} week after birth protective with a relatively high level in lambs and kids, on the other side Abs of lambs and

kids from monovalent vaccinated dams (G5) remain protective till 6th and 4th weeks after birth respectively (Table 2). From the obtained results lambs were received higher level of Abs than kids.

Table 1: Physical parameters of vaccinated groups

*Days post vaccination (DPV)	Parameters			
	Body temperature (^{0}C)		Habitus	
	G1	G2	G1	G2
0	39.5±0.092	39.48±0.037	Alert	Alert
1	39.74±0.302	39.5±0.031	Slight decrease in appetite and animals movement with slightlyincreased respiratory rate	Slightly decrease of animals movement with good respiratory rate
2	39.82±0.102	39.64±0.051	Slightly decrease in appetite and animals movement	Mild decrease in appetite
3	39.86±0.103	39.64±0.024	Animals appetite is acceptable with good movable	Animals became alert with good movable
5	39.66±0.093	39.5±0.032	Alert	Alert
7	39.62±0.073	39.42±0.012	Alert	Alert
14	39.5±0.051	39.4±0.03	Alert	Alert

*Significant difference (P-Value<0.05) between days of post-vaccinal reaction

Figure 1: Post- vaccinal reaction in form of slight redness and swelling under the tail of sheep (A) and goat (B)

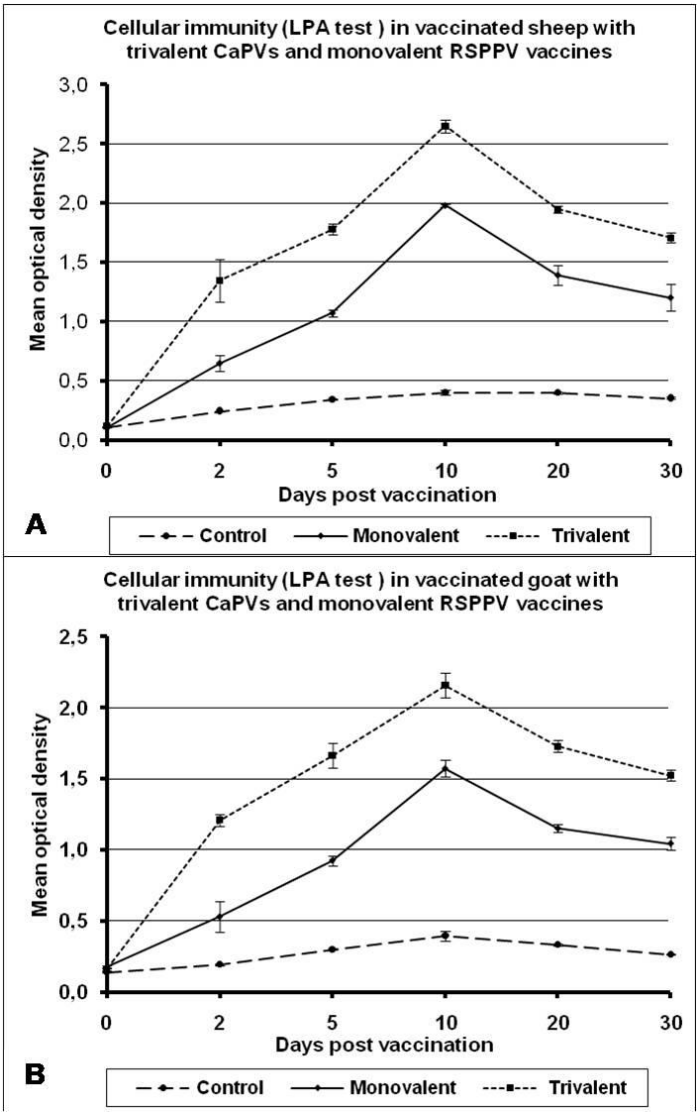

Figure 2: Lymphocyte proliferation index of vaccinated sheep and goats. PBMCs in response to trivalent CaPVs vaccine and monovalent RSPPV vaccine at days 0 (before vaccination), 2, 5, 10, 20 and 30 post-Vaccination of sheep (A) and goat (B). Protective O.D> 1, values were means ± SEM

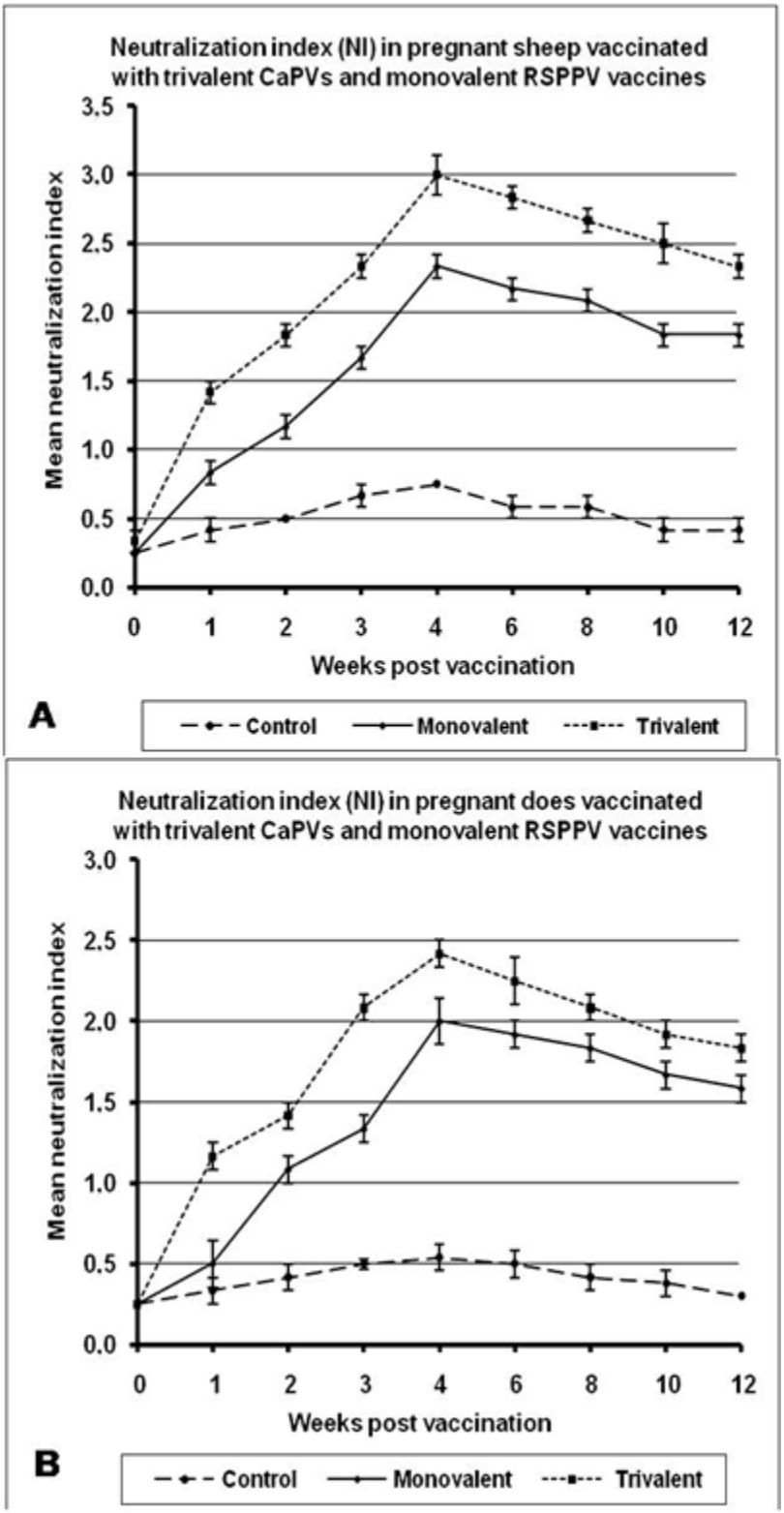

Figure 3: Average NI of vaccinated sheep and goats with trivalent CaPVs vaccine and monovalent RSPPV vaccine from 0 week (Pre-vaccination) till 12 WPV. Sheep (A) and goat (B).Values were means ± SEM. *8 week (time of parturition)

Table 2: Mean neutralization index of antibodies in serum of lambs and kids. The means with different superscript are statistically difference interaction based on Duncan test

Weeks after birth	Lambs		Kids	
	Trivalent	Monovalent	Trivalent	Monovalent
1	$2.38\pm.0.0161^{ab}$	$1.81\pm.0.063^{ef}$	2.50 ± 0.102^{a}	2.07 ± 0.063^{cd}
2	$2.25\pm.0.102^{ac}$	$1.69\pm.0.063^{fgh}$	2.19 ± 0.119^{bcd}	1.63 ± 0.072^{fgh}
3	$2.19\pm.0.1196^{bcd}$	$1.63\pm.0.072^{fgh}$	2.06 ± 0.063^{cd}	1.62 ± 0.068^{fgh}
4	$2.00\pm.0.102^{de}$	1.63 ± 0.072^{fgh}	1.81 ± 0.063^{ef}	1.56 ± 0.063^{ghi}
6	$2.00\pm.0.102^{de}$	1.50 ± 0.102^{hij}	1.8 ± 0.068^{ef}	1.13 ± 0.072^{lm}
8	1.81 ± 0.063^{ef}	1.31 ± 0.063^{jkl}	1.69 ± 0.063^{fgh}	1.11 ± 0.066^{lm}
10	1.75 ± 0.001^{fg}	1.31 ± 0.063^{jkl}	1.63 ± 0.072^{fgh}	1.06 ± 0.063^{m}
12	1.38 ± 0.063^{ljk}	1.07 ± 0.063^{m}	1.25 ± 0.102^{klm}	1.04 ± 0.075^{m}

Discussion

Vaccines have an important role in protection against endemic infectious diseases in Egypt. Vaccination protects the welfare of farm animals and reduces the pain associated with illness, present study was intended to detect the safety of a candidate trivalent CaPVs vaccine in pregnant small ruminants (specific and preferable host) and evaluate the immunity of this vaccine compared with commercial RSPPV vaccine (positive control) as well as the passive immunity in the new-born lambs and kids to determine the most suitable age for the first immunization.

Post-vaccinal reaction in the form of accepted increases in rectal temperature with redness and swelling at the inoculated site are the common characteristic feature of genus CaPVs due to its dermotrophic nature, this reaction was observed exactly in G1 than G2 without statistically significant difference in between ($P>0.05$), this indicate initial immunogenic response of the vaccines, similar results were recorded by (27, 28, 21). Gari et al. (29) said that when the local reactions at inoculation site of vaccine were very low or not observed this indicate that the vaccine viruses was over-attenuated and therefore failed to produce an effective CMI. The close observation of vaccinated groups revealed that both vaccines were found to be safe without adverse reaction on animal's health according to parameters of vaccine safety recorded by (14).

Protective immunity of genus CaPVs depend on both CMI and the humoral immunity, CMI response considered the predominant way to eliminate the infection against CaPVs. In recent years, the immunological studies focused on CMI role in defense mechanism (30). PBMCs after activation with CaPVs antigens were shown higher lymphocyte proliferation than un-activated PBMCs (31). In the present study, cultured PBMCs of both (trivalent vaccinated group) G1 and G2 (monovalent vaccinated group) were analyzed by using the sensitive XTT substance and were shown high proliferation than G3 (un-vaccinated group) with a significant difference (P-value <0.05), lymphocytes proliferation of sheep and goats in G1 were shown higher optical density level than sheep and goats in G2 at all-time points of experiment, these results were in agreement with (21,22,32) who reported that increase of lymphocyte proliferation at 3rd DPV after CaPVs vaccines strains activation till reached its peak on day 10th day then decreased thereafter.

The protective immune system against CaPVs infection not only depends on the cellular immunity but also the specific Abs that related to the durability of protection (33). SNT is a reliable and accurate method for specific Abs detection (34). In this study, (trivalent vaccinated group) G1 and (monovalent vaccinated group) G2 were able to produce Abs in response to CaPVs vaccine strains with significant difference (P-value<0.05). Protective NI> 1.5 against genus CaPVs according to (14) reach its peak level at 4th WPV agreement with (28) with increase its level in sheep and goats of G1 than sheep and goats of G2 all weeks of samples collection till end of experiment (12 weeks). Christine et al. (21) evaluated bivalent (RSPPV and Held GTPV) vaccines in cattle and reported that the bivalent vaccine is effective than RSPPV vaccine with a good protective index till 20 weeks (end of experiment) that could be still later. Trivalent CaPVs vaccine was evaluated in cattle by (22) who recorded that trivalent CaPVs vaccine provide highly protective Abs compared with monovalent RSPPV vaccine with 66% and 0% till 9 month post- Vaccination, respectively. Increases level of LPA and SNT in ruminants animals vaccinated with trivalent CaPVs vaccine than monovalent RSPPV vaccine was explained by (35, 36) who reported that GTPV vaccine induce a good immunogenic response than SPPV vaccine with high level of lymphocyte proliferation and Abs titer. In the present research, the peak level of O.D in vaccinated sheep and goats appeared at 10th day post-vaccination in comparison with the neutralizing protective Abs that reached its peak at 4th weeks post-vaccination this showed that the immunity against pox disease depend at first days after acquired immunity on T- lymphocytes.

Immunoglobulin G (IgG) is transferred through the colostrums and it's the main Ig responsible for neutralizing viruses through binding pathogenic agents and induces the humoral immune response (37), so in this study we should evaluate the new candidate trivalent CaPVs vaccine in pregnant dams and measure the level of protective immunity compared with monovalent RSPPV vaccine. In the actual study, the peak protective Abs was measured in G4 (lambs and kids of trivalent vaccinated dams) and G5 (lambs and kids of monovalent vaccinated dams) after 1st week after birth these results were agreed with (38) who reported that SPPV and GTPV vaccines were effective in producing hyperimmune colostrum that providing new-born lambs and kids during their first seven days. NI titer of SNT was still protective with a relatively good level till 10th weeks after birth in lambs and kids of G4 (1.75±0.001, 1.63±0.072, respectively) while at 12 week of experiment the level of NI is slightly decreased under protective NI of lambs and kids (1.38± 0. 063, 1.25±0.102, respectively) so it´s advisable to start the vaccination program at 2.5 months after birth before the protective Abs decline with CaPVs vaccine while lambs and kids from monovalent vaccinated dams (G5) Abs still protective till (6th, 4th weeks after birth, respectively) with average means (1.500±.0.102, 1.563±0.063, respectively). Effective vaccination of young animals at the appropriate time is cost effective practice for eradication of genus CaPVs diseases in Egypt. Little recent researches display the duration of maternal immunity of SPPV vaccine in sheep and goats, according to (39, 40) who found that maternal immunity in new-born lambs and Kids from vaccinated dams with SPPV vaccine last for 3 and 2 months respectively but in the present study the data obtained that the pregnant dams vaccinated with RSPPV vaccine provide its newborn lambs and kids with Abs protection last for 45 days and 30 days, respectively in comparison with trivalent CaPVs vaccine that provided a relatively protective Abs last for 3 months in both lambs and kids. It is unknown whether the low titre of circulating maternal are sufficient to block vaccine responsiveness, so early onset of lambs and kids vaccination with a safe trivalent CaPVs vaccine will provide more efficient protection by inducing both CMI and humeral immunity. The major mention of using trivalent CaPVs can be used for protection cattle against LSDV due to its induction of a long duration of immunity without generalized post-vaccinal reaction (21, 22).

Conclusion

From this investigation, we concluded that the trivalent CaPVs vaccine gave a good immune response based on both cellular and humoral immunity which represented by the induction of higher level of lymphocytes proliferation and Abs titer respectively, in addition to a high safety degree on pregnant dams and ability to provide relative long protective passive immunity to new-born lambs and kids than the commercial RSPPV vaccine. Trivalent vaccine should be used in vaccination of lambs and kids at 2.5 month of age to avoid the re-occurrence of outbreak. Due to the close antigenic relationship between genus CaPVs strains so we recommended the use of this trivalent vaccine as new candidate in Egypt to control reoccurrence of LSD in cattle with further study to evaluate its efficacy in cattle is also advisable.

Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

ISOLATION OF WHITE SPOT SYNDROME VIRUS (WSSV) IN EGYPTIAN SHRIMP USING CONVENTIONAL PCR AND REAL TIME PCR (QPCR) TECHNIQUES

Yaser M. Hafez[1], Nader Y. Moustafa[2], Asmaa F. Magouz[3], Naglaa F. Al-Maria[1,3]*

[1]EPCRS Excellence Center &Plant Pathology and Biotechnology Lab., Faculty of Agriculture, Kafrelsheikh University, Egypt, [2]Food Control Department, Faculty of Veterinary Medicine, Kafrelsheikh University, 33516, Egypt, [3]Virology Department, Faculty of Veterinary Medicine, Kafrelsheikh University

*Corresponding author, E-mail: naglaafikry5@yahoo.com

Abstract: Shrimp aquaculture industry threated by high mortality rates and severe economic losses as a result of white spot syndrome virus (WSSV) infection. Early-screening and diagnosis of WSSV are great strategies to decrease the economic losses of the disease on shrimp aquaculture. Therefore, this study was carried out to detect of white WSSV infected shrimp under using two molecular based methods, conventional PCR and qPCR. A total number of 90 samples of red (*Aristeus antennatus*) and gray (*Penaeus latisclcatus*) shrimp were collected from Kafr El-Sheikh and Alexandria governorates. External examination of shrimps collected from Kafr El-Sheikh Governorate revealed typical WSSV clinical signs (including loose and easily detached cuticle with appearance of small white spots (3 mm in diameter) and /or larger patches in the external surface of carapace and cephalothorax. The internal examination showed yellowish white, fragile and swollen hepatopancreas and swollen or shrunken lymphoid tissue. Red shrimp showed slightly obvious white spots without any internal lesions. PCR results confirmed the clinical investigation and postmortem (PM) examination and revealed presence of WSSV partial sequences with a size of 190 bp in shrimp samples from Kafr El-Sheikh Governorate. In contrast, samples collected from Alexandria (Borg El-Arab) gave negative results. The result of qPCR confirmed that obtained by conventional PCR and showed that all positive results of WSSV by conventional PCR gave cycle threshold (Ct) values ranged from 34.81 to 40.06. Our results concluded that, WSSV Diseases of shrimp attack shrimp markets of Kafr El-Sheikh Governorate. The conventional PCR and qPCR based methods for isolation and identification of shrimp WSSV, provided accurate results.

Key words: WSSV; PCR; qPCR; shrimp

Introduction

Shrimp are one of the greatest important food sources for human consumption because shrimp have high levels of omega-3 fatty acids (1). The industry income related to shrimp species is about 50 billion dollars annually (2). Production declining of shrimp was observed

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from 1994 to 1997 in India and from 1997 to 1998 in Asia (3-5). In Egypt, the white spot syndrome virus (WSSV) caused high losses in shrimp fields since 2009 (6). WSSV infects shrimp, lobsters, crayfish and crabs belonging to freshwater and marine crustaceans (2, 7).

WSSV is the only member of Whispovirus genus (belong to Nimaviridae family) (8). It is enveloped double stranded circular DNA virus with ovoid to bacilliform shape and a tail like end (9, 10). Shrimp aquaculture industry threated by high mortality rates and severe economic losses as a result of WSSV infection. Early-screening and diagnosis of WSSV are great strategies to decrease the economic losses of the disease on shrimp aquaculture. Conventional polymerase chain reaction (PCR), *in situ* PCR, quantitative PCR, nested PCR as well as loop-mediated isothermal amplification (LAMP) (11-16) has been established for detection of WSSV. The WSSV causes serious economic losses because of high percent of mortality which leads to total crop losses through ten days or less under certain farming conditions (17). The host range of WSSV is at least 78 species, mostly to decapod crustaceans particularly shrimp (18). In coastal area of Egypt, industry depends on shrimp are gradually proceeded to cover the market needs, nevertheless the high risk shrimp farms infected with virus could be disturb the production (19).

To the best of our knowledge, only few researches conducted about WSSV in Egyptian shrimp. Consequently, the goal of this research article was early identification of WSSV in shrimp under Egyptian conditions using of molecular based methods (PCR and qPCR).

Materials and methods

Shrimp samples

A total of 90 shrimp samples were collected from two Egyptian Governorates located on Mediterranean Sea; Kafr El-Sheikh and Alexandria. The red shrimp (*Aristeus antennatus*) and gray shrimp (*Penaeus latisclcatus*) obtained from Kafr El-sheikh Governorate as well as gray shrimp (*Penaeus latisclcatus*) obtained from Borg El-Arab farm (Alexandria Governorate).

Conventional PCR assay

Shrimp samples were prepared for DNA extraction. Carapace and abdominal segments of declining shrimp as well as the control shrimp were split using a scalpel. A part of gills, internal organs, muscle and cuticle are preserved in -20ºC for extraction of DNA. Total DNA was extracted according to the instructions of Gene-spin TM Viral Extraction Kit (Intron, South Korea, Cat. No. 1715) and as previously described (20). The PCR reaction mixture was 25 µl which consisted of 12.5µl of 2X master mix (0.1U/µl *Taq* polymerase, 500 µM dNTP, 20mM Tris-HCl (pH8.3), 100mM KCl, 3mM MgCl2 and Stabilizer and enhancer), 1 µl of 10 pmol of each primer, and 2 µL of template DNA (50 µg/ml). The primers were designed as previously described (19). Amplification was performed in a thermocycler (Bio-Rad, C - 1000). The amplified products were examined on 1.5% agarose gel. Ten µL of amplified product, negative control were injected into the well then run with 50 bp DNA ladder in 1X TAE electrophoresis buffer (5 volts/cm2 for 45min.). At the end of the run of electrophoresis, the gel was captured by a gel documentation system. The expected DNA fragments were 190 base pairs (bp) in length.

Real time PCR assay

A single tube qPCR reaction was adjusted according to the kit manual instruction. In brief, the reaction mixture was 25µl which consisted of 12.5µl of 2X SYBR Green qPCR Master Mix, 1 µl of 10 pmol of each primer (5-AATGGTCCCGTCCTCATCTCA-3) as well as (5-GCTGCCTTGCCGGAAATT-3) specific for WSSV (15), and 2 µL of template DNA (50 µg/ml). PCR was conducted in an eppendorf thermal cycler amplification was performed in a thermocycler (Real time PCR-Agilent Technologies - Stratagene MX300P). Early denaturation at 94 ^{0}C for 5 min, then cyclic condition was 35 cycles at 94 ^{0}C for 30 sec, annealing at 54 ^{0}C for 1 min as well as extension at 72 ^{0}C for 1 min. The final extension at 72 ^{0}C for 10 min (15, 20). The melting temperature for all obtained products was 80ºC indicating

the specificity of primers annealing to the template.

Results

Results of gross pathology

The collected shrimps collected from Kafr El-Sheikh Governorate were suspected to be infected with White Spot Syndrome virus (WSSV) based on the following main symptoms: loose and easily detached cuticle with appearance of white spots (3 mm in diameter) in external surface carapace and cephalothorax (Fig.1). These spots were not easy to be removed and in some region, they collected forming large patches of different sizes with whitish circular spots. The internal examination of these shrimps revealed yellowish white, fragile and swollen hepatopancreas and swollen and shrunken lymphoid tissue (Fig.1). In addition to accumulation of the fluids that caused swelling of bronchiostegites. On the other hand, red shrimp showed slightly obvious white spots without any internal lesions (Fig.1).

Results of conventional PCR

PCR results confirmed the clinical investigation and postmortem (PM) examination and revealed presence of WSSV partial sequences with a size of 190 bp in shrimp samples which had WSSV gross lesions obtained from Kafr El-Sheikh Governorate (Fig. 2). In contrast, samples collected from Alexandria (Borg El-Arab) gave negative results. PCR products of infected shrimp samples of different species revealed that 54 out of 60 shrimp samples from Kafr El-sheikh Governorate were PCR positive. Meanwhile, 30 samples collected from Borg El-Arab Alexandria Governorate gave negative (–ve) results *(*Fig. 2).

Our results cleared that, there was a significant differences of the incidences of WSSV ($P < 0.01$) among the type of shrimp and among different regions. The results cleared that, the degree of WSSV infected shrimp from Kafr El-sheikh markets showed high incidences to WSSV infection however, no disease incidence were recorded in Alexandria gray shrimp (Table 1).

qPCR results of WSSV in shrimp samples

The 90 samples were examined with real time PCR using specific primers for WSSV. The result showed that all positive results of WSSV by conventional PCR gave cycle threshold (Ct) values ranged from 34.81 to 40.06 (Table 2, Fig. 3).

Table 1: Incidences of isolated WSSV among examined shrimp after conventional PCR

Number of sample	Number of positive sample	Locality	Site of DNA extraction	Appearance of symptoms	Results	% of infection
15	14	Kafr-Elsheikh	Cuticle of red shrimp	Have symptoms	+ve (WSSV)	93.3 %
15	13	Kafr-Elsheikh	Cuticle of gray shrimp	Have symptoms	+ve (WSSV)	92.9 %
15	13	Kafr-Elsheikh	Internal organs of gray shrimp	Have symptoms	+ve (WSSV)	92.9 %
15	14	Kafr-Elsheikh	Internal organs of red shrimp	Have symptoms	+ve (WSSV)	93.3 %
15	0	Alexandria	Cuticle of gray shrimp	No symptoms	-ve (WSSV)	0
15	0	Alexandria	Internal organs of gray shrimp	No symptoms	-ve (WSSV)	0

$Chi^2 = 11.14$ ** ** = Significant at ($P < 0.01$)

Table 2: Mean of Cycle Threshold of samples examined for WSSV using qPCR

Samples NO.	Site of DNA extraction	Mean of Cycle Threshold
1	Cuticle of red shrimp from Kafr-Elsheikh	40.06
2	Cuticle of gray shrimp from Kafr-Elsheikh	36.01
3	Internal organs of gray shrimp from Kafr-Elsheikh	34.81
4	Internal organs of red shrimp from Kafr-Elsheikh	37.60
5	Cuticle of gray shrimp from Alexandria	0.0
6	Internal organs of gray shrimp from Alexandria	0.0

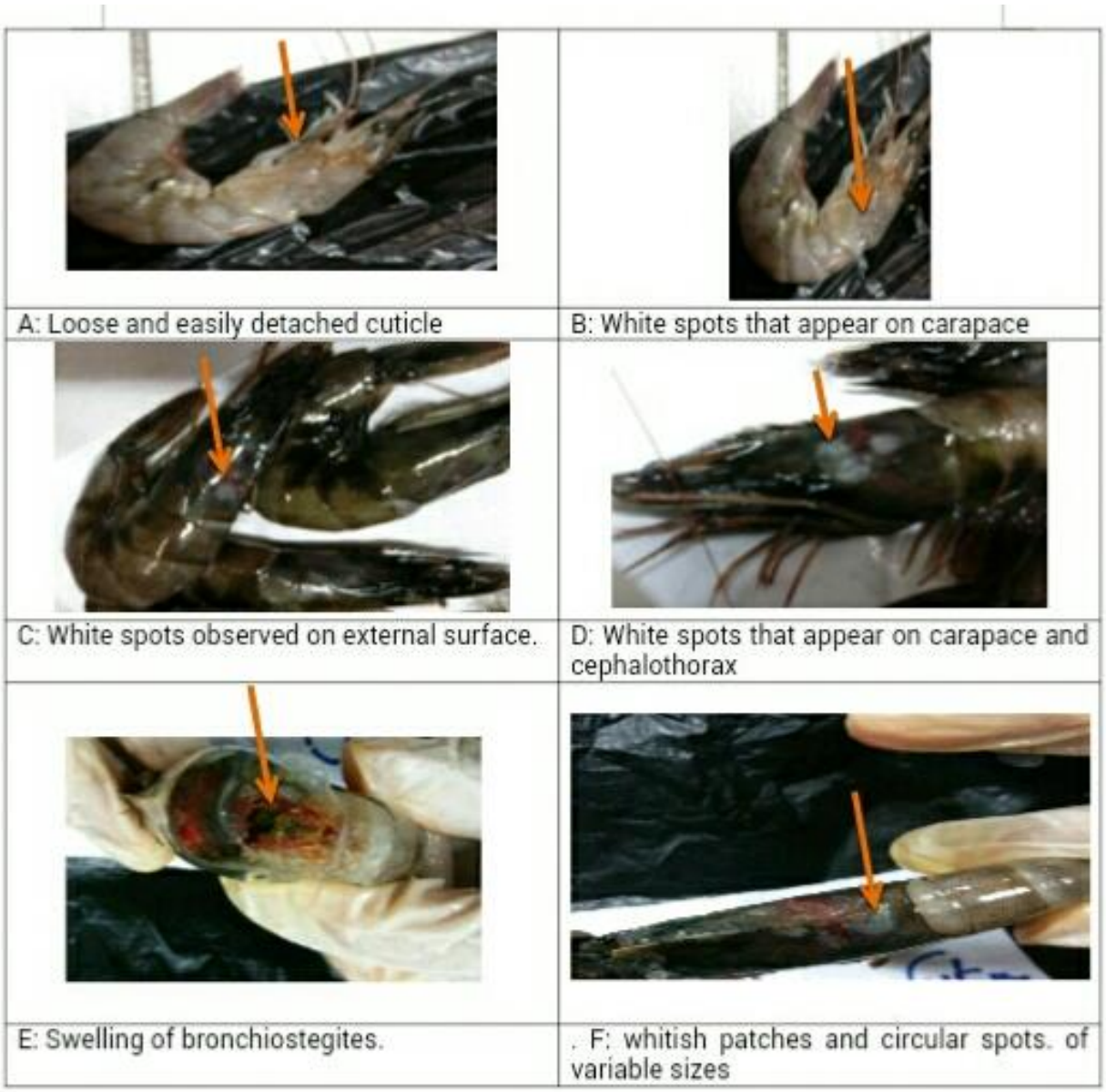

Figure 1: White spot syndrome virus (WSSV) infection gross lesion on shrimp. A, B: from red shrimp. C- F: from gray shrimp

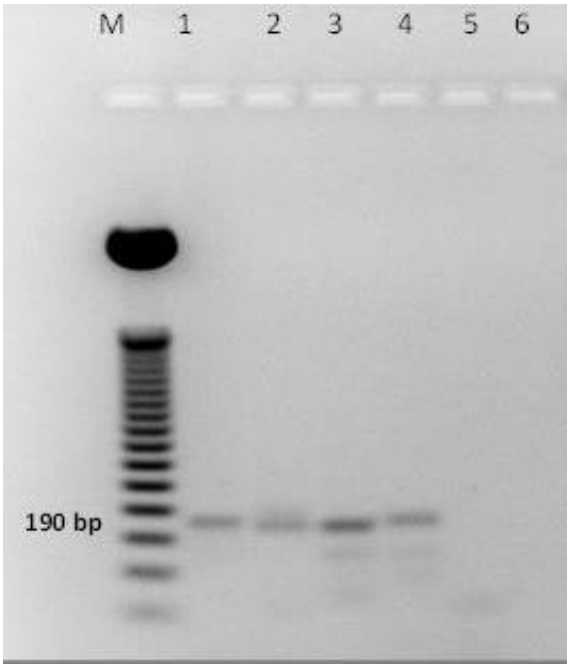

Figure 2: PCR results of WSSV on agarose gel. M= 50 bp ladder, 1= (sample from cuticle of red shrimp), 2= (sample from cuticle of gray shrimp), 3= (sample from internal organs of gray shrimp), 4= (sample from internal organs of red shrimp), 5= (sample from cuticle of gray shrimp), 6= (sample from internal organs of gray shrimp). 1-4 samples collected from Kafr EL-sheikh, 5-6 samples collected from Alexandria

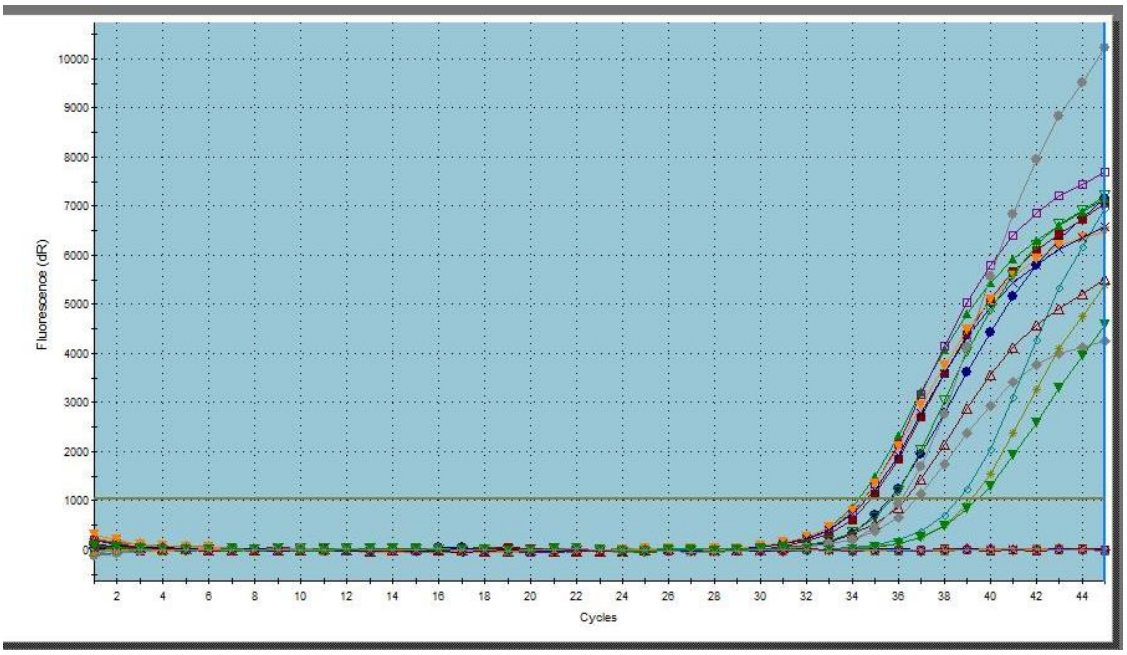

Figure 3: Amplification curves of real time PCR results of WSSV

Discussion

WSSV is one of the most serious shrimp disease which does not only attack shrimp farms in Egypt and causes economic losses but also infects other freshwater and marine crustaceans, mostly crayfish and crabs (1, 9). The virus is so dangerous so that can lead to 100% mortalities during only 2-10 days from appearance of symptoms (2, 21). Little information and knowledges about the incidences and prevalence of this virus in shrimp aquaculture have been reported in Egypt. Although the shrimp aquaculture

became more advanced in Egypt, the infected shrimp threaten shrimp farming. Thus, control WSSV is required to avoid shrimp losses. The aim of this study was to throw the light on the occurrence and incidences of WSSV in some two species of the shrimp present in Egypt.

Our results revealed that 54 out of 60 shrimp samples collected from Kafr El-sheikh Governorate showed positive results for WSSV however, no positive results were obtained from the 30 total shrimp samples collected from Alexandria Governorate. These results supported by the results obtained by Megahed et al 2019, Eissa et al 2009 and Salama et al 2008 (6, 19, 22) and confirming WSSV identification among Egyptian shrimps.

Herein, gross pathological examination revealed presence of white spots on the shrimp body after removal of cephalothorax cuticle. Our results agreed with those obtained by (2, 23-26) who also found cuticle chromophores and calcium deposition. Although we did not find softening of exoskeleton, in many epizootics of this disease, this softening could be observed. Changes in the structural integrity of the exo and pro cuticle could be the most vital reason for WSSV syndrome (28-30). The obtained internal examination results agreed with (2, 27, 31) and proved that white spot virus infected hepatopancreatic sheath.

PCR (both conventional and real time) confirmed presence of WSSV in shrimps collected from Kafr El-sheikh Governorate. In the present study, 90 shrimp samples were examined with conventional PCR using specific primers of WSSV. The result showed that 54 out of 60 gray and red shrimp samples of Kafr El-sheikh Governorate were PCR positive. Meanwhile 30 gray shrimp samples collected from Borg El-Arab, Alexandria Governorate gave negative (–ve) results. Results from conventional PCR were further confirmed by qPCR which showed amplification curves with Ct values ranged from 34.81 to 40.06 in all conventional PCR positive samples. PCR has been applied for the diagnosis of WSSV infections in clinical samples and shown to be rapid, sensitive and specific diagnostic method (32-35).

Conclusion

To the best of our knowledge, this may be the first study to detect shrimp WSSV in Egypt using qPCR. The conventional PCR and qPCR based methods are successful methods for early identification of WSSV in clinical samples of infected shrimp that delivers accurate tool for identification of this virus.

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Original Research Article

OCCURRENCE AND RISK FACTORS OF DIGITAL DERMATITIS IN EGYPTIAN DAIRIES

El-SayedA.El-Shafaey[1,3], Mohamed Z. Sayed-Ahmed[2,4], Sherif M. Shoeib[5], Sabry A. El-khodery[2]*,

[1]Department of Surgery, Anesthesiology and Radiology, Faculty of Veterinary Medicine, Mansoura University, Mansoura 35516, Egypt, [2]Department of Internal Medicine and Infectious Diseases, Faculty of Veterinary Medicine, Mansoura University, Mansour 35516, Egypt, [3]Department of Veterinary Medicine, College of Agriculture and Veterinary Medicine, Qassim University, Buraydah 51452, Qassim, Saudi Arabia, [4]Department of Clinical Pharmacy, College of Pharmacy, Jazan University, Jizan45142, Saudi Arabia, [5]Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Mansoura University, Mansoura 35516,Egypt

*Corresponding author, E-mail: khodery@mans.edu.eg

Abstract: In the present study, the prevalence of digital dermatitis (DD) and the related risk factors in dairy cows was investigated. For 12 months, information about the establishment of DD lesions and other claw disorders in dairy cows was collected. A questionnaire was designed to get data about age, milk yield, season, parity, transition period, concurrent diseases and biosecurity. The hind claws were more affected than fore claws (95.24 vs 4.76%) particularly the left hind claws (56.67%). However, The DD more prevalent during the second lactation than the third lactation (42.85 vs 36.50%) and first lactation (42.85 vs. 19.06%) which gradually decreased reaching the lowest incidence in heifer cows (42.85 vs 1.59%). Furthermore, DD was more prevalent during summer and autumn. Based on cow level, the final step of logistic regression analysis revealed a significant relationship between DD and cow' age (P <0.05; Odds Ratio [OR]: 1.01; 95% confidence interval [CI]: 0.67–1.47), parity (P <0.05; OR: 1.01.11; 95% CI: 0.47– 401.93), season (P < .05; OR: 1.36; 95% CI: 1.01–1.81), and transition period (P <.05; OR: 1.53; 95% CI: 0.48–4.83). The recognition of factors which influence occurrence of DD may permit changes in herd management or breeding policies which can be applied to diminish the prevalence of DD, and can establish the appropriate preventive procedures.

Key words: digital dermatitis; dairy cows; risk factors; epidemiology

Introduction

Digital dermatitis (DD) is a global emerging digital skin disease of dairy cows, causes lameness with subsequent economic losses (1-3). Digital dermatitis decreases reproductive performance, milk production, as well as productive lifespan and animal welfare (4, 5). The lesions of DD are mainly oval or circular, with strawberry-like appearance, with presence of a circumscribed erosive purulent epidermitis, which can develop to an ulcerative granulomatous lesion. The predilection site of typical lesions of DD are the posterior part of digits, , and

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the mid-way between the heel bulbs, and adjacent to the coronet (6,7). The precise cause and factors which predispose to DD occurrence in herds are still unclear, but it seems a multifactorial disease (8).

Environmental, farm-management and individual animal factors are the suggested risks for this disease (9, 10). These risk factors can be split into two groups; cow-level factors (breed, parity, stage of lactation, calving season, transition period and other claw affections) (11-13). The transition period in dairy cows is the time extending from three weeks pre-calving, to three weeks post-calving. Minor alteration in the management system of dairy cows induces a dramatic effect on feed intake, health condition, locomotion and final profitability (14). Prompt alteration in both metabolic and hormonal systems occur during this period induces a stress in the cow, which is a critical determinant of the high risk of diseases in a dairy herd. Lameness may be triggered during the transition period. Physiological and behavioral changes during transition can damage the corium. This damage is not immediately apparent but results in poor hoof growth inducing DD, sole ulcers and hemorrhage (15). The pathogenesis and clinical findings of DD lesions have been well presented. However, there are no standard features of classifying the lesions, while trials have been made to characterize the lesions based on the stage and severity (16-18).

Scoring system has been presented to reflect the stages of DD (6, 19). Scores 1 and 2 are the initial stage of lesions, which has intact epidermis. But the score 3 is characterized by presence of ulcerative lesion. Scores 4 and 5 are the late lesions in the course of healing (19). In a study, it has been suggested that, animals could be classified based on their DD to no lesions, single lesions or recurring lesions (20, 21).

Reducing the occurrence of via early diagnosis and treatment is an crucial management system (22, 23). There are a lot of treatment options for DD; systemic antibiotics, individual topical treatment, and mass topical therapy using a footbath (24). Topical treatment of DD worldwide includes antibiotic and non-antibiotic preparations (sprays or pastes) applied with/without bandage (25, 26, 27). Furthermore, when treatment of DD is in progress, thorough claw trimming should be done (28). In this study and epidemiological investigation on the occurrence and factors associated with digital Dermatitis in Egyptian dairies.

Materials and methods

Area

The area of study is present in the northeastern part of Egypt, at the Damietta, along with branch, of the River Nile, fifteen kilometers from the Mediterranean Sea. The study region is 200 kilometers north to Cairo. It represents approximately 5% of the total area of Delta, but 1% of the total area of Egypt.

Study animals

For 12 months, an epidemiological investigation was achieved in one dairy herd in Damietta governorate. We constructed a questionnaire to include age, milk yield, season, parity, transition period, concurrent diseases and biosecurity was provided by veterinary medical officer. A total of 63 (14%) of 450 dairy cows were randomly selected and examined weekly for DD lesions using risk factors scores for subjective assessment of risk parameters (Table 1).

Management system

The animals were housed in a cubicle (free-stall/feedlot) barn with straw-bedded stalls, and a slatted floor which was automatically scraped. The cows were tied in a long-stall barn with a daily replaced chopped straw was used as bedding on the top of rubber mats. The feeding system was a total mixed ration (TMR) for all cows, and milking was twice/day. The intake of concentrates was increased 4 weeks before calving and peaked 3 weeks after. The annual milk production per cow in the farm averaged 8500 kg energy-corrected milk during the year. A professional hoof trimmer trimmed the cows, feet routinely twice a year, in autumn and spring using a transportable hydraulic trimming chute. Footbaths were not used on this dairy farm during the study. Based on the approval of the Committee of Animal Welfare and Ethics,

Mansoura University, this study was carried out.

Case identification

The clinical signs of DD in the 63 Holstein-Friesian dairy cows were recorded weekly and over 4 week after treatment. At each examination, cows were placed in a hoof trimming chute, the feet were washed manually with a water hose and a brush, and a detailed examination of the feet of each cow was performed for observation of DD lesions. Visual inspection and digital palpation were applied to evaluate the lesions of DD according to Döpfer et al. and Laven et al. (29,30). On the basis of clinical presentation, location, shape, depth, color, the diameter of the lesions and lesion maturity score (stage) were noted. The lesion diameter was measured at the widest part of the lesion in the examination stanchion using measuring tape.

Statistical analyses

For analysis of data, a commercial software program was used (SPSS version 21.0, SPSS Inc, USA). As an initial step, the descriptive statistics were presented and the distribution of risk factors among cows with DD was identified. The Association between the incidence of DD and the related factors was assessed by logistic regression on cow level. Two levels of statistics were performed, the univariate then multivariate. In univariate step, the status of cows with DD was the dependent, but the risk factor was the independent. When the significance level was at (P<.1), further backward stepwise multiple logistic regression was conducted. The standard parameters of logistic regression were presented finally. The association between DD and variables of clinical symptoms was assessed by chi-square test. In all steps, at $P < .05$, the outcome was recorded as significant.

Results

Prevalence

Sixty-three (33.2%) Egyptian dairy cows were proved to be infected with DD. The hind claws were significantly higher than fore claws (95.24 vs. 4.76%) particularly the left hind claws (56.67%). DD was significantly higher in young age 3-5 years than older cows (≥5 years old) (33; 52.4 vs. 10; 15.9%) ($P < .05$; OR: 1.01; 95% CI: .67-1.47). It was significantly higher during the second lactation than third lactation (27; 42.85 vs. 23; 36.50%) (P<.05; OR: 8.01; 95% CI: 6.5-6.5) and first lactation (12; 19.06%), which gradually decreased reaching the lowest incidence in heifer cows (1;1.59%) as showed in Fig.1A.There was a decrease in milk production in cows with DD than control cows. DD prevalence was affected by the transition period of the affected cows. It was progressively increased from calving till 3 weeks after calving. However, cows at time 0 of calving showed a significant (P<0.04) retraction of the lesion score. On the other hand, after calving, a significant increase (P<0.05), particularly during the first 3 month after calving was recorded. The seasonal prevalence of DD was summer (26, 41.27%); autumn (21;33.34%); winter (9;14.29%); spring (7;11.12%) as showed in Fig.1B.

Univariate statistics

The results of univariate analysis were presented in Tables 2, with the distribution of suggested risk factors associated with DD on cow levels.

Multivariable analysis

The results of this final step indicated that Hosmer and Lemeshow's goodness of fit model adequately fits the data on cow level ($\chi 2$, 7.89; P= 0.20). On cow level, age, parity, season and transition period were the potential risk factors (Table 3).

Clinical findings

A total of 63 of 190 Holstein-Frisian dairy cows were diagnosed. Sixty lesions were present on the hind claws and three on the fore claws, especially on the plantar skin over the bulb of the heels (45; 71.43%), and occasionally on the anterior aspect of the interdigital cleft (10; 15.87%) or adjacent to the dorsal coronet (8;12.70%).Thirty-four lesions were recorded on left hind claws, 26 on the right hind claws, 2 left fore claws and1right fore claw

cows. while, the DD lesions were located in a cow on both hind claws. In 63 examined claws, the skin lesion appeared as strawberry (33; 52.38%), oval (18; 28.57%) and circular spots (12; 19.05%) on the plantar aspect of the feet (Fig.2D). Most of them were distal, but adjacent to the bulbar skin-horn junction. Small lesions (< 1 cm) were (16; 25.39%)and lesions that were assumed to be early cases were located at the skin-horn junction (commonly in direct proximity to the interdigital cleft/plantar commissure). While, the medium lesions (1-2cm) were (21, 33.33%) and larger lesions (>2cm) were (26; 41.27%) were situated anywhere along the junction and sometimes merged to cover the entire bulbs. Lesions located exclusively on the skin of the interdigital cleft were elongated with a layered appearance. The pili in the area bordering the skin-horn junction were erected, elongated approximately 3 to 4 times their normal length, and matted. There were 5 M1 lesions (7.94%), 54 M2 lesions (85.71%), 3 M3 lesions 4.76%),and1M4lesions(1.59%)asshowedinFig.2A.TheM2lesionswerelocated over the bulb of the heel while, M1 lesions were found within the interdigital space. The M2 lesions were circumscribed areas of ulcerative, erosive dermatitis which often painful, and easily bleed when they were manipulated than other stages. The surrounding skin was reddened and the follicles appeared to be hypertrophied. Severely affected feet had a massive soft-tissue swelling in the bulbar area. There was as strongest association between M1 and M2 DD lesion. The M1 lesion has the liability to be M2 than negative one (M0). While, M2 lesions usually becomes M3-M4 in their way for recovery. There was a close link between the stage and color of lesion. Therefore, that 40 (63.49%) cows showed pink/red (erosive) lesions were observed during the clinical examination. While, 10(15.87%) cows showed brown/gray lesion and creamy/yellow (granulomatous) lesions were identified in 13 (20.63%) cows asshowedinFig.2C.The lesion depth was varied between shallow to deep lesions. Most of the evaluated feet showed proliferative lesion 37 (58.73%) cows and deep lesion in9(14.29%) cows. However, 15 (23.81%) cows showed shallow lesions (Fig.2B).

Table 1: The risk factors scores for subjective assessment of risk parameters in 63 Holstein-Frisian Dairy cows with digital dermatitis

Risk factors scores	Score and Description	
Age (Year)	0 = 1-3	1 = >3-5
	2 = >5-7	
Milk yield (kg/Y)	0 = 2100- 4000	1 = 4200
	2 = 6300	3 = 6000- 8500
Season	0 = Winter	1 = Spring
	2 = Summer	3 = Autumn
Lactation number/parity	0 = Heifer	1 = 1st
	2 = 2nd	3 = 3rd
	4 = 4th and over	
Transition period/wk	0 = Before calving	1 = After calving
Concurrent disease	0 = Negative	1 = Positive (mastitis, metritis, claw affections)
Affected limb	0 = Left fore claw	1 = Right fore claw
	2 = Both fore claw	3 = Left hind claw
	4 = Right hind claws	5 = Both hind claws

= Means equal

Table 2: Odds ratios (OR; including 95% confidence interval), frequencies, and digital dermatitis (DD) prevalence for the variables associated with DD among 63 cows in Egypt

Category	Description	Frequency	Prevalence	OR	CI	P Value
Age	1- 3 Years	20	31.7			
	> 3 - 5 Years	33	52.4	3.86	.7- 21.2	.1
	> 5 -7 Years	10	15.9	2.86	.5- 14.01	.1
Milk yield	2100- 4000 Kg/Y	27	42.9			
	4200 Kg/Y	25	39.7	1.08	0.1- 0.8	1.
	6300 Kg/Y	11	17.5	0.56	0.0- 0.7	1.
Season	Winter	9	14.29			
	Spring	7	11.12	.035	.001- 1.2	.06
	Summer	26	41.27	17.7	17.6- 17.7	.50
	Autumn	21	33.34	.44	.04- 4.6	
Parity	Heifer	1	1.59			
	1st lactation	12	19.06	5.6		
	2nd lactation	27	42.85	8.1	6.5- 6.5	1.0
	3rd lactation	23	36.50	6.5		
Concurrent Diseases	Negative	0	0			
	Positive	63	100			
Limb	Left fore claw	20	31.7			
	Right fore claw	3	3.73	3.5		
	Right hind claw	33	56.67	3.5		
	Left hind claw	5	7.9			

Abbreviations: CI, confidence interval at 95%; OR, odds ratio

Table 3: Final logistic regression model the risk factors associated with digital dermatitis in Egyptian dairy cattle on cow level

Variable	ß	SE	P	OR	Cl
Age	.006	0.20045	.98	1.01	0.67-1.47
Parity	.051	0.36224	.89	1.01	0.47-1.93
Season	.304	0.14894	.04	1.36	1.01-1.81
Transition period	.423	0.58770	.47	1.53	0.48-4.83
Constant	.271	0.75946	.72	56.65	------

Abbreviation: ß, regression coefficient; Cl, confident interval at 95%; OR, odds ratio; P, P value; SE, stander error

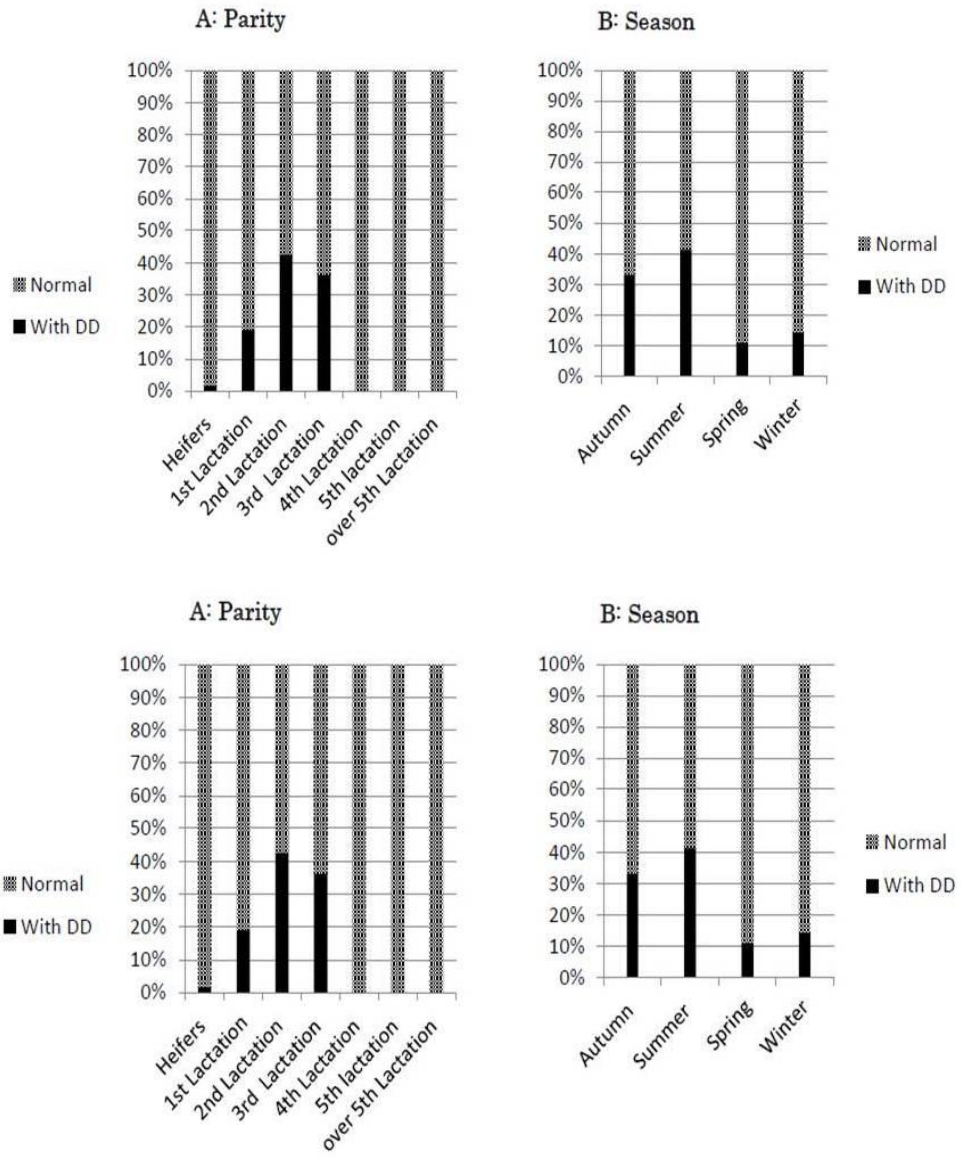

Figure 1: Effect of Parity and season on digital dermatitis in Egyptian dairies

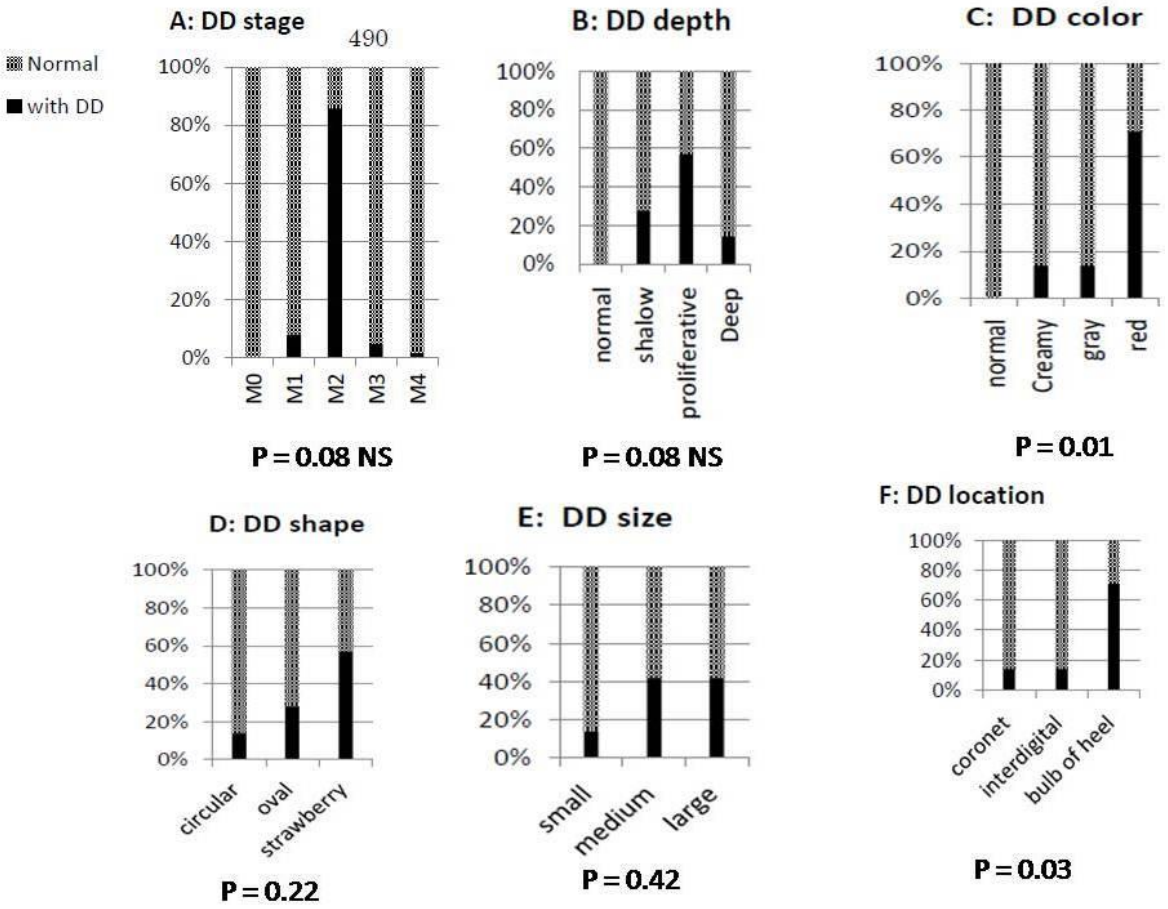

Figure 2: Effect of management system on digital dermatitis in Egyptian dairies

Discussion

Proper understanding of the predispositions factors of digital dermatitis in cattle may aid the improvement of management strategies to avoid or diminish the disease and its economic outcome (31). In our study, cow level risk factors were studied. The breed predisposition is one of the exciting causes of DD in dairy cows. Holstein cows are more susceptible to DD than other dairy breeds (32,33). The present study revealed that Holstein cows were vulnerable to DD. Moreover, DD is recognized to be more common in the pelvic feet than in forefeet (34,35).

In our results, the lesions of DD were observed exclusively in the hind claws more affected than fore claws (95.24 vs.4.76%) particularly the left hind claws (56.67%). This could be attributed to longer periods of cows recumbency on such side with their left hind leg positioned under the abdomen in a somewhat contaminated, moist, and warm site (35).

The current results provide an idea about the occurrence and the important associated factors of DD in dairy cows. The disease predominance in the current study was 32.2% in Egyptian dairy cows. Such finding is in concurrence with that of earlier studies in dairy cows (31,36).In Swedish dairy cows, the rate of the disease is

higher (41%) than that of the current findings (27). However, in Netherlands, DD were found less prevalent (20%) than in our study (3). Parity was significantly higher during the second lactation than 3rd lactation (27; 42.85 vs 23; 36.50%) and these results agree with Holzhauer et al. (3), who reported that there was a negative relationship between prevalence of DD and parity. At 30-60 days in milk and at the third parity cow, the odds ratio is higher than other times. The presence of concurrent claw affection were increased the prevalence of DD in dairy cows up to 100%. This results was agree with Holzhauer et al. (3), who found that other claw diseases, as interdigital phlegm on, hyperplasia, and heel horn erosion predispose to the disease. Incidence of DD was higher in dairy cows in the second and third lactation number than in subsequent lactations or heifer. This is in agreement with results presented by (12,3,37) who stated that, DD is an infectious disease and decreased incidence in older cows might be related to increased local immunity. Limited research has focused specifically on the relationships among DD prevalence and the transition period. The results of the present study showed that, throughout the transition period, the cows are more liable to develop lesions of DD than in other lactating months. At transition period, the period of high risk for DD, the cows are commonly liable to external influences, and clinical diseases (38).Abrupt environmental and metabolic changes during the transition period may explain the increased probability of hoof and horn diseases during this period.

The seasonal changes in calving-related management and environment are the main stressors, which play a significant role in the occurrence and severity of DD. The seasonal prevalence of DD in the present study was higher in summer and autumn in comparison with winter and spring. Interestingly, the housing system has been also found a main risk factor for DD (33,35). The housing system of selected farm for this study was free-stall with the cows tied in a long-stall barn, so there might be an association with prolonged housing periods and the increased prevalence of DD.

There is a strong association between wet/dirty claw environments and the occurrence of DD. It has been observed that housing on concrete floor has higher odds than on a slatted floor with manure scraper (40). In this study, dairy cows were housed in a cubicle barn with mattress-bedded stalls, and a slatted floor which was automatically scraped. Frequent removal of cows manure would diminish the undesirable effects of the unhygienic and wet claw environment, via increasing the resistance of the claw capsule to erosions and subsequent bacterial infection (40,41). In this study, most of the DD lesion definition such as diameter, shape, color, depth and location were evaluated as shown in table/figure. In dairy cows, M1, M2have been reported the major types of lesions. The M2 lesions are circumscribed areas of ulcerative, erosive dermatitis. Due to involvement of the dermis, the lesions are painful and affected animals are lame. Digital dermatitis lesions were assed everywhere on the solar aspect of the claws of the affected dairy cow included in this study. The heel bulbs are close together which more prone to continually being moist, which favors the development of DD especially M2 lesions (42). However, the M1 lesions were located in the interdigital space which has some aeration and dryness than the heel bulbs.

The rate of development and resolution of DD lesions were evaluated along the weekly observation of the affected claws. Moreover, the changes in the lesion from stage to another in the same claw were also assessed weekly. The shift among various stages of DD can progress within one month. Therefore, weekly observation would be beneficial in understanding the changes in DD lesions stages without missing even small lesions. The majority of transitions between different stages of DD have steady order, where the transitions between M2 and M4 are the most common, though transitions between M4 and M1 have also been recorded. These present findings coincide with those reported in previous studies (43,44,45).

Conclusions

The results of this study provide an idea about the prevalence and risk factors of digital dermatitis in dairy cows in Egypt, Because of the interrelation between infectious causes of claw diseases, an effective interference against DD should directed to an integrated approach to the control of all infectious claw diseases. Furthermore, the recognition of factors which influence occurrence of DD may permit changes in herd management or breeding policies which can be applied to diminish the prevalence of DD, and can establish the appropriate preventive procedures.

Conflict ofinterests

None of the authors of this article has a financial or personal relationship with other people or organizations that could appropriately influence or bias the content of the article. There are no funding sources for this study.

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Original Research Article

OCCURRENCE AND MOLECULAR CHARACTERIZATION OF EXTENDED SPECTRUM BETA-LACTAMASE PRODUCING *Enterobacteriaceae* IN MILK AND SOME DAIRY PRODUCTS

Walaa Gaffer[1], Mayada Gwida[2*], Randa Abo Samra[3], Maha Al- Ashmawy[1]

[1]Department of Food Hygiene and Control, Faculty of Veterinary Medicine, Mansoura Universit, Mansoura 35516, Egypt, [2]Department of Hygiene and Zoonoses, Faculty of Veterinary Medicine, Mansoura University, Mansoura 35516, Egypt, [3]Food Inspection Laboratory, Animal Health Research Institute, Damietta Port, Egypt

*Corresponding author, E-mail: mayada_gwida@mans.edu.eg

Abstract: In the present study a total of one hundred samples were randomly purchased from different supermarkets in Damietta city, Egypt, including 25 samples each of raw milk, ice cream, Kareish and Domiati cheese. The collected samples were screened initially for cefotaxime-resistant bacteria using selective enrichment. The suspected colonies were further characterized by analysis of resistance genes in ESBL-producing isolates. A total of 59 ESBLs producers colonies (24 from raw milk, 15 from kareish cheese, 13 from Domiati cheese and 7 from ice cream) were recovered and identified as *Escherichia coli* (n = 29), *Klebsiella pneumonia* (n = 8), *Klebsiella oxytoca* (n = 5), *Enterobacter aerogenes* (n =8), *Citrobacter diversus* (n = 8) and *Serratia liquefaciens* (n = 1). Resistance to two or more antibiotics was observed among the recovered isolates. *E. coli* isolates showed high resistance pattern against cefaclor (100%), cefoxitin (100%), cefalaxim (96.5%), ceftazidime (93%), pencillin (93%) and 79% for Pipercillin/Tazobactam. All the recovered *Klebseilla* spp. isolates were resistant to Cefepime and Pipercillin/Tazobactam (84.6% each) and exhibited 100% resistant to other antimicrobials agents. Meanwhile, all the recovered isolates were sensitive to imipenem and meropenem. Gene encoding *blaCTX-M1* was mostly predominant among screened genes, being present in 13 (48%) dairy samples for *blaTEM* and *blaSHV,* they were detected in 12 (44%) and 4 (14.8%) isolates, respectively. In conclusion, there is clear evidence of circulation of antibiotic-resistant food borne ESBL producing *Enterobacteriaceae* in the examined dairy samples. The concern about increasing the risk of dissemination of such multi-drug resistant pathogens rises with a potential asymptomatic colonization and complication of systemic infection in human subjects. There is also a possible interface for the exchange of resistance genes within and across species and with commensal bacteria of the human and animals.

Key words: dairy products; ESBL producing *Enterobacteriaceae;* Egypt; public health

Introduction

The emergence of antimicrobial resistance foodborne zoonotic bacteria, particularly *Enter-*

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obacteriaceae that carry ESBLs, has been recognized as one of the most important global problems in both veterinary and human medicine (1). As a matter of growing concern, livestock and animal-derived foods especially milk and its products are the most incriminated foods of animal origin that is linked to disease outbreaks around the word and represent important reservoirs for ESBLs-producing *Enterobacteriaceae* (2). *Escherichia coli* and *Klebsiella pneumoniae* are considered the most predominant species of ESBLs producing *Enterobacteriaceae* worldwide and are being the causal of urinary tract infections, pneumonia and sepsis in human patients (3). In most of developing countries involving Egypt, there are no restricted rules for the use of antibiotics for the treatments of dairy ailments particularly third and fourth generations of cephalosporin due to their therapeutic effectiveness or their short withdrawal periods. Hence, massive and indiscriminate use of these antibacterial agents is critically important to dairy farming and could have the potential to the spread of ESBLs-producing bacteria or even multi drug resistance (MDR) pathogens (4). Resistance to β- lactamase in the family *Enterobacteriaceae* has been reported to be linked to the production of class A and C β-lactamase enzymes which able to hydrolyze and inactivate the β-lactam ring and confer different degrees of resistance to various β- lactam classes (5). ESBLs are bacterial enzymes that degrade oxyiminocephalosporins with the most clinically important class A β-lactamase enzymes and plasmid-mediated Temoniera (TEM), sulfhydryl variable (SHV), and Cefotaxime-Munich (*CTX-M*) types are the three main families of ESBLs (6).

Raw milk can be contaminated with ESBLs producing *Enterobacteriaceae* in several entities such as mastitis, directly by animal feces or indirectly during milking (7). Unfortunately, the vast majority of the population in Egypt's, especially in rural families, still consume raw dairy products without pasteurization including traditional Egyptian cheese as Kareish and Domiati cheese (most popular soft white cheese) with a general believe that pasteurization would drastically affect the milk quality (8). To date, very limited information existing regarding ESBLs producing *Enterobacteriaceae* isolated from dairy cattle (3). Therefore, it is of utmost significance to address the potential occurrence of ESBLs producing *Enterobacteriaceae* from raw milk and some dairy products which marketed in different localities in Damietta Governorate, Egypt and to highlight the threats to human health posed by consumption of raw milk and dairy products.

Materials and methods

Sampling and sample preparation

A total of 100 samples (25 each of market raw milk, Kareish cheese, Domiati cheese and small scale ice cream) were randomly purchased from groceries, retail outlets, supermarkets in Damietta city, Egypt in March 2018. All the collected samples were transported immediately in coolers in its original package under aseptic conditions to the laboratory for bacteriological examinations which were started quickly after receiving the samples according to the previously described protocol (9).

Isolation and identification of cefotaxime-resistant bacteria

Twenty five ml or g of each dairy samples were diluted with 225 ml of buffered peptone water (BPW). In case of cheese, samples were homogenized in a stomacher for through mixing. All the prepared samples were immediately plated onto MacConkey agar plates (Oxoid, Basingstoke, UK) supplemented with 1 mg /L cefotaxime (Sigma-Aldrich) and incubated for 24 h at 37 °C. At least three grown colonies were selected and sub-cultured onto MacConkey agar supplemented with 1 mg/L cefotaxime at 37 °C for 24 h to obtain pure cultures for subsequent analysis. Bacterial characterization was performed using Gram staining, and conventional biochemical testing including Oxidase, indole, methyl red, vogus-prauskaur, citrate, and urease.

Serotyping of identified E. coli

Serotyping of biochemically confirmed *E. coli* isolates were performed by agglutination tests by using rapid diagnostic *E .coli* antisera

sets according to the previously described protocol (10) (Denka Seiken Co., Japan**)** at Food Analysis Center, Faculty of Veterinary Medicine, Benha University, Egypt.

Antimicrobial susceptibility testing and ESBLs detection

All recovered isolates were screened for susceptibility test using 13 antimicrobial agents on Mueller-Hinton agar using disc diffusion method and evaluated according to CLSI criteria (11). The following antimicrobial discs were applied: Penicillin G. (P 10 µg), Ampicillin (AM10 µg), Imipenem (IPM 10 µg), Meropenem (MEM 10 µg), Cefalaxin (CL 30 µg), Cefaclor (CEC30 µg), Cefoxitin (FOX 30 µg), Ceftazidime (CAZ 30 µg), Cefotaxime (CTX 30 µg), Cefepime (FEP 30 µg), Azteonam (ATM 30 µg), Amoxicillin-clavulinic (AMC 20/10 µg), Piperacillin-tazobactam (Tpz 100/10). Each isolate that exhibited resistant to one or more of the third and fourth-generation cephalosporins were confirmed for ESBL production by Double Disk Synergy Test (DDST) according to CLSI guidelines.

Characterization of β-lactamases

Genomic bacterial DNA was extracted from the identified ESBLs producers *E. coli* (n= 17) and *Klebseilla* spp (n= 10) using the QIAamp® DNA Mini Kit Qiagen according to the manufacturer`s guidelines. The purified DNA was further analyzed by PCR targeting ESBL encoding genes including *blaTEM, blaSHV and blaCTX-M* using specific oligonucleotide primers sequences and PCR conditions as described previously (Table 1).

Results

Out of hundred dairy samples, 30 yielded bacterial growth on MacConkey agar supplemented with cefotaxime : 8 (32%) from raw milk, 8 (32%) from Domiati cheese, 9 (36%) from kareish cheese and 5 (20%) from ice cream. Overall, 59 ESBLs producers colonies (24 came from raw milk, 15 from kareish cheese, 13 from Damietta cheese and 7 from ice cream) and were biochemically identified as *E. coli* (n = 29, 49%), *Klebsiella pneumonia* (n = 8, 13.5%), *Klebsiella oxytoca* (n = 5, 8.5%), *Enterobacter aerogenes* (n =8, 13.5%), *Citrobacter diversus* (n = 8, 13.5%) and *Serratia liquefaciens* (n = 1, 1.7%) (Table 2&3). The most prevalent phenotype was *E.coli* (n = 17, 17%), *Klebsiella pneumonia* (n = 6, 6%), 5 (5%) for each of *Enterobacter aerogenes* and *Citrobacter diversus*, 4 (4%) for *Klebsiella oxytoca* and 1 (1%) for *Serratia liquefaciens* (Table 3).

The pattern of antimicrobial drug susceptibility for the 59 ESBLs producers are presented in Table 4&5. Briefly, *E. coli* isolates showed high resistance pattern against cefaclor (100%), cefoxitin (100%), cefalaxim (96.5%), ceftazidime (93%), pencillin (93%) and 79% for Pipercillin/Tazobactam. All the recovered *Klebseilla* spp. isolates (n =13) were resistant to Pencillin, Ampicillin, Cefalaxim, cefaclor, Cefoxitin and Ceftazidime (100% each) and to Cefepime and Pipercillin/Tazobactam (84.6% each). On the other side, all recovered isolates were sensitive to imipenem and meropenem. Gene encoding *blaCTX-M1* was mostly predominant among screened genes (fig 1&2), being present in 13 (48%) dairy samples. for *blaTEM* and *blaSHV*, they were detected in 12 (44%) and 4 (14.8%) isolates, respectively. Three isolates harbored the three screened β-lactamase genes; *blaCTX-M1* and *blaTEM* were detected in four isolates; while *blaSHV* and *blaCTX-M1* were observed in one isolate.

Table 1: Oligonucleotide primers sequences used for amplification of B-lactamase resistance genes

Target gene	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
blaCTX-M1 (F)	5′ TTAGGAAGTGTGCCGCTGTA ′3	655	12
blaCTX-M1 (R)	5′ CGGTTTTATCCCCCACAAC′3		
blaSHV (F)	5′ AGCCGCTTGAGCAAATTAAAC ′3		13
blaSHV (R)	5′ ATCCCGCAGATAAATCACCAC ′3	713	
blaTEM (F)	5′ CATTTCCGTGTCGCCCTTATTC ′3		13
blaTEM (R)	5′ CGTTCATCCATAGTTGCCTGAC ′3	800	

Table 2: Distribution of ESBLs producers Enterobacteriacea spp. from the examined dairy samples

Sample number	Origin	*Number of isolated Enterobacteriacea spp.* from the positive samples					
		E.coli	*K. penumonia*	*K. oxytoca*	*Enterobac-ter*	*Citrobacter*	*Serratia*
	Milk						
1	Pos n= 8/25 (32%)	2					
6		3			1		
10		3		1			
11						3	
12					2	1	1
13		1		1			
14					3		
21			2				
	Damietta chesses						
23	Pos n= 8/25 (32%)			1			
27		1	1				
29		2					
31			1				
35					1		
41		1	1				
43		1					
49						1	
	Kariesh cheese						
51	Pos n= 9/25 (36%)			2			
54		1					
58						2	
59		1				1	
61		2					
69		1					
70		3					
74		2					
75			2				
	Ice Cream						
79	Pos n= 5/25 (20%)		1				
80		2					
83		2					
94		1					
97					1		
	Total pos n = 30/100 (30%)	29	8	5	8	8	1

Table 3: Occurrence and distribution of different ESBLs producers *Enterobacteriaceae* spp. in examined dairy samples

Enterobacteriacae spp.	Milk n =25		Kariesh cheese n = 25		Damietta cheese n = 25		Ice Cream n =25		Total Dairy samples	
	Pos samples	N of colonies	Pos samples	N of colonies	Pos samples	N of colonies	Pos samples	N of colonies	Pos samples	N of colonies
E.coli	4 (16%)	9	6 (24%)	10	4 (16%)	5	3 (12%)	5	17 (17%)	29
Klebseilla .pneumoniae	1 (4%)	2	1 (4%)	2	3 (12%)	3	1 (4%)	1	6 (6%)	8
Klebseilla .oxytoca	2 (8%)	2	1 (4%)	2	1 (4%)	1	0	0	4 (4%)	5
Enterobacter aerogenes	3 (12%)	6	0	0	1 (4%)	1	1 (4%)	1	5 (5%)	8
Citrobacter diversus	2 (8%)	4	2 (8%)	3	1 (4%)	1	0	0	5 (5%)	8
Serratia liquefaciens	1 (4%)	1	0	0	0	0	-	0	1 (1%)	1
Total number of ESBLs n=59		24		17		11		7		59

Table 4: Characteristics of ESBL-positive *Enterobacteriaceae* identified from dairy samples

Sam-ple num-ber	Origin	Species	b-lactam antibiotic resistances												
			P	AM	IPM	MEM	CL	CEC	FOX	CAZ	CTX	FEP	ATM	AMC	TPZ
1	Milk	E.col (O128:H2)	R	R	S	S	R	R	R	R	R	S	R	R	R
1	Milk	E.coli (O91:H21)	R	R	S	S	R	R	R	R	R	S	S	R	R
6	Milk	E.coli (O55:H7)	R	S	S	S	R	R	R	S	R	S	S	R	R
6	Milk	E.coli (O121:H7)	R	R	S	S	R	R	R	R	R	R	S	S	R
6	Milk	E.coli (O146:H21)	R	S	S	S	R	R	R	R	R	R	S	S	R
10	Milk	E.coli (O111:H2)	R	R	S	S	R	R	R	R	R	R	S	S	S
10	Milk	E.coli (O111:H2)	R	S	S	S	R	R	R	R	R	S	S	S	R
10	Milk	E.coli (O114:H4)	S	S	S	S	R	R	R	R	R	S	S	S	S
13	Milk	E.coli (O78)	R	S	S	S	R	R	R	R	R	S	S	S	S
27	Domiate cheese	E.coli (O111:H2)	R	R	S	S	R	R	R	R	R	R	R	R	R
29	Domiate cheese	E.coli (O121:H7)	R	R	S	S	R	R	R	R	R	R	R	R	R
29	Domiate cheese	E.coli (O26:H11)	R	S	S	S	R	R	R	R	R	R	S	S	R
41	Domiate cheese	E.coli (O26:H11)	R	S	S	S	R	R	R	R	R	S	S	S	R
43	Domiate cheese	E.coli (O114:H4)	R	R	S	S	R	R	R	R	R	R	S	R	R
54	kareish cheese	E.coli (O124)	R	R	S	S	R	R	R	R	R	R	S	R	R
59	kareish cheese	E.coli (O111:H2)	R	R	S	S	R	R	R	R	R	R	S	R	R
61	kareish cheese	E.coli (O91:H21)	R	S	S	S	R	R	R	R	R	S	S	S	R
61	kareish cheese	E.coli (O121:H7)	R	S	S	S	S	R	R	R	R	S	S	S	S
69	kareish cheese	E.coli (O91:H21)	R	S	S	S	R	R	R	R	R	S	S	S	R
70	kareish cheese	E.coli (O126:H21)	R	S	S	S	R	R	R	R	R	R	S	S	R
70	kareish cheese	E.coli (O128:H2)	R	R	S	S	R	R	R	R	R	S	R	R	R
70	kareish cheese	E.col i (O111:H2)	R	R	S	S	R	R	R	R	R	S	R	R	R
74	kareish cheese	E.coli (O127:H6)	R	R	S	S	R	R	R	R	R	S	R	S	R
74	kareish cheese	E.coli(O146:H21)	R	R	S	S	R	R	R	R	R	R	S	S	S
80	Ice cream	E.coli (O26H11)	R	R	S	S	R	R	R	R	R	R	S	S	S
80	Ice cream	E.coli (O91:H21)	R	R	S	S	R	R	R	R	R	R	S	S	R
83	Ice cream	E.coli (O111:H2)	R	R	S	S	R	R	R	R	R	R	S	R	R
83	Ice cream	E.coli (O121:H7	R	R	S	S	R	R	R	R	R	R	R	S	R
94	Ice cream	E.coli (O26:H11)	R	S	S	S	R	R	R	R	R	R	S	S	S

Table 4: Continuation

Sample number	Origin	Species	b-lactam antibiotic resistances												
			P	AM	IPM	MEM	CL	CEC	FOX	CAZ	CTX	FEP	ATM	AMC	TPZ
21	Milk	*k. pneom*	R	R	S	S	R	R	R	R	R	R	R	S	S
21	Milk	*k. pneom*	R	S	S	S	R	R	R	R	R	S	S	R	R
27	Domiate cheese	*K.pnemo*	R	R	S	S	R	R	R	R	R	R	S	S	R
31	Domiate cheese	*K.pnemo*	R	R	S	S	R	R	R	R	R	S	S	S	S
41	Domiate cheese	*k.pnemo*	R	R	S	S	R	R	R	R	R	R	S	S	R
75	kareish cheese	*k.pnemo*	R	R	S	S	R	R	R	R	R	R	R	S	R
79	Ice cream	*k.pnemo*	R	R	S	S	R	R	R	R	R	R	S	S	S
10	Milk	*k. oxy*	R	R	S	S	R	S	R	R	R	R	S	S	R
13	Milk	*k. oxy*	R	R	S	S	R	S	R	R	R	R	S	R	R
23	Domiate cheese	*K.oxy*	R	R	S	S	R	S	R	R	R	S	S	R	R
51	kareish cheese	*k. oxy*	R	R	S	S	R	R	R	R	R	R	S	S	R
51	kareish cheese	*k.oxy*	R	R	S	S	R	R	R	R	R	R	S	S	R
6	Milk	*Enterobacter*	R	R	S	S	R	R	R	R	R	R	R	R	R
12	Milk	*Enterobacter*	S	S	S	S	R	R	R	R	R	S	S	S	S
12	Milk	*Enterobacter*	R	R	S	S	R	R	R	R	R	S	S	S	R
14	Milk	*Enterobacter*	R	R	S	S	R	R	R	R	R	S	S	R	R
14	Milk	*Enterobacter*	R	S	S	S	R	R	R	R	R	S	S	S	R
14	Milk	*Enterobacter*	R	S	S	S	R	R	R	R	R	S	S	S	R
35	Domiate cheese	*Enterobacter*	R	R	S	S	S	S	R	R	R	R	S	S	R
97	Ice cream	*Enterobacter*	R	S	S	S	R	R	R	R	R	S	S	S	R
11	Milk	*Citerobacter*	R	R	S	S	R	R	R	R	R	R	S	S	R
11	Milk	*Citerobacter*	R	R	S	S	R	R	R	R	R	S	S	R	R
11	Milk	*Citerobacter*	R	S	S	S	R	R	R	R	R	S	S	S	R
12	Milk	*Citerobacter*	R	R	S	S	R	R	R	R	R	S	R	R	R
49	Domiate cheese	*Citerobacter*	R	R	S	S	R	R	R	R	R	S	S	R	R
58	kareish cheese	*Citerobacter*	R	R	S	S	R	R	R	R	R	R	S	S	R
58	kareish cheese	*Citerobacter*	R	R	S	S	R	R	R	R	R	S	R	S	R
59	kareish cheese	*Citerobacter*	R	S	S	S	R	S	R	R	R	S	S	S	S
12	Milk	*Serratia*	R	S	S	S	R	R	R	R	R	S	S	R	R

P= Pencillin , AM = Ampicillin, IPM = Impipenem, MEM = Meropenem, CL=Cefalaxim, CEC=Cefaclor, FOX = Cefoxitin, CAZ = Ceftazidime , CTX= Cefotaxime, , FEP = Cefepime, ATM=Azteronam, AMC=Amoxycillin/clavulinic, TPZ = Pipercillin/Tazobactam,

Table 5: Antimicrobial resistance profiles in the identified *Enterobacteriaceae* spp.

Used anti-biotic		Raw milk						Damietta Cheese					Kareish Cheese				Ice cream		
		E.coli	*K. P*	*k.O*	*E*	*C*	*S*	*E.coli*	*K. P*	*k.O*	*E*	*C*	E.coli	*K. P*	*k.O*	*C*	E.coli	*K. P*	*E*
p	**% of Resistance**	88.88 (8/9)	100 (2/2)	100 (2/2)	83.33 (5/6)	100 (4/4)	100 (1/1)	100 (5/5)	100 (3/3	100 (1/1)	100 (1/1)	100 (1/1)	90 (9/10)	100 (2/2)	100 (2/2)	100 (3/3)	100 (5/5)	100 (1/1)	100 (1/1)
AM		66.66 (6/9)	100 (2/2)	100 (2/2)	50 (2/4)	75 (3/4)	0 (0/1)	60 (3/5)	100 (3/3	100 (1/1)	100 (1/1)	100 (1/1)	50 (5/10)	100 (2/2)	100 (2/2)	66.66 (2/3)	80 (4/5)	100 (1/1	0 (0/1)
CL		100 (9/9)	100 (2/2)	100 (2/2)	100 (6/6)	100 (4/4)	100 (1/1)	100 (5/5)	100 (3/3	100 (1/1)	0 (0/1)	100 (1/1)	90 (9/10)	100 (2/2)	100 (2/2)	100 (3/3)	100 (5/5)	100 (1/1)	100 (1/1)
CEC		100 (9/9)	100 (2/2)	0 (0/2)	100 (6/6)	100 (4/4)	100 (1/1)	100 (5/5)	100 (3/3	0 (0/1)	0 (0/1)	100 (1/1)	100 (10/10)	100 (2/2)	100 (2/2)	100 (3/3)	100 (5/5)	100 (1/1)	100 (1/1)
FOX		100 (9/9)	100 (2/2)	100 (2/2)	100 (6/6)	100 (4/4)	100 (1/1)	100 (5/5)	100 (3/3	100 (1/1)	100 (1/1)	100 (1/1)	100 (10/10)	100 (2/2)	100 (2/2)	100 (3/3)	100 (5/5)	100 (1/1)	100 (1/1)
CAZ		77.77 (7/9)	100 (2/2)	100 (2/2)	100 (6/6)	100 (4/4)	100 (1/1)	100 (5/5)	100 (3/3	100 (1/1)	100 (1/1)	100 (1/1)	100 (10/10)	100 (2/2)	100 (2/2)	100 (3/3)	100 (5/5)	100 (1/1)	100 (1/1)
CTX		100 (9/9)	100 (2/2)	100 (2/2)	100 (6/6)	100 (4/4)	100 (1/1)	100 (5/5)	100 (3/3	100 (1/1)	100 (1/1)	100 (1/1)	100 (10/10)	100 (2/2)	100 (2/2)	100 (3/3)	100 (5/5)	100 (1/1)	100 (1/1)
FEP		33.33 (3/9)	100 (2/2)	100 (2/2)	16.66 (1/6)	25 (1/4)	0 (0/1)	80 (4/5)	66.66 (2/3	0 (0/1)	100 (1/1)	0 (0/1)	40 (4/10)	100 (2/2)	100 (2/2)	33.33 (1/3)	100 (5/5)	100 (1/1)	0 (0/1)
ATM		11.11 (1/9)	50 (1/2)	0 (0/2)	16.66 (1/6)	25 (1/4)	0 (0/1)	40 (2/5)	0 (0/3	0 (0/1)	0 (0/1)	0 (0/1)	30 (3/10)	50 (1/2)	0 (0/2)	33.33 (1/3)	20 (1/5)	0 (0/1)	0 (0/1)
AMC		33.33 (3/9)	0 (0/2)	50 (1/2)	33.33 (2/6)	50 (2/4)	100 (1/1)	60 (3/5)	0 (0/3	100 (1/1)	0 (0/1)	0 (0/1)	40 (4/10)	100 (2/2)	0 (0/2)	0 (0/3)	20 (1/5)	0 (0/1)	0 (0/1)
TPZ		66.66 (6/9)	50 (2/2)	100 (2/2)	83.33 (5/6)	100 (4/4)	100 (1/1)	100 (5/5	66.66 (2/3	100 (1/1)	100 (1/1)	100 (1/1)	80 (8/10)	100 (2/2)	100 (2/2)	66.66 (2/3)	80 (4/5)	0 (0/1)	100 (1/1)

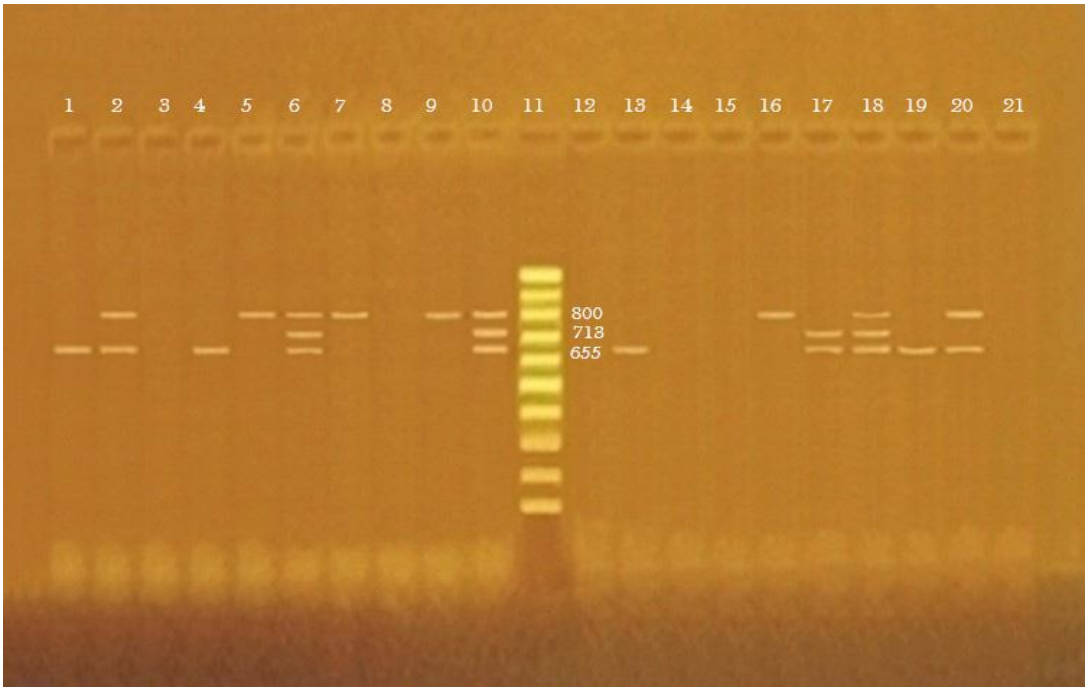

Figure 1: Agarose gel electrophoresis of multiplex PCR amplification of *blaCTX-M1*, *blaSHV* and *blaTEM* in *E. coli* with expected amplicon size ~ 655, 713, 800 bp respectively. Lane 11 is DNA ladder; lane 10 is positive control while lane12 is negative control

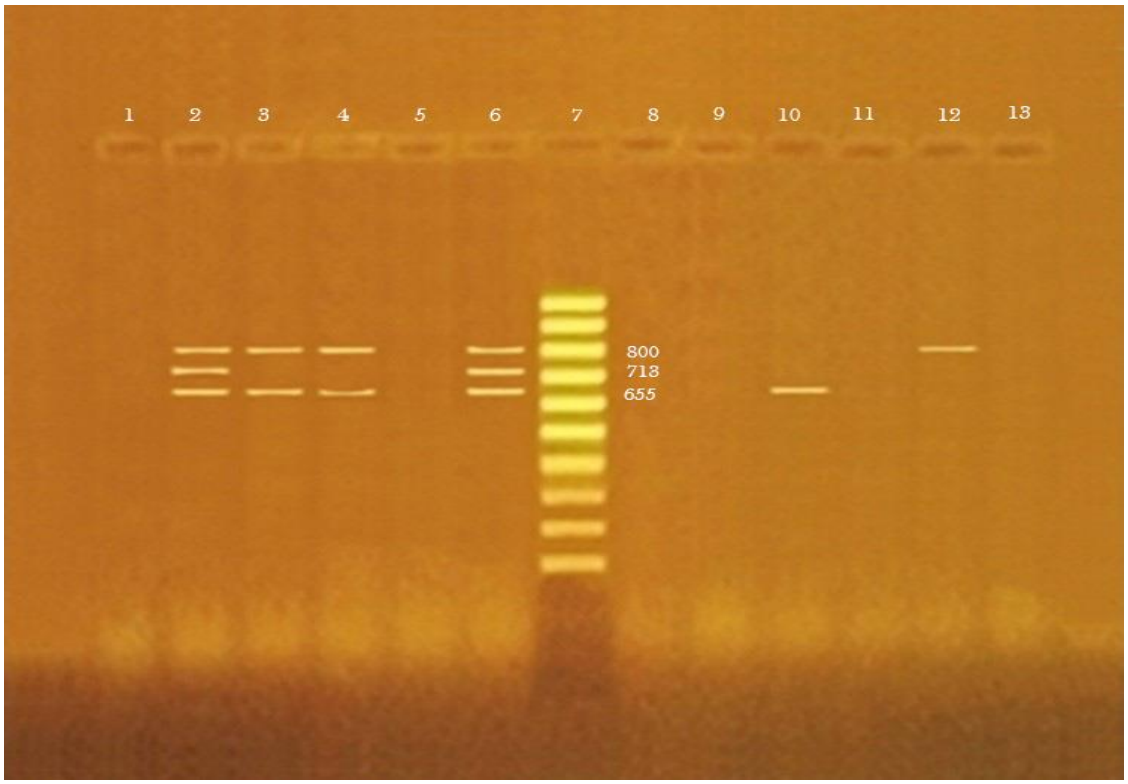

Figure 2: Agarose gel electrophoresis of multiplex PCR amplification of *blaCTX-M1*, *blaSHV* and *blaTEM* in *Klebseilla spp.* with expected amplicon size ~ 655, 713, 800 bp respectively. Lane 7 is DNA ladder; lane 6 is positive control while lane 8 is negative control

Discussion

In Egypt, there is a lack of data regarding ESBLs producing *Enterobacteriaceae* from milk and dairy product with a limited report about the surveillance of ESBLs producing *E. coli* in dairy cattle (3). Therefore, the present study was set to characterize ESBLs producing *Enterobacteriaceae* in some dairy products sold in Damietta city. In this study, the overall detection rate of ESBL producer *E. coli* in the examined dairy samples was 17%. A nearly similar detection rate was previously reported from bulk tank milk samples from German dairy farms (1); while a low frequency of ESBLs (0.7%) was reported in raw milk from the Czech Republic (14). In contrast, a higher detection rate 42.8% (114/266) of ESBL-producing *E.coli* was previously reported in Egypt from dairy cattle (3). It is really difficult to compare studies from Egypt and other countries due to lack of reports and different methods being used for determining the presence of ESBL producing *Enterobacteriaceae*. In one study, the authors failed to determine ESBL-producing isolates in bulk tank milk from 100 different Swiss dairy farms (15). In another study from Turkey, the authors identified ESBL-producing *Enterobacteriaceae* in 100 raw milk with the percentage of 43.6% (16). In a recent study from Sudan, the authors detected ESBL production in 17 out of the 22 isolated *E. coli* (29.3%) from 70 raw cow milk samples collected from different villages in Aljazira state (17). A prevalence of 42.22% ESBL-positive *E. coli* isolates out of 45 *E. coli* strains was identified from 24 typical Slovak cheeses made from raw milk (18).

In the present study, 13.5% of the identified isolates and 6% of the examined samples were classified as ESBL producing *Klebsiella pneumonia*. Different detection rate was reported from recent studies in India (19) and Sudan (17). In those studies, detection rate of 1.5% and 44.8% of *K. pneumonia* were identified as ESBL producing *Klebsiella pneumonia* from raw milk. ESBLs producing *Enterobacteriaceae* has been described for the first time from

hospitalized humans but recently, several researchers from different countries reported its dissemination in the community and also in healthy food producing animals worldwide (1, 3. 5, 15).

Resistance to antimicrobial agents represents a real challenge in Egypt, with high burden on the Egyptian health care system (20). In that study, the authors found that 151 out of 355 (42.5%) of the *Klebsiella* spp. and 47 of 87 (54%) of *Escherichia coli* from the patient in intensive care units were identified as ESBL producers. Several studies have discussed the resistance spectra of different ESBL producers regardless their origin (i.e. human, animal or food origins) (5,15-16). Globally, ESBL-producing microorganisms are one of the most severe problematic multi-resistant and are being identified with increased frequency (4). Our results showed that resistance to two or more antibiotics was frequently encountered among the recovered isolates. As the production of most ESBLs is plasmids encoding, co-resistance to other groups of antibiotics is a common sequel (15). Remarkably, the range of resistance in our study was sufficiently worrisome. For instance, if an infection caused by any identified strains (as that observed in this study) it could represent a great concern due to limited therapeutic options. According to latest report of Infectious Diseases Society of America, ESBL-producing *E coli* and *Klebsiella* spp. were recognized as one of the six drug-resistant microbes that urgently required new remedies (21). Our genotypic characterization of the positive dairy samples by PCR assays revealed that 48% of the positive dairy samples were possessed *bla*CTX-M1encoding CTX-M broad-spectrum β-lactamases, while other genes like *blaTEM* and *blaSHV* were detected in 44% and 14.8%, respectively. This finding was in agreement with that previously reported (22, 24, 15, 23, 16). Unlike to our finding, several authors reported the absence of the *bla*CTX-M gene but identified only *bla*TEM and *bla*SHV genes in 15 and 4 isolates (18). The *bla*SHV-110, *bla*SHV-111, and *bla*CMY-41 genes have been identified from Domiati cheese from small producers in El Behera and Alexandria governments, Egypt (5). The production of broad-spectrum lactamases could provoke a significant clinical consequences with a resultant threaten the successful treatment of infectious diseases resulting in exacerbate public-health concerns (18).

Conclusion

In conclusion, there is clear evidence of circulation of antibiotic-resistant food borne ESBL producing Enterobacteriaceae in the examined dairy samples. As the majority of Egyptian population still consume raw milk and its product like soft cheese e.g. Domiati and Kareish cheese which contained ESBL producing Enterobacteriaceae , the concern about increasing the risk of a spread of such multi-drug resistant pathogens raises with a potential asymptomatic colonization and complication of systemic infection in human subjects and this could be achieved with safe use of antibiotics, reinforced sanitary measures, and a continues investigation of the phenomenon of antibiotic resistance in food producing animals.

Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

SESAME OIL MITIGATES INITIATION STAGE OF DIETHYNITROSAMINE HEPATOCARCINOGENESIS IN RATS

AbdAllah A. Mokh[1], Doaa H. Abdelhady[1], Emad W. Ghazy[1], Hadeer Aboumosalem[1]*, Wael M.Goda[2]

[1]Faculty of Veterinary Medicine, Department of Clinical Pathology, Kafrelsheikh University, [2]Faculty of Veterinary Medicine, Department of Clinical Pathology, Damanhour University, Egypt

*Corresponding author, E-mail: hadeeraboumosalem@gmail.com

Abstract: Diethylnitrosamine (DEN) induced hepatocarcinogenesis in experimental animals through triggering reactive oxygen species (ROS) release and subsequent induction of oxidative stress dependant liver damage. This study was conducted to estimate the protective role of sesame oil (SO) in the initial phase of DEN induced hepatocarcinogenesis. Forty five male Wistar rats were randomly divided into five groups groups (*n* = 9 each). In the first group (control), rats were orally administrated normal saline. Rats of second group (DEN) were intraperitoneally (i.p) injected with a single dose of (200mg/kg body weight, DEN) at the 8thday of the experiment. The third, fourth, fifth groups orally administrated SO at a dose (2.5, 5, 10 mL/kg b.w), respectively 1 week before i.p injection of DEN and continued for 4 successive weeks. DEN- induced hepatotoxicity as detected by normocytic normochromic anemia with marked increase in white blood cells and significant increase in hepatic damage enzymatic markers (alanine transaminase (ALT), aspartate aminotransferase (AST), γ-glutamyl Transferase (γGT) and alkaline phosphatase (ALP)) with significant decrease in serum total protein. Hepatic malondialdehyde (MDA) was increased significantly while hepatic antioxidant biomarkers superoxide dismutase (SOD), catalase (CAT) and hepatic reduced glutathione (GSH) were significantly decreased. Histological examination of hepatic tissue of DEN treated rats proved centrilobular necrosis associated with bile duct and oval cell proliferation. This was accompanied with over expression of *CYP2E1* and down regulation in *BAX* gene expression in liver. Administration of SO minimized the harmful effects of DEN on hematological, biochemical, antioxidant and histopathological parameter as well as on gene expression. The degree of improvement was in dose dependant manner. Our findings revealed that SO supplementation can mitigate the toxic effects of DEN via their potent antioxidant and free radical-scavenging activities.

Key words: diethylnitrosamine, sesame oil, antioxidant, gene expression, rats

Introduction

Liver cancer considers one of the most frequent causes of death. Hepatocellular carcinoma (HCC) is a malicious tumor of liver cell originating from hepatocyte and considered as

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the most common third cause of cancer death worldwide (1, 2). The main risk factors in liver cancer include hepatitis C virus, feed additives as (BHA BHT, nitrates/nitrites), mycotoxins, air and water pollutants (3). In Egypt, The prevalence of liver cancer has been growing in latest years (4).

Nitrosamines are potent environmental carcinogens because of their mutagenic and carcinogenic abilities. Nitrosamines found in water, industrial product, tobacco, cured cheese and smoked meats (5). Diethylnitrosamine (DEN) is commonly used to instigate hepatocellular carcinoma in experimental rat models (6, 7) probably through induction of oxidative stress, resulting in liver damage with increased deleterious free radicals formation (8). Chemoprevention may help to decrease the incidence or severity of carcinogenic insult. They can be used as approaches for liver cancer treatment with less toxic effects.

Sesame oil (SO) extracted from *Sesamum indicum* seeds which has phenolic lignans as sesamol, sesamin, sesamolin and tocopherol (vitamin E) all of which give sesame oil the significant free radical-scavenging ability (9). Sesame oil has the ability to minimize ROS production and lipid peroxidation in various animal model through its antioxidant ability (10). Thus, the current work aimed to estimate the hepatoprotective effect of sesame oil on initial stage of HCC induced by DEN in rat models.

Materials and methods

Preparation of DEN and sesame oil

DEN (purcashed from Sigma Aldrich) was dissolved in normal saline. Sesame oil was obtained from Harraz Company (Cairo, Egypt 100% pure) in a solution form, given at different concentrations (2.5, 5, 10 ml/kg b.w) by stomach tube according to animal body weight.

Animals

Forty-five healthy male Wistar rats (weighing 90±20 g/rat and at age of 1 month old) were obtained from the Alexandria Organization for Biological Products. The rats were housed in metallic cages with thermally controlled temperature ranged from 22to25 °C, relative humidity 50–60%, with12 h photoperiods and12 h dark. During the entire period of study, the rats were provided with a semi-purified basal diet and water *ad libitum*. The experiment protocol followed the Guide for the Care and Use of Laboratory Animals at Kafrelsheikh University. All safety measures had been taken to minimize animal stress.

Experimental design

After two weeks acclimatization period, random classification of rat into 5 different equal groups (9/each) was done. Rats in the1st group were kept as control and were only given normal saline by gastric intubation during the whole period of the experiment. A single ip injection of normal saline was also given to these rats at the 8th day of the experiment to initiate hepatocarcinogenesis (11). Rats of the 2nd group (DEN) were i.p injected with a single dose of DEN (200 mg/kg b.w) at the 8th day of the experiment (11). The 3rd group rats orally administrated sesame oil (2.5 mL/kg b.w) 1 week before i.p injection of DEN (at the first day of the experiment) and continued for 4 successive weeks. The 4th group orally received sesame oil at a dose of (5 mL/kg b.w) 1 week before i.p injection of DEN (at the first day of the experiment) and continued for 4 successive weeks (10). The 5th group received sesame oil at a dose of (10 mL/kg b.w, orally) 1 week before i.p injection of DEN (at the first day of the experiment) and continued for 4 successive weeks (12).

Blood and liver sampling

Blood samples were collected from rats eyes by retro-orbital venus plexus bleeding under effect of mild ether anesthesia by using of clean capillary tubes and immediately grouped into two groups the first with anticoagulant for hematological parameters determination, the second group without anticoagulant for serum biochemical estimation. Later, rats were slaughtered and livers were rapidly removed then trimmed from excess tissues and washed by normal saline and distilled water, cut apart into three parts; The 1st part was cut into slices and

directly put in liquid nitrogen, then stored at-80 °C for molecular analysis. The 2nd portion used to prepare tissue homogenate for antioxidant examination as previously described (13). The last portion was directly put in 10% formalin for histopathological analysis.

Hematological examination

Blood samples collected in tubes coated with EDTA (1mg/ml blood) were used for measuring of complete blood count (RBCs, Hb, PCV, MCV, MCHC, WBCs, and platelets count) using exigoautomated cell counter (Exigo BM800, USA)

Biochemical parameters

The serum level of (ALT) and (AST) enzymes were kinetically determined as previously described (14), while the serum level of (γGT) and(ALP) enzymes were Calorimetrically estimated by (15). Serum total protein and glucose were calorimetrically detected as previously detailed (16, 17). MDA level and activities of catalase, SOD and GSH were determined in liver homogenate as method described by (18-22).

Histopathological analysis

Liver tissue specimens were preserved in 10% neutral formalin, fixed in paraffin**,** sectioned and stained by H&E (23).

Molecular investigation of BAX and CYP2E1 genes

Total RNA was extracted from liver with Trizol reagent (total RNA isolation reagent, INTRON Biotechnology, Inc). Complementary DNA (cDNA) was synthesized using cDNA synthesis kits as previously described (24). PCR tubes containing SYPR green master mix (Enzynomic company, Cat number RT500), cDNA, primers (Table1) and RNase free water were inserted in BIoRad IQ2 real time thermal cycler. β- actin was used as a housekeeping gene. Thermal cycling conditions were: incubation at 94 °C for 15 min then 94 °C for15 s (40 cycles) followed by 60 °C for 30s and 70 °C for 30s. The data reported at extension step. Melting curve analysis used to determine specificity of PCR products. IQ5 software was used to detect amplification curves and Ct values. Gene expression variation of different samples determined through comparing between the Ct values of all groups by using "$2^{\Delta\Delta Ct}$" method. The PCR products were confirmed by using 1.5% agarose gel.

Data statistical analysis

Graph pad prism version 5.0 was used to analyze the resulted data. Differences in values were analyzed by one-way analysis of variance (ANOVA), then Tukey's-compare all pairs of columns. All data were exposed as mean ± standard error of the mean (SEM) with citation of significance level at $p < 0.05$.

Results

Hematological finding

Data explored in table (2) showed deleterious impacts of (DEN) and the ameliorative effects of Sesame oil on the erythrogram. A significant decrease in hematological parameters (RBCs, Hb, PCV and platelets) without significant change in the values of (MCV, MCH and MCHC) was noticed in DEN treated group as compared to the control group. Opposing to these results WBCs count was significantly increased. SO treated group showed a marked ($P \leq 0.05$) decrease in hematological parameters (RBCs, Hb, PCV and platelets) as compared to DEN-treated group, but with a significant ($P \leq 0.05$) decrease in WBCs count.

Serum Biochemical assays

Data demonstrated in (Fig. 1A) showed toxic effects of DEN and the protective role of sesame oil on serum biochemical measurements. Group II (DEN) showed significant increases in the activity of hepatic damage enzymatic markers (ALT, AST, ALP, γGT) and serum glucose when compared with the control group. Group III, IV, V which supplied by sesame oil and (DEN) showing significant decrease in the activity of serum liver function marker enzymes (AST, ALT, ALP, γGT) and serum glucose when compared with the DEN group. The degree of improvement was in dose dependant manner.

Data illustrated in table (2) showed deleterious effects of (DEN) and the ameliorative effects of sesame oil on Serum proteins. Group II (DEN) showed significant decreases in total proteins, albumin and globulins concentration when compared with control group. Group III, IV, V which supplied by sesame oil and DEN showing significant increase in total proteins, albumin and globulins concentration when compared with DEN group.

Data demonstrated in (Fig. 2) revealed the harmful effects of DEN as well as the challenge effects of Sesame oil on lipid peroxidation and antioxidant biomarkers. Group II (DEN) showed significant increase in hepatic MDA content, while liver CAT, GSH, and SOD activities were statically decreased when compared with control group. Group III, IV, V (DEN -SO) showed significant decreases in hepatic MDA content but liver CAT, GSH, SOD activities had increased markedly in comparing to DEN group.

Histopathological finding

Effect of DEN and *Sesame oil* on histopathological features of liver is presented in (Fig. 3). Histological examination of the hepatic tissue sections of control negative rats revealed normal hepatocellular architecture mainly consisting of normal hepatocytes with normal cytoplasm and small uniform vesicular-shaped nuclei which arranged in a radial pattern around the central vein of the hepatic lobules (Fig. 3A). Liver of control positive animal (DEN) revealed centrolobular necrosis associated with severe ballooning of hepatocytes. Moreover, severe ballooning of hepatocytes were associated with bile duct and oval cell proliferation (Fig. 3B). Liver of diseased animal treated with (2.5 ml Sesame oil) showed decreased hepatic vacuolation, bile duct hyperplasia and centrolobular necrosis (Fig. 3C).The fourth and fifth groups treated with (5-10 ml sesame oil) respectively, showing reduction of hepatic vacuolation with subsequent marked reduction the altered hepatocytes as well as the number of oval cells and the necrobiotic changes associated with DEN treatment appearing the hepatic tissues mostly within normal limits (Fig.3D, E), respectively.

Molecular analysis

The expression of *CYP2E1* and *BAX* genes was determined by Real time PCR that reveals the transcription levels changes of these genes in liver of rats after i.p injection of DEN alone or in combination with *Sesame oil* in three doses (2.5, 5, 10 ml/kg b.w). DEN injection showed marked ($P \leq 0.05$) increase in *CYP2E1* gene expression and significant decrease in *BAX* gene expression in comparison with the control, while supplying of Sesame oil before DEN injection at a dose of (2.5, 5, 10 ml/kg b.w) decreased the *CYP2E1* gene expression and increased the expression of *BAX* gene when compared with animals treated with DEN only as showed in (Fig. 4**)**. The degree of improvement was dose dependent.

Table 1: Primers used in qPCR

Gene	Primer sequence (5'-3')
Rat ß-actin	F 5′-TCCTCCTGAGCGCAAGTACTCT -3′
	R 5′-GCTCAGTAACAGTCCGCCTAGAA -3′
BAX	F 5′-CACCAGCTCTGAACAGATCATGA -3′
	R 5′-TCAGCCCATCTTCTTCCAGATGGT -3′
CYP2E1	F 5′-CTCCTCGTCATATCCATCTG -3′
	R 5′-GCAGCCAATCAGAAATGTGG -3′

Table 2: The impact of SO supplementation against DEN on hematological parameters and Serum protein profile.

	RBCs 10^6/ µl	HB g %	HCT%	MCV/fl	MCHC%	Pl($\times 10^3$/ µl)	WBCs 10^3/ µl	Total potien gm/dl	Albumin gm/dl	Globulin gm/dl
Control	6.9± 0.10^a	13.4±0.36^a	46.6± 1.14^a	66.9±1.63	28.8±0.40	714.8±6.5^a	10.2 ±1.12^{dc}	6.86 ± 0.1^a	3.86 ± 0.08^a	3 ±0.05^a
DEN	5.9± 0.05^b	10.3± 0.04^b	36.9± 0.10^b	62.2±0.67	27.8 ± 0.13	303.8±8.5^d	24.0 ±0.85^a	5.14± 0.12^c	3 ± 0.07^c	2.28± 0.14^b
DEN+ Sesame oil (2.5ml/kg)	7.3± 0.31^a	13.7± 0.10^a	48.3 ±0.98^a	64.9±0.31	28.7± 0.36	455.8±5.3^c	20.5 ±0.51^{ab}	6.1 ± 0.08^b	3.4 ± 0.07^b	2.78 ± 0.03^a
DEN+ Sesame oil (5 ml/kg)	7.0± 0.21^a	13.0 ±0.26^a	47.1 ±0.66^a	67.4±1.72	27.6± 0.23	571.2±4.6^b	14.8 ±0.62^c	6.58± 0.03^a	3.66 ± 0.04^{ba}	2.92 ± 0.02^a
DEN+ Sesame oil (10ml/kg)	7.2 ± 0.10^a	13.5 ± 0.18^a	48.0± 1.32^a	66.4±1.33	27.7± 0.61	700.2±3.2^a	12.5± 1.10^c	6.7± 0.05^a	3.7 ± 0.07^a	3 ± 0.1^a

Values are means ± standard error. (n=9) Mean values with different letters at the same column differ significantly at ($p \leq 0.05$). RBCs: red blood cells, Hb: hemoglobin, HCT%: hematocrite, MCV: mean corpuscular volume, MCHC: mean corpuscular hemoglobin concentration, WBCs: white blood cells, Pl: platelets.

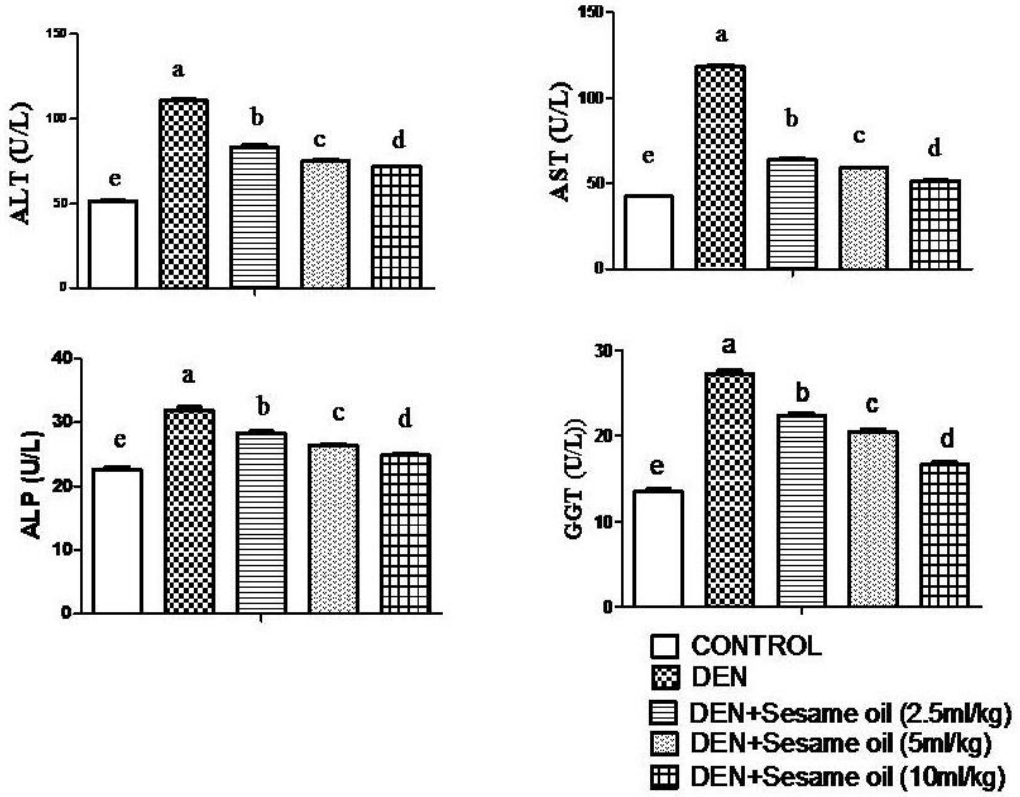

Figure 1: Effect of different concentrations of sesame oil on serum levels of liver enzymes (ALT, AST, ALP, GGT) of DEN-intoxicated rats. Values are means ± standard error. (n=9). Mean values with different letters in each graph significantly differ at ($p \leq 0.05$)

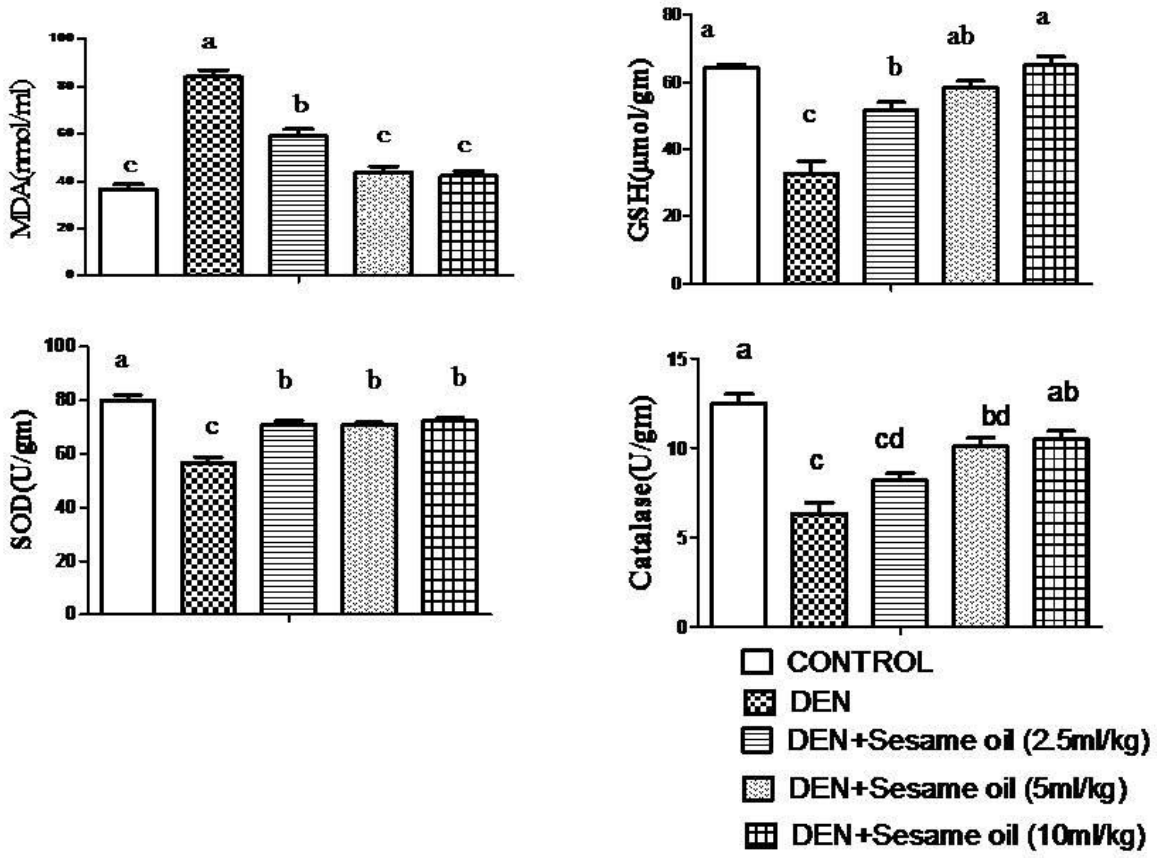

Figure 2: Effect of different concentrations of sesame oil on hepatic level of lipid peroxidation marker MDA, and endogenous antioxidants (GSH, SOD, CAT) of DEN-intoxicated rats. Values are means ± standard error. (n=9). Mean values with different letters in each graph significantly differ at ($p \leq 0.05$)

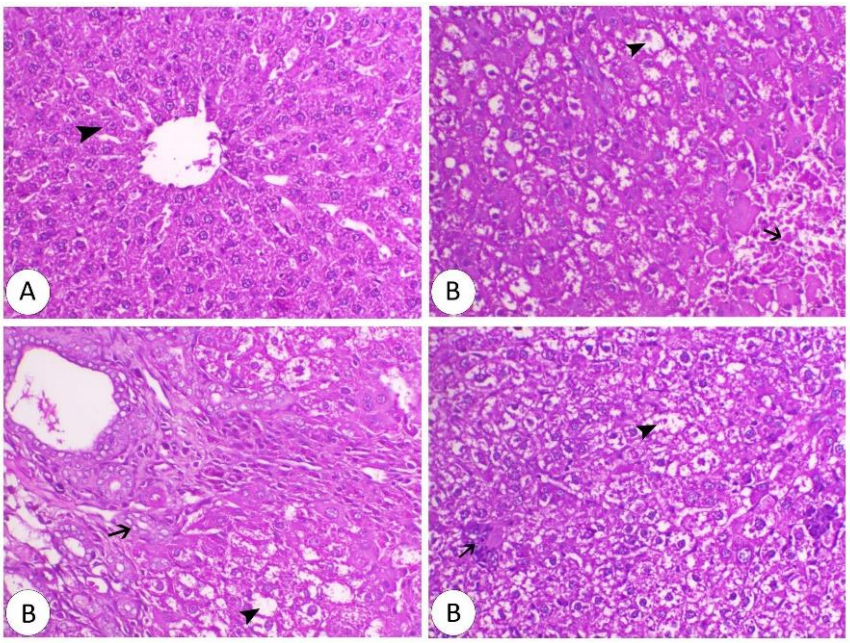

Figure 3: A. Liver of control animal showing normal hepatocytes arranged in cords around the central vein, H&E, X 200. **B**. Liver of control positive animal showing centrolobular necrosis (arrows) associated with severe ballooning of hepatocytes (arrowheads), H&E, X20

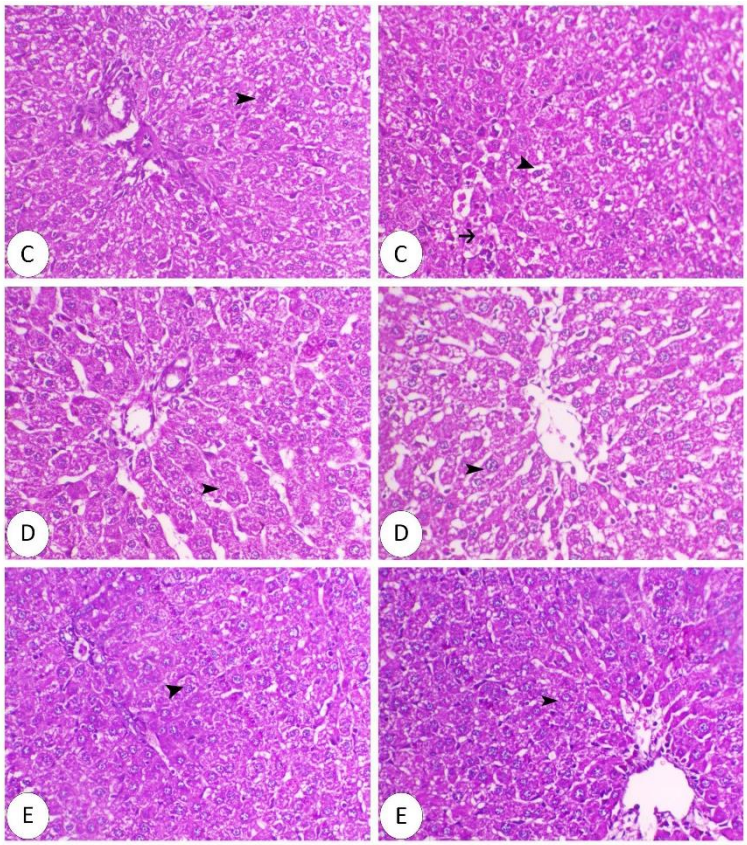

Fig.3. C. Liver of animal treated with *DENA and Sesame oil* (2.5 ml) showing decrease hepatic vacuolation (arrowhead) and centrolobular necrosis (arrow). **D.** Liver of animal treated with *DENA and Sesame oil* (5 ml) showing marked decrease hepatic vacuolation (arrowhead). **E.** Liver of animal treated with *DENA and Sesame oil* (10 ml) showing significant decrease hepatic vacuolation (arrowhead). H&E, X 200

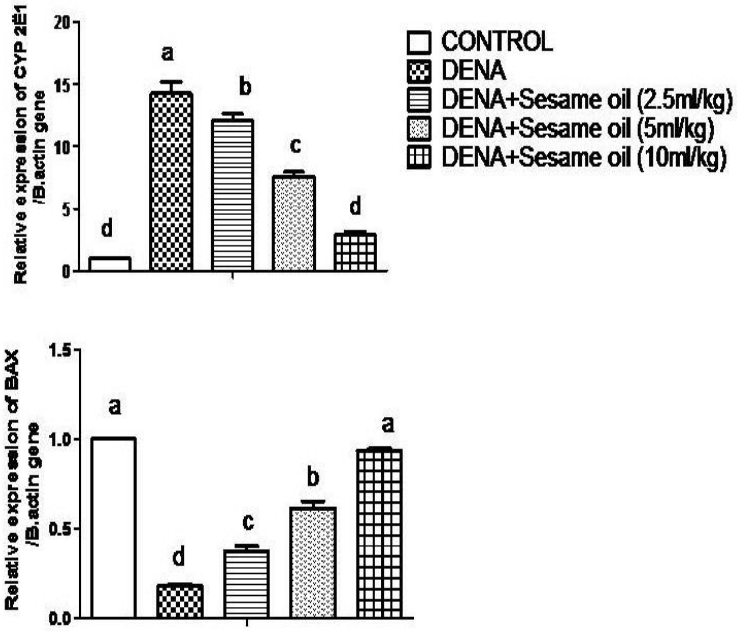

Figure 4: Effect of different concentrations of sesame oil on relative expression of *CYP2E1* and *BAX* genes in liver of DEN-intoxicated rats. Values are means ± standard error. (n=9). Mean values with different letters in each graph significantly differ at ($p \leq 0.05$).

Discussion

T Hepatocellular carcinoma induced by several factors such as food supplement(25), endogenous or environmental stimuli (26). It happens stepwise through initial stage alterations, proliferation resulting in malignant transformation (27). DEN could be metabolized by cytochrome P450 to produce active ethyl radical, which can interact with DNA causing mutagenicity and consequent carcinogenesis (28, 29). Research on plants helped in the detection of compounds (30)with antitumor activity from non-traditionally useful plants that clinically used as effective drugs(31). The current study has been initiated to investigate whether sesame oil supplementation could inhibit the initiation stage of hepaticcarcinogenesis induced by DEN in rats.

Hematopoietic system is a very sensitive systems to estimate the danger impacts of drugs and toxins on our health (32). The current work revealed that i.p injection of DEN caused marked bad effect on the eyrthrogram of DEN rats. SO pretreatment had improved the disturbances of hematological parameters caused by DEN in an effective manner. The damaging consequences of DEN on hemogram was proved by significant reduction in RBCs, Hb%, HCT and platelets counts without significant changes in MCV or MCHC. Opposing to these results, a significant elevation in TLC count was also detected in control positive group (DEN-treated). These results explained the etiological relationship between anemia (normocytic normochromic anemia) and DEN treatment which might be produced as a result of different mechanisms including either bone marrow cells damage or increase osmotic fragility of RBCs and damage of cell membrane (33). furthermore, (34)reported that DEN not only resulted in reduction in RBCs and platelets count but also induced an increase in the TLC count. The reduction in platelets count could be due to either inhibition of bone marrow activity by DEN which consequently decrease its production or increased its consumption(35). On the other hand, elevation in the total leukocytic count could be due to inflammation occurs during DEN treatment and bioactivation in the experimental animals. SO has a good role in counteracting DEN – hazard effects on the erythrogram by increasing RBCs and Hb count. SO has antioxidant ability to improve erythrocyte deformability markedly (10).

The liver condition is indirectly examined through the determination of serum ALT, AST, ALP and γGT. This study reported that The elevations of these enzymes could be attributed to their leakage from damaging cell membrane which induced as aresult of several pathological conditions as hepatic cellular injury (36, 37). DEN treated group resulted in increase in ALT, AST, ALP and γGT serum activities which proved hepatic cell damage. These results were reported elsewhere(38, 39).In the current experiment treatment with sesame oil caused a significant reduction in ALT, AST, ALP and γGT activities which indicate significant recovery of hepatic cell function. The hepatoprotective role of sesame oil may be due to its antioxidant active componentas (sesamin, sesamol, sesamolin, andtocopherol). Which reduce ROS production and lipid peroxidation, by this means the membrane permeability was alleviated and the leakage of these enzymes into the blood was minimized (40).

Sesame oil treated group caused a significant reduction in level of blood glucose when compared with DEN treated group as sesame oil contains monounsaturated fatty acids and bioactive compounds which enhanced β cells to produce insulin and regulate the blood glucose(40). The fat-soluble lignans (sesamin, sesamolin, and sesamol) help in decreasing of the hepatic oxidative destruction as a result the blood sugar level was reduced (41).

Concerning to evaluation of serum proteins such as albumin and globulin which concern a good criteria for assessing the synthetic function of the liver(42). In the present study it was noticed that the reduction of total protein level indicate diseased and bad health condition(43).This present study showed considerable decrease in blood serum total protein, albumin, and globulin concentrations in rats with DEN induced hepatopathy that lead to severe liver damage correlated with tissue histoarchitecture. When *sesame oil was* administrated at

different doses with i.p injection of DEN result in significant elevation in total protein, albumin and globulin concentrations which proved the recovery of synthetic function of the liver, These results agree with (44, 45).

DEN injection enhances the oxidative damage through elevation of hepatic MDA and depleted enzymatic (SOD and CAT) and non enzymatic (GSH) antioxidant markers(46, 47). ROS have a critical role in the initiation of lipid peroxidation(48). Which produced in high amount as a result of cellular damage (49). generation of ROS and LPO help in initiation of tumorigenesis (50). DEN enhances hepatic oxidative destruction resulted in HCC formation(51). This study agreed with(46, 47). The administration of sesame oil resulted in marked decrease in MDA level and elevation in the activities of (SOD, catalase and GSH) when compared with the DEN-treated rats. The antioxidant effect of SO is due to the non-fat antioxidants content as (sesamol, sesamin, sesamolin and tocopherol (vit E) (52), which protect the cells from oxidative damage.

The histopathological changes in liver tissues caused by DEN revealed the increased percentage of degenerated hepatocytes manifested as areas of perivascular inflammatory infiltrates with diffuse ballooning degeneration, severe ballooning of hepatocytes associated with bile duct and oval cell proliferation. This may be due to the hepatotoxic effect of DEN which causes oxidative stress and liver tissue damage. Hepatic cell degeneration induced by DEN administration is in harmony with those obtained, (25, 53, 54). Sesame oil administration decreased oxidative damage in HCC rats through reducing production of ROS and LPO leading to significant improvement of hepatic tissue which evidenced as marked decrease in hepatic vacuolation, inflammatory infiltrates, oval cell proliferation and centrolobular necrosis.

Molecular gene expression (*CYP2E1* and *BAX*) are considered important genes expression in cancer. The cytochromes *P*450 (CYPs) are main enzymes in development and treatment of cancer. They enhance the metabolic activation of several carcinogenic substances as (benzene, CCL_4,chloroform, styrene, *N*-nitrosodimethylamine, NNK)(55). Thus, *CYP2E1* might be an essential gene in detection toxicity and carcinogenicity susceptibility to human from environmental and industrial chemicals (54, 56). *CYP2E1* is one of CYPs *Class one* which activated during pre-carcinogens and drugs metabolism. In this study liver of DEN-treated rats produced an over expression of *CYP2E1*gene opposing to control group results,(57) supported these findings.*CYP2E1* has an important role in metabolism and activation a number of chemicals, solvents, cancer producing agents. Sesame oil treated animals showed a significant down regulation in *CYP2E1* gene expression when compared with DEN-treated group. Sesame oil has a methylenedioxypheny compounds which are potent inhibitors or inactivators of *CYP* isoforms. These compounds could interact with the *CYP450* isozymes and affect the drug metabolisms resulting in inhibition in the activity of this gene(58).

BAX is a pro-apoptotic gene that regulates cell death. It is an important indicator of mitochondrial dysfunction and one of the essential pro-apoptotic members of the *Bcl-2* family proteins. It manages the apoptosis process within normal and cancer cells. Apoptosis Dysfunction makes the cancer treatment more difficult and helps tumorigenesis to progress. Activation of *BAX* gene increases permeability of the mitochondrial membrane; result in releasing of apoptotic factor cytochrome *c* which causes cancer cell death. In the current study DEN-treated rats showed significant down regulation in the expression of *BAX* gene when compared with control negative group. DEN inhibits apoptosis, promoting the proliferation of cancer cell and increase cell survival. These results agreed with(59).Sesame oil treated rats revealed a significant over expression of *BAX* gene in comparing with DEN-treated animals as Sesame oil enhanced cytochrome releasing from mitochondria leading to promote caspase-3 cleavage (the initiator- and important caspases in the intrinsic pathway of apoptosis)induce apoptosis (60), which subsequent arrest proliferation of cancer cell and cause death to it.

Conclusion

The data in the current study conclusively demonstrated that oral administration of sesame oil exert significant protective effects against DEN induced oxidative and liver damage by increasing host antioxidant defense mechanisms. This could be attributing to the improvement of anemia, decrease in serum liver enzyme activity, reduced the degree of hepatic vacuolation and necrosis of by DEN, down regulation of CYPE21and up regulation of BAX gene which enhance cancer treatment.

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Original Research Article

EFFECT OF SOME FEED-ADDITIVES ON THE GROWTH PERFORMANCE, PHYSIOLOGICAL RESPONSE AND HISTOPATHOLOGICAL CHANGES OF RABBITS SUBJECTED TO OCHRATOXIN-A FEED CONTAMINATION

Maha S. A. Salama[1], Wael A. M. Morsy[1], Radi A. Mohamed[*2], Samy A. El-Midany[3]

[1]Animal Production Research Institute, Kafr El-Sheikh branch, Ministry of Agriculture, Dokki-Egypt, [2]Department of Aquaculture, Faculty of Aquatic Sciences and Fisheries, Kafrelsheikh University, El-Geish Street, 33516 Kafrelsheikh, Egypt, [3]Hygiene and Preventive Medicine Department, Faculty of Veterinary Medicine, Kafrelsheikh University, (P.O. 33516) Kafr El-Sheikh, Egypt

*Corresponding author, E-mail: r.mohamed.vet@gmail.com

Abstract: This experiment aimed to evaluate the toxic influence of ochratoxin- A (OTA) feed contamination and the effectiveness of some feed-additives (Humic acids, Bio-Plus 2B, Bio-Mos) in detoxifying ochratoxicosis. Thirty five day old weaned Animal Production Research Institute (APRI) rabbits were selected and were allocated to four groups. Group 1 (control, OTA group): rabbits were fed basal diet (ochratoxin- A level: 19 µg /kg diet). Group 2: rabbits were fed a basal diet containing 0.2 % humic acid. Group 3: rabbits were fed a basal diet containing 0.4 mg/kg diet *Bacillus subtilis* and *Bacillus licheniformis* (Bio-Plus 2B). Group 4: rabbits were fed a basal diet containing 0.1% Manna oligosaccharides (Bio-Mos). The control group showed significantly reduced feed intake, daily weight gain and growth performance index. Furthermore, there was a significant increase in AST, ALT, malondialdehyde, creatinine, and urea. Moreover, Bio-Mos group revealed significantly increased serum Hemoglobin, PCV, RBCs, MCV and RBCs/ lymphocyte ratio, and significantly decreased serum SOD and CAT activities. Additionally, internal organs (liver, kidney and intestine) of groups received feed additives revealed less deteriorative change in histopathological investigations in comparison with the control group. In conclusion, supplementation of contaminated feed with ochratoxin-A by feed-additives improves feed intake and final body weight and reduces mortality rate of rabbits. In addition, it improves liver and kidney functions and reduces its pathological changes, and restores antioxidant to its normal level.

Key words: rabbits; ochratoxin-A; growth performance; anti-oxidant

Introduction

Animal feed and feed ingredients are commonly contaminated with different types of mycotoxins which produced by some fungal species (1). One of the most dangerous mycotoxins types is ochratoxin-A. It created by some species of *Aspergillus* and *Penicillium*. Presence of ochratoxin-A (OTA) in animal feeds raises concerns in poultry, rabbits and livestock industry due to subclinical intoxications and poor growth in animals (2). Contamination of animal

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feed with OTA is very come and its toxic consequences are focused on the internal organs in form of hypertrophy, increase weight and sever destruction in active cells (3). In case of rabbits and some other poultry, the most affected organ is kidney (39). However, the other organs (liver, intestine, muscle, bone, bone marrow, testes, ovaries and lymphatic organs) may also be influenced by OTA toxicity (4). (3) found that rabbits suffering from OTA toxicity showed lower consumption of feed, reduced weight gain, higher feed conversion ratio, lower profit gain and consequently reduced overall production of rabbit (5).

Due to the severity of OTA toxicity, many trials were done to overcome its bad effects. Using of feed additives is a novel method used for reducing the destructive effects of OTA by reducing its absorption from animal intestine. (6). Humate is a substance originated from the decayed animal and pants in soil and contains many active ingredients such as humic acid, humus, fulvic acid, and some micro-minerals that have strong binding and absorption activity (7). Therefore, humic acid was used as therapeutic agents in the field of veterinary and human medicine for example as a coating agent in diarrhea treatment, antibacterial agent and immune stimulator (8).

Some *Bacillus species* have a strong powerful capability in toxins removal especially in the field of food production (9). (10) found that most of toxins produced by sever pathogenic fungi are biologically controlled by using *Bacillus subtilis*. Moreover, the growth of *Aspergillus species* could be inhibited under effect of *Bacillus subtilis* (11) and the aflatoxin produced by *A. flavus* and *A. parasiticus* and could be prevented under the effect of *Bacillus stearothermophilus* (12, 13). Another example for feed additives is manna oligosaccharides that produced by yeast *(Saccharomyces cerevisiae*) cell wall. It characterized by its strong binding capacity with microbes, chemical agents and toxins preventing its accumulation and absorption from intestine (14).

The aim of this experiment was to evaluate the influence of OTA and the antitoxic efficacy of some feed-additives on feed utilization, performance, hemato-biochemical parameters and histopathological changes of APPRI growing rabbits.

Materials and methods

Chemical analysis of OTA in rabbit feed

The feed was prepared to supply experimental animals with their basic requirements for the growth (Table 1) according to (15). Analysis of feed ochratoxin-A was performed using florometer- antibody column method in Vet. Med. Laboratory, Kafrelsheikh University, Egypt and the value of OTA in basal diet of rabbit was 19 μg/kg diet.

Experimental design

The experiment was conducted in a Sakha Station rabbits farm), Animal Production Research Institute, Agriculture Research Center, Egypt. Animals used in the experiment were weaned and 35-day old APRI-line rabbits. Each animal was housed in a separate box of a larger cage. Animals were arbitrarily allocated to four groups (20 rabbit each), two replicate each. For acclimatization, all rabbits were fed the same feed for a week before starting the experiment. At 35 days of age, each experimental group received its specific feed as follow: Group 1 (control): rabbits were fed only on basal diet (ochratoxin- A level: 19 μg /kg diet). Group 2 (Humic acids): rabbits were fed a basal diet contain 0.2 % humic acids. Group 3 (PLUS): rabbits were fed a basal diet contain 0.4 mg/kg diet *Bacillus subtilis* and *Bacillus licheniformis* (Bio-Plus 2B). Group 4 (MOS): rabbits were fed a basal diet contain 0.1% Manna oligosaccharides (Bio-Mos) (8 - 11). Feed additives were mixed with pelleted feed daily by spraying of feed with molasses mixed with water as a binder for additives and then additives mixed carefully with feed. In case of group 1 (control, OTA group), feed was mixed only with molasses mixed with water. All groups received continuous supply of feed and water. Rabbits feed consumption was recorded on daily bases. Whereas, body weight (BW), weight gain (WG) and feed conversion ratio (FCR) were

measured weekly. Mortality rate (%) was recorded along the experimental period. Economic efficiency (16), relative growth rate [(W2 - W1) x100]/ [1/2 (W2+W1)] and performance index [(final body weight (kg)/ feed conversion ratio) x 100] were calculated for each group (17).Whereas: W1is the initial weight, and W2 is the final body weight.

Blood was aspirated from the marginal ear vein at the end of the experiment. Blood (3 ml) was aspirated and mixed gently in heparinized tubes. Whole blood was used for hematological assay. Plasma was obtained by centrifugation of 2 ml whole blood at 3000 rpm for 15 min. Then, plasma was stored at -20 °C until used for biochemical assay.

At the end of experiment (91 day), six rabbits were randomly selected from group 1, 2, 3 and 4and were slaughtered using sharp knife to obtain internal organs (liver, kidney and intestine) for histopathological examination.

The Ethical Committee, Faculty of Veterinary Medicine, University of Kafrelsheikh and the Agricultural Administrative Authority approved the conduct of this experiment.

Hematological examination

Heparinized whole blood was analyzed after collection by 2 hours for estimation of red blood cells (RBCs) count, packed cell volume (PCV), haemoglobin concentration, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cells (WBCs) according to (18). On two clean microscope slides, thin blood smears were prepared from each blood sample. Slides were left to dry in room temperature. Slides were stained with a modified Wright's stain, and covered. One hundred cells were counted under × 100 lense and the number of neutrophils count, lymphocytes count, monocyte, eosinophil and basophil were calculated.

Biochemical examination

Plasma samples were used for calorimetric estimation of total protein (TP), globulin, albumin (ALB), triglyceride (TG), total cholesterol (TC) , HDL-C, LDL-C, VLDL-C, cholesterol/ HDL ratio, LDL/ HDL ratio, glucose, urea, creatinine (CREA), alanine aminotransferase (ALT), aspartate aminotransferase (AST) by using commercial kits (Bio-Diagnosis Co., Cairo, Egypt).

Antioxidant parameters and lipid peroxidation biomarker

Lipid peroxidation biomarker used in this study was malondialdehyde (MDA). The antioxidant indicators used in this study were superoxide dismutase (SOD) and catalase activity that were estimated as previously described (19), (20) and (21).

Histopathological changes

Kidneys, liver and intestine tissues specimens were fixed in 10% formalin immediately after slaughtering. The fixed tissues were paraffin embedded, sectioned (3 μm) and stained by hematoxylin and eosin (H&E) (22).

Statistical analysis

Data was tested for distribution normality. Data were analyzed using SAS® statistical system, Package v9.2, version 2002. Data were reported as means and SEM, compared by one-way ANOVA and the Duncan's multiple range tests was used as a post hoc test (23) except for mortality rate that was analyzed using Chi-square test. The level of significance was at (P<0.05).

Results

Feed consumption and rabbits performance

Feeding rabbits on diet contaminated with OTA (control group) led to a significant (P<0.05) decrease in feed consumption, WG, final BW and performance index as compared to the other groups (Table 2). On the other hand, administration of MOS, plus and humic groups reduced the negative influence of OTA and significantly (P<0.05) enhanced feed consumption, final BW, relative growth rate, and performance index in comparison with group 1 (control). Rabbits in the control group showed higher FCR and mortality (30 %) rate than other groups (Table 2).

Biochemical parameters

Although TP, ALB and globulin concentration showed no significant differences (Table 3), glucose concentration reached highest level significantly ($P<0.05$) in MOS and plus groups. While, the TG and TC recorded the lowest significant levels ($P<0.05$) in MOS group matched to other groups (Table 3). The LDL and VLDL show lower significant ($P<0.05$) differences in MOS group, while HDL shown higher significant different in Bio-Plus 2B (PLUS) group. The MOS and plus groups revealed a significant reduction ($P<0.05$) in blood serum creatinine and urea compared to humic and control groups. Meanwhile, AST and ALT activities significantly ($P<0.05$) increased in control group compared to other groups (Table 3).

Hematological parameters

Supplementation of MOS and Bio-Plus 2B improved ($P<0.05$) blood hemoglobin, PCV, RBCs, MCV and RBCs/ lymphocyte ratio (Table 4). However, the MCH, WBCs, neutrophils and lymphocyte percentages were significantly ($P<0.05$) higher in the control group (Table 4). While, MCHC, monocyte and eosinophil revealed absence of significant effect of all groups.

Lipid peroxidation and antioxidants enzymes activity

Plasma MDA level was significantly higher in rabbits supplied with basal diet (control group) and was lowered following supplementation with MOS, PLUS and humic acid (Table 5). Plasma CAT and SOD activities were increased significantly ($P<0.05$) with MOS and PLUS supplementation compared to the control group.

Histopathological changes

Histopathological findings of the control group showed marked swelling of hepatocytes with granular vacuolated cytoplasm (Fig. 1a), tubular degeneration and necrosis of kidney (Fig. 1b) and intestinal sub-epithelial lymphocytic infiltration (Fig. 1c). While, liver of the humic (Fig. 2a) fed rabbits showed slightly swollen hepatocytes with granular vacuolated cytoplasm, mild to moderate degree of renal tubular epithelium degeneration of kidney (Fig. 2b) and mild degree of intestinal villi atrophy associated with sub-epithelial infiltration of lymphocytes (Fig. 2c). Yet, in group 3 the liver showed normal hepatocytes (Fig. 3a), only mild degree of renal tubular degeneration of the kidney (Fig. 3b) and slight degree of mononuclear inflammatory cell within the lamina propria and hyperplasia of the covering epithelium of intestine (Fig. 3c). Meanwhile, liver (Fig. 4a), kidneys and intestine (Fig. 4b, c), sections of the MOS group 4 showed nearly normal morphological appearances.

Table 1: Composition and chemical analysis of basal diet

Ingredients	%	Chemical analysis (% as DM):	%
Berseem hay	30.05	Dry matter (DM)	85.81
Barley grain	24.60	Crude protein (CP)	17.36
Wheat brain	21.50	Organic matter (OM)	91.42
Soybean meal (44% CP)	17.50	Crude fiber (CF)	12.37
Molasses	3.00	Ether extract (EE)	2.230
Limestone	0.95	Digestable energy(DE, kcal/kg)(2)	2412
Di-calcium phosphate	1.60	Calcium(2)	1.243
Sodium chloride	0.30	Phosphorus(2)	0.808
Mineral-vitamin premix(1)	0.30	Methionine(2)	0.454
DL-Methionine	0.20	Lysine(2)	0.862
Total	100		

(1) One kilogram of mineral–vitamin premix provided: Vitamin A, 150,000 UI; Vitamin E, 100 mg; Vitamin K3, 21mg; Vitamin B1, 10 mg; VitaminB2, 40mg; Vitamin B6, 15mg; Pantothenic acid, 100 mg; Vitamin B12, 0.1mg; Niacin, 200 mg; Folic acid, 10mg; Biotin, 0.5mg; Choline chloride, 5000 mg; Fe, 0.3mg; Mn, 600 mg; Cu, 50 mg; Co, 2 mg; Se, 1mg; and Zn, 450mg

Table 2: Effect of experimental diets on growth performance of growing APRI-line rabbits from 5 to 13 weeks of age

Parameters	Control	Humic acids	PLUS	MOS	SEM	*P*-value
Initial body weight (g)	674.6	671.7	672.9	676.2	11.53	0.9953
Final body weight (g)	2052.5^{d}	2134.0^{c}	2201.0^{b}	2332.0^{a}	20.75	0.0001
Daily weight gain (g)	24.6^{c}	26.1^{b}	27.3^{b}	29.6^{a}	0.421	0.0001
Feed intake (g/d)	83.9^{c}	85.8^{bc}	87.2^{ab}	89.2^{a}	0.824	0.0007
Feed conversion ratio	3.421^{a}	3.295^{ab}	3.199^{b}	3.025^{c}	0.057	0.0005
Relative growth rate	101.1^{c}	104.3^{bc}	106.3^{ab}	110.1^{a}	1.396	0.0018
Performance index (%)	60.3^{c}	65.1^{bc}	69.0^{b}	77.4^{a}	1.703	0.0001
Mortality rate (%)	30^{a}	20^{b}	10^{c}	10^{c}	-	-

SEM = Standard error of means

a, b, c, d, Means in the same row with different superscripts are significantly different (P<0.05)

Table 3: Effect of experimental diets on some blood parameters of APRI-line rabbits

Items	Control	Humic acids	PLUS	MOS	SEM	*P*-value
Total protein (g /dl)	5.19	5.57	5.71	5.88	0.143	0.1921
Albumin (g /dl)	3.50	3.72	3.75	3.85	0.127	0.4375
Globulin (g /dl)	1.69	1.85	1.95	2.03	0.113	0.2178
Glucose (mg/ dl)	89.3^{b}	93.0^{b}	102.3^{ab}	114.7^{a}	4.631	0.0259
Triglycerides (mg/ dl)	97.3^{a}	86.7^{a}	86.0^{a}	70.0^{b}	3.844	0.0048
Cholesterol (mg /dl)	67.0^{a}	53.0^{b}	47.0^{bc}	38.0^{c}	3.055	0.0007
HDL (mg /dl)	13.9^{b}	13.0^{b}	18.0^{a}	14.5^{b}	0.888	0.0089
LDL (mg /dl)	26.3^{a}	18.3^{b}	17.2^{b}	9.47^{c}	2.210	0.0019
VLDL (mg /dl)	27.7^{a}	21.3^{b}	18.7^{bc}	14.0^{c}	2.517	0.0063
Kidney function:						
Creatinine (mg/ dl)	1.37^{a}	1.27^{a}	1.20^{ab}	1.08^{b}	0.061	0.0346
Urea (mg/ dl)	33.7^{a}	31.3^{a}	29.6^{ab}	26.7^{b}	1.167	0.0213
Liver function:						
AST (U/L)	125.3^{a}	101.3^{b}	83.3^{b}	86.3^{b}	6.960	0.0076
ALT (U/L)	71.0^{a}	69.7^{a}	62.3^{ab}	51.3^{b}	3.464	0.0138

SEM = Standard error of means, a, b, e, Means in the same row with different superscripts are significantly different (P<0.05)

Table 4: Effect of experimental diets on blood hematological values of APRI-line rabbits

Items	Control	Humic acids	PLUS	MOS	SEM	*P*-value
Hemoglobin (g/ dl)	10.5^{c}	11.7^{b}	12.1^{ab}	12.4^{a}	0.200	0.0005
PCV $^{(1)}$ (%)	32.7^{c}	35.7^{bc}	37.1^{b}	38.8^{a}	0.623	0.0007
RBCs (x10^{6}/ µl)	5.28^{b}	5.49^{ab}	5.60^{ab}	6.20^{a}	0.240	0.0748
MCV$^{(2)}$ (fl)	60.1^{c}	61.3^{bc}	63.8^{ab}	66.1^{a}	0.800	0.0030
MCH$^{(3)}$ (pg)	20.4^{a}	19.6^{ab}	19.3^{bc}	18.5^{c}	0.300	0.0152
MCHC$^{(4)}$ (g/ l)	32.3	32.0	32.1	31.5	0.384	0.4456
WBCs (x10^{3}/ µl)	9.23^{a}	9.03^{a}	7.67^{b}	6.30^{c}	0.351	0.0011
Neutrophils (%)	52.0^{a}	51.7^{a}	32.0^{b}	35.7^{b}	3.283	0.0016
Lymphocyte (%)	53.7^{a}	44.3^{b}	39.3^{bc}	34.3^{c}	2.333	0.0012
RBCs/ Lymphocyte ratio	0.10^{c}	0.13^{bc}	0.14^{b}	0.18^{a}	0.012	0.0041
Monocyte (%)	9.00	8.67	9.67	8.33	1.155	0.8640
Eosinophil (%)	5.33	5.33	4.67	3.67	0.333	0.1617
Basophil (%)	0	0	0	0	-	-

SEM = Standard error of means, a, b, e, means in the same row with different superscripts are significantly different (P<0.05).[1] Packed cell volume, [2]Mean corpuscular volume, [3]Mean Corpuscular Hemoglobin, [4]Mean Corpuscular Hemoglobin Concentrations.

Table 5: Effect of experimental diets on anti-oxidant parameters of APRI-line rabbits

Items	Control	Humic acids	PLUS	MOS	SEM	*P*-value
MDA (N/ mol)	2.45^{a}	2.05^{ab}	1.90^{ab}	1.65^{b}	0.250	0.0903
CAT (U/ l)	1.75^{b}	1.87^{ab}	2.05^{a}	2.09^{a}	0.087	0.0585
SOD (U/ ml)	17.5^{c}	19.5^{bc}	21.6^{ab}	22.4^{a}	0.722	0.0034

SEM = Standard error of means, a, b,….e, Means in the same row with different superscripts are significantly different (P<0.05)

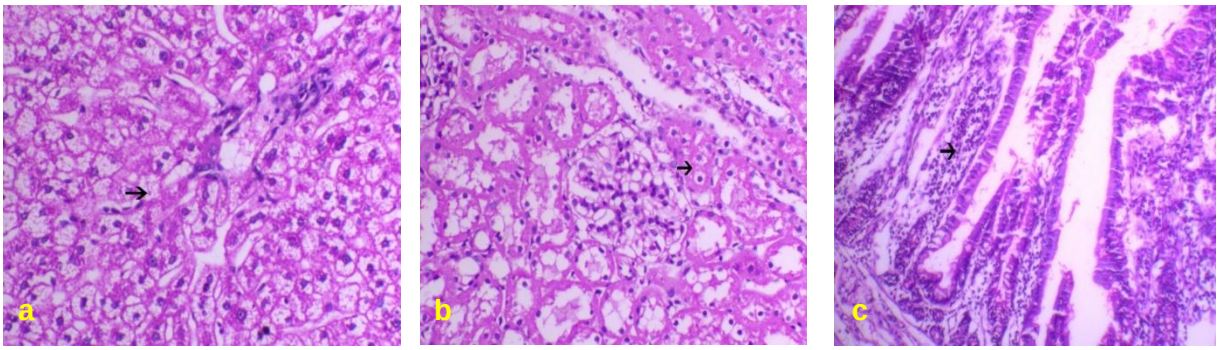

Figure 1: Control group 1 showing (a) liver showing marked swelling of hepatocytes with granular vacuolated cytoplasm (arrow), (b) the kidney showing tubular degeneration and necrosis (arrow), and (c) the Intestine showing marked sub-epithelial lymphocytic infiltration. (H&E, X 200)

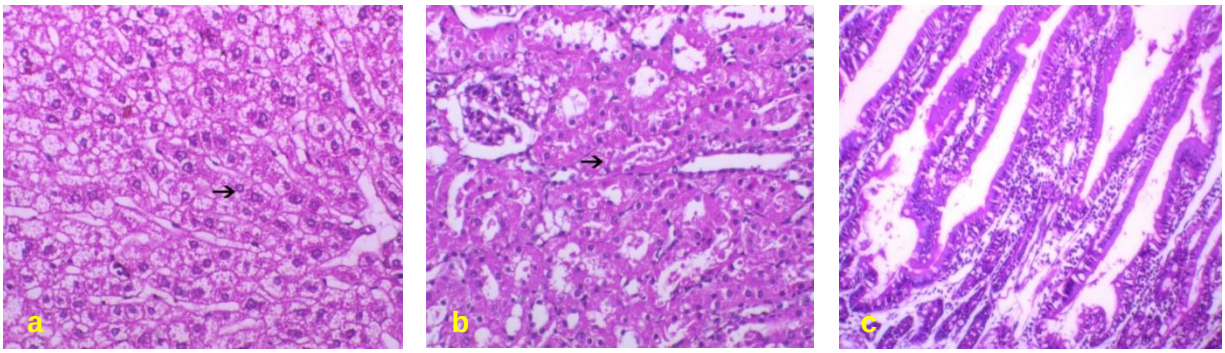

Figure 2: Humic group 2 showing (a) liver showing swelling of hepatocytes and with granular vacuolated cytoplasm (arrow), (b) the kidney showing mild to moderate degree of renal tubular epithelium degeneration (arrow), and (c) the Intestine showing mild degree of intestinal villi atrophy associated with sub-epithelial infiltration of lymphocytes. (H&E, X 200)

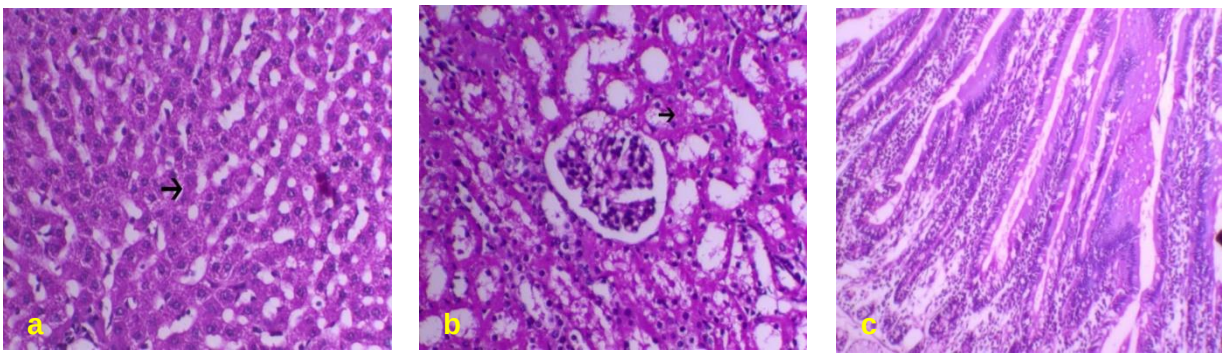

Figure 3: Plus, group 3 showing (a) liver showing normal hepatocytes (arrow), (b) the kidney showing mild degree of renal tubular degeneration (arrow), and (c) the Intestine showing slight degree of mononuclear inflammatory cell within the lamina propria and hyperplasia of the covering epithelium. (H&E, X 200)

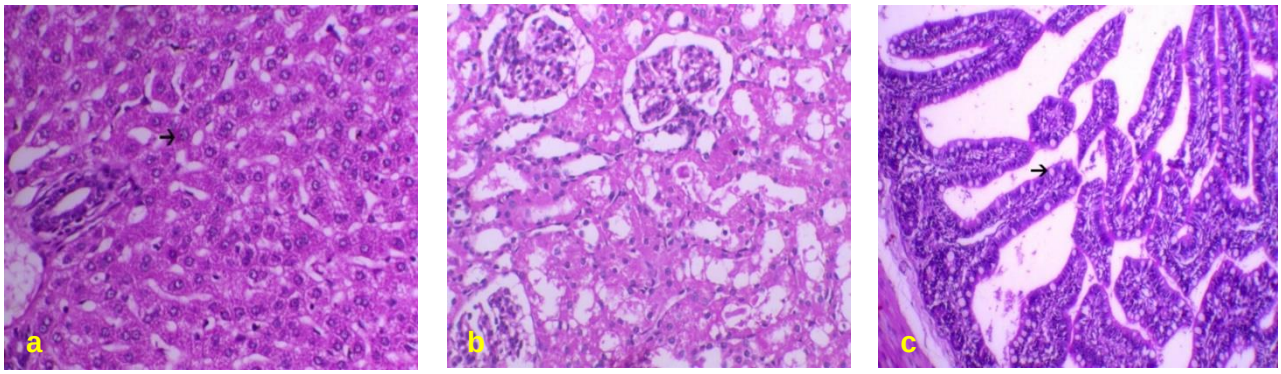

Figure 4: MOS group 4 (a) normal hepatocytes arranged in cords around the portal area (b) the kidney showing normal renal glomeruli and tubules, and (c) the Intestine showing normal branched intestinal villi with normal pseudo stratified epithelium with goblet cells. (H&E, X 200)

Discussion

To Feeding rabbits on feed contain OTA reduced final BW, WG and daily feed intake and performance index observed in the control group. These findings are in agreement with earlier studies recording the adverse effects of OTA on feed utilization and performance (3). These adverse effects might be attributable to phenylalanine moiety that present in OTA, which inhibit synthesis of phenylalanine-t-RNA and consequently reduce protein synthesis (24). Furthermore, OTA reduce carbohydrate metabolism, mainly glucogenolysis through inhibition of DNA, RNA and protein synthesis. While, feed additives especially MOS improve feed consumption, WG, final BW and decrease FCR. These results agreed with (25) who observed that using of MOS increase the height of intestinal villi. Accordingly, the surface area of intestinal villi increased and the absorption capacity improved that may improve feed consumption and performance.

Rabbits in control group showed higher levels ALT and AST. These findings are in concordance with the previous studies conducted by (26, 27). Higher level of ALT indicates changes in the hepatocyte membrane permeability due to the vascular congestion induced by OTA toxicity and finally hepatic destruction (28). While, higher level of AST secretion implies skeletal muscle destruction (29).

In present study, the control group showed an increase in the concentration of creatinine and urea that may give an indication about kidney damage due to OTA toxicity. These findings are similar to that stated in rabbits and some domestic animals (26, 28). (30) reported that toxicity by OTA is a direct cause of nephrotoxicity. Furthermore, due to nephrotoxicity urea and creatinine are accumulated in destructed nephrons resulting in further damage and kidney failure.

Supplementation of feed additives especially MOS and PLUS improved the reduced blood hemoglobin, PCV, RBCs, MCV and RBCs/ lymphocyte ratio those were elevated in the control group. These results are in consistence with (31) who reported internal bleeding, hemorrhages or bruising, stomach ulcers in ochratoxicosis rabbits that resulted in lower hemoglobin and RBCs. Control group appeared in exhausted condition due to the signs of anemia that may be due to increased anaerobic metabolism and reduced aerobic one. (32) reported that during OTA toxicity, rabbits showed lower RBCs count, haemoglobin content and PCV which might lead to an increase in anaerobic metabolism and decrease in oxygen supply to tissue and cells. Furthermore, RBCs cytotoxicity that may resulted from destruction of RBCs and may be occur in case of OTA toxicity (33).

Glucose concentration reached the lowest level in the control group. This result agreed with (34) who reported ochratoxicosis induced hypoglycemia; this finding may be due to intestinal villi destruction and subsequently poor absorption. In addition, it may be due to destruction in the membrane of hepatocytes. On the other hand, kidney failure and nephrotoxicity may lead to sever loss of glucose while using

of feed additives protects the intestinal villi, hepatocytes membrane and kidney nephrons from destruction (35, 26). In some cases of OTA toxicity, low glucose levels may be the cause of nervous manifestations observed in rabbits before death (36).

Triglycerides and cholesterol are significantly increased in control group compared to other groups.it may be due to destruction of hepatocytes and changes in metabolism of lipid which was prevented by using feed additives. (37) observed similar results in broiler subjected to ochratoxicosis.

Rabbits in the control group showed high oxidative destruction and changes in the plasma biochemical levels. While, rabbits supplied with feed additives showed high resistance to the OTA induced oxidative damage. Toxicity with OTA causes destruction in liver, kidney, intestine and muscle cell membranes that may be due to the increased lipid peroxidation (38). Therefore, activities of antioxidant were investigated due to its protective action in form of superoxide anion production, which protect cell membranes from OTA induced destruction that may be due to the effect of feed additives (38).

Kidney is the primary target organ for ochratoxin-A (39). The control group showed kidney tubular degeneration and necrosis while, Kidney histopathological lesions included mild to moderate degree of renal tubular epithelium degeneration of plus and humic groups. Liver histopathological lesions showed marked swelling of hepatocytes with granular vacuolated cytoplasm of control group. Meanwhile, intestine showing slight degree of mononuclear inflammatory cell within the lamina propria and hyperplasia of the covering epithelium, and mild degree of intestinal villi atrophy associated with sub-epithelial infiltration of lymphocytes while control group marked sub-epithelial lymphocytic infiltration. These indicate the feed additives improve liver, kidney and intestinal histology and function. The results of histopathological examination in kidney, intestine and liver confirmed by biochemical profile of examined rabbits were consistent with those reported by (40).

Toxicity with OTA resulted in impaired feed utilization and performance of rabbits. In addition, it caused high mortality rate. Consequently, the outcomes of this study represented by higher relative growth rate and performance index in grouped received feed additives may be reflected on the rabbit's economic production. Therefore, using feed additives especially MOS might improve feed utilization, WG, FCR and performance resulting in high economic revenue.

Conclusions

Supplementation of contaminated feed with ochratoxin-A by feed-additives especially MOS improves feed intake and final body weight and reduces mortality rate of rabbits. In addition, it improves liver and kidney functions and reduces its pathological changes, and the antioxidant biomarkers.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Original Research Article

EFFECT OF USING CHICORY ROOTS POWDER AS A FAT REPLACER ON BEEF BURGER QUALITY

Tamer El Zeny, Rowida Y. Essa*****, Badia A. Bisar, S. M. Metwalli

Food Technology Department, Faculty of Agriculture, Kafrelsheikh University, Egypt

*Corresponding author, E-mail: rowida.eisa@agr.kfs.edu.eg

Abstract: Chicory roots powder is considered as a rich source of fiber and polyphones. It can be interfered in the formation of many functional foods that play an important role in maintaining human health from many diseases such as obesity and diabetes. So, this research was carried out to determine the chemical composition of the chicory roots and to evaluate assess the quality properties of reduced- fat burger as influenced with chicory roots powder. The prepared burger samples contained chicory roots powder as fat replacer with substitution ratio of 25, 50 and 75% of animal fat. Cooking quality and sensory evaluation were measured in burger samples. The results revealed that adding Chicory roots powder lead to an improvement in burger nutritional value and cooking properties. In burger contained chicory roots powder, there was an increment in cooking yield meanwhile, shrinkage and feeder number were decreased. Adding chicory roots powder to burger as a fat replacer does not cause any negative effects on its sensory properties.

Key words: fat replacer; burger; dietary fiber

Introduction

Consumers now have good knowledge about their health and food. Therefore, healthy processed meat product must have low fat, cholesterol, and calories (1). Burger is considered as one of the highest popularity food in Egypt and all over the world (2). It has a high acceptability and consuming rates because it is a cheap quick meal (3). On the other hand, it has some harmful effect due to high content of saturated acids (20-30%) (4). Fat has an important role in burger increasing emulsion stability of meats as well as water holding capacity; decreasing loss during cooking process and improving organoleptic characteristics (5). However, the presence of fats in meat products leads to a high content of cholesterol and saturated fatty acids (6). The increment of saturated fats intake levels causes harmful diseases such as, cardiovascular disease, stroke, obesity and cancer (7). Obesity is one of the most serious diseases in the world, especially as it is linked to other diseases such as heart disease and diabetes (8).

According to the American Heart Association (2002), fat should be shared with about 15% to 30% of the total calories taken daily. WHO also recommended that saturated fat should not exceed 10% of daily supplemented calories (9, 11).

Therefore, many studies have been conducted to produce healthy meat products and reducing the proportion of fat. On the other hand, it must be borne in mind that this may lead to some problems with the acceptance of the prod-

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uct, because fat is a main component that effects on meat product properties such as sensory attributes, texture and flavor (12, 13). Reducing the proportion of fat by adding substances that are based on non-meat proteins, carbohydrate and dietary fiber is a scientific way to solve this problem (14).

Dietary fiber is a part of plant food that is not fully digested by digestive enzymes, and it is very important for human health. On the other hand, human consume dietary fiber less than recommended by the WHO (23–38 g/day) (15, 16). In processed foods, part of the fat is replaced by dietary fiber (17). In meat processing, crude fiber has good applications in minimizing formula cost cooking yield enhancement, and texture improvement (18). Several studies have shown the importance of fiber to human health, it helps to reduce cholesterol, also reduce high blood pressure and reduce the chances of colon cancer as well as obesity (19, 20).

Cichorium intybus L. (chicory) is a Mediterranean plant species belonging to the Asteraceae family. Cichorieae tribe includes approximately hundred genera and many hundreds species of which some genera are used as salad vegetables (21). On the other hand, the word 'Chicory'is likely to be derived from the Egyptian word 'Ctchorium'. All parts of this plant are pharmacologically useful due to the presence of a number of medicinally and nutritionally important compounds such as inulin, flavonoids, caffeic acid derivatives, terpenoids, sesquiterpene, vitamins, steroids oils, lactones, volatile compounds, and coumarins, It possesses antibacterial, antioxidant and anti-inflammatory (22).

There is not enough information about using chicory as a fat replacer in meat products; therefore, the main aim of this study was to evaluate addition of chicory powder at different levels as fat replacer on burger quality attributes.

Materials and methods

Chicory (*Cichorium intybus*) was obtained in January, 2017 from the local field of Kafr El-shiekh governorate, Egypt. The chicory roots were free of physical damage and injury of insects and fungi infection. Beef meat and other components used for burger preparation were obtained from local market at Kafr El-shiekh city, Egypt.

Chemicals

All chemicals and reagents used in this study were obtained from Sigma Aldrich Chemical Co. (St. Louis, M.O, USA). All other chemicals and solvents were of analytical grade.

Preparation of Chicory roots powder

The plant of chicory were transferred directly to the laboratory of food technology department, Faculty of Agriculture, Kafrelshiekh University then it was washed with tap water to removeremaining soil and other impurities. The chicory roots were cut into small pieces and dried at 40ºC for 2 days in an air oven. The dried roots were crushed using a laboratory mill, then, sieved (100 meshes) and finally, the powder was stored in sealed bags at 4± 2ºC (23).

Burger preparation

The beef burger was formulated to contain the following ingredients 80% red beef meat, 20% kidney fat, 18% (w/w) water (ice), 1.5% (w/w) salt, ground black pepper (0.3%), red pepper (0.2%) and cumin (0.2%) according to Aleson-Carbonell, Fernández-López (24). Aforementioned ingredients were used to prepare the control sample while 25, 50 and 75% of control fat content were replaced by chicory roots powder to prepare burger supplemented with chicory roots powder as a fat replacer.

Meat burgers weighed approximately 30 g each. The beef burgers were stewed using an electrical grill (Genwex GW-066) at 220°C (the space between heat source and the samples was 4 cm) for 8 min (4 min for each side of beef burgers).

Chemical analyses

Gross chemical composition of chicory roots and burger was analyzed according to A.O.A.C. (25).

$$\text{Cooking yield (\%)} = \frac{\text{Cooking weight}}{\text{Raw weight}} \times 100$$

$$\text{Shrinkage (\%)} = \frac{\text{Raw diameter - Cooking diameter}}{\text{Raw diameter}} \times 100$$

Cooking loss (%) = ((Raw weight - Cooking weight)/ Raw weight)*100

$$\text{Feeder number} = \frac{\text{Moisture content \%}}{\text{organic nonfat content \%}}$$

Where, % organic non fat = 100 – (fat % + ash content + moisture %)

Sensory evaluation

Sensory evaluation of twenty panelist have been assessment burger samples for their sensory properties (taste, color, odour, texture, tenderness and overall acceptability) using a hedonic scale of 1-10 according to the method of Badr and El-Waseif (28)

Statistical analysis

General linear model of SPSS (Ver. 16.0, 2007) was used to conduct ANOVA for determination of differences between means. The probability levels of $P \leq 0.01$and $P \leq 0.05$ were considered to be significant for statistical procedures. All measurements and trials were done in triplicate.

Results and discussion

Chemical composition of chicory roots powder

Moisture and protein content of chicory roots powder were 6.84±0.89 and 9.01±0.93 (Table1) .On the other hand, data in Table (1) showed that, chicory roots had a high ash content (5.60%) and ether extract (1.60%), crude fiber (5.92%) and antioxidants (78.02). These results in the same trend as the results obtained previously by (29). From the previous by results obtained, one can record that chicory roots could be considered as a good source of fiber, so it can be used as an alternative ingredient to fats in many processed foods.

Chemical composition of prepared beef burger

Table (2) showed that chemical composition of the cooked burger formulated with chicory roots powder, the chemical analysis of cooked burger showed that the percentage of moisture, protein, ash, crude fiber and available carbohydrates content were increased by increasing the amounts of chicory roots powder replacement in the burger. The increment of moisture content may be due to the capability of chicory roots powder rich with fiber to hold more water via preparation and cooking process. Meanwhile, the increment in other constituents may be a reflection of the quantity of these constituents in chicory roots.

On the other hand, the data in the same table revealed that ether extract content values in cooked burger were decreased significantly with chicory roots powder addition ($p \leq 0.05$) in comparison with the control sample. Maximum ether extract content percentage was noticed in control sample while, treatment contained (CRP) with 75% of animal fat showed the minimum percent. These obtained results were in harmony with those reported by Gök, Akkaya (7), Kılınççeker and Kurt (26) and Yousefi, Zeynali (27) who stated that beef burger integrated with different types of fat replacers were highly in some constituents such as moisture, ash, protein, fiber and available carbohydrates contents and lower in fat than in the control group.

Burger cooking properties

Moreover, data in Table (3) revealed that burger samples which replaced by chicory roots have cooking loss percentages lower than control. This decrement is due to the ability of chicory roots fiber to hold a large amount of water. There was a significant decrement in the loss via cooking process as a function of the increment in fat substitution level with chicory roots. Also, the results declared that adding chicory roots showed a positive influence on burger cooking yield. These results are in agreement with Kassem and Emara (30) and Namir, Siliha (31) who stated that there was a decrement in the cooking loss values of low fat burger when the levels of high fiber substances was increased.

Preventing shrinkage considered as one of the most important factors to maintain the quality levels of burgers because some consumers related to shrinkage and adding a high amount of water. Control beef burger sample had a high percentage of shrinkage after cooking process in a comparison with burger integrated with

chicory roots powder. These results are in conformity with the finding stated by Namir, Siliha (31).

Feeder number is applied to assess the meat products physical characteristics. Feeder number was 0.70 for control samples, which decreased gradually with the increment of chicory roots powder level. It was for all laboratory samples was lower than 4.0. as stated by Pearson (32) who recorded that feeder number in good products should be lower than 4.0.

Sensory evaluation

In the present study, the sensory evaluation of cooked burgers containing Chicory roots powder with 25%, 50% and 75% of control sample fat were recorded in Table (4).

Concerning the data in Table (4), one can be noticed that there were a slightly difference between control sample and that contained chicory roots powder with percentages of 25%, 50% and 75% of animal fat for all sensory characteristics. While the sensory scores of burger contained date chicory roots powder with ratio of 75% of fat were low compared with control sample, however they were in the acceptable limits (more than 6).

Conclusion

Chicory roots powder is considered as a rich source of fiber so that it can be used as fat replace in beef burger. Also, this study revealed that substituting 75% of animal fat in burger with chicory roots powder showed manufacture no negative effects on physical and cooking quality of processed burger.

Table 1: Gross chemical composition of chicory roots powder (% on dry weight basis)

Component	Chicory roots powder
Moisture (%)	6.84±0.89
Protein (%)	9.01±0.93
Ash (%)	5.60
Ether extract (%)	1.60
Crude fiber (%)	5.92

Table 2: Proximate chemical composition of burger with different concentrations of chicory roots powder as a fat replacer (on dry weight basis)

Treatments / Component%	Cooked burger			
	Control	CRP 25% of fat	CRP 50% of fat	CRP 75% of fat
Moisture	32.09^{b}	30.15^{d}	31.33^{c}	35.12^{a}
Crude protein	26.17^{d}	28.00^{c}	28.27^{b}	28.52^{a}
Ether extract	20.01^{a}	16.54^{b}	11.97^{c}	7.63^{d}
Ash	1.97^{c}	1.99^{c}	2.10^{b}	2.19^{a}
Crude fiber	1.23^{d}	2.98^{c}	3.78^{b}	4.21^{a}
available carbohydrates	50.62^{c}	50.49^{cd}	53.88^{b}	57.45^{a}

CRP means chicory roots powder, Values followed by the same letter in the same column are not significantly different at $P \leq 0.05$.

Table 3: Cooking properties of burger with different concentrations of chicory roots powder as a fat replacer

Treatments Properties	Control	CRP 25% of fat	CRP 50% of fat	CRP 75% of fat
Cooking yield (%)	47.14[d]	53.08[c]	57.82[b]	61.90[a]
Cooking loss (%)	52.46[a]	46.92[b]	42.18[c]	37.70[d]
Shrinkage (%)	25.15[a]	21.86[b]	18.89[c]	15.89[d]
Feder number	0.70[a]	0.59[b]	0.57[b]	0.64[b]

CRP means chicory roots powder

Values followed by the same letter in the same row are not significantly different at $P \leq 0.05$.

Table 4: Effect of Chicory roots powder percentage as a fat replacer on the sensory properties of burger*

Treatments Sensory	Control	CRP 25% of fat	CRP 50% of fat	CRP 75% of fat
Taste	8.0[a]	7.23[b]	7.05[b]	6.99[bc]
Colour	8.04[a]	7.20[b]	7.11[c]	6.95[c]
Odour	8.13[a]	7.14[b]	7.09[c]	6.96[cd]
Texture	8.12[a]	7.10[b]	7.07[c]	6.88[d]
Tenderness	8.21[a]	7.05[b]	7.01[b]	6.70[c]
Total acceptability	8.1[a]	7.14[b]	7.06[c]	6.89[d]

*All data are the mean±SD of twenty replicates. Mean followed by different letters in the same row differs significantly ($P \leq 0.05$)

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Original Research Article

ASSESSTMENT OF MOULD CONTAMINATION OF TILAPIA NILOTICA AND *Mugil cephalus* FISH AND TRIALS TO REDUCE USING NATAMYCIN

Nader Y. Mostafa[1], Ghada A. K., Kirrella[1], Hoda A. M. Aideia[2], Jeena M. M. Abo Shaisha[3]*

[1]Food Control Department, Faculty of Veterinary Medicine, Kafrelsheikh University, [2]Microbial Toxin Unit, Animal Health Research Institute, Dokki, Giza, [3]Food Control Department, Animal Health Research Institute, Kafrelsheikh

*Corresponding author, E-mail: jeena.aboshaisha@yahoo.com

Abstract: Contamination of fish by fungi is considered one of the most serious causes of losses in aquaculture. Therefore, our study was firstly aimed to screen the fungal status of two commonly consumed fish species in Egypt, Tilapia nilotica and Mugil cephalus. Secondly, an experimental trial to investigate the antifungal effect of natamycin on Tilapia nilotica. A total of 60 fish samples including Tilapia nilotica and Mugil cephalus (30 of each) were randomly collected from various retail markets and shops at dissimilar sanitation levels at Kafrelsheikh Governorate, Egypt during the winter season, 2018. All samples were examined for fungal contamination. Tilapia nilotica and Mugil cephalus samples showed average mould counts of 3.63×10^2 and 1.65×10^2 CFU/g, respectively. Nine fungal species were isolated from two fish species. Seven and five species were isolated from Tilapia nilotica Mugil cephalus, respectively. The highest prevalent fungal species isolated from the two fish was *Aspergillus flavus*. Natamycin showed significant antifungal properties in a concentration- dependent manner. Thus, efficient hygienic handling, rapid cooling of fish can reduce the fungal contamination of fish. In addition, we highly recommend soaking or spraying fish with natamycin solution as an efficient strategy in reducing the fungal load of raw fish.

Key words: *Tilapia nilotica*; *Mugil cephalus*; fungi, natamycin

Introduction

Fish is a significant source of animal protein, vitamins, minerals and omega-3-fatty acids, however, on the same time, fish is a rapidly decomposed food if not properly handled and stored. With the growing demand for fish as human food, fish farming is rapidly extending all over the world to recover the need for animal protein. Tilapia nilotica and Mugil cephalus are considered as two of the most popular fishes in Egypt.

Fish diseases and hypoxic stress play an important role in lowering fish production in aquacultures (1). Source of fungal infection in aquaculture may be contributed to the utilization of contaminated feed as revealed by positive correlation between decomposition of such feed and fungal infections (2). Fungal diseases consider a serious cause of lowering

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fish production. Most fungi affecting fish considered opportunistic which infect fish when they are under stress or reduced immunity due to unfavorable ecological conditions. Fungal infections occur secondarily to viral or bacterial infections, or at mucosal loss resulting from trauma or severe handling (3). Intensive aquaculture conditions may cause transmission of fish diseases, especially fungal diseases, resulting in drastic economic losses.

Assessment of fungal contamination of fish by using mould count could evaluates the quality and shelf life of fish (4). Contamination of fish by different fungal species as *Aspergillus glaucus, A. sulpheus, A. ochraceus, A. candidus, A. versicolor, A. wentii, A. oryzae, A. melleus, Mucor racemosus, Torula fulignea* and *Cladosporium herbarum* was reported (5). Additionally Aspergilli observed to be the most widely recognized soil growth found in high numbers in water connected with biofilms and residue (6).

Thus, there is a great need for proper handling, storage and preservation of this precious food source. Using food preservatives to prolong the shelf life of fish and to control fungal contamination is another alternative way for fish preservation. Natamycin is a natural polyene macrolide antifungal compound generated from fermentation of bacteria such as *Streptomyces natalensis*. It had an antimicrobial effect, considerably fungicidal and active against all food spoilage moulds and yeasts with most strains. Despite its long-term use, resistance does not naturally occur unlike the chemical organic propionate and acid sorbate as preservative for which a number of resistant moulds and yeasts have been detected (7). Furthermore, natamycin is applied, in over 150 countries, in the food industry as a preservative (8). It can be added in a different ways: as an aqueous suspension (such as mixed into brine) sprayed on the product or into which the product is dipped, or in powdered form (along with an anticaking agent such as cellulose) sprinkled on or mixed into the product (9).

Therefore, this study was conducted to check the mycological contamination of fresh Tilapia nilotica and Mugil cephalus and to find out the most suitable methods for decreasing of the fungal contamination of Tilapia nilotica using natamycin with different concentrations.

Materials and methods

Samples Collection

Sixty random fish samples of Tilapia nilotica and Mugil cephalus (30 each) were collected from different retail markets and shops at various sanitation levels at Kafrelsheikh Governorate, Egypt. The collected samples were packed in a sterile polyethylene bags, closed and cooled in an insulated box contained crushed ice, then immediately transported to the laboratory for further examination. The fish samples were subjected to mycological examinations.

Mycological examinations

Preparation of samples

All fish samples were prepared under complete aseptic conditions, 25 g of each sample cut-off, weighed and homogenized with 225 ml of 0.1% of sterile buffered peptone water (LAB104 , LAB M , UK) for 1-2 minutes at 2000 rpm using sterile homogenizer (type M-p3- 302, mechanic, precyzina, Poland), such homogenate represents the dilution of 10^{-1}. The homogenate was kept for 5 minutes at room temperature. One ml from the original dilution (10^{-1}) was aseptically transferred to another sterile tube containing 9 ml of sterile buffered peptone water 0.1% and further tenfold decimal serial dilutions were prepared (10).

Determination of the total mould counts

The total mould counts were determined by culturing duplicate plates of Sabouraud's dextrose agar media (Oxoid, Basingstoke, UK) supplemented with chloramphenicol 100 mg/L followed by dark incubation at 25˚C for 5-7 days. During incubation time, the plates were examined daily for the fungal growth. Estimation of total mould was obtained by direct counting of the cultured agar plates (10).

Identification of some isolated moulds

Colonies of different fungal species inspected under microscope. Macroscopic and microscopic characteristics of the developing cultures were utilized (11, 12, 13 and 14).

Experimental work

In a trial for reduction of mould contamination of Tilapia nilotica fish using diluted solutions of natamycin (food grade- China), different concentrations were used under 0.5, 1and 2.5g/l. A total number of 40 Tilapia nilotica fish (weigh about 200 g/each) were divided to 4 groups (n=10 fish). The first group was soaked in sterile distilled water for 30 min and kept as a control (non- treated) group. The second, third and fourth groups were soaked with repeated shaking for 30 min at room temperature in 0.5g/l ,1g/l and 2.5 g/l natamycin solutions, respectively. Total mould count was conducted as mentioned before.

Statistical analysis

All results were expressed as (means ± SE). The values were analyzed statistically by Graph Pad Prism5 software.

Results and discussion

Evaluation of fungal status of Tilapia nilotica and Mugil cephalus

Contamination of fish by fungi may be due to presence of microorganisms on their skin surfaces, intestine and in the gills. In many parts of Egypt and other developing countries, fish is sold in the fish markets in open air, kept in wooden boxes and covered with ice. Poor handling of the fish might lead to rupture of the intestinal tract and spoilage of the fish body with the fish intestinal contents. Thus, in the first part of this study, the fungal status of the examined fish species was investigated via estimation of total mould counts. During catching, handling, transportation and processing, contamination may occur which leads to introduction of pathogens into the fish meat. The sources of these pathogens may be from the surrounding environment (15). Many factors affect the microbiological characters of different sea food types like species differences, environment, methods of catching, on board, handling, fishing vessels, sanitation, processing, preservatives and packaging (16). Tilapia nilotica is commonly contaminated with mould and yeast than other types of fish which may fed on feed contaminated with moulds and yeasts (17). Also, the presence of high number of fish in limited areas encourages the growth, multiplication of mould and enhances spread of food borne outbreak infections.

In the present study, Tilapia nilotica had higher total mould count than Mugil cephalus. The mean values of the total mould counts were $3.63\times10^2 \pm 8.75\times10$ and $1.65\times10^2 \pm 4.78\times10$ CFU/g in the examined Tilapia nilotica and Mugil cephalus, respectively (Table 1 and Fig. 1). This may be attributed to the higher moisture content of Tilapia nilotica than Mugil cephalus which may cause a higher contamination with mould (18). These results were in agreement with (19) who recorded higher mould counts in Tilapia nilotica sold in Ed Dueim, Sudan. Furthermore, some studies (20, 21) revealed higher mould counts in Tilapia nilotica and Mugil cephalus collected from different fish farms and natural water channels in Kafrelsheikh and Dakahlia Governorate, Egypt. This fungal contamination may be indicate inadequate sanitary measures performed starting from the point of catching. The conditions of the environment in the refrigerators, cooling boxes, anglers' hands and clothes are very suitable for the development of mould spores (22 and 23). Fungal contamination of fish may lead to their spoilage and production of mycotoxins with potential health hazards to human due to their carcinogenic effects, liver diseases and organ damage (24).

Fungal species isolation from fish

Moreover, to verify the most common fungi in both *Tilapia nilotica* and *Mugil cephalus* phenotypic identification of fungal isolates was carried out. Results in table (2), showed 9 fungal species were isolated from two fish species. Seven species was isolated from *Tilapia nilotica* while five species was isolated from *Mugil cephalus*.

Figure (2) showed that Tilapia nilotica had the highest value of isolation percentage of *Aspergillus flavus* (86.95%) followed by *A. niger* (73.91%), however, the lowest values were obtained for *A. ochraceus, A. parasiticus* and *Alternaria* species as (8.69%). Mugil cephalus had *A. flavus* as the highest value of isolation percentage (78.94%) followed by *Cladosporium* species (47.36%), while the lowest values were obtained for *A. niger* (10.52%) (Fig. 3). These findings were in concurrence with detailed information by (2). It was found that aspergillomycosis in African Tilapia (*Oreochromis species*) is caused by *A. flavus, A. japonicas and A. terreus* (25). Also, we found *Cladosporium* species at a lower rate as described beforehand (26). The incidence of contamination of fish feed by Aspergillus species, especially *A. flavus* at high moisture levels lead to increase fungal growth during the storage period of feed at increased dampness levels (27-29). The polluted water supply, worker's hands and feeds, represent very important role on the health status of fish (30, 31).

In agreement with our results, (32) isolated *A. niger, A .flavus, A. versicolor, A. parasiticus, Rhizopus spp., Mucor spp., Phoma herbarum* and *Trichoderma hamatum* from Tilapha nilotica. Moreover, (9) isolated 14 and 10 fungal species from Tilapia and Mullet, respectively. The highest was *A. niger* (100%) in Tilapia, while *A. flavus* was the highest (83.3%) in mullets. (33) also isolated 7 genera of mould from different types of fish. Variety in the results are most added to contrasts in land territory, temperature, dampness, and hygienic conditions (34). Therefore, the fungal contamination of fish could be due to incorrect sanitation during transportation, handling, catching, storage, marketing and manufacturing of fish (35 and36).

Reduction of mould contamination in Tilapia nilotica fish using natamycin

Fungal contamination of fish had several implications starting from organoleptic changes may lead to fish spoilage, which is an unacceptable metabolic process which causes foods to be rejectable and undesirable for human consumption due to changes in sensory characteristics. Fish is also very liable to spoilage due to enzymatic and chemical activities, the breakdown of protein, carbohydrates and fat of fish result in the progress of off-odor, off flavor and formation of slime which make the fish rejectable for human consumption. Thus, one major task of the food hygienists and microbiologists is to find ways to prolong the shelf life of fish and to decontaminate or decrease fungal contamination levels of fish. Thus, in the second part of this study, trials to control the outgrowth of moulds contamination of Tilapia nilotica fish using natamycin was evaluated.

Natamycin could significantly reduce fungal contamination of Tilapia nilotica fish and extend the shelf life of fish in a concentration-dependent manner (Table 3 and Fig. 4). For instances, natamycin 2.5g/l significantly reduced total mould count (77.09%). Similarly, (37) also declared the anti-fungal activity of natamycin in Saloio cheese. Fungal infections in fish have expanded probably due to an absence of a strong anti-fungal therapy, and for the development of fungicidal resistant strains (38, 39) and the poisoning of the most relevant anti-fungal components (40). So, there is a mandatory need to look for new strategies against fungal contamination in fish (40 and 41). As natamycin is approved for surface treatment of cheese, sausages, yoghurt and many foods all over the world as a food additive and its use is considered to be safe, it could be used to control mould growth on fish.

Table 1: Total mould count (cfu/g) of the examined fish samples (n=60)

Fish type	No.of positive samples No.	%	Min.	Max.	Mean±SE.M
Tilapia nilotica	23	76.66	3.4×10	9.8×10^2	$3.63\times10^2\pm8.75\times10$
Mugil cephalus	19	63.34	1.8×10	2.6×10^2	$1.65\times10^2\pm4.78\times10$

Table 2: Incidence of fungal species in examined fish samples

Identified mould spp	***Tilapia nilotica***		***Mugil cephalus***	
	No. of positive samples(23)	**%**	**No. of positive samples(19)**	**%**
A. flavus	20	86.95	15	78.94
A. niger	17	73.91	2	10.52
A.ochraceus	2	8.69	5	25.31
A.parasiticus	2	8.69	0	0
A.versicolor	0	0	6	31.57
Alternaria spp.	2	8.69	0	0
Cladosporium spp.	0	0	9	47.36
Rhizopus spp.	8	34.78	0	0
Mucor spp.	4	17.39	0	0

The table expressed as the percentage was calculated in relation to the number of positive examined fish samples

Table 3: Effect of natamycin with different concentrations in *Tilapia nilotica* fish

Concentration	Total mould count	Reduction%
Control(nontreated)	$3.63\times10^2\pm8.75\times10$	0
Natamycin 0.5g/l	$2.73\times10^2\pm3.7\times10$	24.83
Natamycin 1g/l	$1.76\times10^2\pm1.45\times10$	51.4
Natamycin 2.5g/l	$0.83\times10^2\pm3.71\times10$	77.09

Reduction % = Control-After treatment/ Control×100

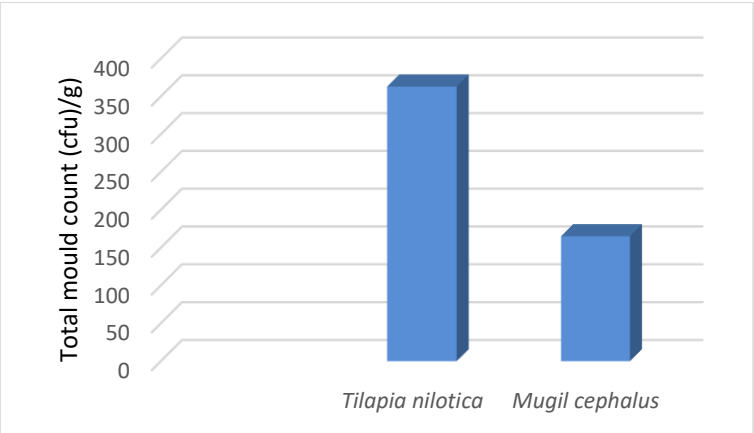

Figure 1: Total mould count (cfu/g) of the examined samples

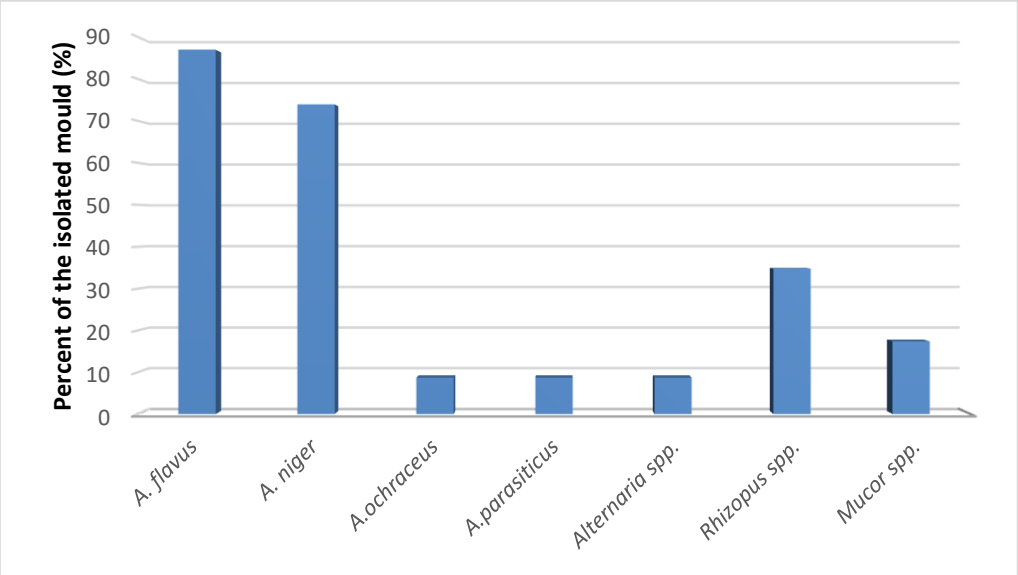

Figure 2: Percent of the isolated mould from *Tilapia nilotica* fish

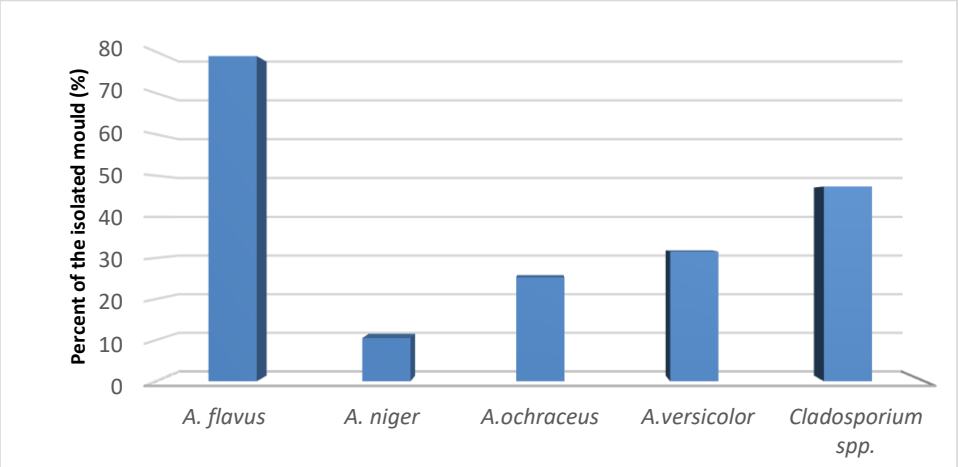

Figure 3: Percent of the isolated mould from Mugil cephalus fish

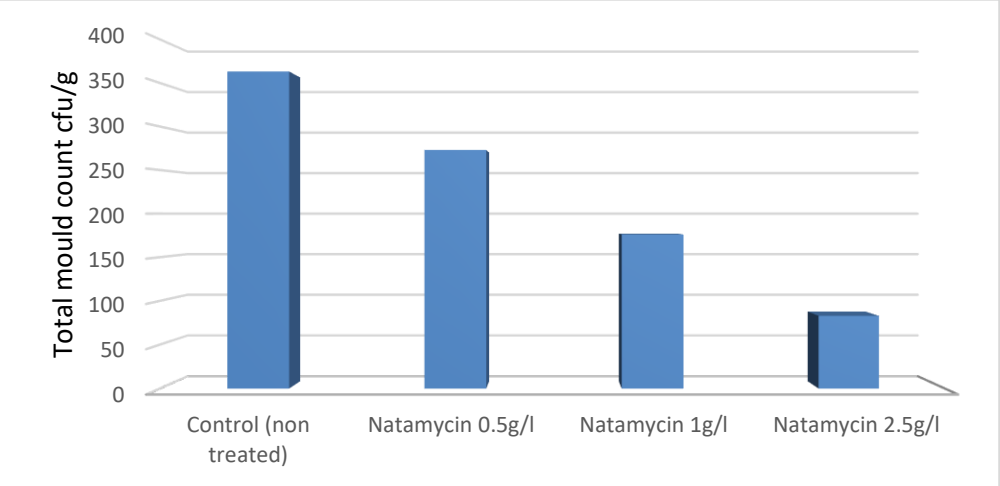

Figure 4: Effect of natamycin with different concentrations in *Tilapia nilotica* fish

Conclusion

Efficient hygienic handling, rapid cooling of fish, using of clean water during fish wash could reduce the initial fungal load of fish. It is critical that the proprietors of the fish's ranches and administrative specialists ought to adjust a superior technique to counteract fungal contamination of fishes amid developing periods and amid taking care of, preparing, transportation and processing. In addition, we highly recommend soaking or spraying of fish with natamycin solution as an efficient strategy for reduction of the fungal load of raw fish.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

EFFECT OF SUPPLEMENTATION OF AROMATIC PLANTS OILS ON IMMUNITY, UDDER HEALTH AND MILK PRODUCTION OF FRIESIAN COWS

Atef Y. Salem[1]*, Hassan G. El–Awady[1], Mohamed A. Tag EL-Dein[2], Dina A. Eisa

[1]Department of Animal Production, Faculty of Agriculture, Kafrelsheikh University 33516, Egypt,
[2]Animal Production Research Institute, Egypt

*Corresponding author, E-Mail: dinaeisa2030@gmail.com

Abstract: Eight weeks prior to expected calving date, 32 Friesian cows were assigned according to parity to four homogenous groups (8 animals each). Cows were fed a basal ration alone as a control group 1 (CON G1), or basal ration supplemented with 25 mg black seed oil per kg body weight, (BSO G2), 11 mg chamomile flower oil (CFO G3), or 25 mg oregano leaves oil (OLO G4). Biochemical and hematological parameters were measured and there were within the normal ranges of cattle. Relative to the control group, aromatic plant oils significantly reduced plasma lipids and increased plasma concentration of IgG. Feed intake was similar among groups. Economic evaluations were in favor of BSO group. BSO significantly ($P<0.05$) improved daily milk yield (DMY), fat and protein, but reduced lactose content of milk. CFO increased ($P<0.05$) daily milk yield. Only fat-corrected DMY was increased with OLO supplementation. The three supplements reduced ($P<0.05$) milk electric conductivity (EC) and somatic cell count (SCC). Overall, our results indicated that supplementing diets of Friesian cows with aromatic plant oils, particularly black seed oil, improved milk yield, udder health, and some immune parameters.

Key words: aromatic oils; cows; blood parameters; milk production and immunity

Introduction

Essential oils are a diverse group of secondary plant metabolites that contain naturally occurring volatile components that support smell and taste of plants (1). The volatile aromatic compounds have an oily appearance and they are extracted from plants (2). As a product, they are extracted from a plant or some parts through hydrodistillation, steam distillation, or dry distillation, without heating by a mechanical process (3). Essential oils have unique properties and offer a huge potential benefit for animal performance. One potential benefit that gained a lot attention is the antibacterial properties of these compounds. There is interest that essential oils could be a potential approach to improve feed efficiency, nutrient utilization, and animal health as an alternative to antibiotics. However, at this point we still need further research to provide information for practical feeding recommendations.

Fatty acids, volatile oils and trace elements combination contributes black seed effectiveness. Little is known about the activity of volatile oil in *N. sativa* (4). Black seed oil (*Nigella sativa*) contains a substance called Nigellone which is a natural antioxidant that can modulate and regulate the early activation steps in the acquired immune response (5).

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Oregano *(Origanum vulgare L.)* is an herb with high antioxidant capacity–compared to several other medicinal herbs (6, 7). Oregano oil can be defined as plant extract that contains mainly carvacrol and thymol, which are the major phenols constitute about 78-82% of essential oil. Moreover, it has antifungal (8) and antimicrobial properties (9) which make it an appropriate organic alternative for antibiotic as well as a promising feed additive in order to prevent meat lipid oxidation (10). Furthermore, 50% of essential oil of chamomile flowers (*Chamomila recutita*) contains alphabisabolol (trepenoid) and azulene these compounds showed anti-inflammatory, antibacterial, antimycotic and ulcer protective properties (11). Electrical conductivity (EC) of milk was indicator parameter for mastitis and is measured by the presence of ions (12). The current work was amid to effects of black seed, oregano and chamomile oil supplementation on blood parameters, immunity, udder health, and yield and composition of milk of lactating Friesian cows.

Materials and methods

This study was carried out at Sakha Animal Production Research Station, belonging to Animal Production Research Institute (APRI), Agricultural Research Center, Ministry of Agriculture, Egypt.

Animals and experimental groups

Thirty-two Friesian cows with live body weight (LBW) of 547.50±15.75 kg at eight weeks before parturition were classification into four groups (8 each) there to LBW and season of lactation (2^{nd} to 8^{th}) continued until four months postpartum. Cows fed a basal ration contained (on DM basis) 40% concentrate feed mixture (CFM), 25% fresh berseem (FB), 20% corn silage (CS) and 15% rice straw (RS) without any supplement in G1, which was served as control. The ration was supplemented with 25 mg black seeds oil (5) per kg LBW in G2 (13), 11 mg chamomile flower oil (14) per kg LBW in G3 (15) and 25 mg oregano leaves oil (16) per kg LBW in G4 (17). Composition of feedstuffs and calculated composition were show in Table (1). The CFM composed of 20% soybean meal, 24% wheat bran, 34% yellow corn, 12% rice bran, 5% linseed, 3% molasses, 0.5% premix and 1.5% common salt. Chemical analysis of samples of feedstuffs, were carried out according to the methods A.O.A.C (18).

Management

Cows housed in semi-open backyards under sheds and were fed their rations to cover their recommended requirements (19). The CFM was offered two parts at 8 a.m. and 4 p.m. daily. All cows had free access to fresh drinking water throughout the day.

Samples of milk yield (MY)

Animals were machine milked twice daily at 6 a.m. and 5 p.m. Daily milk yield was recorded individually and corrected for 4% fat contact (FCM): 4% FCM = 0.4 x MY (kg) + 15 x fat yield (kg) (20). Milk samples were taken from cows every two weeks and were analyzed for milk composition by Milko-Scan (model 133B). Milks of EC were measured for all quarter using "Dramtnski Electronic in agriculture, Mastitis detection, Italy". Furthermore, SCC analyzed milk sample with a Fossomatic 5000 "Foss Electric A S 69, Slangerupgade DK 3400 Hilleroed, Denmark Comp".

Samples of blood

Samples of blood were taken all cows before partition at days 45**,** 30 and 15 and after partition at 7^{th} day. Blood samples were taken from the jugular vein by clean sterile needle in the clean dry plastic tube after 4 hours from the morning feeding in two parts. First part centrifuged at 4000 r.p.m for 15 min and stored at -20^{o}C. Some boichemical parameters of blood were determined calorimetrically by using commercial kits (Diagnostic System Laboratories, Inc., USA). Second part used for determining hematological parameters in whole blood samples with EDTA (anticoagulant). Hematological analysis was performed by Medici Vet. (21). Hematological

analysis (Medonic CA 620, Sweden) was performed within 1-2 hrs after samples collection. Hematological variables were red blood cells (RBC's), hemoglobin (HGB), hematocrit (HCT), platelet (PLT). Leucocyte variables were white blood cells (WBC's) and differential white cells (lymphocytes, monocytes and neutrophils). The concentration of immunoglobulin G (IgG) in the blood serum samples determined using the quantitative ELISA Bovine (IgG), ELISA Quantitation Kit, Bethyl laboratories, UK.

Economic evaluation

Economic efficiency calculated between the price of milk yield 4% FCM and the cost of daily feeding consumed. The prices in Egyptian pound (LE) per ton were 4750 LE for CFM, 320 LE for fresh berseem, 690 LE for corn silage and 250 LE for rice straw. Also, the prices per kg were 110 LE for black seed oil, 90 LE for chamomile oil, 90 LE for oregano oil and 5 LE for 4% FCM produced during year 2017.

Statistical analysis

The obtained data were statistically analyzed system using SAS (22). One way ANOVA was used. The significant differences among treatment groups were using Duncan's New Multiple Ranges Test (23).

Table 1: Chemical composition and calculated feeding values of feed ingredients and basal ration.

Item	DM %	Composition of DM %						Calculated %	
		OM	CP	CF	EE	NFE	Ash	TDN	DCP
CFM	91.01	91.48	16.52	8.37	2.46	64.13	8.52	68.9	12.8
Fresh berseem	20.01	91.19	15.63	24.54	2.88	48.21	8.83	64.4	12.6
Corn silage	33.60	90.97	9.45	17.32	2.55	61.65	9.03	69.9	8.4
Rice straw	90.30	83.59	2.56	31.79	1.09	48.15	16.41	48.2	0.5
Basal ration	61.67	90.12	12.79	17.72	2.37	57.24	9.88	64.87	10.03

DM: dry matter; OM: organic matter CP: crude protein; CF: crude fiber; EE: ether extract; NFE: nitrogen free extract; TDN: total digestible nutrients; DCP: digestive protein. CFM: Concentrate feed mixture

Table 2: Blood serum biochemicals of cows supplemented with aromatic plant oils.

	Experimental groups			
Item	G1	G2	G3	G4
Total protein (g/dl)	5.90±0.43^{b}	7.55±0.32^{a}	6.83±0.15^{ab}	6.82±0.33^{ab}
Albumin (g/dl)	3.78±0.21^{a}	3.54±0.11^{b}	3.56±0.11^{ab}	3.64±0.14^{a}
Globulin (g/dl)	2.50±0.16^{b}	4.10±0.28^{b}	3.27±0.21^{b}	3.06±0.35^{b}
Total lipids (mg/dl)	221.0±0.46^{a}	191.0±0.29^{b}	184.0±0.43^{c}	183.0±0.40^{c}
Cholesterol (mg/dl)	71.6±0.32^{a}	68.9±0.17^{b}	65.5±0.24^{c}	64.0±0.21^{d}
Triglyceride (mg/dl)	93.0±0.21^{a}	87.8±0.21^{b}	84.8±0.18^{c}	83.7±0.18^{c}
IgG (mg/ml)	37.4±0.93^{b}	50.9±3.96^{a}	46.5±3.70^{a}	45.7±1.45^{a}

Values are represented as the mean ± SE.

^{abc}Within-row different superscript letters denote significant difference ($P<0.05$).

G1: basal diet. G2: basal diet supplemented with 25 mg black seed oil per kg body weight.G3: basal diet supplemented with 11 mg chamomile oil per kg body weight.G4: basal diet supplemented with 25 mg oregano oil per kg body weight.

Table 3: Blood hematological parameters of cows supplemented with aromatic plant oils

	Experimental groups			
Item	G1	G2	G3	G4
WBC's (10^3/µl)	9.63±0.36^{c}	13.7±0.89^{a}	11.6±0.41^{b}	11.2±0.92^{b}
Lymphocytes %	52.02±0.44^{c}	58.1±0.88^{a}	54.7±1.72^{b}	52.7±1.72^{c}
Monocytes %	6.96±0.17^{b}	8.93±0.58^{a}	7.33±0.81^{ab}	7.63±0.49^{b}
Neutrophils %	26.9±0.32^{c}	31.5±1.84^{a}	29.7±2.01^{b}	28.2±2.25^{b}
PLT (10^3/µl)	108.0±2.72^{d}	185.0±6.48^{a}	168.0±6.75^{b}	152.0±3.07^{c}
RBCs (10^6/µl)	6.34±0.32	6.46±0.19	6.32±0.40	6.10±0.36
Hgb (g/dl)	10.7±0.72	12.7±0.72	12.5±1.02	12.2±0.32
HCT (%)	28.9±0.53^{b}	31.5±0.53^{a}	30.1±0.87^{ab}	29.5±0.80^{ab}

Values are represented as the mean±SE.
[a] Different superscript letters within the same row denote significant difference (P<0.05).
G1: basal diet. G2: basal diet supplemented with 25 mg black seed oil per kg body weight. G3: basal diet supplemented with 11 mg chamomile oil per kg body weight. G4: basal diet supplemented with 25 mg oregano oil per kg body weight. WBCs: PLT: platelets**;** RBCs: Hgb: Hemoglobin; HCT: hematocrit

Table 4: Average actual yield, milk composition, electric conductivity and somatic cells count of cow's milk supplemented with aromatic plant oils

	Experimental groups			
Item	G1	G2	G3	G4
Actual yield, kg/day	12.9±0.25^{b}	15±0.35^{a}	14.5±0.24^{a}	14.1±0.30^{ab}
Fat corrected milk yield, kg/day	12.3±0.28^{c}	15.9±0.34^{a}	14.8±0.53^{ab}	14.2±0.48^{b}
Fat%	3.66±0.11^{b}	4.42±0.13^{a}	4.15±0.19^{ab}	4.03±17^{ab}
Protein, %	3.01±0.03^{b}	3.11±0.11^{a}	3.03±.05^{b}	3.06±.07^{ab}
Lactose, %	4.85±0.09^{a}	4.19±0.12^{b}	4.20±0.14^{ab}	4.32±0.19^{a}
Electrical conductivity, MS/ml	5.59±0.02^{a}	5.21±0.11^{b}	5.26±0.06^{b}	5.26±0.07^{b}
Somatic cells count, 10^3/ml	393±3.65^{a}	162±5.96^{c}	178±2.61^{b}	183±2.01^{b}

Values are represented as the mean±SE.
Different superscript letters within the same row denote significant difference (P<0.05).
G1: basal diet. G2: basal diet supplemented with 25 mg black seed oil per kg body weight.G3: basal diet supplemented with 11 mg chamomile oil per kg body weight.G4: basal diet supplemented with 25 mg oregano oil per kg body weight.

Table 5: Feed intake and economic efficiency of cows in different experimental groups

	Experimental groups			
Item	G1	G2	G3	G4
Total DM intake (kg/day)	17.64±0.32	17.73±0.35	17.77±0.34	17.75±0.41
Feed cost (LE/day)	51.69±0.56	51.94±0.54	52.06±0.57	52.00±0.50
Feed cost (LE/kg FCM)	4.24±0.03^{a}	3.26±0.04^{c}	3.53±0.07^{b}	3.66±0.05^{b}
Total revenue (LE/day)	60.85±0.74^{c}	79.65±0.68^{a}	73.80±0.65^{b}	71.05±0.75^{b}
Net revenue (LE/day)	9.16±0.10^{c}	27.71±0.08^{a}	21.74±0.11^{b}	19.05±0.09^{b}
Economic efficiency	1.18±0.02^{b}	1.53±0.03^{a}	1.42±0.04^{ab}	1.37±0.03^{ab}
Relative EE %	100.00±2.54^{b}	129.66±1.89^{a}	120.34±2.15^{ab}	116.10±2.28^{ab}

Different superscript letters within the same row denote significant difference (P<0.05).
G1: basal diet. G2: basal diet supplemented with 25 mg black seed oil per kg body weight.G3: basal diet supplemented with 11 mg chamomile oil per kg body weight.G4: basal diet supplemented with 25 mg oregano oil per kg body weight.

Results and discussion

Blood parameters

Blood serum biochemical of cows in experimental groups Table (2) showed that G2 had the higher concentrations of total protein and globulin in serum (7.55- 4.10 g/dl) followed by G3 (6.83-3.27 g/dl) and G4 (6.82-3.06 g/dl), while G1 (5.90- 2.50g/dl) had the lowest values. However, G1 recorded the higher concentrations of albumin, total lipids, cholesterol and triglycerides, but G2 had the lowest albumin and G4 had the lowest total lipids, cholesterol and triglycerides concentrations. Results cleared that BSO supplementation increased the concentrations of total protein and globulin in serum. Oregano oil (OLO) decreased the concentrations of total lipids, cholesterol and triglycerides. The increasing of total protein and lipids in treatments may be due to that aromatic oil increased digestibility coefficients of crud protein and lipids. Blood total protein and their fractions can be used as indicators to evaluate the ruminant nutritional status and physiological changes (24). Moreover, immunoglobulin G (IgG) was higher significantly ($P<0.05$) in G2 (50.9 mg/ml), G3 (46.5 mg/ml) and G4 (45.7 mg/ml), whereas G1 (37.4mg/ml) had the lowest concentration which attributed to contains of minerals and vitamins, which may have a role in improvement immunoglobulin and immunity response (22). Concentrations of total protein, albumin, globulin and glucose in blood serum were higher of animals received chamomile flower, garlic and fenugreek seeds comparison with the control one (26). Plasma total protein, albumin, and plasma immunoglobulin tended to higher ($P<0.05$) in lactating buffaloes received 10 ml BSO/head/day (13). Polyherbal supplementation at the rate of 200–250 mg/kg body weight improved immunity (27).

Blood hematological parameters of Friesian cows in experimental groups are presented in Table (3). Cows in G2 supplemented with black seed oil showed highest ($P<0.05$) total WBC's count and relative percentages of lymphocytes, monocytes and neutrophils (13.7, 58.1, 8.93 and31.5 %), followed by G3 (11.6, 54.7, 7.33 and 29.7 %) and G4 (11.2, 52.7, 7.63, 28.2 %), whereas G1 (9.63, 52.02, 6.96 and 26.9 %) had the lowest values. The increase of immunoglobulin could be credited to B-lymphocyte stimulation (28). The WBCs are soldiers of body and their high counts may be due to increase or development of the immune systems of the animals and the lymphocytes constituted majority of WBCs counts and the cells increase with age in early life of animals (23).

Also, G2 had the higher PLT count and HCT (185 10^3/µl and 31.5%) followed by G3 (168 10^3/µl and 30.1 %) and G4 (152 10^3/µl and 29.5%), but the lowest values were in G1 (108 10^3/µl and 28. 9%). While RBC's and Hgb was nearly similar for the different groups and not significantly affected by oil supplementation. It is clear that black seed oil (G2) was more effective in blood hematological parameters than chamomile oil (G3) and oregano oil (G4). Blood hematological profile reflects the physiological response of the cow to its external environments and internal with by feeding and feeds (29). Package cell value significantly increase ($P>0.05$) with 10 ml (BSO)/head/day of lactating buffaloes (13). Addition of medicinal plant CF significantly increased total RBC's and Hb concentration in lamb's blood (30). Black seed (*Nigella sativa*) contains minerals and vitamins which may have a vital role improving immune system and improve of formation and maturation of RBC's count (13, 25).

Milk production

Actual and 4% FCM yield (Kg/day) for cows of the experimental groups (Table 4) significantly ($P<0.05$) among groups, which have higher in G2 (15, 15.9) followed by G3 (14.5, 14.8) and G4 (14.1, 14.2), while were lower in G1 (12.9, 14.2). Superiority of G2, G3 and G4 compared to G1 in milk production and fat corrected milk as affected by the galactogoetic, which have the positive effect of the active components that aromatic oils may contain (30). Oregano leaf had little increase 3.5% FCM yield in dairy cattle (16).

Milk compositions of cows in experimental groups were presented in Table (4). Results indicated that G2 significantly higher (P<0.05) contents of fat and protein (4.42 and 3.11 %), followed by G3 (4.12 and 3.03 %) and G4 (4.03 and 3.06 %), but G1 (3.66 and 3.01%) had the lowest contents. The increasing of total protein and fat in milk due to the high contain of them on blood plasma; this declares the positive correlation between them.

While, lactose mean was nearly similar in each group. In general, aromatic plant oils supplementation especially BSO improved milk composition of Friesian cows, which might due to improve the udder health. Treatment with NS was improvement milk yield and lactose in buffaloes than in the control and recorded slight increase in milk protein percentage (31). The use of CF and NSS 10 g/ewe/day as natural feed additives increased (P<0.05) total protein (TP). But, lactose not effects in CF or NSS supplementation (30).

Udder health

Aromatic plant oils led to significantly lowest (P<0.05) in EC and SCC than in control Table (4). Black seed oil (BSO) supplement showed the lowest values of electrical conductivity and somatic cells count. The EC of milk has a positive correlation with somatic cell count. The infection with clinical mastitis increased in Friesian cows with the increase of somatic cell count levels (33). The values of EC in our study ranged (5.59 – 5.21 MS/ml) and are within EC of healthy cow milk is 4.0-5.5 milisenses (MS)/cm (12, 34). EC is used as routine test for sub clinical mastitis diagnosis (35) The EC determined anions and cations (Na^+ and Cl^-) ions concentrations increased and K+ concentration and lactose decreased when the cows and buffalos were suffering with mastitis due to inflammation of udder hence increase the EC. Although EC is also affected by some other factors such as bread, lactation stage and milking interval (36). Polyherbal treated at the rate of 200–250 mg/kg live weight improved udder health (27).

Economic efficiency

Feed intake and economic efficiency of cows in experimental groups were presented in Table (5). Total DM intake from the different rations was nearly similar for the different groups indicating that aromatic plant oils supplementation don't have any adverse effect on feed intake. Also, the costs of feed intake were similar for all groups, whereas G2 only significantly (P<0.05) lower feed cost per kg 4% FCM compared to G1 had the higher value, while G3 and G4 were intermediate between them. However, G2 recorded significantly (P<0.05) highest values of total revenue of 4% fat corrected milk, net revenue, economic efficiency and relative economic efficiency, followed by G3 and G4, but G1 had the lowest values. The improvements in economic efficiency with aromatic plant oils supplementation might be due to the increase of both actual fat and 4% fat corrected milk yield. Black seed and pumpkin oils supplementation for growing rabbits didn't affected feed cost, but significantly (P<0.05) increased total and net revenue (37).

Conclusion

These results concluded that aromatic plant oils additives to diets of dairy cows improve production and milk composition, udder health and immunity as well as economic efficiency and that best results obtained was with black seeds oil.

Disclosure statement

The authors declare that they have no conflict of interest.

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Original Research Article

DIAGNOSIS AND PHYLOGENETIC ANALYSIS OF THE CIRCULATING PESTE DES PETITS RUMINANTS VIRUS IN Al-SHARQIA GOVERNATE

Hend E. Elsheikh[1]*, Mamdouh F. El-Mekkawi[1], Abd Allah A. Abou-zaid[1], Amal M. Abd El Raof[2]

[1]Department of Animal Medicine (Infectious Disease), Faculty of Veterinary Medicine, Zagazig University, Zagazig 44511, Egypt, [2]Tissue Culture Unit – Virology Department Animal Health Research Institute Dokki, El-Giza

*Corresponding author, E-mail: hendelsheikh91@yahoo.com

Abstract: Peste des petits ruminants (PPR) is a Morbillivirus within the Paramyxoviridae family which characterized by highly contagious nature with high morbidity and mortality rates in domestic small ruminants. The aim of this study was to investigate an outbreak of P PRV in a flock of sheep and goats in Belbes city, AL-Sharqia governorate in 2018 by virus isolation and conventional RT-PCR. Phylogenetic analysis of N gene sequence of PPRV isolate. Also, compare it with other isolate from previous outbreak in Zagazig city, Al-Sharqia governorate in 2017 as a measurement of the infection status in Egypt. The current study applied on a flock of 55 small ruminants (42 goats and 13 sheep) in Belbes city which suspected to be infected by PPRV. All infected animals were not vaccinated against PPRV and randomly move from place to place. The morbidity rate was 100% and mortality rate was (23.8% in goats and 7.69% in sheep). Diseased animals suffered from fever, mucopurulent ocular discharge, nasal discharge, dyspnea, diarrhea, necrotic tissue and diphtheritic membrane in oral cavity. The ten samples (6 tissue scraping from oral lesions and 4 oculo-nasal swabs) were tested by conventional r everse transcription PCR which revealed 100% sensitivity compared to VI (virus isolation) 70%. Comparative of N gene sequence of both PPRV isolates revealed that homogenous population of PPR virus isolates up to 99%. Also, the PPRV isolate of the current study is related to Ethiopian strain and the previous Egyptian strains (Ismailia 3/ Egy/2010 isolate, El-Kalubeya isolate and Ismailia 1-2014 isolate). The PPRV isolate of present study and all previous Egyptian isolates belong to lineage IV in phylogenetic analysis. The results emphasize the importance of molecular methods for a broader understanding of the epidemiology and development of the virus in the country.

Key words: PPRV; Conventional RT- PCR; phylogenetic analysis; Al-Sharqia

Introduction

Sheep and goats contribute significantly to the economy of farmers in African and Asian nations. Sheep and goats are a source of meat, milk, and wool similarly to their speedy growth and reproduction. Poor man considers goats as cows in developing international locations (1).

The primary isolation of PPRV became recorded in Egypt in 1987 (2). The causative agent

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of PPRV is a member of the genus morbillivirus underneath the circle of relatives of the paramyxoviridae (3). The genome of PPRV is a linear, single stranded, non-segmented, negative sense RNA encoding six structural proteins: nucleoprotein (N), phosphoprotein (P), matrix (M), fusion (F), haemagglutinin (H) and a large polymerase (L) and two nonstructural (C and V) proteins (4, 5).

The incubation period of PPRV is ranged from 2–7 days (6) and the disease is manifested clinically by fever, presence of vesicles, ulcers on tongue and inside oral cavity, ocular discharge, leukopenia, profuse watery diarrhea, and respiratory manifestation (7, 8). PPRV cause high morbidity up to 100% and mortality up to 90% (6, 9). The most extensively used cell culture for virus isolation are Vero cells (10). The cytopathic effects (CPE) produced by PPRV in Vero cells are giant cells, cell rounding, formation of grape-like clusters, and small syncytia (10, 11). Reverse transcription polymerase chain reaction (RT-PCR) accompanied by nucleotide sequencing is the maximum diagnostic method used for PPRV identity (12, 13). Although PPRV is consider as one strain or serotype(14) , genetic type, based at the fusion (F) protein gene and the nucleoprotein (N) gene (15), has diagnosed four wonderful lineages (lineages I, II, III and IV) and is considered a powerful tool for the worldwide spread of virus. Lineage I is disbursed in West African strains in the 1970s and in Central African strains in those years; Lineage II in west Africa in Guinea, Ivory coast, and Burkina Faso strains; Lineage III in Sudan, east African, Oman and Yemen strains and lineage IV in The Arabian Peninsula, south east Asian, middle east, northern and central African strains (16) .

Thus, the aim of this study was to investigate the outbreak of PPRV in a flock of sheep and goats in Belbes city, AL-Sharqia governorate in 2018 by virus isolation and conventional RT-PCR. Phylogenetic analysis of N gene sequence of PPRV isolate and compare it with o ther isolate from previous outbreak in Zagazig city, Al-Sharqia governorate in 2017 as a measu rement of the infection status in Egypt..

Materials and methods

The experimental design was approved by Zagazig University's Animal Care and Use Committee (ZU-IACUC/2/F/19/2019).

Field examination

A flock of 55 small ruminants (42 goats and 13 sheep) in Belbes city In Al-Sharqia governate in August 2018 was suspected to be infected by PPRV. All infected animals were not vaccinated against PPRV. The case history showed that these infected animals were randomly move from place to place.

Ten samples of clinically diseased animals (6 tissue scraping from oral lesions and 4 oculonasal swabs) were collected. Until laboratory testing, the samples were kept at-20 ° C.

Samples preparation

Preparation of oculo-nasal swabs (four samples)

Oculo Nasal swabs were obtained on saline containing 10% pen-strep- amphotericin B to avoid bacterial and fungal contamination. Mixing well by pulse vortexing. The mixture was pipetted into a plain tube. Centrifugation at 2000 rpm for 10 minutes. The supernatant was pipetted into sterilized epindorf tube and stored at - 20°C till laboratory examination.

Preparation of tissue scraping from oral lesions (six samples)

Tissue samples were homogenized using tissue homogenizer. Nine milliliters of phosphate buffer saline was added to make 10 % tissue suspension. Centrifugation at 2000 rpm for 10 minutes was carried out and supernatant was taken into sterilized tube and stored at - 80°C till laboratory examination.

Isolation of PPRV on tissue culture

PPRV grown in Vero cell-culture supplied by Tissue Culture Unit in Animal Health Research Institute according to (17). The cells should be examined daily for 7–14 days for evidence of cytopathic effect (CPE). Infected cells developed a characteristic CPE consisting of retraction of the cell membrane from surrounding cells, and eventually rounding of cells. The cells with typical CPE were freeze thawed three

times to take the virus. If cytopathic effect were not evident after 4-7 days of the third passage, the samples were declared negative.

Purification of PPRV RNA

It was carried out using QIAamp viral RNA minikit (Qiagen, Germany) according to manufacturer instructions.

Conventional RT-PCR

It was carried out for confirmation of PPRV in suspected diseased animals by detecting the viral nucleic acid using Qiagen one step RT-PCR kit (Qiagen, Germany) and primers directed to the highly conserved sequence of nucleoprotein gene of PPR virus as described by (12) and according to instructions of the manufacturer.

PPRV isolates sequence

PPRV, isolate of the current study was compared with PPRV isolate from mesenteric lymph node of dead carcass of goat within a flock infected by PPRV in Zagazig city, Al-Sharkia governorate in 2017 (18). The N gene specific PCR amplicons were sequenced and assessed for sequence variations.

A purified RT-PCR product was sequenced in the forward direction on the automated DNA sequencer Applied Biosystems 3130 (ABI, 3130, USA). Use the Bigdye Terminator V3.1 cycle sequencing kit ready for reaction. (Perkin-Elmer / Applied Biosystems, Foster City, CA), with Cat. No. 4336817. A BLAST ® (Basic Local Alignment Search Tool) (19) was initially performed to determine the identity of the sequence for GenBank accessions. The sequence reaction was done according to the manufacturer's instructions.

Phylogenetic analysis

A comparative sequence analysis was performed using the CLUSTAL W multiple sequence alignment program, version 1.83 of MegAlign of the Lasergene DNAStar software module Pairwise, which was designed by(20) and phylogenetic analyses were performed using maximum likelihood, neighboring joining and maximum parsimony in MEGA6 (21).

Results

Field examination

Clinical examination of diseased animals showed fever, congested mucus membrane, normal lymph nodes, increase heart and respiratory rates.

The clinical signs observed were mucopurulent ocular and nasal discharges, conjunctivitis, stomatitis, ulcers and diphtheritic membrane inside the oral cavity, dyspnea and diarrhea (Fig. 1).

The morbidity rate was 100% and mortality was (23.8% in goats and 7.69% in sheep) (Table 1).

Isolation of virus on Vero cell culture

Out of 10 clinical samples, 7 (70%) samples showed CPE after three successive passage of virus on Vero cell culture as cells exhibited cell detachment, vacuolation and cell rounding (Fig. 2).

Conventional RT-PCR

The result of Conventional RT-PCR revealed all tested 10 samples (6 tissue scraping from oral lesions and 4 oculo-nasal swabs) were positive. (Table 2).

PPRV Sequence and phylogenetic analysis

Sequence analysis of the N gene of the current study (PPRV isolate from infected animals in Belbes City 2018) and isolate from the mesenteric lymph node of dead goat carcass in a flock infected with PPRV in Zagazig City, Al-Sharkia governorate in 2017 showed that the two isolates shared a 99 percent homology. The percentage of our isolate identity to Ethiopian strain was 98% applied from gene bank. Furthermore, the nucleotide isolate identity to the previous Egyptian isolates (Ismailia 3/Egy/ 2010 isolate, El-Kalubeya isolate and Ismailia 1-2014 isolate) were 99%, 98% and 98% respectively (Fig. 3). The PPRV isolate of the present study belong to lineage IV in phylogenetic analysis.

Table 1: The morbidity and mortality rates of infected animals with PPRV

Speaches	Total number of animals	Number of diseased animals	Number of dead animals	Morbidity rate	Mortality rate
Goats	42	42	10	100%	23.81
Sheep	13	13	1	100%	7.69
Total	55	55	11	100%	20%

Table 2: Percentage of positive samples in virus isolation and conventional RT-PCR

Samples	Number of Samples	Viral isolation		Conventional RT-PCR	
		Number of +ve samples	Percentages %	Number of +ve samples	Percentages %
tissue scraping from oral lesions	6	4	66.66%	6	100%
Oculo-nasal swabs	4	3	75%	4	100%
Total	10	7	70%	10	100%

Figure 1: Diseased animals showed (A) Mucupurulant occular discharge. (B) Errosion and diphtheritic membrane of hard palate. (C) Necrotic tissue and ulceration of tongue. (D) Soiling of hind quarters revealing profuse watery diarrhea

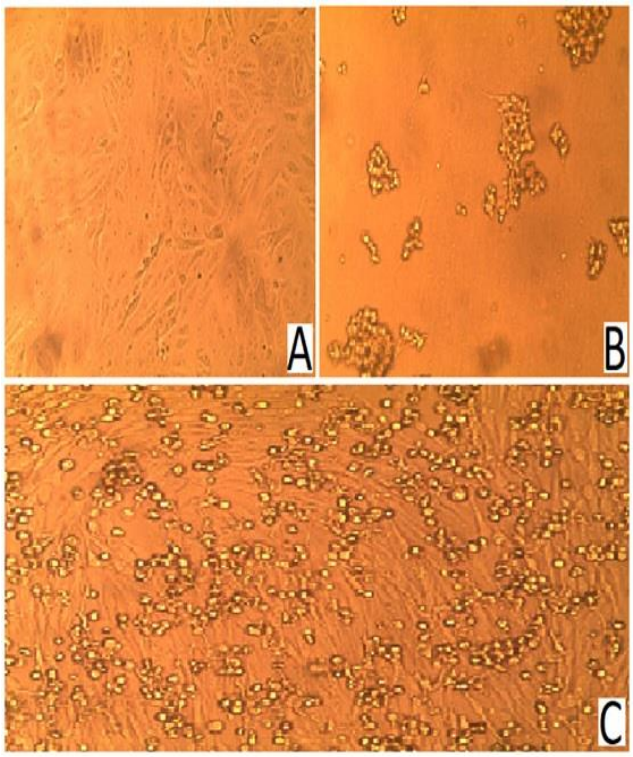

Figure 2: (A) Control Vero cell culture, spindle shape of cells. (B) Cytopathic effect of PPRV on Vero cell culture showed cell detachment. (C) Cytopathic effect of PPRV on Vero cell culture showed cell rounding

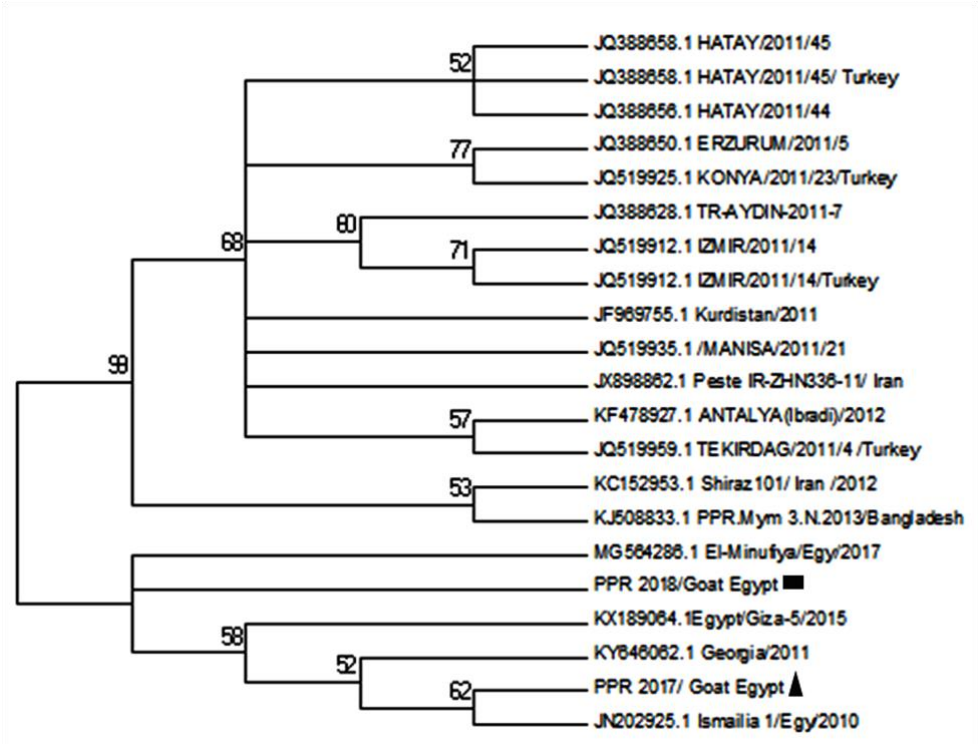

Figure 3: Phylogenetic relationship and comparative of PPR virus isolate of the current study (PPR 2018/goat Egypt ■) with other virus isolate, from previous study in Zagazig city 2017 (PPR 2017/goat Egypt ▲) based on partial sequences of N gene. Also, Neighbor-joining tree of PPRV N gene showing phylogenetic relationships of the PPRV isolates of present study with ather closely releated Egyption isolates available from GenBank

Discussion

Peste des petits ruminants (PPR) is a Morbillivirus within the Paramyxoviridae family which characterized by highly contagious nature with high mortality rates of domestic small ruminants (22). The disease is manifested by sudden onset of depression, fever, mucopurulent ocular and nasal discharges, oral lesions, dyspnea, diarrhea and death (23).

The Food and Agriculture Organization (FAO) reported that approximately 63 percent of small ruminants are highly susceptible to PPRV infection specially in South Africa, Central Asia and Southern Europe (24). In Egypt, PPR was first recorded in 1987(2). PPR has been diagnosed with polymerase chain reactions in various locations in Egypt over the past 10 years (13, 25, 26 and 27). Therefore, PPRV considered one of the most seriously contagious viral disease of small ruminant causing huge economic losses in Egypt.

The present study applied on a flock of 55 small ruminants (42 goats and 13 sheep) in Belbes city in Al-Sharkia governorate in 2018, suspected to be infected with PPRV as infected animals showed fever, depression, off food, mucopurulent ocular discharge, mucopurulent nasal discharge, dyspnea, diarrhea, necrotic tissue and diphtheritic membrane in oral cavity. These symptoms agree with those described by (7, 8).

The present study revealed that the morbidity rate was 100% in both goats and sheep and mortality rate was (23.8% in goats and 7.69% in sheep). The high mortality rate percentage in goats may be due to severe immunosuppression of infected goats (28) as PPRV downregulate CD46 (29), inhibit the leukocyte proliferation (30), and/or apoptosis of mononuclear cells in peripheral blood (31) .These finding were supported by the previous studies of (32) and (33) who observed that the PPRV symptoms in sheep were less severe than goats. Furthermore, mild infection in sheep has been reported in previous studies of (25, 27, 34, and 35). On the contrary, (36) observed significantly higher incidence rate of PPRV in sheep 39.1% than in goats 23%.

The ten samples (6 tissue scraping from oral lesions and 4 oculo-nasal swabs) were tested by conventional reverse transcription PCR which revealed 100% sensitivity compared to VI (virus isolation) 70%. The CPE produced after three successive passage were cells detachment, cell rounding, and formation of grape-like clusters. Our result supported by(10, 11) who noted that (CPE) produced in Vero cells by PPRV consist of giant cells, cell rounding, formation of typical grape-like clusters, and formation of small syncytia.

Despite low sensitivity, virus isolation is the most valuable method for detecting PPRV (37). Low-quality samples, poor transport, poor storage, aging cells and virus isolation in suboptimal cell culture (without the lymphocyte activation molecule (SLAM) that helps to isolate them) are predisposing factors for low VI sensitivity (38). Viral isolation also takes long time and requires further confirmation (39).

This study reported that all tested 10 samples 100% were positive by conventional RT-PCR. These findings endorsed those obtained by (13) who found that the positive samples tested by conventional RT-PCR and real-time PCR were 90% and 100% respectively. (37, 40) observed that conventional RT-PCR could detect virus in eye swabs in experimentally infected goats four days after infection.

PCR is the most favorable and highly sensitive tool for virus identification and molecular epidemiological studies among the various techniques used for PPRV detection (39).

Phylogenetic analysis of N gene sequence is the key method for differentiation between circulating PPRV lineages and help in molecular epidemiology of the disease (41).

The comparative of N gene sequence of the current study (PPRV isolate from Belbes City 2018) and the isolate from the previous study in Zagazig City 2017 showed that the two isolates had a 99 percent homology suggesting that there are no rapid genetic changes to circulating viruses. Nucleotide sequences were aligned with other PPRV sequences available in GenBank and phylogenetic tree were used to determine the genetic lineage of the circulating virus.

Circulating strains have been classified as lineage IV.

The high percent homology between the two isolates may be due to uncontrolled movement of sheep and goats within Al-Sharqia governorate. Also PPRV outbreaks are more common around festivals periods.

In addition, the PPRV isolate of the current study related to Ethiopian strain and the previous Egyptian strains (Ismailia 3/Egy/2010 isolate, El-Kalubeya isolate and Ismailia 1-2014 isolate).

The origin of the circulating viruses may be from Ethiopia. Egypt imports small ruminants from different African countries including Ethiopia because it has meat production deficit.

The similar result demonstrated by (13) who reported that, the cause of outbreak in Ismailia and Suez in period 2014 and 2016 was Ethiopian origin. Another study revealed that the Sudanese strain caused PPRV outbreaks in Ismailia, Egypt during the 2010-2012 (27). Also, this study confirmed that all Egyptian isolates classified as PPRV lineage IV strains circulate extensively from governorate to other without controlled measures.

Conclusion

The study confirms that PPRV has been detected and circulated in a flock of goats and sheep in the city of Belbes, Al-Sharqia. The N gene sequence and phylogenetic analysis confirm that the PPR virus circulating in this region of the country is of lineage IV and closer to PPRV isolated in Zagazig in 2017. Also PPRV isolate of present study revealed high level of homology with majority of the previous Egyptian isolates and Ethiopian strain. The movement of unbarred animals and their trade could be one of the reasons for the transmission of this virus. The results emphasize the importance of molecular methods for a broader understanding of the epidemiology and development of the virus in the country. The information from these studies will help to achieve the goal of the disease control and eradication.

Conflict of interest

There is no conflict of interest.

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Original Research Article

EFFECT OF DIFFERENT CRYOPROTECTANTS ON THE POST-THAW SPERM CHARACTERISTICS AND *IN VIVO* FERTILITY OF BUFFALO (*Bubalus bubalis*) BULL SEMEN

Essam A. Almadaly[1]*, Fady S. Tawfik[2], Ismail I. El-Kon[1], Bassiouni A. Heleil[1], El-Sayed M. Fattouh[1]

[1]Department of Theriogenology, Faculty of Veterinary Medicine, Kafrelsheikh University, El-Geish Street, 33516 Kafrelsheikh, Egypt, [2]Artificial Insemination Center, Veterinary Medicine Directorate, Ministry of Agriculture, Al-Gharbia, Egypt

*Corresponding author, E-mail: dr_essam_2005@yahoo.com; essam.mostafa@vet.kfs.edu.eg

Abstract: This study aimed to investigate the effect of different cryoprotectants, glycerol (GLY) or ethylene glycol (EG) or dimethyl sulfoxide (DMSO) on sperm characteristics, and *in vivo* fertility of frozen-thawed buffalo-bull semen. A total of 85 ejaculates collected by artificial vagina from buffalo-bulls of proven fertility were used in this study. The collected ejaculates were examined for volume, motility, viability, morphology, and sperm cell concentration. The qualifying ejaculates (≥ 3 mass motion, > 70% progressive motility and viability, < 15% abnormal morphology and > 1 x 10^9 sperm cells/mL) were pooled and diluted with Tris-based diluent containing either 7% GLY or 5% EG or 5% DMSO. After 4 h equilibration time, the diluted semen was loaded in 0.5 mL straws, labeled, sealed and frozen stored until analysis. Frozen straws were thawed and evaluated for progressive motility, viability, hypo-osmotic swelling test (HOST), acrosomal membrane integrity, and acrosome reaction (AR) in response to calcium ionophore A23187. Moreover, *in vivo* fertility was calculated after artificial insemination (AI) of 75 buffalo-cows (25 female/cryoprotectant) treated with double doses of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) 11 days interval. The proportions of progressive motility, viability and intact-acrosome were higher ($p < 0.05$) in extender containing 7% GLY compared to 5% EG and 5% DMSO. The proportion of intact-plasma membrane was comparable ($p \geq 0.05$) between GLY and EG but higher than that of DMSO. A time-dependent increase in the % AR and % relative AR was recorded in the three cryoprotectants with clear significant ($p < 0.01$) difference among them at 30 and 60 min incubation, respectively. Moreover, GLY yielded higher pregnancy rate (52%) than EG (32%) and DMSO (16%). In conclusion, GLY is recommended for preservation of buffalo-bull semen in order to maintain sperm plasma membrane integrity and improve *in vivo* fertility of frozen-thawed buffalo-bull semen.

Key words: buffalo-bull; frozen-thawed semen; cryoprotectant; estrus synchronization; *In vivo* fertility.

Introduction

Buffalo (*Bubalus bubalis*) is the most important and popular livestock animal for milk

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production in Egypt. It was introduced to Egypt from India, Iran and Iraq around the middle of the 7th century. Its lactation season ranged from 210–280 day with annual average of 915 kg milk/animal. The total population of buffaloes in Egypt was decrease from 4.16 million in 2012 to 3.64 million in 2016 (1) with decline of approximately 11% within four years, most of them reared in small herds (1-3 animals). Despite it is the best milk, meat and leather producing animal in our country but it has not received sufficient consideration regarding the improvement of its breeding strategies.

Improvement of livestock production can be achieved using one of the assisted reproductive technologies such as artificial insemination (AI) (2) which is an essential tool for the quick dispersion of valuable germplasm and the feasible method to improve the genetic quality of farm animals (3). Cryopreservation is an essential method for conservation of germ cells and is suitable for the proper genetic management (4). Semen quality and fertility is clearly reduced by freezing-thawing damage compared with fresh semen and it can be compensated by the insemination of larger number of sperm cells. Generally, 40 – 50% of sperm cells die after cryopreservation even with optimized methodology (5) due to an inevitable chemical, osmotic, thermal, and mechanical stresses which lead to lower post-thaw motility and viability (6). Intracellular ice crystallization is implicated in this inevitable damage of spermatozoa during cryopreservation (7). Moreover, buffalo-bull spermatozoa are more vulnerable to freezing-thawing damage compared with other mammalian species (8).

The fertility rate of frozen-thawed buffalo-bull spermatozoa is ranged from 33% (9) to 45% (10). Sudden cooling of diluted semen from 30 °C toward 0 °C result in a lethal stress called cold shock in some of the sperm cells which was proportional with the cooling rate, the temperature interval and range (11). This sharp variation in temperature induces damage in sperm cell membranes. It is possible that these effects are related to phase change in lipids and altered functional state of membranes (6).

The composition of semen extender containing proper cryoprotectants is critical for successful semen cryopreservation (12) where the cryoprotectant minimize the intracellular ice crystals formation and/or damage. The penetrating cryoprotectants such as glycerol (GLY), ethylene glycol (EG) and dimethyl sulfoxide (DMSO) have the ability to pass through the sperm plasma membrane. Accordingly, it acts both intra and extra-cellular, rearrange the membrane proteins, reduce intracellular ice crystals formation and thus, protect sperm cells from freeze-thaw damage (13).

Addition of penetrating cryoprotectants to sperm cell suspension, the cells first shrink due to water efflux then swell as the cryoprotectant get intracellular with water to maintain chemical potential. During removal of cryoprotectant the cell will initially swell due to water influx and then return to isosmotic size due to efflux of cryoprotectant with water. This osmotic stress adversely affects sperm motility, viability and function (14).

Conventionally, GLY is used at concentration ranged from 5 to 8% to cryopreserve bovine sperm. However, it has both osmotic and toxic effects on the plasma membrane and metabolism of frozen-thawed semen with subsequent reduced motility and fertility (12). Also, higher concentration of GLY may lead to cell death (15). Recently, the high membrane permeability of EG was shown to reduce the plasma membrane damage of bovine sperm compared with GLY (16). Thus, apparently EG has less detrimental effect on viability and motility and offer protective effect to sperm acrosome than GLY (17). Nowadays, biotechnology of cryoprotectants play substantial role in improving the quality of frozen-thawed semen. Incorporation of efficient and less toxic cryoprotectant within semen extender can make a substantial impact in improving the quality of frozen-thawed buffalo-bull spermatozoa. Therefore, the present study aimed to investigate the effect of different cryoprotectants (GLY, EG and DMSO) on sperm characteristics and *in vivo* fertility of frozen-thawed buffalo-bull semen.

Materials and methods

Animals

The present study was carried out at Mehalet-Mousa Research Station, Kafrelsheikh, Egypt. Five healthy Egyptian buffalo bulls (5–6 years-old) of proven fertility, acceptable libido and of good semen quality were used for semen collection. Seventy five Egyptian buffalo-cows (4–5 years-old) having history of normal parturition was used for *in vivo* fertility experiment. All animals were kept indoors all over the year and fed on ration that met both maintenance and production requirements. Buffalo-cows were fed on concentrates plus corn silage and straw in summer and concentrates plus green clover and straw in winter.

Chemicals, media and reagents

Unless otherwise stated, all chemicals used were of high purity and procured from Sigma–Aldrich Chemical Company (St. Louis, MO, USA). Freshly prepared Tris-based extender consists of 3.028 g Tris, 1.678 g citric acid and 1.0 g fructose, 20% (v/v) egg yolk, 1000 IU/mL penicillin G sodium, 1 mg/mL streptomycin sulfate and the same extender containing either 7% GLY (Sigma-Aldrich, USA) or 5% EG (SDFCL, India) or 5% DMSO (Fluka, Germany) was used for semen dilution and freezing (5). Saline medium consists of 142 mM NaCl, 2.5 mM KOH, 10 mM glucose and 20 mM Hepes and adjusted to pH 7.55 at 25 ºC with NaOH (18) was used for dilution and incubation of spermatozoa. Saline medium containing 275 mM sucrose in place of NaCl was used for washing spermatozoa and designated as sucrose medium (18). Both media contained 0.1% (w/v) polyvinyl alcohol (PVA, molecular weight 30,000–70,000) and 0.1% (w/v) polyethylene glycol (PEG). A fixative used was 2% Glutaraldehyde in 0.165 M of sodium cacodylate buffer, adjusted to pH 7.3 at 25 ºC. Sperm counting medium [(SCM) 0.9% (w/v) NaCl, 0.1% (v/v) formaldehyde, 0.1% (w/v) PEG and 10 mM ethylenediaminetetraacetic acid in H_2O] was used for counting of sperm cells. Calcium ionophore A23187 (free acid; Calbiochem-Novabiochem Corp., San Diago, USA) was prepared at 100 mM in DMSO, and frozen stored at - 30 ºC in small aliquots wrapped from light. Immediately before use, an aliquot was thawed and used at a 1:100 dilution in saline medium to give a final concentration of 1 mM.

Semen collection

Ejaculates were collected from well-trained buffalo-bulls using an artificial vagina of inner sleeve temperature 40 °C according to Rasul et al. (19). Ejaculates were collected twice a week early morning with 10 min interval. Immediately, after collection the collected ejaculates were kept in water bath of 37 ºC during initial semen evaluation.

Semen evaluation

As soon as possible the collected ejaculates were observed for color, consistency, hygienic quality and volume. An aliquot (200 µL) from each ejaculate was used for evaluation of the following sperm characteristics:

1. Sperm motility

1.1. Mass motility

As quick as possible the fresh semen samples were examined on pre-warmed glass slide on a warm stage (38.5 ºC) under phase contrast microscope (Olympus BX20, Tokyo, Japan) at 100 × magnification. The mass motility was scored from 0–5 grade depending upon the speed and intensity of waves. All ejaculates of mass motility less than 3 were excluded.

1.2. Individual motility

An aliquot (5 µL) of fresh semen was placed on warm glass slide and diluted with 20 µL isotonic sodium citrate dihydrate 2.9%. The diluted semen was covered with a warm cover slip and examined under phase contrast microscope at 200 and 400 × magnifications. At least, five microscopic fields were examined to calculate the average in increments of 5%. Ejaculates of less than 70% progressive motility were excluded.

2. Sperm viability

Alive sperm percentage was determined in Eosin-Nigrosin (EN) stained semen smear according to Bjoerndahl et al. (20). Briefly, 5 µL of fresh semen was mixed with 20 µL pre warmed EN stain and smeared on warm glass

slide, dried and examined at 400 and 1000 × magnification of phase contrast microscope. At least, two microscopic fields containing 200 spermatozoa were examined. The proportion of unstained spermatozoa was expressed as viability%.

3. Sperm cell concentration

Sperm cell concentration was determined by direct cell count using Neubauer haemocytometer (Marienfeld, Germany) (21). Exactly, 10 µL of fresh semen was diluted with 3000 µL SCM and thoroughly mixed. An aliquot (12.5 µL) of sperm suspension was spotted into counting chamber and count sperm cells under phase contrast microscope at 400 × magnification.

Semen dilution and cryopreservation

Immediately before dilution, Tris-based extender was centrifuged at 3310 x g for 20 min and the upper portion of supernatant and sediment were discarded (22). Extenders (intermediate portion after centrifugation) were prepared one day before semen collection and kept overnight at 4 °C. The ejaculates of at least 3 score mass motility, 70% progressive motility and 800 x 10^6/mL sperm cell concentration were diluted. Both, ejaculate and extender were maintained in water bath of 37 °C. The selected ejaculates were pooled together to avoid the bull effect before being diluted with cryoprotectant free extender stepwise at room temperature. The semen was diluted gradually 1:10 (each straw had at least 20 x 10^6 motile sperm). Diluted semen was cooled to 4 °C for 2 h and further diluted (1:1) with cryoprotectant containing extender (23). The cryoprotectant was either 7% GLY (24) or 5% EG (3) or 5% DMSO (19). Diluted semen was equilibrated at 4 °C for at least 4 h (25) before being loaded in 0.5 mL polyvinyl French straws (IMV, France) with a suction pump at 4 °C in a cold cabinet (Minitub, Germany). The filled straws were plunged above liquid nitrogen vapor (-120 °C) for at least 10 min then immersed in liquid nitrogen (-196 °C) for storage.

Evaluation of frozen-thawed semen

Two straws for each treatment were thawed in water bath of 39 °C for exactly 1 min, thoroughly dried and gently evacuated in 1.5 mL vial which kept on a warming plate (38.5 °C) during evaluation. Frozen-thawed semen was examined for the following sperm characteristics:

1. Progressive motility

The proportion of progressive motility was determined in wet mount using 10 µL of frozen-thawed semen on pre-warmed glass slide covered with warm cover slip (18 x 18 mm), and examined at 400 × magnification under phase contrast microscope. Five microscopic fields were examined for the proportion of progressive motile sperm according to Chutia et al. (26).

2. Sperm viability

The proportion of live sperms was determined in EN-stained smears similarly as mentioned before in fresh semen analysis. At least, 200 spermatozoa were examined for each semen sample to calculate the proportion of unstained spermatozoa which expressed as viability %.

3. Sperm plasma membrane integrity

Plasma membrane integrity was determined by using hypo-osmotic swelling test (HOST) (27). Hypo osmotic solution was prepared by dissolving 0.73 g sodium citrate and 1.35 g fructose in 100 mL Milli-Q water (osmotic pressure ~190 mOsmol/kg). For evaluation, 50 µL of frozen-thawed semen was suspended in 500 µL of pre-warmed HOST solution and incubated at 37 °C for 30 – 60 min. After incubation, 50 µL of HOST exposed sperm suspension was fixed with an equal volume of 2% Glutaraldehyde in sodium cacodylate buffer for at least 30 min at room temperature. Two wet mount (2 µL/each) were prepared and examined for the proportion of sperm cells showing tail swelling under (1000×) phase contrast microscope. In total, 200 spermatozoa per each wet mount were examined for their swelling characterized by coiled tail. The proportion of HOST-positive sperm cells (% intact-membrane) was determined from the following equation (HOST-positive % = Y − X/400 ×

100), where X was spermatozoa having tail abnormalities and Y was spermatozoa having swelled tails according to Fukui et al. (28).

4. Acrosomal membrane integrity

Acrosomal membrane integrity was determined in EN-stained semen smears according to the method described by Bamba [29]. At least, 200 spermatozoa were examined in each semen sample under phase contrast microscope at 1000 × magnification. Sperm cells having crescent shape apical ridge considered intact-acrosome otherwise consider damaged-acrosome.

5. Stimulation of frozen-thawed spermatozoa with Ca^{2+} and Ca^{2+} ionophore A23187

Spermatozoa were incubated with Ca^{2+} and Ca^{2+} ionophore A23187 as previously described by Almadaly et al. (18). Two straws were thawed at 39 °C for 1 min and evacuated in 1.5 mL vials. The two vials were centrifuged at 830 x g for 6 min at room temperature and remove the supernatant. The sperm pellets were resuspended in saline medium then was overlay on sucrose medium containing 0.1% PVA and 0.1% PEG and centrifuged at 400 x g for 5 min then at 1000 x g for 10 min at room temperature. After centrifugation the supernatant was discarded and sperm cell concentration was adjusted to 6 x 10^6/mL. Spermatozoa were resuspended in saline medium containing 3 mM $CaCl_2$ and incubated in the presence and absence (DMSO vehicle, control) of 1 mM Ca^{2+} ionophore A23187 at 37 °C for up to 60 min in air. At 0, 5, 10, 15, 30, and 60 min subsamples were collected and fixed with 1% Glutaraldehyde in 0.165 M cacodylate buffer at room temperature for at least 30 min. After incubation, wet mounts (2 μL/each) were prepared and examined under oil immersion lens of phase contrast microscope.

Sperm having a clear, dense apical ridge on the head were considered acrosome-intact, but sperm cells with ruffled or vesiculated acrosome or without acrosome were considered acrosome reacted. The raw data expressed as % acrosome reaction (AR) was transformed to percentage of spermatozoa that were acrosome-intact at the onset of stimulation but subsequently have an AR in response to Ca^{2+} and Ca^{2+} A23187 (% relative AR) with the following formula: (%AR at each time point of stimulation - percentage at 0 min) / (100 - percentage at 0 min) x 100 (30).

Estrus synchronization and artificial insemination

This experiment was applied on 75 pluriparous buffalo-cows maintained at the same farm of buffalo-bulls and of more than 90 days postpartum with normal genitalia. Before the onset of experiment all buffalo-cows were examined by rectal palpation and/or ultrasound (Mindry, Germany) for judging the ovarian activity which indicated by the presence of follicles and/or corpus luteum. Cyclic buffalo-cows received double dose of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) with 11 days interval (31) where each animal received 750 pg $PGF_{2\alpha}$ analogue (Cloprostenol sodium, Estrumate, Berkhamsted, England) intramuscularly on days 0 and 11. Exactly, 48 h after the second dose of $PGF_{2\alpha}$ animals were carefully observed for the signs and/or the reflexes of estrus throughout the day and night. Estrus buffalo-cows were inseminated with frozen-thawed straws (two straws/animal) containing either GLY (n = 25) or EG (n = 25) or DMSO (n = 25). All animals were intrauterine inseminated (recto-vaginal technique) by the same inseminator using am-pm regime.

Pregnancy diagnosis

All inseminated buffalo-cows were rectally palpated and/or ultrasound examined for positive findings of pregnancy on day 45 post-insemination. Presence of either amniotic vesicle or fetal membrane slip was considered a reliable sign for the pregnant buffalo-cow.

Statistical analysis

The results were presented as mean ± standard error of mean (SEM). All analyses were achieved using a statistical software program (Graphpad Prism Version 6.0; Graphpad Software, San Diego, CA, USA). Analysis of variance (ANOVA) with Turkey's multiple com-

parison test was used subsequently for comparison of means at a significance level of $p < 0.05$ (32). The proportion of pregnant females was analyzed by Chi-square test at $p < 0.05$.

Results

Sperm characteristics of fresh semen

The obtained results revealed that all ejaculates of the five buffalo-bulls used for processing of frozen semen had acceptable mass motility (4.5 ± 0.2), progressive motility (78.7 ± 1.3), viability (79.5 ± 0.8) and sperm cell concentration (1.6 ± 0.1 x 10^6/mL) as well as % abnormal morphology (9.6 ± 0.7, Table 1).

Sperm characteristics of frozen-thawed semen

1. Sperm motility

The mean ± SEM of % progressive motility of frozen-thawed buffalo-bull spermatozoa frozen-stored in the presence of one of the three cryoprotectants have been presented in Table 2. The progressive motility were extremely ($p < 0.001$) different among the three cryoprotectant where it was greater in GLY (41.3 ± 1.2) followed by EG (31.4 ± 0.9) and lower (25.4 ± 1.1) for DMSO.

2. Sperm viability

Regarding the results of sperm viability (Table 2), GLY yielded the greater ($p < 0.001$) sperm viability (47.7 ± 1.1) than both EG (42.3 ± 0.8) and DMSO (39.2 ± 1.2) without significant difference between EG and DMSO.

3. Plasma membrane integrity

The proportion of intact-plasma membrane of spermatozoa frozen-stored in the presence of GLY or EG (Table 2) were greater ($p < 0.001$) than that of DMSO (46.3 ± 0.9) without significant difference between GLY (58.4 ± 1.5) and EG (58.7 ± 0.8).

4. Acrosomal membrane integrity

The proportion of intact-acrosome was significantly ($p < 0.001$) different among the three cryoprotectant with greater % (61.7 ± 1.2) in GLY than EG (58.4 ± 0.8) and DMSO (48.4 ± 0.5) as shown in Table 2.

5. Induction of AR with Ca^{2+} and Ca^{2+} ionophore A23187

Incubation of spermatozoa with Ca^{2+}/A23187 resulted in a time-dependent increase in the % AR (Table 3), in the three cryoprotectants without significant ($p \geq 0.05$) difference at 0 and 5 min but with significant ($p < 0.01$) difference at 10, 15, 30 and 60 min. Regarding % relative AR (Table 4) it was similar among the three cryoprotectants at 5, 10 and 15 min but was extremely different among them at 30 and 60 min. Almost in all incubation times GLY was predominant in both % AR and % relative AR (Tables 3, 4).

Pregnancy rate

The pregnancy rate was different ($x^2 = 7.32$, $p < 0.05$) among the three cryoprotectants as depicted in Figure 1, where it was greater (52%) with GLY than EG (32%) and DMSO (16 %).

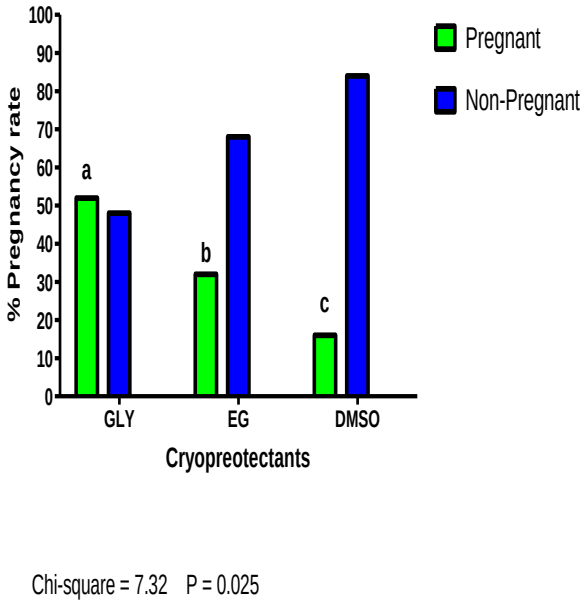

Figure 1: Effect of GLY, EG and DMSO in *Tris*-based extender on the *in vivo* fertility of frozen-thawed buffalo bull spermatozoa
GLY = Glycerol, EG = Ethylene glycol, DMSO = Dimethyl sulfoxide.

Table 1: Fresh semen quality (mean ± SEM) of buffalo-bulls*****

No. of replicates	Mass motility (0 – 5)	Progressive motility (%)	Sperm viability (%)	Abnormal morphology (%)	Sperm cell concentration (x 10^9/mL)
12	4.5 ± 0.2	78.7 ± 1.3	79.5 ± 0.8	9.6 ± 0.7	1.6 ± 0.1

*****For each replicate ejaculates were collected and examined in duplicate for each semen parameter.

Table 2: Characteristics (mean ± SEM) of frozen-thawed buffalo-bull spermatozoa frozen-stored with different cryoprotectants*****

Cryoprotectant	No. of replicates	Progressive motility (%)	Viability (%)	Plasma membrane integrity (%)	Acrosomal membrane integrity (%)
GLY	17	41.3 ± 1.2^a	47.7 ± 1.1^a	58.4 ± 1.5^a	61.7 ± 1.2^a
EG	17	31.4 ± 0.9^b	42.3 ± 0.8^b	58.7 ± 0.8^a	58.4 ± 0.8^b
DMSO	17	25.4 ± 1.1^c	39.3 ± 1.2^b	46.3 ± 0.9^b	48.4 ± 0.5^c
P value	-	< 0.001	< 0.001	< 0.001	< 0.001

*****Two straws were thawed and pooled together for each replicate and examined in duplicate for each sperm characteristic. Means bearing different superscript within the same column were significantly different at $p < 0.05$.

Table 3: The % AR (mean ± SEM) of frozen-thawed buffalo-bull spermatozoa frozen-stored with different cryoprotectants in response to Ca^{2+} and Ca^{2+} ionophore A23187*

Cryoprotectant	No. of replicates	Incubation time (min)					
		0	5	10	15	30	60
GLY	6	21.2 ± 0.7^a	28.8 ± 0.6^a	36.2 ± 1.2^a	46.0 ± 1.3^a	62.7 ± 1.4^a	76.3 ± 1.1^a
EG	6	19.3 ± 0.6^a	30.2 ± 0.8^a	33.7 ± 0.8^{ab}	42.5 ± 0.8^{ab}	52.2 ± 1.1^b	66.7 ± 1.5^b
DMSO	6	19.2 ± 0.5^a	28.3 ± 0.7^a	31.7 ± 0.7^b	41.5 ± 0.8^b	47.5 ± 0.8^c	59.2 ± 0.8^c
P value	-	0.06	0.13	0.008	0.01	<0.001	<0.001

*****Two straws were thawed and pooled together for each replicate and examined for the % AR in response to calcium ionophore. Values bearing one similar superscript within the same time point were similar ($p \geq 0.05$, repeated measures ANOVA followed by Tukey's multiple comparison test).

Table 4: The % relative AR (mean ± SEM) of frozen-thawed buffalo-bull spermatozoa frozen-stored with different cryoprotectants in response to Ca^{2+} and Ca^{2+} ionophore A23187*****

Cryoprotectant	No. of replicates	Incubation time (min)				
		5	10	15	30	60
GLY	6	10.0 ± 0.7^a	20.0 ± 2.1^a	31.4 ± 1.9^a	52.6 ± 2.0^a	72.1 ± 1.5^a
EG	6	13.4 ± 1.4^a	17.7 ± 1.0^a	28.7 ± 0.9^a	40.6 ± 1.4^b	58.6 ± 1.9^b
DMSO	6	11.2 ± 1.3^a	16.2 ± 1.0^a	27.6 ± 1.3^a	34.9 ± 1.1^b	49.5 ± 1.0^c
P value	-	0.06	0.16	0.17	<0.001	<0.001

* The proportion of relative AR = %AR at each time point of stimulation - percentage at 0 min / (100 - percentage at 0 min x 100. Values bearing one similar superscript within the same time point were similar ($p \geq 0.05$, repeated measures ANOVA followed by Tukey's multiple comparison test).

Discussion

It is imperative to mention that buffalo-bull sperm are more susceptible to freeze-thaw damage than other domestic animals. This poor freezability of buffalo-bull sperm might be related to the lower content of membrane phospholipid and/or its loss during freezing and thawing procedures (33). Also, intracellular ice crystal formation during cryopreservation was the main cause of sperm cell damage. Thus, the composition of diluent containing proper cryoprotectants is critical factor for successful semen preservation (34) as the cryoprotectant minimizes intracellular ice formation (12). Moreover, the ideal cryoprotectant must have low molecular weight, high water solubility, permeated cell quickly and of low toxicity (35).

On the basis of existing information on freezing of buffalo-bull sperm in the presence of different cryoprotectants, GLY improved the post-thaw sperm characteristics including motility, viability, plasma membrane integrity, acrosomal membrane integrity, % AR and % relative AR in response to calcium ionophore A23187 as well as yielded higher pregnancy rate in comparison with EG and DMSO. These findings were regular with the findings of Tasdemir et al. (34) who reported that there was no advantage in using EG or DMSO spermatozoa where GLY yielded the best post-thaw sperm characteristics in comparison with EG and DMSO. Also, Guerrero (36) found that the post-thaw progressive motility and % intact-membrane of bull sperm frozen in 7% GLY-*Tris*-based extender were significantly higher than those of sperm frozen in 7% EG-*Tris*-based extender. On contrary, % intact-acrosome was similar between 7% GLY and 7% EG.

Moreover, the findings of this study were in harmony with Rasul et al. (19) who concluded that GLY is still an efficient cryoprotectant for buffalo-bull sperm and DMSO antagonized the action of GLY and thus reduced the post-thaw quality of buffalo-bull sperm. Moreover, 6% GLY added at 37 °C, provided better cryoprotection to the motility and plasma membrane integrity of buffalo-bull sperm (19).

Our findings were in agreement with Rohilla et al. (25) who reported that 6.8% GLY greatly improve post-thaw buffalo-bull sperm motility, viability, and intact-acrosome in comparison with 5% EG. Furthermore, Gabr (37) reported that higher concentration of cryoprotectant for instance, > 7% GLY resulted in gradual reduction in motility. Post-thaw sperm motility with GLY and DMSO didn't differ significantly and there is a beneficial effect of 7% GLY on improving sperm motility of buffalo-bull semen extended in *Tris*-based extender in comparison with different (5, 7, 10%) concentrations of DMSO and EG (37).

Noteworthy, our findings were partially inconsistent with the findings of Swelum et al. (3) who reported that 5% EG resulted in greater post-thaw intact-acrosome, intact-plasma membrane and conception rate of buffalo-bull spermatozoa than 7% GLY. On contrary, the post-thaw sperm motility was lower with 5% EG than 7% GLY which was in agreement with our results but sperm viability didn't affected with the different cryoprotectants either GLY or EG. El-Harairy et al. (38) found that post-thaw motility and the conception rate of bull spermatozoa frozen in lactose-yolk-citrate extender containing 7% GLY or 7% DMSO was similar. This discrepancy might be due to different animal species, breed, and semen extender as well as different cooling and freezing protocols. For instance, permeability of sperm plasma membrane to cryoprotectant and the consequent osmotic damage was species-dependent because each animal species has different structure and composition of sperm plasma membrane.

It is well known that the sperm plasma membrane considered being the foremost site of freeze-thaw damage due to its high content of poly unsaturated fatty acids that render it more vulnerable to the intracellular ice crystals formed during the freezing process. This membrane damage lead to loss of lipids and phospholipids (39), cholesterol and consequently, the ratio of poly unsaturated fatty acids and saturated fatty acids is disturbed. On the basis of results obtained in the present study that GLY provide higher proportion of intact-plasma membrane, % AR and % relative AR post-thaw

thus, GLY have utmost cryoprotective effect on buffalo-bull sperm plasma membrane in comparison with EG and DMSO.

Incorporation of GLY in semen diluent used for frozen storage of buffalo-bull semen significantly improved its post-thaw quality and function than both EG and DMSO might be due to EG has toxic effect on sperm cells where it badly affects the motility of fresh and frozen-thawed semen (40). Furthermore, EG could not reverse the capacitation like changes (cryocapcitation) of frozen-thawed sperm cells (41) which might be the plausible explanation for the improved function (in terms of % AR and % relative AR) of sperm cells frozen in the presence of GLY compared to those frozen in the presence of EG.

Since the true test to evaluate a semen sample is to estimate the fertility rate after its *in vivo* insemination in a routine AI program under field conditions (42). Then GLY was efficient in cryopreservation of buffalo-bull sperm as the higher *in vivo* fertility was recorded with extender having 7% GLY compared to 5% EG and 5% DMSO. This high fertility is a logical result for the improved sperm characteristics viz; progressive motility, plasma and acrosomal membranes integrity, viability, % AR and % relative AR suggesting that all the sperm characteristics used to assess the semen quality do have substantial role in the fertilization process (43). In conclusion, incorporation of 7% GLY in the semen extender significantly improves *in vitro* quality and *in vivo* fertility of buffalo-bull semen.

Conflict of interest

All the authors declare no conflict of interest.

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Original Research Article

SYNERGISTIC AMELIORATIVE EFFECT OF *Lactobacillus* AND *Spirulina platensis* AGAINST EXPERMINTAL COLITIS IN ALBINORATS: ANTIOXIDANT, HISTOPATHOLOGICAL AND MOLECULAR STUDIES

Emad W. Ghazy[1], Abd-Allah A. Mokhbatly[1], Samah S. Keniber[1*], Khaled M. Shoghy[2].

[1]Clinical Pathology Department, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt, [2]Anatomy & embryology Department, Faculty of Veterinary Medicine, University of Sadat city, Egypt

*Corresponding author, E-mail: samoha.saber@yahoo.com

Abstract: Ulcerative colitis (UC) considers one of inflammatory disorders which affect colon mucosa cause a substantial burden on human day life. In the past, treatment of UC depended on aminosalicylates and antibiotics but due to their adverse side effects and incomplete effectiveness, antioxidant anti-inflammatory agents are used nowadays to ameliorate UC. The aim of this work is to evaluate the modulatory effect of *Lactobacillus* and/or *Spirulina* oral administration in acetic acid induced colitisin albino rats. Rats were divided randomly into (6) groups. 1stgroup (negative control), 2nd group (acetic acid), 3rdgroup (Mesalazine) at dose 20mg/kg orally was used as positive drug control. 4th group (*Lactobacillus*) at dose1×10^9 CFU/rat daily. 5th group (*Spirulina*) at dose500mg/kg daily. 6thgroup (*Lactobacillus* at dose 1×10^9 CFU/rat + *Spirulina* at dose 500mg/kg). Results revealed that the experimental colitis group showed significant increase in DAI, macroscopic damage, colon weight, colonic MDA, NO, molecular expressions (iNOS and COX-2) and significant decrease in colon length, GSH level and CAT activity. *Lactobacillus* and/or *Spirulina* supplementation revealed significant improvement in macroscopic and microscopic finding, increase antioxidant biomarkers, significant inhibitions of MDA and nitric oxide. Furthermore, significant decline in COX-2 and iNOS expressions were reported. In conclusion, the protective effects of *Lactobacillus* and/or *Spirulina* in UC are due to their ability to reduce iNOS and COX-2 expressions, increase antioxidant biomarkers and significant inhibition of lipid peroxidations. Furthermore, *Lactobacillus* and *Spirulina* have synergistic protective effect on colon tissue and could be used in combination to ameliorate UC.

Key words: colitis; acetic acid; *Lactobacillus*; *Spirulina platensis*; Mesalazine; antioxidant, anti-inflammatory.

Introduction

Inflammatory bowel disease (IBD) is a an inflammatory disorder of the gastro intestinal tract, including ulcerative colitis (UC) and Crohn's disease(CD) (1). Ulcerative colitis is restricted to the colon mucosa, while any part of the whole gastrointestinal tract can be af-

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fected in Crohn's disease (2). It's occurrence and prevalence are common in any age and different area around the world, that means its emergence global disease(3). Ulcerative colitis pathogenesis is not completely understood. There are many factors affected these diseases, including immune genetic factors (4), abnormal micro biota (5), epithelial barrier disruption, broken of intestinal microbiota and other environmental factor (6).

The most clinical signs of colitis are abdominal tenderness, bloody mucous stool, purulent stool, and relapse. Furthermore, diarrhea, interrupted digestion, loss of body weight and an extensive burden on daily life (7). These clinical manifestation were established by scoring of disease activity index (DAI) (5).

Acetic acid is considered one of the main chemical widely used animal model to induced ulcerative colitis (8). Pathogenesis, histopathological features and inflammatory mediator profile to this type of colitis is phenotypically more identical to human IBD (9).

Specialized intestinal epithelial cells (IECs) are considered the physical barrier of luminal microbiota and has important role in maintaining intestinal homeostasis. So, any disturbance in the epithelial layer and intestinal permeability lead to dysregulated intestinal immune homeostasis and lead to IBD (2). Ulcerative colitis induced an increase reactive free radicals inductions and pro-inflammatory cytokines production. Furthermore, it showed significant intestinal epithelial cells apoptosis which disintegrate intestinal mucosal and barrier function. At the same time, inducible nitric oxide synthase (iNOS) (10) and cyclooxygenase-2 (COX-2) production are increased that playing a critical function in the incidence of this disease (11).

Probiotics known as "live microorganisms when supplemented in sufficient numbers, they induce health benefits to the host "(12). *Lactobacillus* is considered one of probiotics species which improving the component of intestinal micro flora(13), preventing action on IBD, and relieving colic symptoms(14). Furthermore, *Lactobacillus* has pronounced antimicrobial effect (15) and may change gut pH by producing some acids which prevent growth of another pathogenic bacteria(16).

Spirulina platensis is one of marine blue green microalgae with high nutritional values(17). It is rich source for many minerals, vitamins, protein and antioxidant anti-inflammatory compounds such as carotenoids, and phycocyanin pigment (18).Regarding to several effects of *spirulina*, it includes anticancer (19), antiviral (20), anti-allergic (21), antimutagenic (22), cytoprotective (23) and cardioprotective effects (24). Moreover it induces blood vessel-relaxing effect (25), hypocholesterolemic effect(26), hypolipidemic actions (27). In addition, hepatoprotective (28), neuroprotective, reduced concentrations of tumor necrosis factor (TNF-alpha) (29)and immune-enhancing action (30) were also reported.

In the past, treatment of UC depended on aminosalicylates, antibiotics, steroids, and immune modulators but incomplete effectiveness and their adverse side effects, natural antioxidant anti-inflammatory agents are used nowadays to ameliorate UC (10).

For that reason, the present work aimed to judge the modulatory effects of oral administration of *Lactobacillus* and/or *Spirulina platensis* in experimental colitis models in rats by estimating antioxidant parameters as well as molecular and histopathological investigations.

Materials and methods

Chemicals

Lactéal fort (*Lactobacillus* LB) capsules purchased from Tenth of Ramadan for pharmaceutical industries &diagnostic reagents (Rameda), Egypt. *Spirulina* purchased from the Algal unit of Biotechnology (National Research Center, Dokki, and Cairo, Egypt). Marsalaz tablet purchased from Marci pharmaceutical industries El- Obour City, Egypt. Acetic acid obtained from El-Nasr Pharmaceutical Chemicals Company (Cairo, Egypt). Diethyle ether obtained from (spinreact) Spin. EDTA from (Salix). Malondialdehyde (MDA), Catalase (CAT), Nitric oxide (NO) and Glutathione reduced (GSH) purchased from BIO-

DIGNOSTIC Company kits-Egypt. All chemicals utilized in this study was of analytical grade.

Animals and feed management

Forty-nine male albino rats of average weight (100 g/rat) were purchased from Animal House Colony of the Tanta Center. The rats were adapted to standard laboratory conditions (temperature 22–25°C, relative humidity 50–60%), rats were fed a balanced diet and water *ad libitum*.

Experimental design

Rats after adaptation period (2 weeks) were distributed randomly into six (6) groups (8 rats in each except control positive 9 rats). The 1st group (negative control), received saline orally for 10 days then followed by normal saline instillation at tenth day per rectum. The 2nd group (positive control), received saline orally for 10 days followed by 2ml of acetic acid (4%) intrarectally (10)at tenth day. The 3rd group (Mesalazine group) received 2ml acetic acid (4%) rectal instillation on day 10, then given Mesalazine orally at dose (20mg/kg) (31)for 5days after induction of colitis. The 4th group (*Lactobacillus* group) received daily *Lactobacillus* at dose (1×10^9CFU) orally/rat (14)for 10 days, then rectal instillation of 2ml acetic acid (4%) at tenth day. The 5th group (*Spirulina* group) received *Spirulina* daily at dose (500mg/kg) orally(32)for 10 days, then rectal instillation of 2ml acetic acid (4%) at tenth day. The 6th group (*Spirulina* + *Lactobacillus*) (combination group) oral received both *Spirulina* (500mg/kg) and *Lactobacillus* (1×10^9CFU) daily for 10 days, then rectal instillation of 2ml acetic acid (4%) at tenth day. All groups were observed daily for clinical signs, mortality rate, body weights, food and water intakes of the rats from each group. The rats were sacrificed at 5thday from induction of colitis, rats were slaughter under diethyl ether anesthesia. Colon segments were dissected, removed adipose tissue, washed with normal saline, for macroscopic and microscopic examination. Colon samples were preserved immediately at-80°C for molecular and oxidative stress analysis.

Induction of colitis

Colitis was produced by intra-colonic administration of 4% acetic acid (2ml)(33). Through a lubricant rectal pediatric urinary catheter under low-dose of ether anesthesia. Briefly, animals were restricted from food for 24h (34). The instillation site was about 8cm from the anal margin into the rectum. After instillation directly rats were maintained in vertical position for about 1 min to prevent acid leakage (35). Animals in the group one exposed to the same practice with saline instead of the acetic acid solution.

Assessment of disease activity index

According to (36)**,** clinical signs in colitis were determined by evaluating the disease activity index (DAI)(table 1). The disease activity index includes (stool consistency, presence of rectal hemorrhage and decrease in body weight), DAI in all the tested groups were observed daily after induction of colitis by 4% acetic acid.

Assessment of scoring severity of colitis

For macroscopic damage score (Table 2), the colon was examined visually either for adhesions or gross morphological alterations immediately after death (37).

Determination of colon weight and colon length

After separated the colon from adipose tissue and remind intestine must be determine the colon weight (g) and colon length (cm).

Tissue sample for histopathology and antioxidant biomarkers

Tissue specimens of colon were rapidly taking and equally divided into three parts : proximal part for molecular investigation (rapidly stored at -80°C) (38)**,** middle part used for histopathology, and distal part of colon stored at -20°C and used for oxidative stress and antioxidant parameters.

Estimation of antioxidant biomarkers of colon tissue homogenates

Prior to dissection, distal part of colon were rinsed with phosphate buffered saline (PBS) solution, pH 7.4, containing 0.16 mg/ml heparin to get rid of any red blood cells and clot. Then tissue homogenize in 5-10 ml buffer (i.e. 50 Mm potassium phosphate pH 7.4, 1Mm EDTA and 1 ml/l triton x-100 /g. tissue and Centrifuge at 4.000 rpm for 15 min. at 4^{o}C. The supernatant was separated into an Eppendorf tube and was preserved at −80^{o}C into aliquots for the spectrophotometric analysis of lipid peroxidation content by Malondialdehyde (MDA)(39), nitric oxide (NO) (40), Catalase (CAT)(41), and reduced Glutathione (GSH) estimation (42).

Detection of gene expression quantitatively by real time PCR

Forward and reverse primers sequence for *iNOS*, *COX2* and *β-actin* genes are presented in Table 3**.** Tissue RNA was extracted with Trizol (total RNA isolation reagent, iNtRON Biotechnology, Inc). Complementary DNA (cDNA) was synthesized by using Oligo (dT) primer HiSenSripte TMRH cDNA synthesis kits (IntROn) as described by the manufacturer's directions. The SYBR green was performed using BIoRad IQ2 (Japan) and the following protocol was used (43).The mRNA expression levels were normalized using β-actin.

Histopathological studies

Histopathological tissues preparation and examination was done according to (44)using H&E.

Statistical analysis

Data were presented as means ± S.E. using one-way ANOVA followed by Newman-keuls multiple comparisons using graph pad prism 7 software. Statistical significance was acceptable to a level of P ≤0.05

Results

Mortality rate

Control negative group, fed on standard diet, showed no abnormal clinical signs or mortality during the whole period of experimental. Mortality rate showed in table 4. Generally, the control positive group revealed obvious increase in mortality rate (4/9) (44.4%). Meanwhile, administration of *Lactobacillus* alone decreased mortality rate (2/8) (25%). *Spirulina* alone also decrease mortality rate (2/8) (25%). Furthermore, the co-administration of *Lactobacillus* with *Spirulina* led to pronounced decrease in mortality rate (1/8) (12.5%), similar to Mesalazine group which used for ulcerative colitis treatment.

Disease activity index

The effect of *Lactobacillus* and/or *Spirulina* in experimental colitis on DAI was explained in figure1. Data demonstrated significant ($p \leq 0.05$) elevation in DAI in acetic acid group as compared with control one. Mesalazine group revealed insignificant decrease in DAI in comparison with control positive group. Similarly, *Lactobacillus* group caused pronounced decrease in DAI but this reduction was still insignificant as contrasted with control positive group. Meanwhile, *Spirulina* alone or in combination with *Lactobacillus* revealed significant ($p \leq 0.05$) improvement in DAI as matched with control positive group.

Scoring severity of colitis

The effect of *Lactobacillus* and/or *Spirulina* on macroscopic damage score was illustrated in figure 2. Data explored that significant ($p \leq 0.05$) increase in damage score in acetic acid positive colitis as compared with the negative none treated one. Whereas, all other treated groups reported significant ($p \leq 0.05$) reduction in score damage in comparison with the positive control group.

Colon weight and length

The effect of *Lactobacillus* and/or *Spirulina* on colon weight and colon length was showed in table 4 and figure 1. Concerning to colon

weight, the obtained data illustrated significant ($p \leq 0.05$) increase in the control positive group as contrasted with control negative one. On the other hand, Mesalazine group showed a decrease in colon weight but still insignificant as matched with control positive group. At the same time, simultaneous supplementation of *Lactobacillus* and/or *Spirulina* in acetic acid (4%) induced colitis revealed a significant ($p \leq 0.05$) improvement in colon weight as compared with control positive group. Furthermore, control positive group showed significant ($p \leq 0.05$) decrease in colon length in comparison with control negative group. Meanwhile, Mesalazine group revealed a significant ($p \leq 0.05$) increase in colon length as contrasted with control positive group. Similarly, a significant ($p \leq 0.05$) increase in colon length was detected in *Lactobacillus* and/or *Spirulina* groups as matched with control positive group.

Lipid peroxidation and antioxidant biomarkers:

The effect of *Lactobacillus* and/or *Spirulina* on Lipid peroxidation and antioxidant biomarkers were portrayed in figure 2. The data illustrated a significant ($p \leq 0.05$) increase in MDA and nitric oxide in colon tissue homogenate in the control positive group as compared with control negative one. Meanwhile, control positive group showed marked decrease in GSH and CAT but this decrease still statically insignificant in comparison with control negative group. Nevertheless, Mesalazine group revealed a significant ($p \leq 0.05$) decrease in MDA and NO content as contrasted with control positive group. Meanwhile, treatment by Mesalazine revealed increase in GSH and CAT but still insignificant as matched with control positive group. Regarding, *Lactobacillus* or *Spirulina* supplementation in acetic acid (4%) induced colitis group showed a significant ($p \leq 0.05$) decrease in colon tissue content of MDA and NO as compared with control positive group. Meanwhile, increase in GSH and CAT were observed but still insignificant in contrast with control positive one.

Similarly, the co-administration of *Lactobacillus* with *Spirulina* revealed a significant ($p \leq 0.05$) decline in MDA and NO as contrasted with control positive group. On the other hand, obvious enhancement in GSH and CAT were observed as matched with control positive group.

Molecular investigation

The effect of *Lactobacillus* and/or *Spirulina* in acetic acid (4%) induced colitis on the quantitative gene expression by real time PCR were illustrated in figure 2. The data reflect a significant ($p \leq 0.05$) increase iniNOS and COX2expressionin the control positive group as matched with the control negative one. On the contrary, Mesalazine group showed a significant ($p \leq 0.05$) reduction in iNOS and COX2 expression as compared with control positive group. Gastric intubations of *Lactobacillus* or *Spirulina* showed a significant ($p \leq 0.05$) improvement in iNOS and COX2expressionin comparison with control positive group. At the same time, simultaneous administration of *Lactobacillus* with *Spirulina* showed a significant ($p \leq 0.05$) decline in iNOS and COX2 expression as contrasted with control positive group.

Histopathological findings

The effect of *Lactobacillus* and/or *Spirulina* in acetic acid (4%) induced colitis on histopathological features was showed in figure 3. Colon of rats in control negative group demonstrated normal epithelium, intestinal glands and intestinal lumen. Colon of rats in none treated positive group (severe colitis) revealed necrosis of surface epithelial and enterocytes, deposition of irregular collagen fibers, crypt distortion, and loss of intestinal glands, mononuclear cell infiltration and apoptosis of mononuclear cell. Colon of rats in the Mesalazine group (colitis) revealed patchy mucosal necrosis, proliferation of submucosal lymphoid elements such as, lymphocytes, macrophages and plasma cells with submucosal edema and necrosis together mononuclear cell infiltration. Colon of rats in the *Lactobacillus* group (moderate colitis) showed mono-

nuclear cell infiltration in the mucosa and submucosa, in addition to, congestion of submucosal blood vessels and submucosal edema. Meanwhile, *Lactobacillus* group showed cell infiltration between the mucosal glands with necrosis of the surface enterocytes. Colon of rats in *Spirulina* group (mild colitis) showed mononuclear cell infiltration in the mucosa and between the mucosal glands, edema in tunica muscularis, and normal surface epithelium. Colon of rats in *Lactobacillus* with *Spirulina* group (mild colitis) showed only mononuclear cell infiltration between the mucosal glands with normal surface epithelium.

Table 1: Scoring of disease activity index (DAI)

Score	Weight loss %	Stool consistency	Occult/gross bleeding
0	0	Normal	Normal
1	1-5%	-	-
2	5-10%	Loose stools	Occult blood
3	10-15%	-	-
4	> 20%	diarrhea	Gross bleeding+ mucous

Table 2: Primers used for qPCR

Gene	primer sequence (5/ ------3/)	Reference
iNOS	F:CCTCCTCCACCCTACCAAGT R: CACCCAAAGTGCTTCAGTCA	(Villarán et al., 2010)
COX2	F:TGCGATGCTCTTCCGAGCTGTGCT R:TCAGGAAGTTCCTTATTTCCTTTC	(Bhatia et al., 2008)
β-actin	F: TGTGATGGTGGGAATGGGTCAG R: TTTGATGTCACGCACGATTTCC	(Villarán et al., 2010)

Table 4: colon lesion parameters (mean ± SE) in control and different treated groups

Groups / parameters	C-ve	C+ve	M	L	S	L+S
Mortality rate%	0%	44.4%	12.5%	25%	25%	12.5%
Body weight change	104.2 ± 2.392^{a}	81.6 ± 1.97^{b}	103 ± 2.43^{a}	102.9 ± 1.96^{a}	93 ± 2.77^{a}	95.5 ± 3.6^{a}
Colon weight	1.28 ± 0.09^{b}	1.7 ± 0.15^{a}	1.45 ± 0.08^{ab}	1.36 ± 0.08^{ab}	1.43 ± 0.13^{ab}	1.4 ± 0.04^{ab}
Colon length	14.54 ± 0.20^{a}	12 ± 0.58^{c}	13.2 ± 0.2^{b}	13.75 ± 0.48^{ab}	13.67 ± 0.33^{ab}	13.8 ± 0.37^{ab}
weight/length ratio	0.09 ± 0.01^{b}	0.15 ± 0.02^{a}	0.11 ± 0.0^{b}	0.10 ± 0.01^{b}	0.11 ± 0.02^{b}	0.10 ± 0.01^{b}

C-ve: Control negative, C+ve: Control Positive, (M): Mesalazine group, L: *Lactobacillus* group, SP: *Spirulina* group, L+SP: *Lactobacillus* +*Spirulin*a. Data were statistically analyzed as mean ± SEM. Rows carrying different superscript letters are significantly different at $p\leq0.05$.

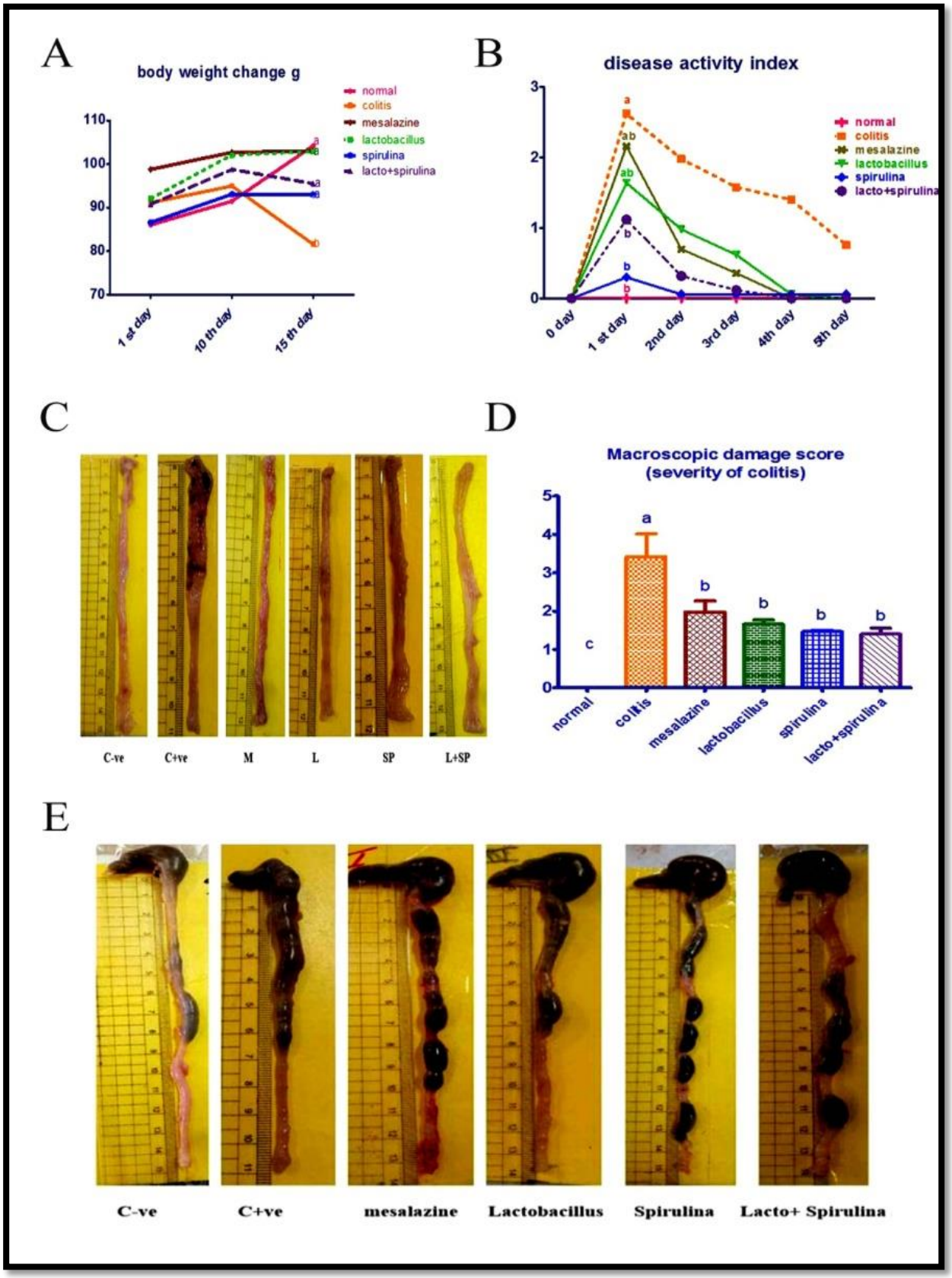

Figure 1: A) refer body weight change (g), B) disease activity index, C) Macroscopic damage in control and different treated groups, D) Colon length in control and different treated groups

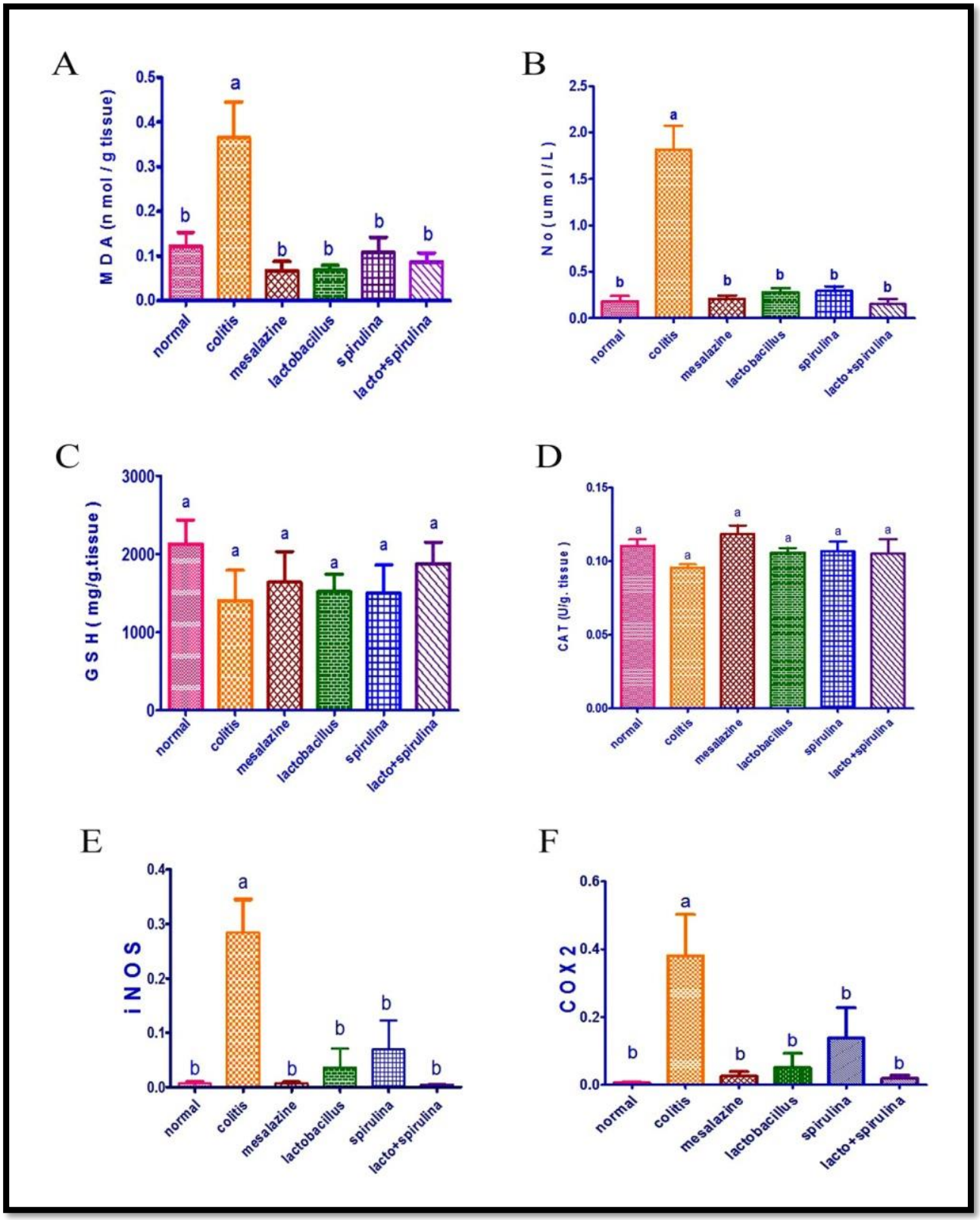

Figure 2: A, B) Lipid peroxidation, C,D) antioxidant biomarkers and E,F) molecular gene expression in control and different treated groups. Data were statistically analyzed as mean ± SEM. Rows carrying different superscript letters are significantly different at $p \leq 0.05$

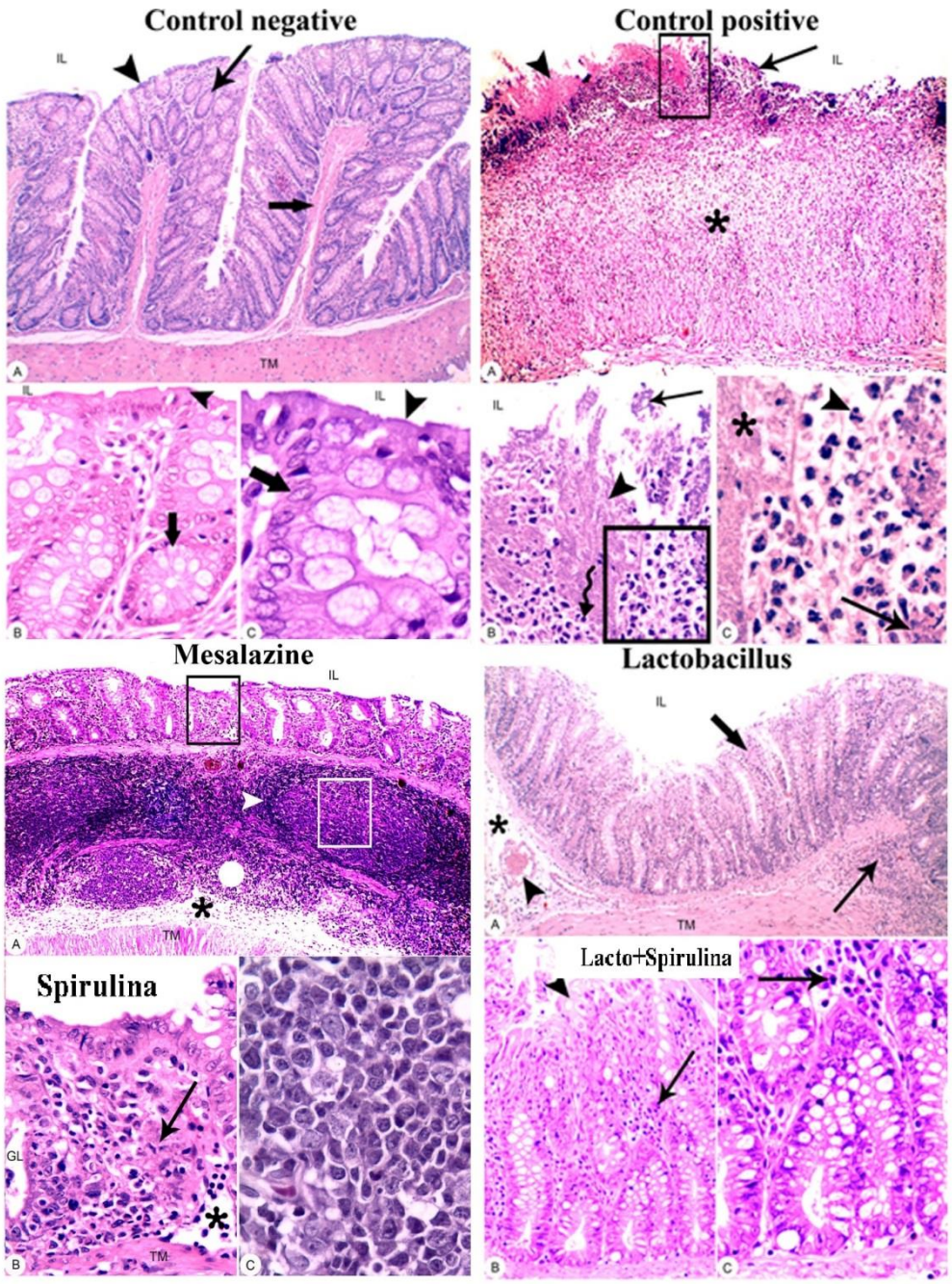

Figure 3: Histopathological feature in control and different treated groups. Microscopic features of colon rats by H&E stain. 1. Normal group, normal histologic architectures. 2. Acetic acid group (acute chemical colitis): showing necrosis of epithelial surface with deposition of irregular collagen fibers, crypt distortion, and loss of intestinal glands. 3. Mesalazine treated group, colitis: showing patchy mucosalnecrosis, proliferation of submucosal lymphoid elements (lymphocytes, macrophages and plasma cells), and submucosal edema.4. *Lactobacillus* treated group moderate colitis: showing mononuclear cell infiltration in the mucosa and submucosa, congestion of submucosal blood vessels and edema. 5. *Spirulina* treated group, mild lymphocytic colitis: showing mononuclear cell infiltration in the mucosa, edema with normal surface epithelium. 6. *Lactobacillus+Spirulina* treated group, mild lymphocytic colitis: showing mononuclear cell infiltration in the mucosa, with normal surface epithelium

Discussion

Ulcerative colitis is an inflammatory condition which cause abdominal pain, bloody diarrhea and mucous in stool(7). Furthermore, weight loss and other many symptoms that differ from person to person and it may be lead to colorectal cancer (45). The study designed to evaluate the effect of some natural agents (*Lactobacillus* and/or *Spirulina*) to ameliorate the clinical manifestation of acetic acid (4%) induced colitis in rats.

The present study exhibited significant increase in DAI and macroscopic damage in control positive rats as compared with the normal negative one. This increase could be referred to loss of appetite, decrease food consumption and feverish conditions which resulted in body weight reduction(46). Others referred this increase in DAI due to high inflammatory response and occasional ulceration that induced bloody diarrhea as confirmed by elevated mortality rate percent (44.4%), histopathological lesions and high macroscopic damage score and these results parallel with (9, 47).

At the same time, rats of control positive group showed significant increase of colon weight with significant reduction in colon length as compared with non treated one that might be referred to cellular swelling which resulted from shift of extracellular water into the cells associated with neutrophils and macrophage infiltration. Furthermore, cells turgor, submucosal edema, vascular dilatation and goblet cell hyperplasia. Similar result was obtained by(48, 49).

Oxidative stress and lipid peroxidation play acritical role in pathogenesis of ulcerative colitis (50, 51). Control positive animals revealed marked reduction in CAT and GSH, meanwhile, a significant elevation of MDA and NO was recorded as contrasted with control negative group. These results run parallel with those obtained by (52)

Generation of ROS and free radicals from migrated neutrophils attack the cellular macromolecules and lead to epithelial cell disruption with extensive colon damage(53, 54). Furthermore, ROS lead to massive oxidation of cell membrane phospholipids, proteins, and DNA. This oxidation cause further stimulates of more neutrophils and macrophage infiltration to damaged tissue (49).Consequently, intestinal mucosa to regulate ROS levels, have enzymatic and non-enzymatic complex antioxidant defenses mechanism such as reduced glutathione (GSH) and catalase (CAT), which try to heal and repair the damaged cells. Moreover, GSH and CAT were consumed by inflamed colon tissues to neutralize oxidative stress(55, 56).

Malondialdehyde (MDA) considered the end result of lipid peroxidation which caused by ROS. Based on that, the elevated MDA in acetic acid (4%) induced colitis rats referred to the increased lipid peroxidation and high cell damage(57, 58).

Nitric oxide (NO) is produced by some inflammatory cells such as granular leukocytes (neutrophils) or granular leukocytes (monocytes, and macrophages) as well as extravascular compartment as epithelial cells from inflamed colon tissue. Thereby, elevated NO content in experimental colitis considered as an index of inflammation(59).

Molecular genes expressions (iNOS and COX-2) are considered important gene expressions in acetic acid induced colitis, and they have synergistic effect to augmented the inflammatory reaction(60). Furthermore, recent investigations reported close interrelationship existing between iNOS and COX-2 expressions at sites of inflammation, and leading to excessive induction of inflammatory mediators which may causing the development of intestinal damage(61).

In this study, colon tissues exposed to acetic acid produced an over expression of iNOS and COX-2messenger as compared with the control negative one, these results were supported by findings of (62, 63).

Concerning to inducible nitric oxide synthase (iNOS), it is an enzyme usually expressed during inflammatory reactions(64). Meanwhile, synthesis of large amounts of nitric oxide (NO) content is demonstrated in acute or chronic inflammation, and it produced largely when iNOS expression is increased.

Overall, elevated of iNOS expression may be an indicator of inflammation progression(10).

Enhancement of COX-2 (inducible cyclooxygenase enzyme) expressions from inflamed colon tissues play an integral role in the pathogenesis of ulcerative colitis (65, 66). Moreover, excessive over expressions of COX-2 lead to prostaglandin (PGE) releasing (67), and ROS production which cause further cell injury (65).

Histopathological finding go side by side with the obtained macroscopic damage score, oxidative and anti-oxidative biomarkers in addition to molecular gene expressions, these data were in harmony with those obtained by(68).

Concerning to Mesalazine, it considered positive drug control which inhibit oxidative stress and ROS releasing from inflamed colon tissue. Moreover, its antioxidant (31) anti-inflammatory effects (69)reduced tissue damage and modulated mortality rate (12.5%).

The current data declared that, gastric intubation of *Lactobacillus* on acetic acid induced colitis showed marked decrease in DAI in comparison with control positive group. This improvement of DAI could be owed to either increase body weigh by increasing energy efficiency (70) or decrease bloody diarrhea and these results were confirmed by decrease mortality rate percent (25%). On the other hand, *Lactobacillus* supplementation revealed significant decline in macroscopic damage score which was confirmed by reduce colon weight associated with significant enhancement of colon length as contrasted with control positive group (71).

The damage score was ameliorated due to the ability of *Lactobacillus* to modulate inflammatory response of colon tissue through interferes with innate immune system and adaptive immunity. Therefore, it enhances anti-inflammatory cytokine production (72). Others attributed the reduction of inflammation by *Lactobacillus* to bacteriocins secretions which act as broad spectrum antimicrobial substances and protect against secondary infections, Consequently decreasing the duration of diarrhea (73). In the other hand, *Lactobacillus* may changepH of the gut flora which leading to reduce inflammatory state of colon(74).

At the same time, lipid peroxidation and antioxidant biomarkers evaluate ability of *Lactobacillus* to reduce ROS and free radicals from inflamed colon (75). In this study, supplementation of *Lactobacillus* revealed an improvement of antioxidant biomarkers (GSH and CAT) and decreased in oxidative stress parameters (MDA and NO) as matched with control positive group, these facts were confirmed by result of (76). Multiple experimental studies demonstrated the antioxidative activities of *Lactobacillus* by secreting enzymatic and non enzymatic anti-oxidant substance and promoting its release from the inflamed colon tissue (77).

Moreover, *Lactobacillus* enhance the production of particular antioxidant biomolecules, for example, exopolysaccharides (EPSs) which probably useful for elimination of oxidative stress from intestine (78). Consequently decrease lipid peroxidation (MDA) and NO marker from inflamed colon tissue. Finally, it exhibited metal chelating activities which get together with the pathogenesis of most chronic diseases(79). These results were confirmed by significant decrease in iNOS andCOX-2 expression in *Lactobacillus* supplemented group as matched with control positive group, these data are in harmony with those obtained by(80).

Consistent with this mechanism, histopathological findings of *Lactobacillus* group were showed moderate colitis. This finding agrees with (12). Therefore, *Lactobacillus* had many therapeutic benefits and was used as vehicles for treatment of gastrointestinal diseases (81)

In this experiment, *Spirulina* supplementation played great role in relieving the incidence of induced colitis. Significant enhancement in DAI and macroscopic damage score were recorded in *Spirulina* group as compared with control positive group, this effect could be explained by the high protein content, amino acids, vitamins (vitamin B complex) and folic acid which induce an increase in nutri-

tional value of this algae and promote weight gain (27).

Similarly, a significant decline in bloody diarrhea was found in *Spirulina* group reach to (0%) may be due to mucopolysachharids content which makes building blocks of colon cell membranes and accelerates healing of colon tissue (30) These results were confirmed by decreased mortality rate to 25% and histopathological findings. Furthermore, gastric intubation of *Spirulina* in acetic acid induced colitis showed a marked decline in colon weight with significant increase in colon length as contrasted with control positive group. This improvement could be referred to ability of *Spirulina* to manage the inflammatory conditions and oxidative damages (82). Phycocyanin present in *Spirulina* considers anti-inflammatory ingredients of it, which decrease production of intracellular ROS and histamine from mast cell, inhibit inflammatory cell infiltration specially neutrophil and reduced edema index in the induced inflammation(83, 84). Besides that, *Spirulina* has excellent antioxidative properties and preservative effects to structural integrity of colon tissue(85). In present study, *Spirulina* supplementation showed improvement in CAT and GSH and significant decrease in NO and MDA in comparison with control positive group. These results confirmed by molecular RNA expression of iNOS and COX-2 which showed significant decrease in contrast with control positive group. The antioxidant anti-inflammatory effects of *Spirulina* could be attributed to its content of phycocyanin and β-carotene which have capability to scavenge free radicals and ROS from inflamed colon tissue (86).

In the same way, *Spirulina* decreased inflammatory mediators and inflammatory cytokines (IL-1β, IL-6, and IL-12) releasing from inflamed colon (87). Therefore, it plays a fundamental role in UC improvement. Moreover, histopathological finding of *Spirulina* showed mild colitis, and this result supported by finding of (32).

Noteworthy, the combination of *Spirulina* and *Lactobacillus* in acetic acid induced colitis afforded a higher protection and more effectiveness than each one alone, this result was confirmed by recording the lowest mortality rate (12.5%), molecular expressions and histopathological findings similar to positive drug control (Mesalazine), that owed to an improvement growth performance and health condition (88). *Spirulina* not only modulate inflammatory response of acetic acid induced colitis, but also it consider as importance nutritional point for *Lactobacillus* due to rich source of protein content, amino acids, vitamins etc which could be needed to nourishment of *Lactobacillus* and improve intestinal colonization (89, 90). Over all, *Spirulina* has growth promoting effect on *Lactobacillus* to reduce the inflammatory effect of ulcerative colitis.

Conclusion

In conclusion, the protective effect of *Lactobacillus* and /or *Spirulina* against experimental colitis in rats could be directly attributed to scavenging ROS, inhibiting lipid peroxidation and suppressing NO releasing. Furthermore, *Lactobacillus* and *Spirulina* have synergistic protective effect on colon tissue and could be used in combination to ameliorate ulcerative colitis.

Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

HEMATOLOGICAL AND BIOCHEMICAL PROFILE IN FEMALE CAMELS (*CAMELUS DROMEDARIUS*) DURING THE TRANSITION PERIOD

Eman A.Ebissy[1], Ahmed A. El-Sayed[1]*, Ragab Hassan Mohamed[2]

[1]Department of Animal health and Poultry, Animal and Poultry Production Division, Desert Research Center (DRC), Matariya, Cairo, Egypt, [2]Theriogenology Department, Faculty of Veterinary Medicine, Aswan University, Aswan, Egypt

*Corresponding author, E-mail: decernes@yahoo.com

Abstract: The aim of the present study was to evaluate the hematological and biochemical alterations in female camels during the transition period. Ten apparently healthy camels were randomly selected and subjected to clinical examination. The investigated camels demonstrated no detectable clinical illness and kept under veterinary supervision throughout the study period. A blood sample was collected from each camel for laboratory investigation before and after 2 and 4 weeks of parturition and at time of parturition. Our findings revealed a significant (p=0.001) high values of neutrophils, MCH and MCHC at +14, and a significant (p=0.001) low values of lymphocyte count at -14 and at time of parturition. The globulin concentration increased significantly (p=0.029) at +28. For serum phosphorus and magnesium concentrations, their values were significantly (P< 0.05) elevated at -14 and +28, respectively, while enzymatic activity of liver enzymes including aspartate aminotransferase (AST), γ-glutamyl transferase (GGT) and alkaline phosphatase (ALP) were significantly (P< 005) elevated at +14, while serum activity of GGT and ALP continued at the same pattern at +28. For serum glucose and cortisol concentrations, their values were significantly elevated at (0), while serum creatinine concentrations were significantly (p=0.023) decreased at +14. Estrogen concentration increased significantly at -14 and 0, but decreased significantly at +14 and +28. Progesterone concentrations increased significantly at -14 and decreased at 0, +14 and +28. Other tested parameters did not differ significantly pre- and post-partum. The data presented in this study could be used as a reference guide for female camels during the transition period.

Key words: Biochemistry; camel; hematology; transition period

Introduction

Dromedary camels are characterized by a seasonal activity and induced ovulators. Reproductive physiology of Arabian dromedary camels has gotten little interest compared to other animal species, especially in relation with changes in blood constituents. Changes in several biochemical parameters have been reprimanded for reproductive disappointments (1).

Severe economic losses can result from suboptimal transition of pregnant animal from the late-pregnancy to lactation; this could probably to impaired production and reproductive performance (2, 3). Hence, optimal transition

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requires a comprehensive understanding of biochemical alterations that occurs during the transition period (4).

The transition period, defined as three weeks around the time of calving, is characterized by distinct endocrine alterations that are much more dramatic than at any other time stage of lactation–gestation cycle (2, 5). In Egypt, only limited data on serum biochemistry and hematology of one humped camel are available in the literature (6) and most of these literatures encompass limited laboratory measurements (7, 8). Therefore, the present study is an attempt to provide an insight into the dynamics of selected haematobiochemical alterations in the transitional one humped camel in order to provide potentially new and useful information about the guidelines for the management strategies during different physiological phases.

Materials and methods

Animals

The present study included ten apparently healthy female dromedary camels reared at Mariut Research Station, Desert Research Center, El-Amria, Alexandria, Egypt. Their mean body weight was 415 kg (range: 313 - 590 kg) and their mean ages were 12.6 years (range: 10 -15 years). All procedures were performed in accordance with the guidelines of Desert Research Center (Egypt) and approved by its Ethical Committees. The camels were considered clinically sound on the basis of physical examination of heart, lungs, rumen and intestine and other vital signs (9, 10) in tandem with the preliminary findings of hematological examination. Camels were housed in an open yard and fed on a maintenance ration composed of a concentrate mixture including 50% corn, 47% barley, 2% minerals, 1% salt which given at rate of 3 kg/head/day, while Egyptian clover hay (Trifolium alexandrinum) and fresh water were offered *ad libitium*.

Blood sampling

Ten milliliter of blood was collected from each animal at −28,−14, 0, +14 and + 28 days of expected date of delivery via jugular vein puncture using vacutainer tube containing anticoagulant (EDTA or sodium fluoride) and without anticoagulant to yield whole blood or serum, respectively. The tubes containing EDTA were used for prompt hematologic examination. The hematological indices included total leucocytes (WBC) and differential leukocyte count (lymphocytes, neutrophils, monocytes, and esinophils), red blood cells (RBC), hemoglobin (HGB), hematocrit, mean erythrocyte volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) which were carried out on automatic blood cell counter (Exigoeos veterinary Hematology system, Boule Medical AB, Sweden). On the other side, tubes containing sodium fluoride were used for quantifying concentrations of glucose which were measured spectrophotometrically using a commercial test kit supplied by Spectrum Egypt (Ref: 250-001).The plain tubes were kept at room temperature overnight to be centrifuged at 3000 rpm for 15 minutes. Only clear sera were collected then aliquoted and kept frozen at -20 0C for subsequent biochemical analyses using commercial test kits according to the standard protocols of suppliers. For the total protein, albumen, calcium, phosphorus, magnesium and creatinine, commercial test kits supplied by Bio-Med Egypt were used (Ref: ALB100250; TP116250; CAL103100; PH123100, MG122050 and CRE 106100), respectively. For AST, ALP, γ GT and cholesterol, commercial test kits supplied by Spectrum Egypt were used (Ref: AST260001; ALP214001;γ GT246001 and CHOL230001), respectively. For BUN, commercial test kits supplied by BioScien Egypt were used (Ref: BSU117100). For cortisol and estradiol, commercial test kits supplied by Parameter^{TM} USA were used (Ref: KGE008B and KGE014), respectively. For Progesterone, commercial test kit supplied by Oxford Biomedical Research USA was used

(Ref: EA 74). For creatinine kinase, commercial test kit supplied by Bio Chain USA (Ref: Z5030048) was used.

Statistical analysis

Statistical analyses were carried out using a statistical software program (SPSS, ver.20, Inc., Chicago, USA). Descriptive statistics were performed for all parameters. Repeated measures ANOVA was used to test the effect of physiological status during the transitional period on blood metabolic profile. Results were considered statistically significant at $P < 0.05$.

Results

An overview of serial measurements of hematological and serum biochemical profile in the studied camels during the transition period was illustrated in Tables 1 &2. Clinically, the investigated animals showed no detectable clinical alterations throughout the study period and remained clinically healthy. All camels demonstrated normal laboring and delivered a single calf without obvious clinical illness.

Table 1 illustrates the hematological variables in examined camels. The total white blood cells revealed non-significant differences (P = 0.296) through the different time points (-28, -14, 0, +14 and +28). Lymphopenia was observed at – 14 and 0 time points (P = 0.001) and neutrophilia at +14 and +28 postpartum (P = 0.001). There was no significant difference between Monocyte and eosinophils during the periparturient period (P = 0.730 and P=0.447, respectively). There was no significant difference between the red blood cells, haemoglobin, hematocrit and MCV (P>0.05) throughout the periparturient period. On the other side, the MCH and MCHC increased significantly at +14 after parturition (P=0.001).

Table 2 summarizes the biochemical variables in examined camels. Serum concentrations of the total protein and albumin concentrations did not differ significantly among all the tested time points pre- and post-partum (P>0.05). However, globulin concentrations were significantly (P=0.029) increased at +28. Calcium concentration did not differ significantly among all the tested time-points pre- and post-partum (P=0.201). While concentrations of serum inorganic phosphorus and magnesium were significantly (P=0.004 and 0.023) increased at -14 and +28 respectively. For serum activity of AST, GGT and ALP, their values showed a significant increase (P>0.05) at +14. The concentration of ALP and GGT remained also at a significant (P = 0.001) high pattern at +28 after parturition. In contrast the serum activity of creatinine kinase did not differ significantly pre- and postpartum (P = 0.125).

Concentrations of serum glucose were significantly (P = 0.001) elevated at the time of parturition. The serum concentration of BUN did not show any significance at the tested time points (P = 0.370). However, creatinine concentrations were significantly (P = 0.023) decreased at +14 post-partum. Serum concentration of cortisol showed a significantly (P = 0.001) elevation at the time of parturition. hand the concentration of serum estrogen was increased significantly at -14 and 0, but decreased significantly at +14 and +28 (P = 0.001). The serum progesterone level increased significantly at -14 and decreased at 0, +14 and +28 (P = 0.001).

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Table 1: Hematological parameters (mean ± SEM) in female dromedary camels during the periparturient period (n=10)

	- 28	- 14	0	+ 14	+ 28	P value
WBC ($\times 10^9$/L)	11.24 ± 0.49	8.52 ± 1.29	9.96 ± 1.22	11.75 ± 1.65	9.50 ± 0.68	0.296
RBC ($\times 10^{12}$/L)	10.17 ± 0.71	11.65 ± 0.25	10.74 ± 0.42	9.09 ± 1.23	10.90 ± 0.44	0.218
Hb (g/dl)	10.98 ± 0.82	11.64 ± 0.46	10.62 ± 0.55	12.30 ± 0.45	11.64 ± 0.96	0.414
PCV%	33.70 ± 1.24	30.56 ± 1.31	32.10 ± 1.44	33.00 ± 1.22	29.98 ± 1.64	0.319
MCV (fL)	31.42 ± 1.06	30.36 ± 1.71	30.06 ± 1.53	30.66 ± 1.25	28.14 ± 1.50	0.583
MCH (pg)	9.00 ± 0.61	12.90 ± 0.78	8.98 ± 0.54	14.74* ± 0.53	10.58 ± 0.46	0.001*
MCHC (g/dl)	31.95 ± 3.04	36.74 ± 2.19	26.65 ± 0.95	44.38 *± 0.24	32.45 ± 1.71	0.001*
Lymph ($\times 10^9$/L)	6.69 ± 0.32	3.01* ± 0.66	1.92* ± 0.31	3.69 ± 0.50	6.16 ± 0.52	0.001*
Monocyte ($\times 10^9$/L)	0.28 ± 0.04	0.31 ± 0.08	0.25 ± 0.04	0.23 ± 0.07	0.19 ± 0.07	0.730
Neutrophil ($\times 10^9$/L)	3.68 ± 0.31	3.18 ± 0.74	5.31 ± 0.15	8.20* ± 0.96	5.67* ± 1.14	0.001*
Eosinophil ($\times 10^9$/L)	0.25 ± 0.05	0.19 ± 0.02	0.26 ± 0.12	0.33 ± 0.07	0.21 ± 0.01	0.447

*Values with an asterisk within the same raw are statistically significant (P<0.05).
WBC: White blood cells; RBC: Red blood cells; Hb: Hemoglobin; PCV: Packed cell volume; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; Lymph: Lymphocyte

Table 2: Biochemical parameters (mean ± SEM) in female dromedary camels during the transition period (n=10)

	- 28	- 14	0	+ 14	+ 28	P value
Ca (mmol/l)	1.99 ± 0.09	2.18 ±0.11	2.22 ± 0.08	2.26 ± 0.05	2.16 ± 0.03	0.201
P (mmol/l)	1.76 ± 0.14	2.07* ± 0.05	1.70 ± 0.11	1.38 ± 0.07	1.76 ± 0.09	0.004*
Mg (mmol/l)	1.02 ± 0.06	1.02 ± 0.04	1.03 ± 0.03	1.00 ± 0.03	1.18* ± 0.02	0.023*
AST (U/l)	102.5 ± 4.11	109.75 ± 0.85	114.25 ± 2.39	124.25* ± 2.17	111.5 ± 4.99	0.005*
ALP (U/l)	33.75 ± 4.21	39.5 ± 4.99	49.5 ± 5.8	81.50* ± 4.09	65.75* ± 4.71	0.001*
GGT (U/l)	17.45 ± 0.77	16.45 ± 0.84	26.50 ± 1.71	39.90* ± 0.95	33.25* ± 3.92	0.001*
CK (U/L)	107.25 ± 3.3	104.75 ± 2.78	113.75 ± 1.49	116.00 ± 1.47	110.75 ± 2.17	0.125
Glucose (mg/dl)	194.5 ± 3.4	162.0 ± 12.86	228.0* ± 5.21	155.75 ± 14.6	160.0 ± 5.18	0.001*
TP (g/l)	5.62 ± 0.14	5.51 ± 0.18	5.92 ± 0.17	5.66 ± 0.06	6.07 ± 0.10	0.156
Alb (g/l)	2.43 ± 0.09	2.82 ± 0.04	2.65 ± 0.19	2.52 ± 0.04	2.39 ± 0.11	0.186
Globulin (g/l)	32.25 ± 0.81	32.73 ± 2.19	29.53 ± 2.10	31.38 ± 0.58	40.68* ± 3.89	0.029*
BUN (mmol/l)	29.15 ± 5.04	30.25 ± 6.95	17.09 ± 0.46	26.50 ± 4.34	28.28 ± 5.41	0.370
Creatinine (mg/dl)	1.00 ± 0.06	1.12 ± 0.04	1.05 ± 0.11	0.69* ± 0.11	0.97 ± 0.07	0.023*
Cortisol (ug/dl)	2.59 ± 0.32	1.76 ± 0.13	5.05* ± 0.66	2.80 ± 0.59	1.53 ± 0.14	0.001*
Progesterone(ng/ml)	3.86 ± 0.10	4.73* ± 0.17	2.76* ± 0.24	2.41* ± 0.25	2.41* ± 0.26	0.001*
Estrogen (pg/ml)	202.8 ± 22.5	303.3* ± 22.6	657.5* ± 36.4	126.8* ± 13.3	102.5* ± 17.2	0.001*

*Values with an asterisk within the same raw are statistically significant (P<0.05).
Ca: Calcium; Ph: Phosphorus; Mg: Magnesium; AST: aspartate aminotransferase; TP: Total protein; Alb: Albumen; ALP: Alkaline phosphatase; GGT: Gamma Glutamyl Transferase; BUN: Blood Urea Nitrogen; CK: Creatinine Kinase

Discussion

The transition period in dromedary camels has gained very little attention relative to that of dairy cattle although a limited report has exist in guanacos (Lama guanicoe) and in the llama (Lama glama) (7, 8). Indeed, laboratory profiling, can detect sick animals, and help identify herds at a potential risk of having metabolic and reproductive ailments. To fulfill this research gap, we sampled ten female pregnant dromedary camels at four weeks prior to the anticipated time of parturition to four weeks after calving. In this study, the most noticeable hematological changes in the female camels during the transition period were neutrophilia which was occurred at +14 and +28. This finding was in accordance with that reported previously in transition camel (11) and in cattle during the periparturient period (12). The authors

attributed such finding to stress being associated with parturition and the beginning of lactation stage.

The current study revealed that the serum concentration of calcium did not show significance pre- and post-partum. This finding was in agreement with that previously given in camel (11, 13) but away from that reported by other researchers (14, 15) who found lower calcium levels at the end of pregnancy in dromedary camel and buffaloes, respectively. The authors attributed such findings to the impairment of absorption of these metabolites from the alimentary tract and to the excessive losses through urine, colostrum.

In the current study, there was a significant increase in the serum phosphorus level prepartum and declined thereafter. This finding was in constant with that given by (11) in camel and that reported in buffalo (15) who found lower phosphorus levels during early stage of lactation in buffaloes. The authors attributed such finding as a result of the important role of phosphorus in the colostrums synthesis (16) and enhanced carbohydrate metabolism.

Magnesium plays a vital role during the metabolism of carbohydrates, lipids, nucleic acids and proteins. In present investigation, there was a significant increase in the serum magnesium at +28 days postpartum. Our finding was similar to that obtained by (11) in transition camel and by (17) in Ongole cows but away from that, reported by (18) who recorded lowered magnesium mean values in cattle and buffalo during early lactation.

The alterations of serum globulin, but not albumin, could be resulted from the formation of immunoglobulins. This finding was noticed to be in harmony with that given in transition camel (11) and in dairy cows (5), while the decrease of serum albumin levels could be associated with hepatic diseases. This finding is in accordance with previous reports in transition camel (11, 21). However, in this study its reduction was not significant.

In the present study, there was a significant decrease in the serum creatinine level at +14 days postpartum. This finding is in constant with that reported previously in periparturient camel (11) and in transition cattle (12). The creatinine level (marker of kidney function) is dependent on total body content of creatinine, which in turn depends on the dietary intake, rate of synthesis of creatinine, and muscle mass (19). The creatinine concentrations in the prepartum period were higher than those in postpartum. This finding is line with that reported previously (20) at late gestation camel who found high creatinine levels in dromedary camels during late gestation. The authors attributed such finding to high requirement of protein and lower rate of kidney elimination associated with late stage of pregnancy (13).

In the current study, there was a significant increase in the serum activity of AST, GGT and ALP at +14. The serum activity of GGT and ALP remained also at a significant (P=0.001) high pattern at +28 after parturition. Our findings were similar to that obtained in periparturient camel (11) and in dairy cows (5) but unlike to that given in late pregnant dromedary camels (22) and in pregnant mares (23) who reported a significant decrease in the GGT activity in the pregnant camels and in pregnant mare. It has been suggested that GGT is involved in the metabolism of glutathione to maintain antioxidative status of the entire body (24).

The increased concentration of glucose at parturition (0) may be due to the stress of parturition and coincide with elevation of cortisol at this period. This finding was in agreement with that given previously in transition camel (11) and in dairy cows (5) but unlike to that given by other researchers (25, 26) in peri-parturient dromedary camel who had found a significant decrease of glucose concentration before parturition compared to the period after. In the former study, the authors attributed such finding to the effect of gestational state on glucose level.

The current study revealed that, the serum level of BUN did not show any significance pre- and post-partum changes. This finding was in agreement with that given in transition camel (11) but in contrary with that reported in pregnancy of dromedary camels (22). The authors reported a significant increase of serum BUN in pregnant camels. The authors attributed such

finding as a result of protein catabolism and high requirement for energy by pregnant camels during the late pregnancy period resulting to an increase in BUN level.

Our findings demonstrated that concentration of serum cortisol was significantly increased at parturition (0) which may be due to stress of parturition due to the increase in the concentration of ACTH secretion from the foetal pituitary (11, 27, 28,29).

The serum activity of estrogen was elevated at -14 and reached its maximum peak at the time of parturition and declined thereafter. Such elevation of serum estrogen is considered as physiological response to potentiate uterine contraction during parturition. These findings were in agreement with that given by (11, 30, 31, 32) in camel and (33, 34) in goat. The authors attributed the dropped of maternal estrogen postpartum to that the estrogen measured in pregnant camel was fetoplacental in origin. On the other side, serum activity of progesterone was lowered at the time of parturition and the time onward (i.e. 0 to +28) which was considered as a physiological response in order to remove the corpus luteum of pregnancy. These findings were in agreement with that given by (11, 33, 34).

Conclusion

The data herein demonstrated a profound haematobiochemical alteration which could be considered as a feature of transition period in peripartum camels. Such alterations are not necessarily indicative of disease but could reflect a physiological variation and could be used as a reference guide for she-camels during the periparturient period.

Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

ASSESSMENT THE SYNCHRONY BETWEEN UTERINE STATUS AND HORMONAL PROFILES IN MODIFIED OVSYNC PROTOCOLS IN RELATION TO FERTILITY IN BUFFALOES

Adel A. Ramoun[1]*, Ismail I. EL- Kon[1], Essam A. Almadaly[1], Aly M. Aly[2], Hasballah A. Abo EL- Ghate[3]

[1]Department of Theriogenology, Faculty of Veterinary Medicine, Kafrelsheikh University, Kafrelsheikh, 33516, Egypt, [2]General Authority for Veterinary Services, Kafr EL-Sheikh, Egypt, [3]Mahallet Mousa Buffalo Research Station, Animal Production Research Institute, Agriculture, Research Center, Egypt

*Corresponding author, E-mail: ramoun2004@yahoo.com

Abstract: A total of 36 pluriparous buffaloes were used to study the uterine morphometry in response to serum estradiol concentrations and associated estrogen receptors (ERs) expression in modified ovsync protocols. The buffaloes were assigned to St-ovsync, CIDR-sync and Insulin-sync (n=12 for each). The St-ovsync consisted of two IM injections of 20µg buserelin on the Day 0 (GnRH1) and on the Day 9 (GnRH2) and an IM injection of 500 µg of Cloprostenol sodium (Estrumate) on Day 7. Buffaloes in CIDR-sync and Insulin-sync protocols were treated as in the St-Ovsync protocol in addition to intravaginal insertion of CIDR from Day 0 to 7 in CIDR-sync and SC injection of Insulin at a dose of 0.25 IU/kg body weight on Days 7, 8 and 9 in Insulin-sync. Blood samples were collected and the uterine wall thickness (UWT) was simultaneously measured by transrectal ultrasonography on Days 0, 3, 5, 7, 8 and 9. Endometrial biopsies samples were collected from five buffaloes in each group to quantify the abundance of estrogen receptors. The UWT on Days 3 and 5 decreased significantly ($P < 0.05$) while serum P_4 concentration on Day 3 was significantly ($P < 0.05$) increased in eventually diagnosed pregnant (EDP) buffaloes in CIDR-sync compared with their counterparts in either St-Ovsync or Insulin-sync. On Day 9, although there was a decrease in serum E_2 concentration in CIDR-sync compared with either St-Ovsync or Insulin-sync in EDP buffaloes, there was an increase ($P < 0.05$) in the ER mRNA expression in CIDR-sync compared with St-Ovsync. It could be concluded that modifying the St-Ovsync by P_4 supplementation through intravaginal insertion of CIDR from Day 0 to 7 or by SC injection of insulin on the Days 7, 8 and 9, could modulate uterine morphometry such conductive to proper fertility response.

Key words: Buffaloes, ERα; uterine wall thickness; Ovsync

Introduction

Optimal uterine environment at the time of FTAI, especially in cows induced to ovulate immature follicles in ovsync programs, is critical to establish and maintain pregnancy (1). The variation in the fertility status of cows kept under the same nutritional and environmental conditions may result from the different response

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of the uterus to steroid hormones during the periovulation time (2). Adequate rise in the proestrous estradiol (E_2) following pre-exposure to progesterone (P_4) prepares the uterus for embryonic development and establishment of pregnancy (3, 1, 2, and 4). The growth and regression of endometrium are synchronized through changes in the circulating and/or local levels of E_2 and P_4 (5).

Endometrial thickness increases during proestrous and reach maximal thickness on the day before ovulation and decreases throughout diestrous (6, 7). Suboptimal uterine environment induced by low level of estrogen during proestrous and progesterone during diestrous decreases infertility in dairy cows (8). Infertility in cows induced to ovulate immature follicles especially in Ovsync-TAI program may be attributed to suboptimal uterine function caused by low steroids at the periovulation time (9, 10, and 1). Not only the availability of steroid hormones with optimal levels in the peripheral blood is adequate to produce the desired effect on the uterus but binding to their specific nuclear receptors is critical (11). The effect of steroid hormones on the uterus depends on tissue and cell specific expression of steroid receptors (12) which reach maximum levels around the time of heat (13, 14). E_2 modulates the expression of its receptors and their function at the cellular level in such way that increase the rate of mitosis and tissues edema (15). Ovsync-TAI programs either GnRHbased or estradiol-based, are used nowadays to improve the reproductive performance of dairy cows (16, 17). In estradiol – based TAI programs, the exogenous E_2, in addition to inducing GnRH/LH surges, it modulates uterine environment. However, the relatively small follicles that might be present prior to second GnRH in case of GnRH-based Ovsync-TAI program may not produce adequate amount of estradiol sufficient to modulate uterine environment such compatible to support pregnancy (1). The US-measured uterine wall thickness (UWT) and horn diameter could be utilized to test if the uterus has been exposed to adequate concentration of steroid hormones compatible with optimal fertility (18).

Scoring the uterine status prior to the breeding time could be useful for predicting fertility in many species including human. Amongst the methods used to evaluate uterine status are cytology of uterine secretion, ultrasonographic (US) examination and uterine biopsy (19). The current study tests the hypothesis that if the US-measured UWT together with the uterine biopsy could be used to predict fertility of buffaloes subjected to modified ovsync programs. Souza et al (2014) (4) utilized endometrial thickness as a predictor of fertility in high producing dairy cows. They concluded that an endometrial thickness less than 7 mm is predictive to failure of conception in dairy cows. However, the associated steroid hormones profile together with estrogen receptors were quantified to correlate between hormonal profiles and uterine morphometry.

Thus, the current study was designed to match US-measured UWT with steroid hormones profiles together with estrogen receptors expression in the endometrial biopsy sample in an attempt to utilize UWT as a predicting measure of fertility following application of modified CIDR- and Insulin- ovsyncs in buffaloes.

Materials and methods

The animals

The study was performed in Mahallet Mousa Buffalo Research Station, affiliated to Animal Production Research Institute, present in Kafrelsheikh province in the northern of the Nile Delta, Egypt. The experiment was performedfrom April to October which coincides withlow breeding season in the Egyptian buffaloes.

A total of 36 pluriparous cyclic Murrah buffaloes having a parity of 2- 4 and an average body condition score of 2.75 to 3.50 (Scoringsystem was 1 = thin to 5 = fat) were used to carry out this study. Buffaloes were kept indoors throughout the year in yards where 50% of the yard area was sheltered. They hadfree access to water. They were milked twice daily and received a diet that covered both maintenance and production requirements according to the Recommendation of Animal Production Research Institute (APRI, 1997unpublished data).

All animals were cyclicand had healthy genital tract on the basis oftransrectal US scanning of the reproductive system. The cyclic activity was assessed onthe basis of detecting a corpus luteum in eitherof two transrectal US examinations of ovariesdone at 10 days' interval (from Day -10 to Day 0). Day 0 was the day of the first GnRH injection.

Experimental design

Buffaloes (N=36) were randomly assigned to three treatment protocols (12 each): standard ovsync (control group, St-Ovsync), modified CIDR-sync (CIDR–sync) and modified Insulin-sync (Insulin-sync). Each buffalo in the St-Ovsync group received IM injections of 20 µg buserelin acetate (GnRH agonist,5 ml Receptal®, Intervet Company, Holland) on the Day 0 (GnRH1), 500 µg of Cloprostenol sodium (PGF2α analogue, 2 ml Estrumate®, Coopers, Schering Plough Company, England) on the Day 7 and a 2nd dose of GnRH agonist similar to GnRH1, on the Day 9 (GnRH2). Buffaloes in the CIDR-syncgroup were treated as in St-ovsync in addition to intravaginal insertionof CIDR (Controlled Internal Drug Release, it contains 1.38 gm of progesterone, Pfizer Company, New Zealand) from the Day 0 to the Day 7. Buffaloes in Insulin-sync were also treated as St-Ovsync in addition to daily s/c injection of insulin (biphasic isophane insulin,0.25 IU / kg of B.W, Mixtard 30 HM®, Nova, Nordisk, Bagsvared, Denmark) on the Days 7, 8 and 9. Each 1 ml of Mixtard 30 HM® contain 30 IU of soluble and 70 IU of isophane biosynthetic human insulin. Buffaloes in all groupswere bred at 16 hr after the second GnRH treatment.

Ultrasonographic scanning

US examination was conducted by using portable ultrasound device (ULTRASCAN MODEL DP 30 VET, Shanghai International Holding Crop GmbH, Europe) equipped with multifrequency (3-10 MHz) linear probe. For transrectal scanning of the uterus, the probe while being carried in the palm of the hand was guided into the rectum. The uterus was identified then the probe was transversely placed on the base of each uterine horn at 3 cm cranial to the intercornual ligament. The UWT was measured by electronic calipers in the cross section of the frozen image. The UWT was measured as the distance between the edge of uterine lumen to the external edge of the perimetrium. The UWT was estimated for each uterine horn then the average value of the two horns was calculated for each cow. The UWT was measured on Days 0, 3, 5, 7, 8 and 9.

Collection of endometrial tissues

On Day 9, endometrial tissue samples were obtained by an endometrial biopsy knife as described by Nielson (7). The endometrial tissues samples were removed by fine forceps and immersed in a microcentrifuge tube containing 30 mm of the lysis buffer supplemented with mercaptoethanol to quantify the expression of mRNAs of estrogen receptor alpha (ERα) gene in the endometrium.

RT-$_q$PCR assay for ERα gene expression

Total RNA was extracted from endometrial biopsy (30 mg) using RNA isolation kit (Thermo-scientific, fermentas Ko731) according to the manufacturer's instructions. The concentration of the total RNA was measured using Nanodrop Spectrophotometer. To obtain cDNA, the total RNA (1 µg) was reverse transcribed using reverse transcription kits (Thermo-scientific, fermentas # EP0451 according to the manufacturer's instructions).

Quantifying of mRNA for ERα in the three protocols was determined by quantative RTPCR using SYBR Green with GAPDH as an internal control reference. The isolated cDNA was amplified using 2x Maxima SYBER Green/Rox qPCR Master Mix (Thermo-scientific, USA, # K0221) and gene specific primers according to the manufacturer's instructions.

The primers for ERα gene were; 5’GAAGTGGGCATGATGAAAGG-3’ forward and AAGGTTGGCACGTCTCATGT reverse and for GAPDH gene were 5’- CCTGGAGAAACCTGCCAAGT-3’ forward and 5’GGTAGAAGAGTGAGTGTCGCT-3’ reverse. The primer for ERα receptor gene was

designed using web based tool (http://www.genome.wi.mit.edu/egiin/primer/primer3www.egi) on the basis of published buffalo sequences to ensure that the primer sequence is unique for the template sequence. Amplicon sequence identity was confirmed with NCBI Blast tool software (Blast http://www.Blast.ncbi.nlm.nih.gov/Blast.egi). The reactions were conducted in a final volume of 25µL using 12.5µL of 2x Maxima SYBER Green/ Rox qPCR Master MIX, 1µL of each primer (forward and reverse), 3µL of cDNA (10-20 ng/µL) and 7.5µL of water nuclease free. The protocol conditions included initial denaturation at 95ºc for 10 minutes and 40 cycles with denaturation at 95ºc for 15sec, annealing at 60ºc for 30sec and extension at 72ºc for 30 sec. The relative expression levels of target gene (ERα receptors) were calculated by using the – ΔΔCT method (20). The house keeping gene (GHPDH) was used as the normalizing reference gene. The cycle threshold (Ct) values calculated for target gene were normalized against reference gene. The St-ovsync (control) group was used as calibrator, while CIDR-sync and Insulin- were considered as test groups for both target and references genes. The mean cycle threshold (Ct) values were used to calculate ΔCT for both target and reference genes in each of test and control groups by the following equation:

ΔCT (test) = Ct (target in test groups) – Ct (ref in test groups)

ΔCT (calibrator) = Ct (target in control) – Ct (ref. in control).

Then ΔCt of the test genes were normalized to the ΔCt of the calibrator:

ΔΔCt = ΔCt (test) – ΔCt (calibrator).

The fold change of the relative gene expression was calculated as follows:

Fold change = $(2^{-\Delta\Delta Ct})$.

Blood sampling and hormonal assay:
Blood sampling

Blood samples were collected by jugular vein puncture on the Days 0, 3, 5 and 9. The samples were centrifuged at 3000 rpm for 15 min. The harvested sera were stored at - 20°C until estrogen and progesterone assays.

Serum progesterone assay

The serum P_4 concentrations were measured by radioimmunoassay using RIA kit (Beckman coulter RIA progesterone IMMUTECH, S.r.o Radiova 1-10227 Prague - Czech Republic) according to the manufacturer's instructions described in the catalog enclosed with the kit. The inter- and intra- assay coefficients of variations were 8.66 and 8.15 respectively. The average sensitivity was 9.58 pg/ml.

Serum estradiol assay

The serum E_2 concentrations were estimated by radioimmunoassay using estradiol kit (Beckman coulter RIA Estradiol; IMMUNTECH, s.r.o Radiova 1-10277 Prauge - Czech Republic) according to manufacturer's instructions described in the catalog enclosed with the kit. The inter- and intra- assay coefficients of variations were 14.5 and 14.4 respectively. The average sensitivity was 9.58 pg/ml.

Serum insulin assay

The serum insulin concentrations were estimated using an IMMUNORADIOMETRIC kit (Insulin (e) IMRA kit; IMMUNOTECH, s.r.o Radiova 1-10227 praque- Czech Republic) according to the manufacturer's instructions described in the catalog enclosed with kit. The inter- and intra- assay coefficients of variations were 8.3% and 5.6% respectively. The average sensitivity was 4.55ng/ml.

Reproductive management

Buffaloes were inseminated at 16 h after the 2^{nd} GnRH treatment (FTAI) with frozen-thawed semen. On Day 30 post TAI, buffaloes were examined by transrectal US of their uteri for pregnancy diagnosis. Conception rates were calculated by dividing the number of buffaloes gets pregnant on the total number of buffaloes submitted to applied ovsync protocols TAI programs in the current study.

Statistical analysis

All data, except conception rates, were presented as means ± SEM. The statistical significance of differences was tested by the analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test using Graphpad prism ver. 6. 0 for Mac (Graphpad software. San Dieogo, USA). *Chi*-Square analysis was used to compare the conception rates among the three protocols.

Results

Ultra sound – Measured UWT

On Day 0, the US-measured UWT did not differ ($P > 0.05$) among St-ovsync, CIDR-sync and Insulin-sync in either eventually diagnosed pregnant (EDP) buffaloes or eventually diagnosed non pregnant (EDnP) buffaloes. It was observed that while the UWT decreased ($P<0.05$) in the CIDR-sync compared with either St-ovsync or Insulin-sync in EDP buffaloes, it did not differ among the 3 protocols in EDnP buffaloes on either of the 3rd or 5th day. Within each protocol, the UWT did not differ ($P > 0.05$) between EDP and EDnP buffaloes in all the investigated days (Table, 1).

Serum progesterone concentrations on the Days 0, 3 and 5

On the Days 0 and 5, the serum P_4 concentrations did not differ among the three protocols neither in EDP nor EDnP buffaloes. In contrary, on Day 3, it increased ($P < 0.05$) in EDP and EDnP buffaloes in CIDR-sync compared with either St-ovsync or Insulin-sync (Table 1).

The uterine wall thickness on Day 9

The UWT did not differ ($P < 0.05$) between either EDP or EDnP buffaloes on Day 9 as well as among EDnP buffaloes on Day 7. However, the UWT decreased ($P < 0.05$) in EDP buffaloes in CIDR-sync compared with either St-ovsync or Insulin-sync group on Day 7 (Table, 2).

Serum estradiol concentration on the Day 9

In EDP buffaloes, serum E_2 concentration on Day 9 showed a decrease ($P < 0.05$) in CIDR-sync compared with either Stovsync or Insulin-sync. In EDnP buffaloes, it showed nonsignificant ($P > 0.05$) variations among the three protocols.

Serum insulin concentration on Day 9

The serum insulin concentration on Day 9 increased ($P < 0.05$) in both EDP and EDnP buffaloes in Insulin-sync compared with either St-ovsync or CIDR-sync group (Table 2).

Estrogen receptor (ER) gene expression

The expression of *ERα* gene was upregulated ($P < 0.05$) in insulin-sync protocol compared with St-ovsync. Also the expression was upregulated in CIDR-sync compared with St-ovsync (Figure 1) regardless the buffaloes were EDP or EDnP. There was nonsignificant difference between insulin-sync and CIDRsync. Relative to the St-ovsync, the fold change of 2.81 and 2.45 were recorded for the upregulation of *ERα* gene expression in the Insulin-sync and CIDR-sync respectively (Figure 1).

The fertility response

Pregnancy diagnosis on Day 30 post- TAI revealed that 5/12 (41.66 %); 6/12 (50%) and 8/12 (66.67 %) buffaloes were diagnosed pregnant in St-ovsync, CIDR-sync and Insulin-sync protocols respectively (Figure 2).

Table 1: Ultrasound-measured UWT and serum P_4 concentrations in EDP and EDnP buffaloes in St-ovsync, CIDR-sync and Insulin-sync protocols on Days 0, 3 and 5

Parameter	Synchronization protocols					
	St-Ovsync		CIDR-Sync		Insulin-Sync	
	EDP	EDnP	EDP	EDnP	EDP	EDnP
UWT (mm)						
Day 0	42 ± 0.02 [a]	42 ± 0.01 [d]	41 ± 0.02 [a]	41 ± 0.03 [d]	42 ± 0.01 [a]	45 ± 0.01 [d]
Day 3	45 ± 0.02 [a]	40 ± 0.01 [d]	34 ± 0.01 [b]	39 ± 0.02 [d]	41 ± 0.01 [a]	38 ± 0.02 [d]
Day 5	42 ± 0.03 [a]	42 ± 0.01 [d]	32 ± 0.01 [b]	38 ± 0.03 [d]	41 ± 0.01 [a]	45 ± 0.02 [d]
Serum P_4 concentration (ng/ml)						
Day 0	3.41 ± 0.83 [b]	1.91 ± 0.29 [e]	3.84 ± 0.66 [b]	2.55 ± 0.28 [e]	3.73 ± 0.98 [b]	2.00 ± 0.34 [e]
Day 3	3.58 ± 0.62 [b]	3.26 ± 0.67 [e]	6.11 ± 0.39 [a]	6.39 ± 1.01 [d]	3.49 ± 0.51 [b]	3.26 ± 0.52 [e]
Day 5	3.25 ± 0.15 [b]	4.05 ± 0.17 [e]	5.95 ± 1.39 [b]	5.14 ± 0.57 [e]	3.04 ± 0.64 [b]	3.98 ± 0.30 [e]

Within the same row, values carrying small letters from a to c in case of EDP and from (d to f) in case of EDnP buffaloes are different at P<0.05. Within the same treatment group, values carrying asterisk are different at P<0.05 between EDP and EDnP buffaloes.

Table 2: Ultrasound - measured UWT on Days 7, 8 and 9 and serum concentrations of E_2 and insulin on Day 9 in St-ovsync, CIDR-sync and Insulin-sync protocols

Parameter	Treatment protocol					
	St-Ovsync		CIDR-Sync		Insulin-Sync	
	EDP	EDnP	EDP	EDnP	EDP	EDnP
UWT (mm)						
Day7	42 ± 0.02 [a]	42 ± 0.01 [d]	33 ± 0.01 [b]	35 ± 0.3 [d]	43 ± 0.02 [a]	44± 0.05 [d]
Day 8	43 ± 0.04 [a]	44 ± 0.01 [d]	35 ± 0.02 [a]	36 ± 0.02 [d]	45 ± 0.02 [a*]	41 ± 0.04
Day 9	34 ± 0.02 [a*]	50 ± 0.03	34 ± 0.02 [b]	49 ± 0.04 [d]	43 ± 0.03 [a]	41 ± 0.04 [d]
Serum estradiol concentration (pg/ml)						
Day 9	17.3 ± 1.55 [a]	9.43 ± 1.23 [d]	12.1 ± 1.45 [b]	9.65 ± 2.11 [d]	22.26 ± 1.31 [a]	12.13 ± 1.90 [d]
Serum insulin concentration (ng/ml)						
Day 9	7.18 ± 1.85 [b]	5.79±2.56 [e]	6.00±0.72 [e]	6.00±0.72 [e]	32.13±2.64 [a*]	15.13±2.21 [d]

EDP = eventually diagnosed pregnant; EDnP = eventually diagnosed non-pregnant. Within the same row, values bearing different letters from a to b in case of EDP buffaloes and from d to e in case of EDnP buffaloes were different at (P<0.05). Within the same protocol, values bearing asterisk were different at P < 0.05 between EDP and EDnP buffaloes.

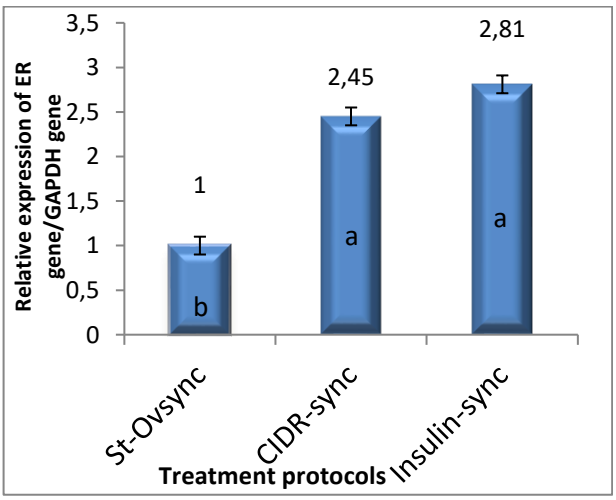

Figure 1: Oestrogen receptor alpha (*ERα*) gene expression in insulin-sync and CIDR-sync groups relative to St-ovsync shown as fold changes ($2^{-\Delta\Delta ct}$). Relative to the St-ovsync (fold change (Fc)=1), the fold changes of 2.81 and 2.45 represented the upregulation of *ERα* gene in Insulin-sync and CIDR-sync groups respectively

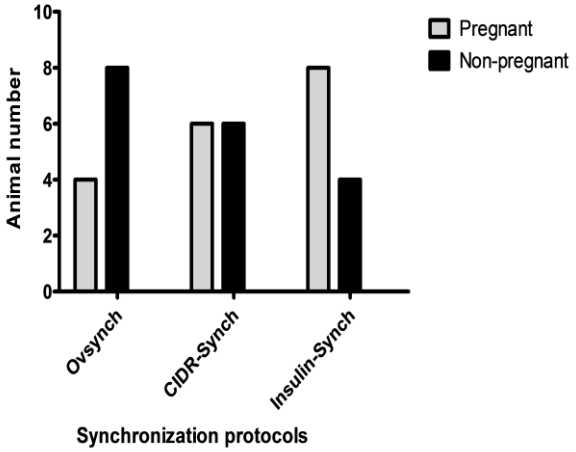

Figure 2: The conception rates on Day 30 post-TAI in buffaloes treated with St-ovsync, CIDR-sync and In sulin-sync. The conception rate was 5/12 (41.66 %); 6/12 (50 %) and 8/12 (66.67 %) in St-ovsync, CIDR- sync and Insulin-sync respectively

Discussion

The present work aimed to study the uterine morphometry in terms of us-measured UWT in relation to steroid hormones profiles and endometrial oestrogen receptors in modified ovsync programs in buffaloes. Since endometrial growth and regression are synchronized with ovarian function through changes in circulating and/or local levels of oestrogen and progesterone(21, 4), ultrasound measuring of the uterine wall thickness, simple and reproducible technique, can be used to evaluate steroid hormone induced uterine environment changes (22).

On the day 3 and 5, the decrease in the UWT in the buffaloes eventually diagnosed pregnant (EDP) in CIDR-Sync compared with either St-Ovsync or Insulin-sync may be attributed to the increase in the serum progesterone concentrations at $p < 0.05$ and $p > 0.05$ on the days 3 and 5 respectively. Taking into account the similarity between the diestrous phase in the estrous cycle and short luteal phase, 2-7 days, in an ovsync protocol (Ref), the reduced UWT, recorded on the days 3 and 5 in the current study, came in line with (23) who reported that the uterine horns had minimal thickness, minimal luminal fluid and maximal horn curl during the period extending from 3rd to 16th day of the oestrous cycle. Sajjan (2014) (23) reported that the thickness of the endometrium, (most responsive layer of the uterine wall to the changes in the serum steroid hormones concentrations) decreased from 5.47 to 5.06 mm with the increase in the serum P_4 concentration from 0.76 to 1.08ng/ml in cows. In the same respect, Jimenez-Krassel et al (2009) (24) attributed the decrease in the endometrial thickness to the increase in the circulating P_4 concentration

The non-significant variations in the UWT among EDP buffaloes on Day 0 as well as among EDnP buffaloes may be explained in the light of non-significant variations in the serum P_4 concentrations on the respective days.

On Day 9, matching the serum E_2 concentrations with UWT in case of EDP buffaloes revealed that the serum E_2 concentrations, being high, were nearly consistent with respective UWT in case of either ST-ovsync or Insulin-sync but not in CIDR-sync. Conversely in EDnP buffaloes, it was inconsistent with respective UWT in all of three groups. In the EDP buffaloes, then insignificant increase in the UWT in the insulin-sync protocol compared with either ovsync or CIDR- sync may be explained in the light of increase in the respective serum E_2 concentration in the insulin-sync compared with its counterpart value in case of either St-ovsync or CIDR – sync protocol. However, the increase in E_2 receptors in CIDR-sync compared with St– ovsync may increase the response of the endo- metrium, which is the main responder part of the uterine wall, to relatively low serum E_2 levels in CIDR- sync thereby increasing UWT that became comparable to its counterpart value in case EDP buffaloes in St-ovsync. In line with this explanation, Xia and Goff (25) reported that the number of endometrial receptors determines the sensitivity of the uterus to the steroid stimulation.

The increase in the concentration of serum E_2 in Insulin-sync compared with the other two groups may be due to stimulatory effect of insulin on the steroidogenic activity of the largest follicle. The exogenous insulin increase both follicular growth and steroidogenic activity in cows (26), buffaloes (27) and goats (28). In CIDR-sync, the lower serum E_2 concentration in EDP buffaloes on Day 9 compared with either St-ovsync or Insulin- sync may be attributed to the presence of persistent follicles that bypassed the effect of the first GnRH and continued to grow and produce E_2 at lower rates under the effect of low tonic level of LH induced by the negative feedback mechanism of P_4 released from CIDR until Day 7 (Day of CIDR removal). Some of these persistent follicles continued to produce E_2 at the previous lower level until Day 9. However, an interval of 48 h (from Day 7 to 9) is not sufficient for the persistent follicle to survive P_4-induced low tonic level of LH during the previous 7 days, thus their steroidogenic activity became compromised. In line with this explanation, Cerri et al (2010) (29) found that the tonic levels of LH were lower in cows having high serum P_4 concentrations on Day 5 of CIDR-sync protocol.

On the other hand, Cerri et al, (29) reported that the decreased concentrations of P_4 during synchronization protocol resulted in high basal LH concentrations, faster growth of dominant follicle (DF) and higher circulating E_2. In spontaneous estrous, a proestrous period of 3-4 days may be sufficient for resumption of proper tonic level of LH that stimulates proliferative and steroidogenic activity of the DF. In accordance with this suggestion, Bridges et al, (2010) (30) reported that reducing the duration of proestrous in cows, induced to ovulate small follicles, resulted in decreased serum preovulatory E_2 (30, 31). Nonetheless, Cerri et al. (29) found concentration was lesser from day 5 to 9 of CIDR-sync protocols in cows having high compared with those having low serum P_4 that the serum E_2 concentrations.

The benefit of studying the abundance of ERα in these three Ovsync protocols comes from the fact that the changes in the uterine morphometry in response to E_2 are an outcome of the interaction of the local/peripheral E_2 concentration with its receptors in the uterine tissues. Okumu et al. (11) reported that not only the availability of a steroid hormone in the peripheral circulation is adequate to produce the desired effect on the uterus but binding to their specific nuclear receptors is critical.

In the current study, the expression pattern of ERα in the endometrial biopsy is compatible with serum E_2 concentrations in either Insulin-sync or St-ovsync but not with CIDR- sync to some extent. The higher E_2 levels in case of Insulin-sync and St-ovsync came in agreement with Sunderland et al (1994) (32) who reported that the expression pattern of ERα is consistent with the circulating concentration of E_2 during oestrous cycle.

In the same respect, Clark et al and Mann et al (33, 34) detected the highest level of ERα and PRs mRNA at the time of oestrous and concluded that the stimulatory effect of E_2 on sex hormone receptors expression. Also Spencer and Bazer (35) reported that E_2 as well as those for P_4 and the functions at the cellular level to increase the rate of mitosis and tissue oedema (15), a finding which explained the increased UWT in either of Insulin-sync or St-ovsync compared with CIDR-sync. However, the reduced UWT in the CIDR-sync may result from the delayed recovery of UW from the effect of high P_4 concentration during period of CIDR insertion. However, the abundance of ERα was higher in CIDRsync compared with St-ovsync but the E_2 concentration was higher ($P > 0.05$) in St-ovsync compared with CIDR-sync. Although the E_2 concentration was lower ($P > 0.05$) in CIDR-sync compared with St-ovsync, the abun- dance of ERs in CIDR-sync was higher than St-Ovsync. This may be attributed to the pre exposure of the uterus in case of CIDR-sync to higher P_4 concentration during the period of CIDR insertion. This explanation may be supported by the results of Shimizu et al (36) who reported that E_2 and P_4 provoke transcriptome changes within the endometrium, with the response to E_2 being greater when the uterus was preexposed to higher P_4.

Conclusion

It is concluded that modifying the St-ovsync by including P_4 through CIDR-insertion from Day 0 to 7 or treatment with insulin on Days 7, 8 and 9 could modulate uterine morphometry, thereby improving fertility outcome of ovsync protocols. Further studies are required in the future to study the effects of the P_4 or insulin-induced transcriptome changes in the uterus of cows treated with modified P_4 or insulin-modified ovsyncs on the uterine histotrophs secretion that may explain its beneficial effects on the animal fertility.

Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

HYPOMAGNESEMIC TETANY IN CAMEL CALVES (*CAMELUS DROMEDARIUS*): CLINICAL CONSEQUENCES AND TREATMENT OUTCOMES

Sherif M. Shoeib[1], Mohamed Z. Sayed-Ahmed[2,3], Sabry A. El-khodery[2]*

[1]VeterinaryTeachingHospital,FacultyofVeterinaryMedicine,MansouraUniversity, Mansoura 35516, Egypt, [2]Department of Internal Medicine and Infectious Diseases, Faculty of Veterinary Medicine, Mansoura University, Mansour 35516, Egypt, [3]Department of Clinical Pharmacy, College of Pharmacy, Jazan University, Jizan45142, Saudi Arabia

*Corresponding author, E-mail: khodery@mans.edu.eg

Abstract: Camels are the top herds wealth in Saudi Arabia, since is considered as heavy animal population, breeding resist more dangerous diseases. This clinical study was delineated to explain the clinical presentation of hypomagnesaemia in camel calves and to evaluate the efficacy of treatment with magenesium therapy. The investigation was performed in central Saudi Arabia during the period from November 2017 to October 2018. Twenty-seven dromedary camels, 3-6months of age were included in this study on the basis of clinical and treatment outcomes during the period from November 2017 to October 2018. Animals were allocated to two groups, the first group (n = 5) was considered as healthy (control) animals. The second group (n = 22) was clinically showing signs of hypomagnesemis. Allanimals were subjected to biochemical analysis of serum and ruminal fluid. Therapeutic response of the clinically affected animals was assessed after intravenous injection of 100 ml calcium-magnesium preparation intramuscularly. injection of 10ml Vitamin B complex, and oral administration of 2gm Mg sulphate orally for five consecutive days. Clinically affected camels revealed tetany, star gazing, episodes of convulsions with erected ears and tail, extension of head and neck, widening and dilatation of nares. Serum and ruminal fluid analysis revealed a significant decreased ($p<0.05$) in Mg, Ca, and Phosphorus (P) in clinically affected cases compared to control and treated groups.Total protein (TP), globulin, albumin, , urea and creatinine were significantly higher in clinical cases compared to treated cases. In conclusion, the hypomagnesemic tetany had a high occurrence in camel calves 3-6 month of age . Furthermore, the treatment regieme of hypomagnesemic tetany had agood threrapeutic response.

Key words: biochemical tests; camel calves; hypomagnesemic tetany; Saudi Arabia

Introduction

Hypomagnesemic tetany is a metabolic disorder that occurs in a wide range of nutritional disorders in ruminants (1). Magnesium deficiency results from a reduced ability of the

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calves to absorb dietary magnesium as exogenous dietary supply (2).

Magnesium homeostasis mainly depends on its absorption from gastrointestinal tract, bioavailability and requirement for milk, but not on a hormonal feedback system. Any extra Mg is excreted via urine. Reduced Mg absorption from the rumen may explain the insufficient inflow (3, 4).Consequently, the level of magnesium in the blood and extracellular fluid depends onthe balance between input and output (4).

The calves affected have concurrent hypocalcemia, as they fed milk with low magnesium (Mg) concentration. The camel calves fed with only milk, will be insufficient to maintain the balance of Mg and calcium (Ca). In lactating mothers and their camel calves, higher serum values of aluminum (Al) and lower values of Mg were observed compared to other groups. It is suggested that the increase in serum Al in calves might be due to feeding Al-rich milk and that the decline in Mg concentration might be a consequence of Al and increased secretion of parathyroid hormone (5).The efficiency of absorption during intestinal transit is reduced in cases of diarrhea (4).

Mild neurological signs with increased excitability occur when there is a critical decrease in the serum Mg level. However, animal may not show clinical signs, although there is low serum Mg level (6). Ingestion of green grasses caused high rumen pH, with subsequent reduction of Mg absorption (7).

The most famous clinical signs of hypomagnesemic tetany are represented by excitations and muscle cramps, which are strongly interrelated with the concentration of Mg in the cerebrospinal fluid. It is suggested that the neurological signs of hypomagnesaemia are induced by activation of neurons in the cerebrospinal fluid (3). Therefore, the present study describes clinical consequences and successful management of hypomagnesemic tetany in camel calves.

Materials and methods

Study area

This clinical study was carried out in central Saudi Arabia (Al-Qassim, Hermla and Shaqra). Each farm was visited twice monthly to explore the presence of clinical disease.

Animals and study design

A total of 27 camel calves with age range from 2-6 months were investigated clinically and biochemically from November 2016 to April 2017. 22 camels had history of nervous signs such as tetany, convulsion, ataxia, staggering gait, muscular tremors and inability to stand up. Most of the clinical cases were recorded in winter and spring seasons. All the clinically affected animals were treated with Mg therapy as mentioned below.

Clinical examination

All calves in the control, and clinical groups were clinically examined as previously documented (4).

Serum sampling and biochemical analysis

Blood samples from control and diseased calves were obtained from the jugular vein and collected into sterile tubes without anti-coagulant. Serum was obtained and stored at -80 °C till analysis. Serum biochemistry was conducted using commercial kits (Bio- Mureix) for spectrophotometric determination of Mg, P, albumin , calcium, potassium, Aluminium, urea nitrogen, and creatinine (8-15), Globulin and Albumin/Globulin ratio were calculated.The parathyroid hormone was measured by radioimmunoassay (16).

Ruminal fluid collection and analysis

Ruminal samples were collected by stomach tube from each animal. The samples were centrifuged at 3000 rpm for 15 minutes. The supernatant layer was transferred to a clean tube and keep in deep freezer for spectrophotometric determination of Mg, Ca, and P using commercial kits (Bio-morieux) and spectrophotometer (spin lab, Spinreact S.A. Model 2003) as previously described (17).

Assessment of therapeutic response

Therapeutic response of the clinical cases was assessed after a five day treatment with IV injection of 100 ml calcium magnesium borogluconate (Cal-Bor-Mag, kela Pharamceuticals, belgium) daily. In addition, 10 ml of Vitamin B complex (corobral, Vetoqunol, france) was given IM and 2gm Mg sulphate were given orally for 5 days (18).

Statistical analysis

Statistical assessment of the clinical parameters, biochemical findings of the control group and of hypomagnesemic calves before and after treatment were done using SPSS Ver. 16.0 package program with Student's paired-t test as previously described (14). All data were given as Mean ± SEM, and results were considered significant at $P < 0.05$.

All the twenty -two animals from twelve different herds in the area were found to express nervous manifestations such as tetany, convulsion, ataxia, staggering gait, muscular tremors and inability to stand up (Fig.1). There was also tachypnea, tachycardia and mild elevation of rectal temperature.

Clinicopathological findingsshowed a significant($P < 0.05$) decrease in the serum values of Mg in camels calves before treatment (0.89±0.09mmol/L) compared with treated camel calves (1.3±0.2 mmol/L). The other examined parameters remained within the normal average in both diseased and normal camels ($P > 0.05$). Ruminal fluid analysis showed a significant reduction ($P<0.05$) in Mg, Ca and P in hypomagnesemic tetany camel calves compared to control animals (Table 1 & 2).

Results

Table 1: Serum biochemical parameters (Mean± SE) of camel calves with hypomagnesemic tetany before and after treatment

Parameter	Control	Diseases	Treated
Calcium (mmol/L)	2.6±0.18	2.31 ±0.3	2.5 ±0.4
Chloride (mmol/L)	110.9±1.3	121.5±1.4	112.2±1.5
Cupper (mmol/L)	82.5±1.5	67.2±2.1	76.6±1.5
Phosphorus (mmol/L)	2.44±0.14	2.25± 0.3	5.7±0.2
Magnesium (mmol/L)	1.27±0.17	0.89±0.09*	1.3±0.2
Aluminium	2.4±0.17	5.4 ± 0.23	2.8±0.07
Zink (mmol/L)	57.8±1.6	62.6±2.1	52.6±1.4
Total protein (g/L)	66.4±0.2	80.1±0.28	76±0.2
Albumin (g/L)	38.4±0.08	38.1±0.13	34 ±0.1
Globulin (g/L)	28±0.2	42±0.15	42±0.16
Urea(mg/dl)	24.9±0.2	22.07±0.5	22.07±0.4
Creatinine (mg/dl)	1.8±0.1	1.3±0.05	1.3±0.07

*: means significantly different at $p<0.05$

Table 2: Calcium, phosphorus and magnesium in ruminal fluid analysis (Mean± SE) of camel calves with hypomagnesemic tetany befor and after treatment

Parameter	Control	Before treat	After treat
Mg (mmol/L)	1.23±0.4	0.98 ± 0.40*	1.2±0.5
Ca (mmol/L)	2.8±0.17	1.6±0.21*	2.84±0.4
P (mmol/L)	1.81±0.12	1.5±0.05*	1.8±0.03

*: means significantly different at $p<0.0$

Figure 1: A camel calves suffered from hypomagnesemictetany showing nervous signs (A), tetanic convulsion and recumbency (B), and star gazing and staggering gait (C)

Discussion

Diseases that cause neurological signs in animals are many and are usually caused by a variety of agents including viruses, parasites, bacteria, neuro-toxic chemicals and plant poisons as well as genetic abnormalities and nutritional deficiencies (19).Clinical findings of clinical hypomagnesemia in camel calves were tetanic convulsions, opisthotonos, star gazing, episodes of convulsions with erected ears, extension of head and neck, erected tail, widening and rotation of the eye ball and dilatation of nares, similar findings were recorded previously (18,20).

It has been demonstrated that slow type of hypomagnesemic tetany is diagnosed in cows for several months, especially during cold season. Critical reduction of Mg levels in the plasma is usually accompanied by clinical signs of hyperirritability and incoordination. Hypomagnesemic tetany is termed the winter tetany, associated with low dietary Mg, low quality of feeds and environmental stresses (21).The clinical parameters showed a significant increase ($P<0.05$) in the heart and respiratory rates in hypomagnesemic cases than control. Body temperature showed mild increased in hypomagnesemic cases compared to control and treated cases. However, ruminal motility was significantly decreased than control. These results were similar to those recorded in previous studies (20, 22, 23). The imbalance between calcium and magnesium ratio in extra and intracellular fluid might induce the abnormal function of nerve fibers in peripheral and/or central nervous system, which lead to increase in heart rate and rapid respiration (24). The reduction of ruminal motility in diseased cases could be attributed to the biological role of Mg in maintaining the ruminal contractions (3).The clinical identification of hypomagnesaemia is usually made by a combination of clinical history, signs and response to Mg therapy. The clinical signs rapidly progress and death occurs within few hours, but most of animals with hypomagnesaemia may found dead (25). In most cases, low dietary magnesium intake not only produces clinical signs, but also may need other predisposing factors. In the present study the Mg level was reduced to 0.89mg/dl in clinical tetanic cases. The hyperesthesia, the tetany and convulsion in hypomagnesaemia could be attributed to the central effect or the disturbance in the neuromuscular junction due to Mg/Ca deficiency (26).This study showed marked increase in aluminum in camel calves with tetany. It has been demonstrated that increase in serum Al in calves might be due to feeding Al-rich milk and that the decline in Mg concentration might be a consequence of Al and increased secretion of parathyroid hormone (5).Moreover, release of adrenal glucocorticoid as a result of stress reaction, causes increased circulating level of K and decrease Mg transport across the

choroidal plexus, with subsequent appearance of the neurological signs (27). Another pathway is that, the decrease of energy intake inhibits the rumen fermentation process, with subsequent decrease of volatile fatty acids and CO_2 concentrations and increase of ammonia concentration. The decrease of energy intake can also increase the rumen pH due to high ammonia and low volatile fatty acid with eventual reduction of the mg absorption (3).

The increased urea level in diseased cases has been observed also in other studies (29). Anorexia and starvation associated with the disease causes protein catabolism and consequently retention of nitrogenous wastes (6). In ruminants, it is known that when there is low protein intake, animals can diminish their urinary urea loss, and increase the availability of nitrogen required for protein synthesis in the rumen. (30).

Ruminal fluid showed a significant reduction ($P<0.05$) in Mg, Ca and P in hypomagnesemic tetany camel calves compared to control These results were comparable to those recorded in goats with experimentally-induced hypomagnesaemia (29). The Mg concentration in the diet is a major determinant of it concentration in the ruminal fluid (31). Therefore, the low Mg in ruminal fluid could be attributed to low Mg intake, which consequently leads to low serum Mg levels. In young calves, it has been known that Mg is absorbed efficiently from the small intestine. But, when the rumen and reticulum are well developed, they are the main site of absorption. (32). The absorption of Mg through ruminal wall depends on its concentration in the ruminal fluid and efficacy of Mg transport action (31). There are 2 mechanisms for Mg absorption from rumen epithelium, the active and passive mechanisms. Active transport of Mg across the rumen wall is necessary when Mg in supplement in diet is little, active transport of the Mg crosswise the wal of rumen is of great importance. However, when there is high rumen fluid Mg, passive transport occurs. Passive transport of Mg allow decrease of concentration gradient into the extracellular fluid (3).The minimum ruminal fluid Mg level that activates the passive mechanism is 9.2 mg/dl (33). Because the Mg concentration in ruminal fluid of clinical hypomagnesemia was lower than that level, the passive transport mechanism of Mg is inactivated.

In conclusion, the results of our investigation indicate that hypomagnesaemia exists in camel calves and it should be considered when confronted with cases showing nervous manifestations.

Conflict of interest

There is no conflict of interest

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Original Research Article

POTENTIAL AMELORATIVE EFFECT OF *PANAX GINSENG* ON HEPATORENAL DAMAGE OCUURED IN HYDROXUREA-TREATED RATS

Samar S. Elblehi*, Dina R. S. Gad El-Karim

Department of Pathology and Clinical Pathology, Faculty of Veterinary Medicine, Alexandria University, 11511, Egypt

*Corresponding author, E-mail: samarsaad30@yahoo.com

Abstract: Hydroxyurea (HU) is an anti-neoplastic drug which is used for treatment of many types of tumors; however, its wide range of deleterious effects may limit its medical usage. This study was conducted to assess the possible protective role of *Panax ginseng* extract (Ginseng) against HU- induced hepato-renal damage in rats. Forty male Wistar albino rats were randomly divided into four equal groups (10 rats each). Group I (control) received 1ml purified water. Group II (Ginseng-treated) received 200 mg/kg body weight (b.wt) of Panax ginseng extract. Group III (HU-treated) received HU at a dose of 500 mg/kg b.wt. Group IV (HU plus ginseng) received HU and *Panax ginseng* extract in similar doses of group II and III. All the treatments were administrated orally. Treatment with *Panax ginseng* failed to ameliorate the hematological alterations caused by HU, but, its ability to alleviate hepato-renal cytotoxic effect was proved through significant reduction in plasma level of hepato-renal injury biomarkers (AST, ALT, GGT, urea and creatinine) which were elevated in HU-treated group. Also, this effect was affirmed by decreased level of plasma pro-inflammatory cytokines (TNF-α and IL-6) and enhanced anti-oxidant state (decreased MDA content and increased GPX activity) of hepato-renal tissues upon *Panax ginseng* extract administration with HU. Histopathologically, HU induced impairments in both hepatic and renal tissues of the treated rats. Conversely, concurrent administration of ginseng with HU partially improved these alterations.

Key words: hydroxyurea; hepato-renal toxicity; Panax ginseng; rats

Introduction

Hydroxyurea (HU) as an anti-neoplastic drug is used in humans and dogs for the treatment of chronic myelogenous leukemia, polycythemia, ovarian cancer, melanoma, and squamous cell carcinoma of the head and neck. Also, it has been used for treatment of renal cell carcinoma, transitional carcinoma of the urinary bladder, prostatic carcinoma, and carcinoma of the uterine cervix in addition to thrombocytosis (1). Moreover, HU has been used for treatment of mast cell tumor and hypereosinophilic syndrome in dogs (2,3). It is the only approved option to be used in humans with sickle-cell disease as it reduces vaso-occlusive crises frequency, blood transfusions, the number of days spent in hospital and mortality of these patients (4). That's because administration of HU to sickle-cell disease patients increases the production of erythrocytes fetal hemoglobin which

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can carry more oxygen than adult hemoglobin (5), decreases circulating neutrophils, enhances the deformability of erythrocytes, decrease adhesion of erythrocytes to endothelium, promotes vasodilation through production of nitric oxide and increasing adenosine deaminase activity in circulating monocytes leading to lower adenosine levels (6, 7).The mechanism of action of HU as an anti-neoplastic drug depends on its ability to arrest rapidly dividing cells at S phase of cell division (8) through inhibition of the class I ribonucleotidereductase enzyme (9) which is responsible for the formation of deoxyribonucleotides from ribonucleotides (10) by inactivating the tyrosyl radical required for enzyme activity leading to massive reduction in the production of deoxyribonucleotides that are necessary for DNA replication (11,12).

HU is generally tolerated however it has a low safety margin due to myelosuppression, as the earliest and most common reported adverse findings in human patients were pan-leukopenia, anemia and thrombocytopenia (3, 5, 13). Additionally, growth retardation spermatogenic arrest, hematopoietic, lymphoid, cardiovascular, and gastrointestinal toxicity associated with hemosiderosis of spleen and liver have been observed in dog toxicity studies (13, 14). In rats administration of HU at high doses induced reduction in the body weight gain, anemia, leukocytopenia, thrompocytopenia associated with hypocellularity of lymphoid organs and bone marrow; epithelial degeneration and/or dysplasia of the stomach and small intestine (14) and decreased fertility in males as itinduced testicular atrophy and decreased sperm production (15), also, it can induce hepatic damage with fatty changes (13). Cardiovascular effects and slight methemoglobinemia were detected in some species ‘‘at doses higher than clinical levels’’ (13, 16), skin and nail lesions have been reported in human and canine patients (2,17,18). In addition, HU can induce lung toxicity with pulmonary edema, lung infiltration and dyspnea (19), and pulmonary fibrosis in a minority of patients (20).

Traditional herbal medicine has become the point of attraction in the clinical and experimental research. *Panax ginseng* (Korean ginseng), commonly known as ginseng is a famous medicinal plant that has been used extensively in traditional Chinese medicine for more than 2,000 years (21-24). Bioactive constituents of ginseng include ginsenosides, polysaccharides, phenolics, flavonoids, and polyacetylenes (25). In particular, the main active components of ginseng are the ginsenosides (or triterpenoid-saponins), aswell as about 38 more types of ginsenosides have been identified approximately (26). These compounds are proved to be responsible for most of the favorable effects of ginseng, which has a wide range of protective or therapeutic effects against many diseases. Ginsenosides have been studied intensively as the main active components of ginseng that have a variety of pharmacological effects, including anti-diabetes (27), anti-oxidant and anti-inflammatory (28), anti-aging (29), anti-obesity (30), immune modulator and anti-tumor effect (31, 32). To the pest of our knowledge, very few detailed reports of HU induced toxicity in lab animals have been published and the published reports were insufficient to demonstrate the hepato-renal effect of HU administration. In this context, this study was conducted to assess the adverse effects of HU administration to male albino rats up to 1 month and the ability of *Panax ginseng* to ameliorate these effects.

Materials and methods

Chemicals

Commercially available formulation of hydroxyurea capsules (Hydrea®500mg, E R Squibb & Sons Ltd., England) was used. Panax ginseng extract were obtained in form of capsules containing 550 mg of Korean red ginseng extract, 8% ginsenosides (Nature's Way, USA).

Animals and experimental design:

Forty male Wistar albino rats, 6–8 weeks old and 180–200 g body weight were obtained from the closed bred colony in Faculty of Agriculture, Alexandria University, Egypt. Rats were housed in metal cages under controlled environmental conditions (24–27 °C temperature, 55% RH, and 12 h light/dark cycle) for 2 weeks.

They were observed carefully during this period to make sure that they were free from any apparent health problem. All animals fed on a standard laboratory diet and received water *ad libitum* during the experiment. This study was approved by committee of Care and Use of Laboratory Animals of Alexandria University.

After the acclimatization period, rats were allocated into four groups (10/each). All groups received daily oral intubation using stomach tube and the treatment lasted for 30 days as the following: Group I (control) received 1ml purified water (the vehicle used for HU). Group II (ginseng-treated) received 200 mg/kg b.wt of *Panax ginseng* extract (33). Group III (HU-treated) received hydroxyurea at a dose of 500 mg/ kg b.wt (34), HU solution was freshly prepared by tablets dissolving in purified water. Group IV (HU + ginseng) received hydroxyurea and ginseng extract in similar doses of group II and III. Twenty four hours after the last dose, the rats of each group were euthanized after light ether anesthesia to be subjected to the following studies.

Hematological studies

Anti-coagulated blood samples (in EDTA containing tubes) were collected from retro-orbital venous plexus for determination of erythrocytic (RBCs) count, and platelets count in addition to total and differential leukocytic count (Exigo® veterinary hematology system, Sweden) and for separation of plasma by samples centrifugation at 1000×g for 10 min.

Biochemical analysis

Plasma activities of AST, ALT and GGT, in addition to levels of urea and creatinine were determined using commercially available kits. Plasma level of IL-6 and TNF-alpha were detected by quantitative sandwich enzyme immunoassay method using Rat spicific ELISA kits (abcam, USA).

Antioxidant studies

About 1 g of kidney and liver of each euthanized animal were separated, perfuse with phosphate buffer saline solution (PBS) solution (pH, 7.4) containing 0.16 mg/ ml heparin to remove any blood or clots, and homogenized in 9 ml PBS solution using tissue homogenizer (Glas-Col. Tissues homogenizer, China). Homogenate aliquots were centrifuged at 10,000 × g for 30 min, supernatant were removed and stored at -80 till the time of assessment of concentration of malondialdehyde (MDA) and activity of glutathione peroxidase enzymes (GPX) using locally available kits (BiodiagnosticEgypt; Biovision, USA) and as previously described (35). Protein content was detected using Bradford's reagent (Sigma-Aldrich, USA).

Histopathological examination

Following necropsy specimens of livers and kidneys of each rat were collected and rapidly fixed for at least 24h in10% neutral buffered formalin. The fixed specimens were processed through the conventional paraffin embedding technique, sectioned at 5 microns and stained with Mayer's haematoxylin and eosin (H&E). Stained sections were examined by light microscope and photographed using digital camera.

Semiquantitative histopathological scoring system

Semi quantitative histological scoring was carried out for the liver and kidneys slides. Five fields (×100) were randomly selected from each rat in each group, and the most important pathological alterations were selected and scored in each organ; the severity of lesion was estimated according to the percentage of affected area/entire section where in: 0 = absence of lesion, 1 = 5–25%, 2 = 26–50%, and 3 = ≥50%

Statistical analysis

The statistical analysis was carried-out using one way analysis of variance (ANOVA) for studying of the effect of different treatment groups on different parameters studied using SAS, (2004) (36).

Results

Hematological results

As presented in Table 1, blood picture of HU-treated rats significantly showed a decrement in RBCs count which coupled by significant decrease in total leukocytic count (TLC), granulocytes, lymphocytes and monocytes

counts in addition to platelets count when compared to control group. Unfortunately, co- administration of ginseng with HU failed to increase the diminished values of the previously listed parameters of blood picture as compared to HU-treated rats. The sole treatment with ginseng did not enhance any of these hematological parameters compared to control group.

Biochemical changes

As shown in Table 2, treatment of rats with HU evoked significant elevation in plasma activity of AST, ALT and GGT enzymes when compared to control group. Administration of ginseng with HDU significantly decreases plasma activity of these enzymes if compared to HU-treated animals. In the same manner, treatment with HU led to significant increase in plasma concentration of urea and creatinine which accompanied by significant decrease in plasma albumin concentration compared to control group. Comparatively with HU-treated group, co-administration of ginseng with HU significantly ameliorated the changes in these parameters. While, treatment with ginseng alone significantly decreased plasma activity of AST and ALT without any significant effect on the previously elucidated plasma hepato-renal function biomarkers compared to control group.

Plasma level of inflammatory cytokines

Plasma level of TNF-alpha and IL6 revealed a significant increase in HU-treated rats in comparison with control group. Meanwhile, their values were significantly diminished following co-administration of ginseng as compared to HU-treated animals. Moreover, administration of ginseng alone did not produce any alteration in plasma level of previously detected pro-inflammatory cytokines as compared to control group. (Table 2).

Oxidative changes

The level of MDA recorded a significant increase in hepatic and renal tissues of HU-treated rats which was accompanied by significant decline in GPX activity in these tissues when compared to control group. These elevations were significantly ameliorated toward normal control level upon co-treatment with ginseng. Treatment with ginseng alone did not reveal any significant changes in oxidant/antioxidant parameters in relation to control group (Table 3).

Histopathological examination

The histopathological changesdetected in livers and kidneys slides were represented in Figs. (1 and 2) and Table (4) which summarized the lesions' incidence and severity reported in HU and HU+ Ginseng-treated groups. No histopathological alterations were detected in either control or Ginseng-treated rats groups.

Liver

Control (Fig.1A) and Ginseng-treated rats' livers showed normal histological structures. Meanwhile, livers of HU -treated rats showed diffuse hepatocytic cytoplasmic vacuolation of both fatty type (sharply outlined vacuoles, Fig.1B, Table4) and hydropic type (hazy border vacuoles). Widening of the hepatic sinusoids, hypertrophy of Kupffer cells, widespread moderate to severe congestion and multifocal areas of hemorrhages (Fig1C) were noticed. Furthermore, multifocal areas of hepatocellularnecrosis with mononuclear cells infiltrations were evident (Fig. 1D, Table 4).The Portal triads were thickened with edema and mononuclear cell infiltrations associated with atrophied hepatic cords (Fig1E, Table4). Conversely, livers of HU+ Ginseng- treated rats showed the previous described lesions but they were less in the severity and distribution (Fig.1F, Table4).

Kidney

Control (Fig.2A) and Ginseng-treated rat's kidneys exhibited normal histological limits of the glomeruli and renal parenchyma. HU - treated rats showed degenerated tubular epithelium with star-shaped tubular lumen. Also, epithelium attenuation and necrosis with desquamated necrotic debris and inter tubular hyaline casts (Fig.2B, Table 4) were noticed. Regarding to the glomerular lesions, compressed capillary tufts with increased urinary spaces and presence of necrotic remnants of capillary tufts (Fig. 2C, Table 4) were recorded. Throughout the renal parenchyma, the interstitial tissue exhibited

multifocal areas of interstitial nephritis with mononuclear cells infiltration, inter tubular edema and marked tubular dilatation (Fig. 2D). In addition, vascular congestion, moderate perivascular edema associated with lymphocytic cells infiltration and moderate fibroplasia (Fig. 2E, Table 4) were noticed. In contrast, the detected renal alternations in HU+ Ginseng-treated group were reduced in the severity and distribution with marked improvement of the renal tissue (Fig. 2 F, Table 4) as compared to HU -treated group.

Table 1: Effect of HU and its combination with Ginseng on blood picture of male Wistar albino rats.

Groups	Control	Ginseng	HU	HU+ginseng
RBCs($\times10^6/\mu L$)	7.86±0.27a	7.85±0.27a	5.40±0.19b	5.28±0.24b
TLC($\times10^3/\mu L$)	14.12±0.23a	14.96±0.28a	8.56±0.26b	8.31±0.25b
Granulocyt($\times10^3/\mu L$)	4.18±0.26a	4.59±0.13a	1.82±0.05b	1.84±0.03b
Lymphocyte($\times10^3/\mu L$)	8.46±0.21a	8.76±0.23a	5.78±0.27b	5.68±0.25b
Monocyte($\times10^3/\mu L$)	1.47±0.16a	1.51±0.15a	0.95±0.07b	0.93±0.06b
Platelets($\times10^3/\mu L$)	555.20±8.01a	552.80±9.32a	330.80±17.31b	329.00±17.42b

All values are expressed as mean ± Std. Error. Means within the same raw of different litters are significantly different at ($P < 0.05$). H: Hydroxyurea, Ginsing: Panax ginseng extract

Table 2: Effect of different treatments on different plasma hepatic and renal function biomarkers and plasma pro-inflammatory cytokins in male Wistar albino rats

Groups	**Control**	**Ginseng**	**HU**	**HU+ginseng**
AST(U/L)	51.40±2.60b	48.40±2.93c	84.70±2.01a	58.30±4.09b
ALT(U/L)	34.20±2.99c	32.40±2.86d	72.60±5.18a	47.60±2.96b
GGT(U/L)	31.00±3.30c	31.20±2.29c	57.00±2.57a	39.90±2.90b
Urea(mg/dl)	54.20±2.51b	50.60±1.98c	80.40±2.94a	53.84±4.00b
Creatinine(mg/dl)	0.54±0.01c	0.53±0.01c	0.76±0.01a	0.63±0.01b
Albumin(g/dl)	6.02±0.06a	5.92±0.13a	4.09±0.08c	5.02±0.18b
TNF(pg/ml)	27.76±2.17c	28.20±2.33c	236.10±17.65a	138.50±6.67b
IL6 (pg/ml)	18.94±1.52c	17.50±1.50c	125.96±5.65a	64.58±8.42b

All values are expressed as mean ± Std. Error. Means within the same raw of different litters are significantly different at ($P < 0.05$).

Table 3: Effect of different treatments on hepatic and renal MDA and GPX in male Wistar albino rats

Groups	Control	Ginseng	HU	HU+ginseng
Liver MDA (μmol/g protein)	8.98±0.93c	7.59±0.53c	26.96±1.44a	26.96±1.44a
Kidney MDA (μmol/g protein)	5.03±0.87c	4.31±0.74c	22.46±2.06a	13.57±0.92b
Liver GPX (U/mg. protein)	3.28±0.29a	3.66±0.37a	1.81±0.12b	2.84±0.28a
Kidney GPX (U/mg protein).	3.04±0.42ab	3.44±0.26a	1.74±0.15c	2.37±0.19b

All values are expressed as mean ± Std. Error. Means within the same raw of different litters are significantly different at ($P < 0.05$).

Discussion

Hydrxyurea is a potent anti-neoplastic drug which is widely used to prevent painful crises of sickle cell anemia (37). Most available toxicity data from animals are present only in product labels, which are incomplete, and are often found only in summary statements (reviewed by Liebelt et al. 2007) (38).This study aimed to evaluate the toxic side effects of HU on liver and kidney tissues of rats and the probable role of *Panax ginseng* in refinement of such effects. HU is a small molecule, which is rapidly taken up by water and distributed widely throughout the body; it may reach vulnerable areas rapidly causing prompt cytotoxicity (39,40).

Myelosupression is one of the major associated complications of the treatment with hydroxyurea (41), as HU can kill S-phase colony-forming unit (CFU) cells of bone marrow and retard the entry of surviving cells into S-phase of division (42) and this may explain the significant reduction in RBCs, leukocytic and platelets count of HU-treated group. Also, HU has the ability to induce damage to erythrocytes membranes due to erythrocytic membrane lipid peroxidation causing lysis of RBC (43) which can be considered as another illustration for the decrement of RBCs count. On the other hand, hydroxyurea inhibit DNA synthesis in germinal center of lymph follicle (44) which may explain the cause of lymphopenia in HU-treated animals. It was suggested that hydroxyurea can induce production of intracellular ROS (42-47) which are involved in cytotoxic effect of hydroxyurea (48,49), as the mechanisms of action of HU depends on its rapid metabolism which produce carbomoylnitroso intermediate which then turned to nitroxide compounds (including nitric oxide) that are responsible for most of toxic effects of HU (50,51). These nitroxides act as free radicals which enhance formation of reactive oxygen species, creating oxidative stress state, interacting with nucleic acids and proteins to offset cellular functions, and altering cell signaling (51). This could be the reason for the elevated plasma activities of different liver enzymes (AST, ALT and GGT) due hepato cellular necrosis and membrane damage causes the release of these enzymes into the circulationin addition to increased plasma level of urea and creatinine. This Indicated oxidative hepato-nephrotoxic effect of HU which was affirmed by our histopathological findings.

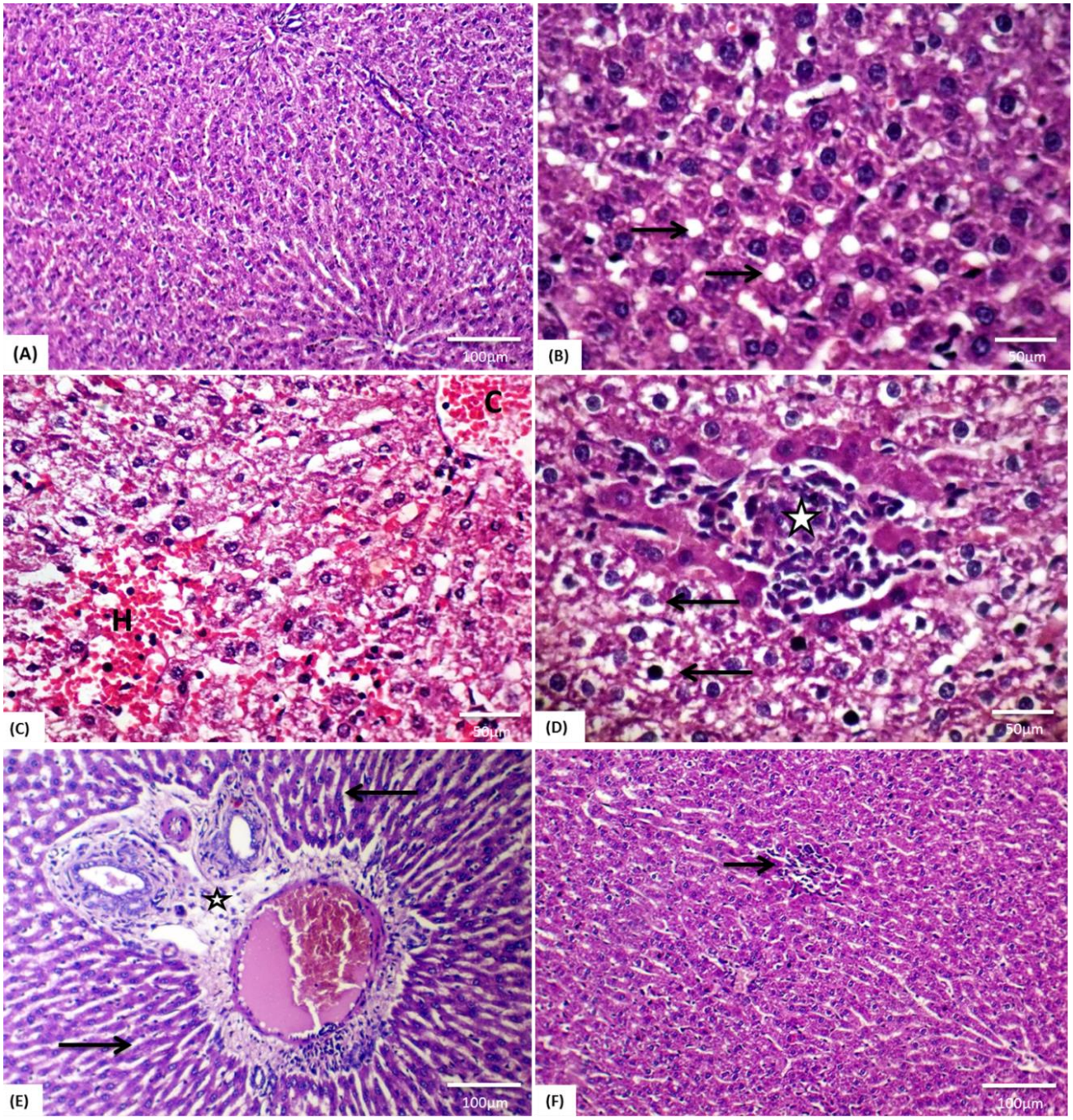

Figure 1: Representitive photomicrograph of rat livers stained with HE: (A) Normal histoarchitecture of a control rat liver (X100). (B,C,D&E) livers of **HD-** treated rats: (B) Hepatocytes showing sharply outlined fat vacuoles (arrows, X400), (C) Replacement of the necrotic hepatocytes with RBCs and mononuclear cells (H) associated with congestion (C, X400), (D) Hepatocyte cytoplasm showing hazy border vacuoles (arrows) in and hepatocellular necrosis with mononuclear cells infiltrations (asterisks, X400), (E) Thickening of the portal areas with edema and mononuclear cells infiltrations (asterisks) with newely formed bile ductules and atophied hepatic cords (arrows, X100). (F) **HD+ Ginseng** -treated rats showed markrd improvement of the hepatic tissue with minute area of hepatocellular necrosis (arrow, X100)

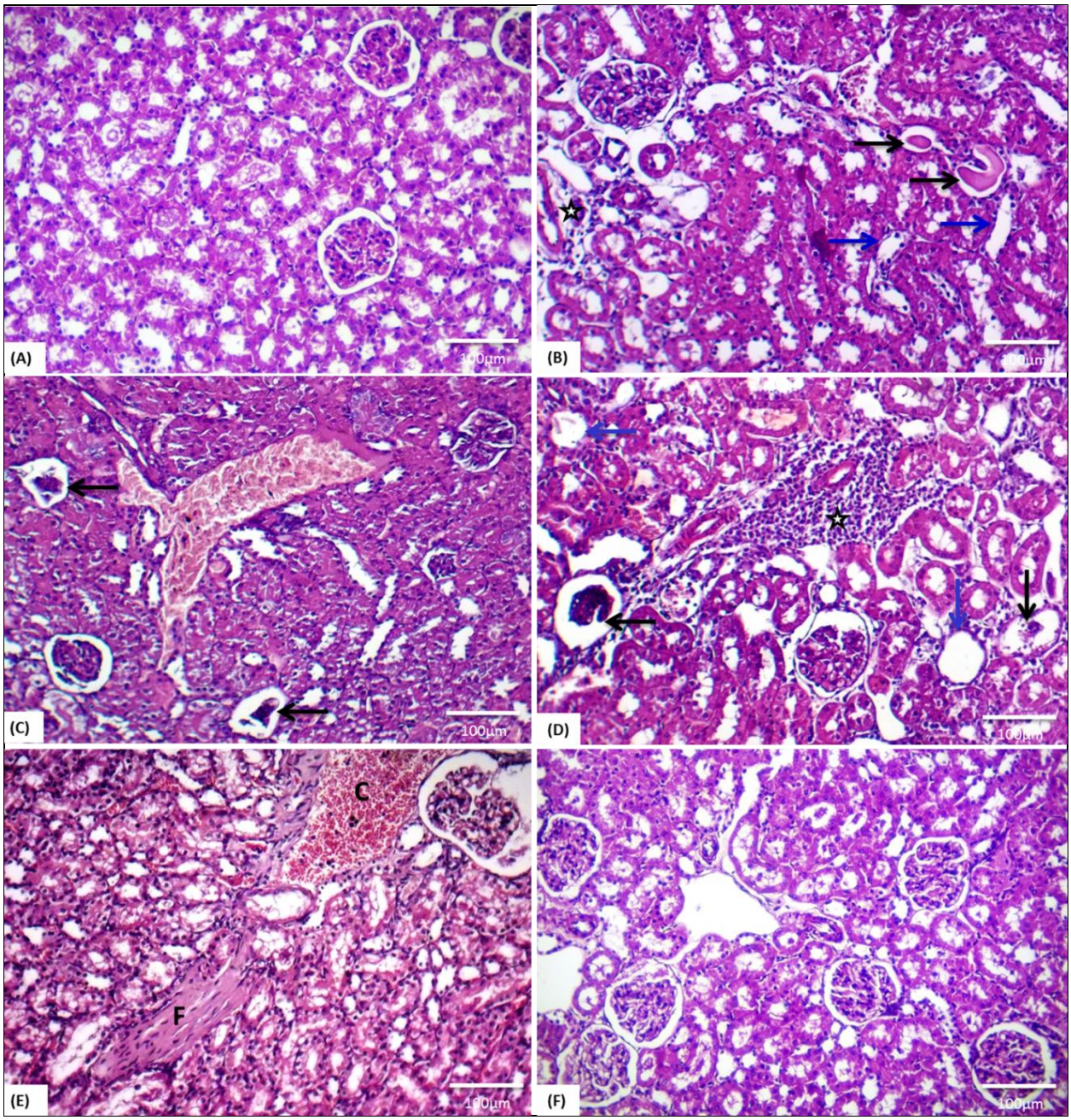

Figure 2: Representitive photomicrograph of rat kidneys stained with HE.X100: (A) Normal histoarchitecture of a control rat kidney. (B,C,D&E) kidneys of HU- treated rats: (B) Entertubular hyaline casts (black arrows), thining and attinuation of the tubular epitheium (blue arrows) and perivascular lymphocytic cells infiltration (asterisk), (C) Necrotic capillary tufts with widening of urinary space (arrows) associated with vascular congestion,(D) Interstitial mononuclear cells infiltrations in the renal cortex (asterisk) with atrophied and necrotic glomerular tufts (black arrows) cystically dilated renal tubules (blue arrows), (E) Vascular congestion(c) and fibroplasia of interstitial tissue (F). (F) HU + Ginseng -treated rats showing marked improvement of the renal tissue.

Table 4: Incidence and Severityof histopathological lesions recorded in livers and kidneys tissyes of HU and HU+Ginseng- treated rats

Scored lesions	**Incidence[1] and Severity[2] of histopathological Lesions**							
	HU-treated rats				HU+ Ginseng-treated rats			
	Ab-sent (-)	Mild (+)	Moder-ate (++)	Severe (+++)	Ab-sent (-)	Mild (+)	Moder-ate (++)	Severe (+++)
Liver								
a-Hepatocytic vac-ular and hydropic degeneration	0	0	4	6	0	0	8	2
b-Hepatocytic fat-tyvaculation	0	0	7	3	0	6	3	1
c-Congestion of cen-tral and portal veins.	0	1	3	6	1	6	4	0
d-Hepatocellular ne-crosis.	0	0	8	2	0	7	2	1
e-Periportal infilama-tory cells infiltra-tions.	0	0	6	4	0	5	3	2
Kidneys								
a-Inter tubular cast formation	0	1	6	3	0	6	3	1
b-Tubular epitheium necrosis	0	0	3	7	0	7	2	1
c-Vascular conges-tion (cortical or me-dullary)	0	0	4	6	0	6	4	0
d-Glomerular lesions	0	0	7	3	0	8	2	0
e-Interstitial mononu-clear cells infiltra-tions	0	0	8	2	0	6	4	0
f-Interstitial fibrosis	0	0	6	4	3	5	2	0

[1]Number of rats with lesions per total examined (10 rats).
[2]Severity of lesions was graded by estimating the percentage area affected in the entire section.
-Lesion scoring: (0) absence of the lesion=0%, (+) mild=5-25%, (++) moderate=26-50% and (+++) severe ≥50% of the examined tissue sections. HU(hydroxyurea) and Ginsing(Panax ginseng extract).

Plasma albumin level is considered as a good indicator for assessment of liver and kidney function as it synthesized by liver and reabsorbed by renal tubules after glomerular filtration, in addition, it is considered one of negative acute phase proteins as its level decreases in response to inflammation (52), so, its level decrement in HU-treated rats may be attributed to the pre-mentioned oxidative hepato-renal affection which could affect hepatic albumin production and increase urinary albumin loss or due to hepato-renal inflammatory state.

Oxidative damage of HU was proved in our study by increased level of MDA (lipid peroxide) and decreased level of GPX in both of hepatic and kidney tissues of HU-treated animals which accompanied by an elevated level of IL6 and TNF alpha in serum of the same animal group. Parallel to this, HU-induced hepato-nephrotoxic damages were affirmed histopathologically by presence of different hepatic and renal lesions that were previously mentioned in HU-treated rat group. Similar lesions were re-

ported by Ahmad et al. (2018) (53). The detected hepato-renal structural distortion may be due to previously mentioned HU-induced oxidative damage. Fortunately, most of biochemical and histopathological alterations caused by HU administration were partially reversed upon co-administration with ginseng. The semiquantitative scoring system demonstrated the protective role of ginseng in HU treated- rats group which was displayed by decreasing the intensity and distribution of the recorded hepatorenal lesions. This effectuation may be attributed to antioxidant activity of *Panax ginseng* that was previously cleared in several studies (54-56). Hepato-renal protective effect of *ginseng* as an antioxidant was previously recorded in different studies (57-60).

Conclusion

Finally, we could conclude that hydroxyurea has an oxidative stress-related deleterious effect on liver and kidneys, in addition to blood cellular components, but, these effects can be partially ameliorated upon administration of ginseng extract as a dietary antioxidant.

Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

NIGHT FEEDING REGIME IMPROVES WATER QUALITY, GROWTH PERFORMANCE AND FEEDING BEHAVIOUR OF EUROPEAN EELS, *Anguilla anguilla*

Abdel-Hay M. Abdel-Hay[1], Sara A. Hamed[1], Mohamed M. Zayd[1], Fatma A. Abouelenien[2], Ibrahim F. Rehan[3], Seham El-Kassas[4], Amany M. Deyab[1], Radi A. Mohamed[1]*

[1]Department of Aquaculture, Faculty of Aquatic Sciences and Fisheries, Kafrelsheikh University, 33516, Egypt, [2]Department of Hygiene and Preventive Medicine, Faculty of Veterinary Medicine, Kafrelsheikh University, [3]Department of Animal Behavior and Husbandry, Faculty of Veterinary Medicine, South Valley University, Qena (83523), Egypt, [4]Department of Animal Wealth Development, Faculty of Veterinary Medicine, Kafrelsheikh University

*Corresponding author: r.mohamed.vet@gmail.com and RadiMohamed@fsh.kfs.edu.eg

Abstract: This study was aimed to explore differences between day and night feeding of farmed European eels (*Anguilla anguilla*) on: (1) the growth and feeding behaviour of eels; and (2) the subsequent impact on the quality of the culture water. A 30 day field experiment was conducted in which 40 European eels (186.4 ± 9.26 g) were arbitrarily allocated into two replicate concrete ponds (200 x 100 x 125cm) that were divided into two treatment groups, one receiving feed by day (DF) and the other receiving feed at night (NF). Results revealed a significant improvement in eel's growth performance (larger final weight and faster growth) of the fish that were fed by night (NF). Moreover, feeding behaviour was also improved in the NF group with a significantly shorter latency to start feeding and shorter feeding duration. As a consequence of improved feeding behaviour, more favourable water quality conditions (higher dissolved oxygen concentrations and lower ammonia concentrations) were observed in the NF group. In conclusion, feeding eels by night could considerably enhance the culturing of eels by promoting enhanced growth and feeding behavior, and simultaneously improving water quality.

Key words: feeding regime; European eel; behaviour; nocturnal feeding; growth; performance

Introduction

The European eel (*Anguilla anguilla*) is a commercially important fish species that is distributed worldwide. It is a catadromous fish which spawns in the Sargasso Sea. For reproduction and spawning, the eel's larvae migrate to the Atlantic coast at which larvae molt several times till reach silver eels stage then return to lake or liver habitat to feed and grow up until it reach partial maturation. At this stage of growth, eels migrate to the marine water for another cycle of proliferation and spawning (1).

Due to a large demand in eels for consumption and a dwindling stock in the wild, the

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farming of eels has become increasingly popular in recent years. Given that the activity is still relatively new, there are many aspects of eel culture that are to date unknown. For example, preferences in feeding time and variations in diurnal and nocturnal feeding patterns.

Most fish species show daily rhythms in locomotion and feeding behaviour. These activities are carried out diurnally or nocturnally according to the species of fish in question (2). Previous studies have described the locomotion and feeding activities of the European eel as mainly nocturnal with increased activity around the time of light shifts; from dark to light and light to dark (1). Feeding at an optimal time of day can yield biological advantages to the species in question such as reducing energy loss (3). The European eel is thought to feed both diurnally and nocturnally and its feeding patterns are thought to be independent of locomotor activity (4).

In eel culture, to avoid feed waste and its bad effects on water quality, animals are provided with feed in a single meal per day and uneaten feed was removed as soon as possible. The feed should be provided daily in the same time to improve the feeding utilization and digestion because fish prepare its digestive secretions just before the upcoming meal that giving the opportunity for better feed digestibility (5).

From this perspective, little is known about the effect of feeding time on European eel (*Anguilla anguilla*) reared for commercial production. Consequently, this study was aimed to investigate the effect of feeding time on the growth performance and feeding behavior of the European eel (*Anguilla anguilla*) reared in concrete ponds in Egypt, as well as the subsequent impact on water quality.

Materials and methods

Animal, management and experimental design

A total of 80 farmed animals were obtained from the General Authority for Fish Resources Development (GAFRD), Cairo, Egypt. The experiment was conducted in 4 equal-sized concrete tanks (200 x 100 x 125 cm). The study was conducted in two treatments; one receiving feed by day (DF) and the other receiving feed at night (NF), two replicates each with continuous air pumping as a source of oxygen. Each tank was supplied with a separate water inlet and outlet covered with mesh to prevent the entrance of undesirable fish and predators and to prevent the escape of the study animals. The bottom of each pond contains crockery and PVC pipes (Figure 1). The eels were placed in a quarantine tank for 30 days to allow them to recover from the stress of transport. The experimental tanks were first disinfected and filled with water before being stocked with 10 randomly selected eels of a similar body weight. The eels in the first two tanks were fed during daylight at 7 am (DF) and the eels in the second two tanks were fed during nighttime at 7 pm (NF). The eels were fed raw fish flesh for 30 day, with a feeding ratio of 10% of body weight. The eels received feed once every 24 hours on a mesh in the corner of each pond to prevent leakage of the feed to the pond bottom. Committee of Aquatic Animal Care and Use in Research, Faculty of Aquatic Sciences and Fisheries, Kafrelsheikh University, Egypt, approved the protocol and conduct of the study.

Water quality analysis

Water quality was analysed once per week at 10 am and 10 pm on the same day to evaluate the quality of pond water. The physicochemical properties of water was assessed in three samples (250 ml each) in each treatment to determine total ammonia, unionized ammonia (UIA) using Martini MI 405 portable photometer. For estimation of temperature, pH, electrical conductivity (EC), total dissolved solids (TDS) and salinity a multiparameter meter was used (HI9829-03042-HANA). Dissolved oxygen (DO) was determined in each treatment at different water depth (20, 80 and 120 cm) using a specific DO meter (AQ 600 Milwaukee, Romania).

Fish performance and growth indicators

Eel feed intake (FI) was calculated everyday along the experiment (30 day). All 40 eels

were weighed at the beginning of the experiment (initial body weight, IBW). At the end of the experiment, the eels were collected from the treatment ponds using a suitable narrow net and retained in different clean plates. The eels were dried using a clean sterile filter paper to remove the excess water from the body before measuring and weighing of final body weight (FBW). Weight gain (WG) was calculated as (FBW-IBW), specific growth rate (SGR): 100[(FBW-IBW)/t]; where (t) is the culture period in days. Feed conversion ratio (FCR) equal to feed intake/ weight gain.

Eel behaviour

The behaviour of eels during feeding was recorded by the observer during the feeding process. Latency to start feeding (the time elapsed between provision of the feed on the mesh and the first eel starting to eat in seconds) and feeding duration (the time elapsed between starting of feed till the ending of feed in minutes) were calculated.

Statistical analysis

The data was subjected to distribution normality test before analysis. Data were analyzed using Graph Pad™ Prism 6. Data were compared by the Student's *t*-test with the significance level $P<0.05$. Results are presented as means ± SEM.

Results

The outcomes of water parameters analysis are presented in Table 1. Dissolved oxygen concentrations were significantly higher in the NF group both during daylight and night except at 10 am at 20 cm depth. Total ammonia, unionized ammonia and pH were significantly lower in the NF group than in the DF group. There were no significant differences in temperature, salinity, TDS and EC between the NF and DF group.

The growth performance parameters are presented in Table 2. The growth performance parameters (FBW, WG, FI, FCR and SGR) were significantly improved by feeding eels at night in comparison to DF.

Feeding behaviour of European eels (*Anguilla anguilla*) is shown in Figures 2 and 3. There was a significant difference in latency to start feeding and the feeding duration between the DF and NF group. The latency to start feeding was shorter in NF than DF group. While the feeding duration was prolonged in DF compared to NF group.

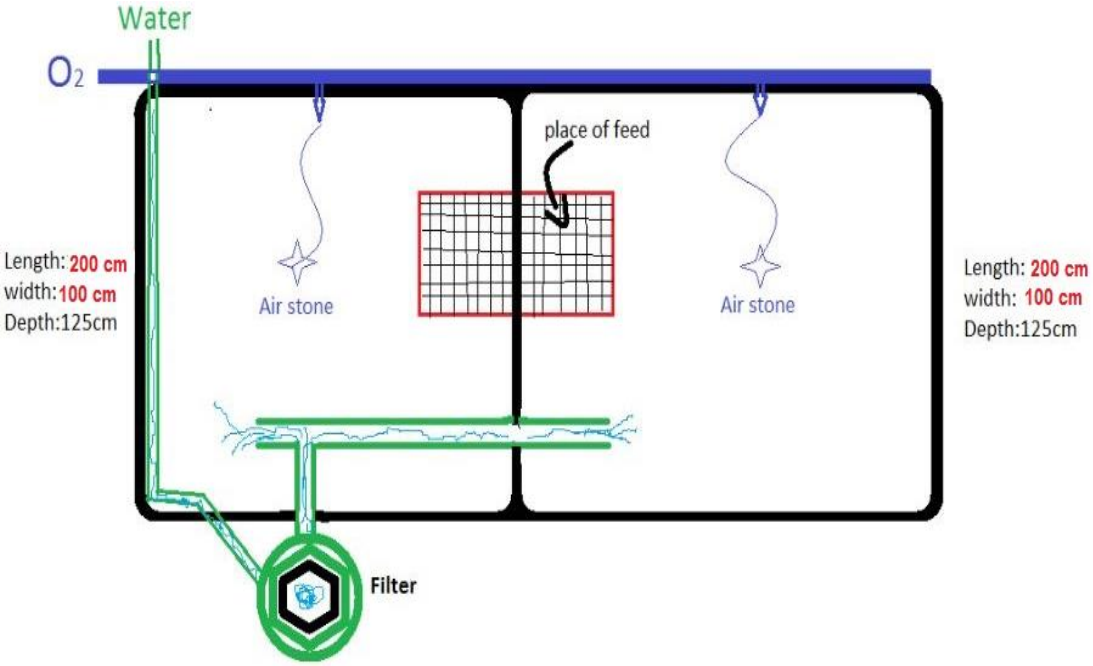

Figure 1: Design of concrete pond used for rearing of European eels (*Anguilla anguilla*)

Table 1: Effect of feeding regime on water quality of European eels (*Anguilla anguilla*)

Water parameter	Monitoring time	Day feeding	Night feeding	*P*-value
DO	10 am			
	20 cm depth	14.370± 0.186	15.030±0.606	0.388
	80 cm depth	7.367±0.273	10.730±0.722	0.031*
	120 cm depth	2.933±0.177	4.933±0.166	0.001*
	10 pm			
	20 cm depth	8.533±0.291	9.900±0.208	0.023*
	80 cm depth	5.233±0.203	6.900±0.300	0.014*
	120 cm depth	1.633±0.203	3.967±0.491	0.028*
Temperature	10 am	24.00±0.058	23.97±0.088	0.770
	10 pm	20.17±0.219	20.20±0.265	0.928
PH	10 am	8.233±0.088	7.577±0.137	0.022*
	10 pm	9.133±0.145	7.900±0.058	0.002*
Total ammonia	10 am	0.027±0.003	0.013±0.004	0.047*
	10 pm	0.034±0.003	0.022±0.001	0.047*
UIA	10 am	0.00237	0.00077	0.001*
	10 pm	0.0119	0.0001	0.001*
Salinity (ppt)	10 am	1.900±0.057	1.900±0.058	0.999
	10 pm	1.900±0.057	1.900±0.058	0.999
TDS	10 am	302.000±9.539	301.700±8.988	0.981
	10 pm	300.000±9.210	300.700±7.951	0.981
EC	10 am	591.300±13.040	592.000±13.230	0.973
	10 pm	590.300±13.100	590.000±13.410	0.974

Dissolved oxygen (DO), unionized ammonia (UIA), total dissolved salts (TDS), Electric conductivity (EC). Asterisks indicate significant differences between experimental groups (Student t-test *p<0.05)

Table 2: Effect of feeding regime on performance of European eels (*Anguilla anguilla*).

	Day feeding	Night feeding	*P*-value
Initial body weight (g)	186.000±3.559	186.800±14.600	0.958
Final body weight (g)	189.100±3.609	203.100±5.258	0.042*
Weight gain (g)	3.050±0.450	16.250±2.822	0.002*
Feed intake (g)	16.600±1.024	66.200±5.017	0.001*
Feed conversion ratio	6.473±0.483	4.632±0.259	0.004*
Specific growth rate	0.09±0.004	0.542±0.003	0.001*

Asterisks indicate significant differences between experimental groups (Student t-test *p<0.05)

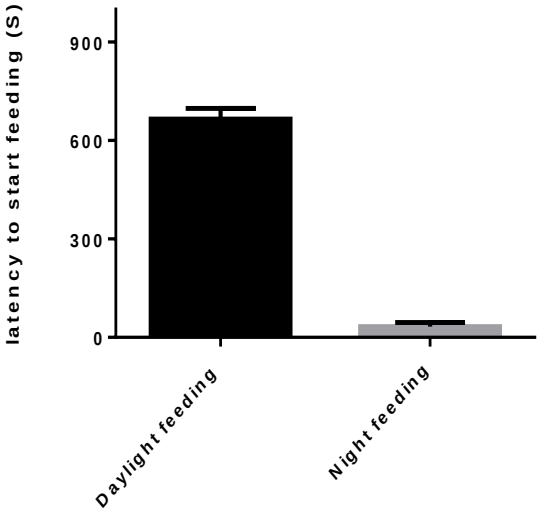

Figure 2: Effect of feeding regime on feeding behaviour (latency to start feeding) of European eels (*Anguilla anguilla*). (Student t-test *p*-value =0.001)

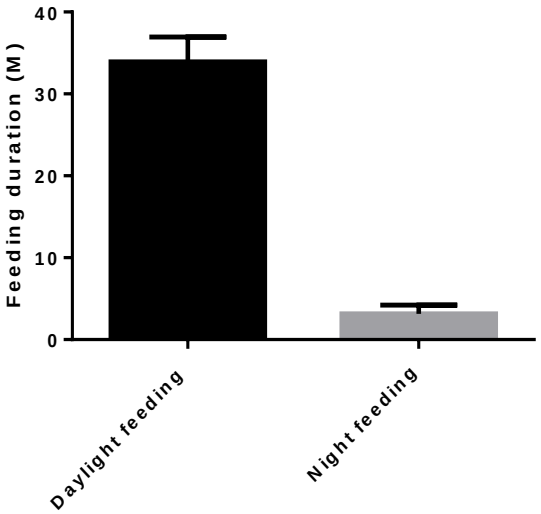

Figure 3: Effect of feeding regime on feeding behaviour (feeding duration) of European eels (*Anguilla anguilla*). (Student t-test *p*-value =0.001)

Discussion

The present study showed that NF regime influenced water quality parameters, growth performance and feeding behaviour of European eels (*Anguilla anguilla*). Water quality is a key factor in the success of any aquaculture system. When stocking eels in high densities, a higher level of DO is needed to attain better growth and to maintain normal activity. Furthermore, pond water exchange by pumping of new water and drain the old one in a regular way is also required (6). Water samples should be analysed regularly for DO, TDS, UIA and temperature. The DO in eel's water should be not less than 3 ppm, UIA level not more than 0.2 mg/L and PH around 7 (7). Additionally many trials were done to improve the quality of pond water and decrease the load of pollutants (8) which if discharged will cause environmental hazard.

The process of NF improved the water quality measurements in form of increased level of DO; which considered the most important determinant for the soundness of aquaculture, (9) both during daylight and night in the three points of monitoring (20, 80 and 120 cm from water surface). Moreover, it reduced the level of PH, total ammonia and UIA both during both monitoring. Generally, low pH will decreased the level of toxic form of ammonia (UIA), which is the most dangerous form. On the other hand, the feeding time has no significant effect on water temperature, salinity, TDS and EC even during daylight and night monitoring. This improvement in the water quality in the NF group has a direct effect on enriching the rearing environmental resource (3). Water quality improvement due to NF will decrease the rate of water exchange, which is more economic and decrease the water pollution load (6). In this study, eels were reared in a temperature ranged from 20.2 to 24°Cwhich considered within the optimum range of rearing temperature in captivity (10). Not only this lower temperature was found to reduce bacterial growth in rearing tanks, but also improved water quality and enhanced survival rates of cultured eels (10). It also reduced the basal metabolic rate of cultured eels and saved the metabolic energy that may have a direct effect in form of improved growth and indirect effect in form of reduced eel's waste products and therefore improving water quality (11). These findings have coincided with Degani et al. (12) who found that the level of DO and UIA concentrations depend on stocking rate, fish metabolic products and pond water quality.

Herein the two groups of European eels displayed different patterns of growth performance. The NF eels showed higher growth performance in form of higher FBW, WG, FI and SGR than DF. While it showed a lower feed conversion ratio than DF. This may be attributed to the increased activity of eels during the night (1) that improved feed intake and consequently improved performance (3). For individual eel weight in the DF group, there was an extensive difference between individuals which may be due to differences in genetic makeup of the individuals within the same treatment or due to their different feeding habits. The same finding was observed by López-Olmeda et al. (4). In addition, the improvement in water quality in the NF group especially high DO and low ammonia, UIA and pH found to have a direct impact on growth performance of eels. Degani et al. (12) reported that DO, total ammonia, temperature and pH are considered an important factors affecting eels performance. Interactions among these parameters greatly affect the rate of growth in eels. Lo´pez-Olmeda et al, (13) found that the feeding time may affect the cortisol level in eels. The lower level of cortisol was observed during the night. Feeding time may be affected by cortisol level and gastric secretion (e.g. gastric PH). Feeding eels once per day may improve digestive secretion that may be increased just before the next meal. This may be the explanation for improved digestion and performance (14). All of these factors may be interrelated with higher WG and FI, lower FCR and improved growth performance in NF group compared to DF group.

Feeding behaviour of European eels was influenced by feeding time. Day feeding group showed prolonged latency to start feeding and feeding duration. This may be due to the reduction of European eel's activity during daylight (1). In addition, the high level of cortisol during daylight period may influence on eels behaviour and the eels become very nervous and stressed that might make differences in the feeding duration or FI which depend on the stress level (4). Furthermore, the prolonged latency to start feeding and feeding duration in the DF group may have a direct impact on the water quality of this group. It may lead to waste of unconsumed feed and then decomposed in pond water (10). This process may be the reason for higher pH, total ammonia and UIA in DF than NF group. Similar findings were observed by Degani et al. (12) and Taufiq et al. (6).

Conclusion

The obtained results suggested that manipulating both environmental and managemental factors can regulate healthy growth performance and feeding behaviour of European eel (*Anguilla anguilla*). Night feeding regime improved water quality, growth performance and feeding behaviour of European eels. Water quality improvement due to NF will reduce the rate of water exchange.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

USING REAL-TIME POLYMERASE CHAIN REACTION TO STUDY THE EFFECT OF SALICYLIC ACID AND SODIUM CHLORIDE ON THE PYOCYANIN PRODUCTION OF *Pseudomonas aeruginosa*

Alaa A. Sherif, Hesham S. Nada, Amgad A. Moawad*, Ibrahim E. El- desouky, Salwa M. Helmy

Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Kafrelsheikh University, 33516, Egypt

*Corresponding author, E-mail: moawadamgad@gmail.com

Abstract: *Pseudomonas aeruginosa* is a bacterium that seizes the opportunity to cause serious infections in both man and animals. Pyocyanin is a strong virulence factor produced by *P. aeruginosa*. The present study was performed to explain the effect of salicylic acid (aspirin) and sodium chloride (NaCl) on *P. aeruginosa* and Pyocyanin using concentrations of 1.5%, 3% and 6% for aspirin and 3%, 6% and 9% for NaCl. The effectiveness of aspirin and NaCl was detected by real time PCR (qPCR). The *P. aeruginosa* isolate was from broilers suffering from septicemia in Zagazig city, Egypt. The study revealed that 6% and 9% concentration of NaCl had a bactericidal effect on the bacterium but 3% concentration of NaCl has an inhibitory effect on both the growth and the expression of Pyocyanin (by 0.5-fold lesser than the untreated sample). Also, data revealed that 1.5%, 3% and 6% concentrations of aspirin inhibited the growth and reduced the expression of pyocyanin with slight various degrees (by 0.7955, 0.4234, 0.1948-fold lesser than untreated sample, relatively). The results of this study could be useful in pharmacological intervention and clinical therapy as aspirin and NaCl could be combined with antimicrobial agents to treat *Pseudomonas* infections.

Key words: *Pseudomonas aeruginosa*; pyocyanin; sodium chloride; salicylic acid

Introduction

Pseudomonas aeruginosa (P. aeruginosa) is a broad, Gram-negative bacterium that seizes the opportunity to infect the immune-compromised hosts including people infected by HIV, patients exposed to chemotherapy and patients suffering from severe burns (1).

The infection with *P. aeruginosa* gives rise to mortality in chickens and clinical symptoms including respiratory signs and septicemia (2). Proteases (elastase, protease, and alkaline protease), toxins (exoenzyme S and exotoxin A), hemolysins and phenazines are virulence factors in *P. aeruginosa*. They have been found to participate in *P. aeruginosa* pathogenicity in man and animals. Pyocyanin is a phenazine compound (5-N-methyl-1-hydroxy phenazine) considered as both a signal molecule and an important

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virulence factor. In cystic fibrosis lungs in vitro, pyocyanin interferes with the ciliary function of the respiratory system and can change the host immune response. Also, pyocyanin is considered as the last signal in the quorum-sensing (QS) system, and organizes many genes in *P. aeruginosa* (3).

Some compounds can inhibit the quorum-sensing system such as salicylic acid (aspirin) (4). Aspirin can inhibit not only biofilm forming of *P. aeruginosa*, but also it can decrease the pyocyanin production (5). It has been reported that salicylic acid cause changes in the membrane of *P. aeruginosa*. Salicylic acid is regarded as an antimicrobial agent in the treatment of *Pseudomonas* keratitis (6).

Many environmental stimuli such as osmolarity, pH, NaCl and the diversity of ions have a distinct influence on *P. aeruginosa* (7, 8). The swarming motility of the bacterium is affected by NaCl. Bacterial growth is inhibited by 6% NaCl solution. Bactericidal action occurs at 10% NaCl solution (8).

16S rDNA-based PCR assays provide fast, plain and dependable identification of *P. aeruginosa a*nd its discrimination from other *Pseudomonas* species which are closely related in phylogenesis (9). Real-Time PCR (qPCR) was applied to study the fluorescence level in a particular selected gene. It is a highly sensitive technique that has remarkable potential for the high-volume analysis of gene expression in both research and routine medical diagnostics. This technique provides real-time quantitation of an initial template of DNA or RNA (10).

The present study was designed to illustrate the effect of NaCl and aspirin on an Egyptian *P. aeruginosa* strain isolated from septicemic broilers.

Material and methods

The origin of the strain

P. aeruginosa was isolated from broilers suffering from septicemia in Zagazig City, Egypt since 2017. *P. aeruginosa* was identified after examining for phenotypic and genotypic characteristics in the laboratory of microbiology department in Zagazig University. The isolate was maintained on Tryptone Soya Agar (Oxoid, CM0131, UK) (TSA) slopes at 5 °C and sub-cultured every month. *P. aeruginosa* was transferred on TSA slopes to Kafrelsheikh University at the laboratory of bacteriology.

Determination of the effect of Sodium Chloride on P. aeruginosa

P. aeruginosa subculture occurred in King A broth (Himedia M1544) supplemented with 30, 60, and 90g/l of NaCl (Bio Jet) to obtain 3%, 6%, and 9% concentrations respectively. Cultures were incubated in a shaker incubator (180 rpm at 30°C) for 48h. The growth was determined visually and compared with the untreated sample of *P. aeruginosa* (3).

Determination of the effect of aspirin

P. aeruginosa subculture was occurred in nutrient broth overnight (PO) at 37 °C. Aspirin (100 mg, BAYER) was used. 2-fold serial dilutions in nutrient broth were prepared resulting in 100, 50, 25, 12.5, 6, 3, 1.5 mg/ml concentrations. Seven tubes each one contains ml from nutrient broth. In the first tube, one tablet from aspirin (aspirin protect 100mg) was added. The concentration became 100 mg /ml in the first tube. 0.5 ml from the first tube was taken to second tube giving concentration 50 mg /ml in the second tube. Repeat the same in other tubes giving the concentrations 25, 12.5, 6, 3, 1.5 mg/ml). Then discard 0.5 ml from the last tube. The last three tubes No. 5(D),6(E) and 7(F) contained 6,3,1.5 % Aspirin respectively. Diluted aspirin (D, E, F) was inoculated with 100 Micron PO containing 6 _ 105 CFU/ml and incubated at 37 ºC for 24 h. Minimum inhibitory concentration (MIC) (the lowest concentration of antibacterial agent that inhibit the apparent growth of the bacteria) was measured. PO was treated with sub-inhibitory concentrations (sub-MIC) of aspirin (1/4 MIC). The number of viable emerged bacterial cells was estimated and collated to the number of untreated PO using the plating

method (12). The growth of both PO treated with 1/4 MIC of aspirin and untreated PO was monitored. Inoculation of an overnight culture of PO into nutrient broth tubes treated with aspirin (1/4 MIC) and control ones without aspirin occurred. Incubation at 37 ºC for 24 h. Every hour, Optical Density 600 nm for treated and untreated samples was measured (4).

Molecular identification

The extraction of DNA was done by QI-Aamp DNA Mini kit instructions (Qiagen, Germany, GmbH, Catalog no. 51304) with some changes. Incubation of 200 µl of the bacterial suspension occurred at 56ºC for 10 min after addition of 10 µl of proteinase K and 200 µl of lysis buffer. Then, 200 µl of ethanol 100% (Applichem) were appended to the lysate. The sample was washed and centrifuged according to the manufacturer's index. Then, elution of the nucleic acid occurred with 100 µl of elution buffer presented in the kit. The reaction contained 25 µl of the following PCR master mix. Emerald Amp GT PCR master mix (2x premix) was 12.5 µl, PCR grade water was 4.5 µl. Forward primer (20 pmol) was 1 µl. Reverse primer (20 pmol) was 1 µl. Template DNA was 6 µl. The amplification of target gene phenazine (phzM) of *Pseudomonas* genus was performed using primer pair of phzM; 5'-ATGGAGAGCGGGATCGACAG- '3 and 5'- ATGCGGGTTTCCATCGGCAG- '3 amplifying 875 bp (13). The used primers were supplied from Metabion (Germany). Amplification cycles (n=35) were performed under these conditions; primary denaturation for 5 min at 94ºC, secondary denaturation for 30 sec at 94ºC, 1 min of annealing at 54ºC and 1 min of extension at 72ºC. Electrophoresis of amplified products in 1.5% agarose gel (ABgene) was done. The sizes of the amplified product were determined by a gene ruler 100 bp DNA Ladder (catalog No. SM0243) (Fermentas, Thermo Scientific, Germany). Photographing was done by Alpha imager (Innotech, Biomedia). A computerized analysis was performed.

RNA extraction for qPCR was done following RNeasy Mini Kit instructions (Qiagen, Catalog no. 74104) with some modifications. To protect RNA from degradation, a double volume (1 ml) of the RNA protect Bacteria Reagent (Qiagen, Germany, GmbH Catalog no. 76506), was added to one volume (0.5 ml) of the broth of *P. aeruginosa* culture, then the mix was incubated at room temperature for 5min. Then add 200 µl of Tris EDTA buffer (TE buffer) (Thermo Fisher, Catalog no. 12090-015) containing 1 mg/ml Lysozyme (Biochemica, Applichem, Catalog no. A3711) and 700 µl of lysis buffer. Then, 500 µl of 96% ethanol (Applichem) were added to the lysate. Washing and centrifugation of the sample were performed according to the manufacturer's index. Then, elution of RNA by 50 µl RNase free water (Quantitect SYBR green PCR kit) (catalog. No .204141). The reaction contained 25 µl of the R master mix according to Quantitect SYBR green PCR kit. QuantiTect SYBR Green PCR master mix (2x premix) was 12.5 µl. RevertAid Reverse Transcriptase (Thermo Fisher, Catalog number: EP0441) was 0.25 µl. Forward primer (20 pmol) was 0.5 µl. Reverse primer (20 pmol) was 0.5 µl. Template RNA was 3 µl. RNase free water was 8.5 µl. The target genes: phenazine (*phzM*) of Pseudomonas genus was performed using primer pair of *phzM* (mentioned previously), a primer pair of 16S rRNA gene;5'- GGGGGATCTTCG-GACCTCA- '3 and 5'- TCCTTAGAG-TGCCCACCCG- '3 (14). The following cycling condition protocol was used: 30 min for reversed transcription at 50 ºC, 5 min for primary denaturation at 94 ºC, amplification cycles (n=40) were performed under the following conditions; 15 min of secondary denaturation at 94ºC, annealing (Optics on) at 52 ºC for 45 min for 16S rRNA but at 54 ºC for 45 min for *phzM*, 30 sec for extension at 72 ºC, dissociation curve (one cycle) conditions; 1 min of secondary denaturation at 94 ºC, 1 min of annealing at 52 ºC for 16S rRNA but at 54 ºC for 1 min for *phzM*, 1 min of final denaturation at 94 ºC. The curves of amplification and cycle threshold (Ct) values

were evaluated by the Stratagene MX3005P qPCR software. To assign the difference in the gene expression on the RNA of the samples, a comparison between the Ct of each sample and that of the control sample occurred according to the "$2^{-\Delta\Delta Ct}$" equation (15).

Results

The effect of NaCl

Growth appeared at 3% concentration NaCl but no growth occurred at 6, 9%. Growth turbidity at 3% concentration of NaCl was less than that of untreated sample.

The effect of Aspirin

Initially, the minimum inhibitory concentration of aspirin against *P. aeruginosa* PO was revealed as 24 mg/ml. The sub-inhibitory concentrations of aspirin (1/4, 1/8 and 1/16 MIC) corresponding to 6, 3 and 1.5 mg/ml concentrations of aspirin respectively were applied to evaluate the inhibitory influence on both quorum sensing system and the virulence factors of *P. aeruginosa* PO. The treated *P. aeruginosa* PO with sub-MIC of aspirin was as similar as the untreated PO in its viability. Example for that, the bacterial number was 154 _ 104 CFU/ml at 1/4 MIC in treated PO but the bacterial number of the untreated PO was 160 _ 105 CFU/ml. Also, both treated and untreated PO attained a stationary phase after incubation for 9 h at 37 ºC, signifying no impact on the growth. But the bluish metallic sheen OF *P. aeruginosa* (11) was changed to some extent.

Turbidity degree was inversely proportional with the concentration of Aspirin. Treated Pseudomonas with 1/4 MIC showed the least turbidity. The turbidity of treated *Pseudomonas* with

1/16 MIC was lesser than the untreated sample.

Polymerase Chain Reaction

PCR reaction was done to diagnose *P. aeruginosa* using 16S rDNA. The results showed that one band 875 bp (product size) as a result of agarose gel compare with DNA marker (100 bp) (figure 1).

Quantitative real-time PCR

The fluorescence light emitted within qPCR is directly proportional to the formed DNA and can be assumed as an amplification plot. As a result of Ct values (table 1), the data revealed that 3% concentration of NaCl reduced the expression of *PhzM* (by 0.5-fold less than the untreated sample). The reduction percentage of *PhzM* was 50%. But for Aspirin, 1.5%, 3% and 6% concentrations caused decrease in the expression of pyocyanin with various degrees (by 0.7955, 0.4234, 0.1948 -fold less than untreated sample relatively), i.e., the reduction in expression of *PhzM* with 80.52 %, 57.66% and 20.45% in sub MIC (1/4 (6mg/ml), 1/8 (3mg/ml) and 1/16 (1.5mg/ml)) of Aspirin respectively (chart1). The experiment had been repeated three times. The amplification curve of 16SrDNA (figure 2) but that of *PhzM* (figure 3) were showed.

Table 1: The Ct values of 16SrDNA and *PhzM* for each sample with the fold change of them were analyzed. The Control sample was the untreated isolated *P. aeruginosa*

Sample No.	Sample ID	*16S rDNA*	*phzM*	
		CT	**CT**	Fold change
B	Control	19.78	22.86	-
C	NACL 3%	20.84	24.92	0.5000
D	Aspirin 6%	20.39	25.83	0.1948
E	Aspirin 3%	19.21	23.53	0.4234
F	Aspirin 1.5%	20.54	23.95	0.7955

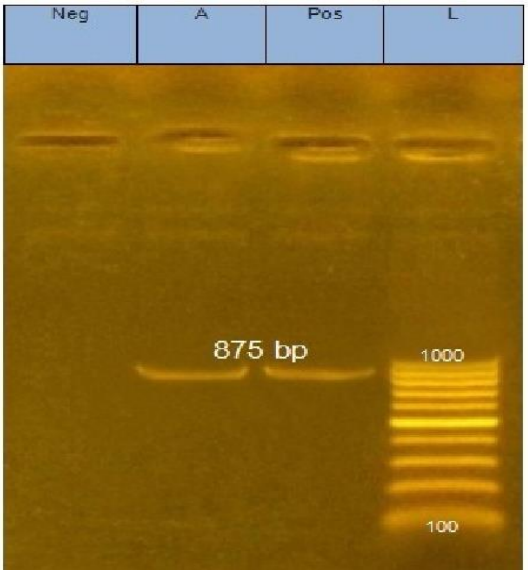

Figure 1: agarose gel electrophoresis of pyocyanin gene amplification of *Pseudomonas aeruginosa* strain. L: 100bp ladder. Lane (A): positive sample. Pos: positive control and neg: negative control. Positive and /or negative controls were represented by field sample that was previously confirmed to be positive or negative by PCR for the related gene in the Reference laboratory of Animal health research institute, Zagazig University. Amplicon size: 875 bp

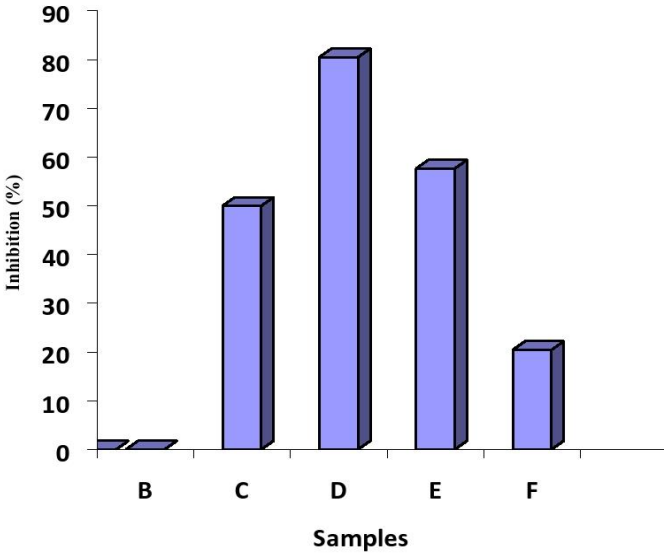

Chart 1: The inhibition percentage of *PhzM* expression in each sample. B: control (untreated sample). C: a sample containing NaCl 3%. D: a sample containing Aspirin 6%. E: a sample containing Aspirin 3%. F: a sample containing Aspirin 1.5%

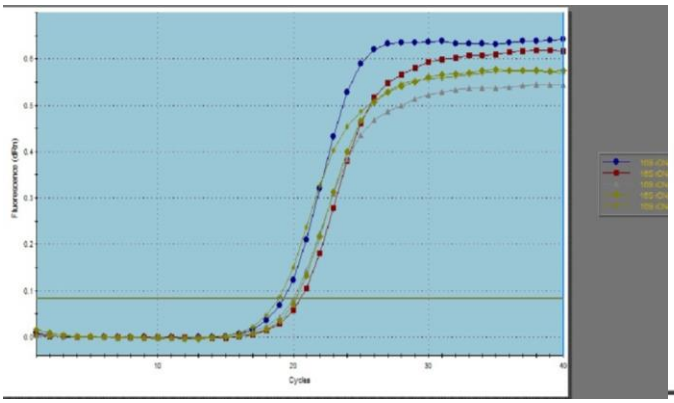

Figure 2: Amplification curve of 16SrDNA. X-axis: amplification cycles. Y-axis: distinctive fluorescence. The axis that was parallel to X axis was a threshold axis. Each point of each sample of the curve which crossed the threshold axis was the Ct value

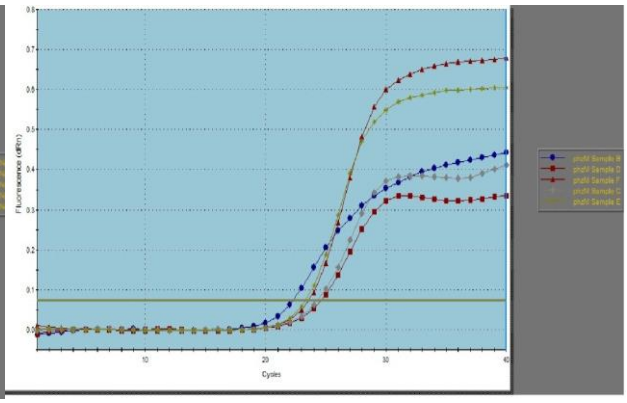

Figure 3: Amplification curve of *PhzM*. X-axis: amplification cycles. Y-axis: distinctive fluorescence. The axis that was parallel to X-axis is threshold axis. Each point of each sample of the curve which crossed the threshold axis was Ct value

Discussion

In the present study, the influence of NaCl and Salicylic acid on the *P. aeruginosa* growth and expression of pyocyanin virulence factor of it was investigated. Results demonstrated that NaCl and Aspirin had a significant impact on the *P. aeruginosa* growth and pyocyanin production. This study revealed that 6% and 9% concentration of NaCl had a bactericidal effect on the bacterium but 3% concentration of NaCl has an inhibitory effect on the growth

and also on the expression of Pyocyanin (by 0.5-fold lesser than the untreated sample). The reduction percentage of *PhzM* was 50%. But for Aspirin, 1.5%, 3% and 6% concentrations caused inhibition of growth and decrease in the expression of pyocyanin with slight various degrees (by 0.7955, 0.4234, 0.1948 -fold lesser than untreated sample relatively), i.e., the reduction in expression of *PhzM* with 80.52 %,57.66% and 20.45% in sub MIC (1/4 (6mg/ml),1/8(3mg/ml) and 1/16 (1.5mg/ml) of Aspirin respectively.

Previous studies revealed that *Pseudomonas* influenced by a different concentration of salinity. Pyocyanin production increased proportionally with salinity concentration. The high amount of pyocyanin (29.57µg/ml) was obtained with 20g/L of NaCl (2% concentration). The result also indicated that salinity above 20g/L have totally affected pyocyanin production but did not affect the bacterium growth which was completely inhibited at 60 g/L of NaCl (6% concentration). The production of pyocyanin was salinity-dependent when *P. aeruginosa* had grown in King A medium supplemented with 2.5 to 20g/l of NaCl. The highest amount of pyocyanin was obtained at 20g/l (2% NaCl) and the growth of *P. aeruginosa* was completely inhibited at 50 g/l of NaCl (5 % NaCl) (3); However, another study showed that maximum of productivity was found in a medium salinity ranging from 5-10g/l. but at concentration 5 g /l or less, there was not significantly inhibition of the growth and the pyocyanin production. (16, 17).

Other studies revealed that the normal growth of *P. aeruginosa* appeared on a medium which contained 0.25 up to 1.25% concentration of NaCl in comparison to control medium. Above 1.75% NaCl concentration in the medium, it can survive up to 2.75% NaCl but the survival number of *Pseudomonas* gradually reduced. The inhibition impact was observable at a solution containing 4% concentration of NaCl. A solution containing 6% concentration of NaCl could inhibit the growth of *Pseudomonas* after incubation for 24 h. The bactericidal effect on *Pseudomonas* occurred by 8% and 10% of NaCl (18, 8).

For patients suffering from cystic fibrosis in the lung, aerosols containing hypertonic saline (7% NaCl) are useful in the treatment due to abolish both motility and growth of *P. aeruginosa* (19).

Salicylic acid can inhibit production of pyocyanin (4,6). Distinct reduction in the virulence factors of *P. aeruginosa* treated with 6 mg/ml (1/4 MIC) and 3 mg/ml (1/8 MIC) concentrations of aspirin. Pyocyanin reduction was 78% due to treating with aspirin (1/4 MIC) (4). Growth in the sub-inhibitory concentrations of salicylic acid resulted in a significant reduction in bacterial cell number, cell density and in quorum-sensing signaling molecules (20). Salicylic acid plays a role in down-regulating some of the virulence factors (especially pyocyanin) in P. aeruginosa and attenuating the virulence of the bacterium on *Caenorhabditis elegans* and *Arabidopsis thaliana*. A 50% reduction of Pyocyanin production was resolved after treatment with 0.1 mM salicylic acid but an 80% reduction of pyocyanin production with no visible influence on the bacterial growth was determined after treatment with 1.0 mM SA (20, 21).

Another study revealed that *P. aeruginosa* 1604 was still able to produce a considerable amount (0.44 µgml-1) of pyocyanin after a 72 hours lag period in the presence of 0.1 % aspirin. At 0.3 % concentration, the inhibitory effect was evident for 120 hours. For long-term inhibitions, a higher amount of applied salicylic compounds (0.3 and 0.4 %) are necessary (5). Salicylic acid can reduce bacterial numbers and its virulence during keratitis so it is effective as an antimicrobial substance to treat *Pseudomonas* keratitis (6).

Conclusion

In this study, *P. aeruginosa* isolate was from broilers suffering from septicemia in Zagazig city, Egypt. By using PCR and qPCR, the data revealed that Aspirin and Sodium chloride inhibit the *P. aeruginosa* growth and decrease pyocyanin production. This new therapeutic approach can be used in combination with antimicrobial agents to treat *P. aeruginosa* cases.

Conflict of interest

None of the authors disclose any conflict of interest.

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Original Research Article

PRENATAL DEVELOPMENT OF THE VOMERONASAL ORGAN IN RABBIT

Saleh A. M. A. ALomaisi[1,2]*, Hanaa M. El-Ghazali[2], Hamed M. Nosseur[2], Salah El-dein A. Ahmed[2], Mervat M. Konsowa[2]

[1]Departments of Animal Research Regional Research Station, Agriculture Research and Extension Authority, 87148 Thamar Yemen, [2]Departments of Anatomy and Embryology, Faculty of Veterinary Medicine, Zagazig University, 44511, Egypt. Paper extracted from PH.D. thesis of Saleh Ahmed Mohammed Ali Al-Omaisi

*Corresponding author: elemesy18@vet.zu.edu.eg, alomisy78@yahoo.com

Abstract: The vomeronasal organ (VNO) is the peripheral receptors, with the ability to detect pheromones, and so it has a role in social behavior, sexual reactions and reproduction. The aim of this study was to designing, categorize, define and demonstrate the normal explaining of the onset time of first appearance and origin as well as developmental changes of vomeronasal organ of the rabbit and its allied structures. This study was carried out on 116 rabbit embryos and fetuses of both sexes. The specimens were obtained, from 14 normal and apparently healthy adult female rabbits. At days (D) 9-11 of pregnancy the nasal placode was invaginated to form the nasal (olfactory) pits. The primordia of the VNO was formed at the ventromedial area of this nasal pit. At D13, the VNO appeared as bilateral un-differentiated epithelial thickenings of the rostroventral part of nasal septum. At D14, the dorsomedial part of the epithelium was about a twice as thick as its ventrolateral part. At D16the VNO opened into the nasal cavity the miniature VNO nerve fascicles were appeared. At D18, VNO immature glands appeared in the dorsolateral part of the VNO the chondral plate was differentiated into chondroblasts. The lumen of the VNO was extremely increased in wide and closed to be oval lumen. At D20, the rostral opening of VNO duct opened directly into the floor of nasal cavity. The medial wall was thicker and had about 8-10 layers of stratified columnar and the lateral one consisted of 3-4 pseudostratified columnar cell layer. At D22, the of acini of the VNO glands determined at the dorsal commissure of the VNO duct. At D28, the lumen was lined by a thick medial mucosa with stratified olfactory like epithelium and thin ventrolateral respiratory epithelium.

Key words fetus; vomeronasal organ; nerve; glands; olfactory epithelium; respiratory epithelium

Introduction

The mammalian vomeronasal organ (VNO) is the site for peripheral receptors of the accessory olfactory system. By detecting pheromones, the VNO has an important role in social behaviour and reproductive or sexual reactions. In mammals, the latter occurs due to its

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ability to detect pheromones, and so VNO has an important role in many social and sexual behaviour. The olfactory system is well developed in rabbit (1). The vomeronasal complex (vomeronasal organ, cartilage, vessels, glands and nerves) is the most outstanding of the peripheral sensory structures found in the nasal septum of mammals. Although recent findings suggest it could be involved in pheromone-mediated behavior (2), VNO subserved basic chemosensory functions in rodents, mainly related to sexual behavior (3). It also plays a major role in the perception of stimuli related to social and/or reproductive behavior in many species of vertebrates (4). The vomeronasal system (VNS), an olfactory neural network that participates in the control of reproductive physiology and behavior, is sexually dimorphic (5).

The real function of VNO is still controversial, a further developmental studies on this may give some more useful information to explain its function. However, there have been so far published only a little reports and details of morphogenesis remain unknown (6). There were several papers discussed the development of the VNO in rabbit (7, 8), rat (9), mouse (10), hamster (11), pig (12-14), mammals (15), primates (16), human (17-19), bovine (20), goat (21), camel (22), and domestic animals (23). The aim of this study was to categorize, define and demonstrate the normal explaining of the onset time of first appearance and origin as well as developmental changes of VNO of the rabbit and its allied structures.

Materials and methods

This study was carried out on 116 rabbit embryos and fetuses of both sexes. The specimens were obtained from 14 normal and apparently healthy adult female rabbit. The rabbits were obtained from the rabbit farm of the Faculty of Agriculture, Zagazig University. They were housed for one week before experiment for acclimatization standard pellet ration (El-Nasr Chemical Company, Cairo, Egypt) and were given free accesses to water *ad libitum*. All animals managed according to Animal Ethical Committee of Faculty of Veterinary Medicine, Zagazig University approval number ZU-IACU/2/F/109 /2018.

The female were served by natural mating and each female housed individually in stainless-steel cages in environmentally controlled rooms and each maternal rabbit was given about100g-day of certified rabbit nitration free access to water. The pregnant rabbit were tested at age 9-28th days of pregnancy. The age of embryo was estimated by the pregnancy records and age of pregnancy depended on the time of mating. Just after slaughtering, evisceration and evacuation of their uteri.

The obtained embryos and fetuses were classified into two group representing the all ages of pregnancy. Group (A) were immersed as a whole in 10% neutral buffered formalin and the other group (B) were immersed as a whole in Bouin's solution for 3-24 hours and then washed carefully with distilled water and transferred to 70% ethyl alcohol. Then the specimens were subjected to the following techniques:

Histological technique

The heads of fetuses over 20 days were immersed in EDTA 5.5% buffered to 7.0 PH with sodium hydroxid and neutralized in 5% sodium sulphate. The time taken for decalcification depended on the age of fetuses according to (24). After all specimens assembled for normal histological technique, all specimens dehydrated in ascending grades of alcohols, cleared in three changes of benzene and embedded in paraffin wax. Paraffin sections of 5-7μ thickness were obtained and stained by different histological stains such as: Hematoxylin and Eosin (H&E) stain for general histological demonstration and silver impregnation (24, 25). The slides were examined by using both light and stereo (Zeiss, Germany) microscopes and the observations were recorded.

Scanning electron microscope

The specimens were delivered at hourly post conception. Specimens were trimmed and fixed in glutaraldehyde for 12-24 hour and then post fixed in 1 % osmium tetroxide for 90-120 min (26). The palates were dehydrated

through an ascending concentration of ethyl alcohol followed by 2.5 % buffered glutaraldehyde + 2 % paraformaldehyde, in 0.1 M sodium phosphate buffer pH 7.4. The specimens were washed 3 x 15 min in 0.1 M sodium phosphate buffer + 0.1 M sucrose and refixed in 2 % sodium phosphate buffered osmium tetroxide pH 7.4 for 90 min. Following washing and dehydration, the specimens were incubated overnight in 70 % acetone + 0.5 % uranyl acetate + 1 % phosphotungstic acid (at 4° C for 15 min), 80 % ethanol (2 x 15 min), 90 % ethanol (2 x 15 min), 96 % ethanol (3 x 20 min), and 100 % ethanol. The specimens were coated with gold-palladium membranes and observed in a Jeol JSM-6510 L.V SEM, The microscope was operated at 30 KV at EM Unit, Mansoura University, Egypt.

The nomenclature used in this manuscript was adopted by Nomina Anatomica Veterinaria (27), Nomina Embryologica Veterinaria (28) and Nomina Histologica Veterinaria (29).

Results

Rabbit embryo of 11 days old

The primordia of VNO performed at the medial aspect of the nasal pit (olfactory pits) as a thickening of the epithelium. The epithelium lined the pit was similar to that of the placode, and merged steadily with the general ectoderm contiguous the outside opening, which migrate towards the mesenchyme and causes a slight recess on the olfactory purse. Later on, cellular bud grew dorsally, caudally, and to the midline on both side and formed the primordia of the vomeronasal groove (Figs. 1A-C).

Rabbit embryo of 13 days old

The first appearance of VNO was in the arrangement of bilateral undifferentiating epithelial thickenings on rostroventral region of the nasal septum. The organ was enclosed via the immature vomeronasal cartilage. The ventral part of the primitive nasal cavity had an invagination of the epithelial covering the ventral part of the nasal septum giving rise to the future vomeronasal duct (Fig.1D).

Rabbit embryo of 14 days old

The primordia of VNO appeared as narrow luminal tube at the base of nasal septum (Fig. 2A). The primordial vomeronasal tube was lined laterally by a layer of stratified columnar epithelium with darkly stained and basely located elongated nucleus. The medial lining epithelium was thicker than the lateral one (Fig. 2B).

Rabbit embryo of 16 days old

The duct of future VNO was in contact with the nasal cavity (Fig. 2C). The rostral part of the future vomeronasal duct was lined by stratified cuboidal to stratified columnar epithelium (Fig. 2D). The lumen of the middle part of the vomeronasal duct was slit like. The dorsomedial wall of this duct was thicker than the ventrolateral one. Vomeronasal cartilage appeared more condensed than in the previous stage (Fig. 3A).

Rabbit fetus of 18 days old

The lining epithelium of vomeronasal duct was differentiated into thick olfactory like epithelium and thin respiratory epithelium. The C-cartilage of the vomeronasal organ was built up of chondroblastic cellular aggregation (Fig. 3B).

Rabbit fetus of 20 days old

The rostral opening of vomeronasal organ opened directly into the floor of nasal cavity (Fig. 3C). While at the middle region, the future vomeronasal duct had slit-like lumen and its medial wall was thicker and had about 8-10 layers of stratified columnar and the lateral one consisted of 3-4 cell layer (Fig. 3D)

Rabbit fetus of 22 days old

The vomeronasal duct showed two types of epithelium, the medial one resembled the olfactory form, while the lateral one appeared to be formed of respiratory type. The Primordia of acini of the vomeronasal glands were determined at the dorsal commissure of the vomeronasal duct (Fig. 4A).

Rabbit fetus of 28 days old

VNO opened rostraly in the nasal vestibule (Fig. 4B). The mucous membrane was of stratified columnar, aggregations of the acini of vomeronasal glands were determined at the dorsal commissure, some of them were luminized and others were still obliterated (Figs. 4C and D).

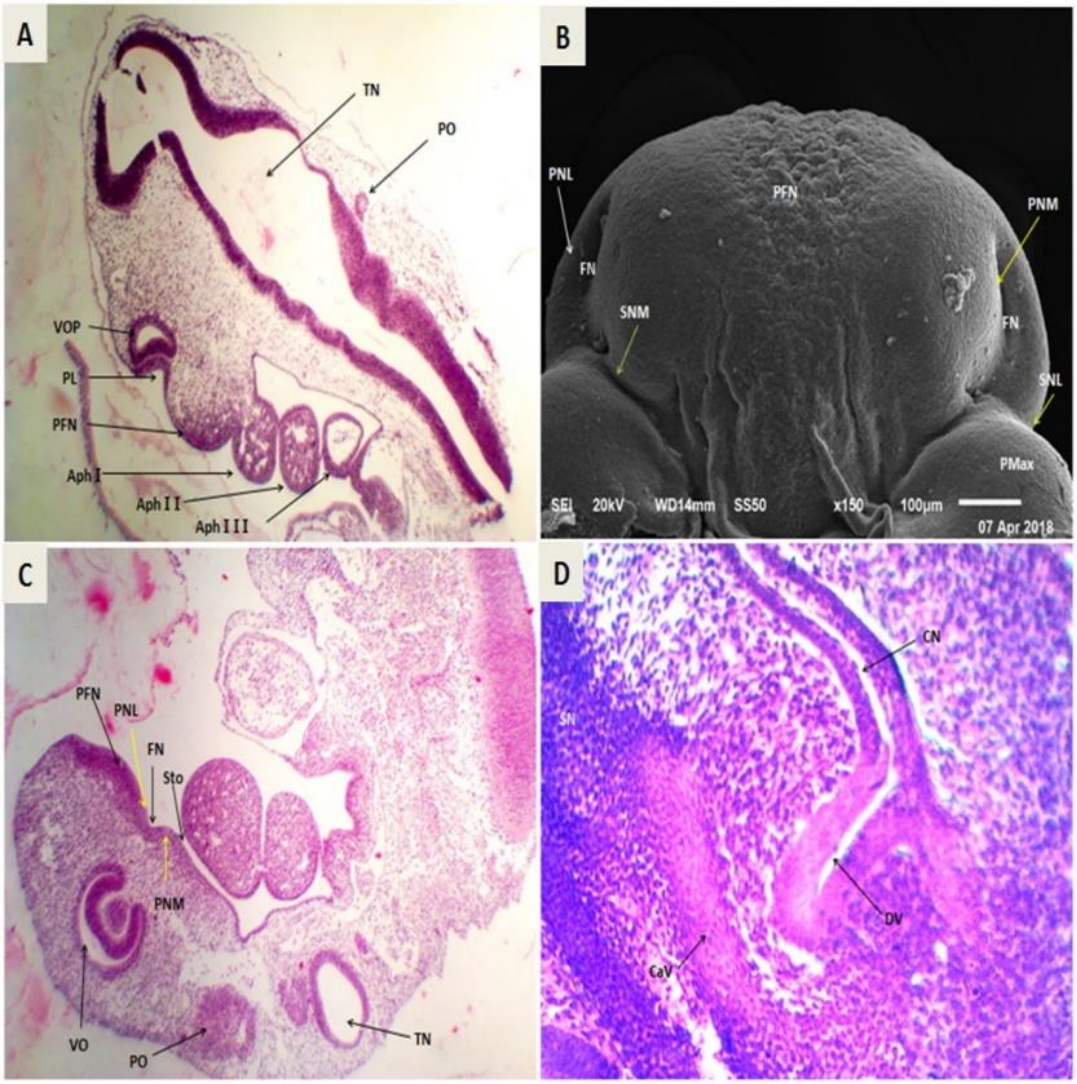

Figure 1: (A) A photomicrograph of L. S. of the rabbit Embryo of 9 days old showing; Prominentia frontonasalis (PFN), Placoda lentis (PL), Vesicula optica (VOP), Placoda otica (PO), Tubus neuralis (TN) with existence of the Arcus pharyngei [branchiales] I- I I I (APh I, APh I I, APh I I I). (H.E. stain 40X).**(B)** A photomicrograph of scanning electron micrograph (SEM) of the head of rabbit Embryo of 11 days old showing; the nasal placodes as a Fovea nasal is (FN), Prominentia nasalis lateralis (PNL), Prominentia nasalis medialis (PNM), Prominentia frontonasalis (PFN), Processus maxillaris (PMax), Sulcus nasolacrimalis (SNL) and Sulcus nasomaxillaris (SNM). **(C)** A photomicrograph of L. S. of the rabbit Embryo of 11 days old showing; Fovea nasalis (FN), stomodaeum (Sto), Prominentia frontonasalis (PFN), Placoda otica (OP), Prominentia nasalis lateralis (PNL), Prominentia nasalis medialis (PNM), Tubus neuralis (TN), Vesicula optica (VO) (H.E. Stain 40X). **(D)** A photomicrograph of C. S. of the head of the rabbit Embryo of 13 days old showing; the primordia of Ductus vomeronaslis (DV), primordia Cartilago vomeronaslis (CaV), Cavum nasi (CN) and Septum nasi (SN). (H.E. stain 100X)

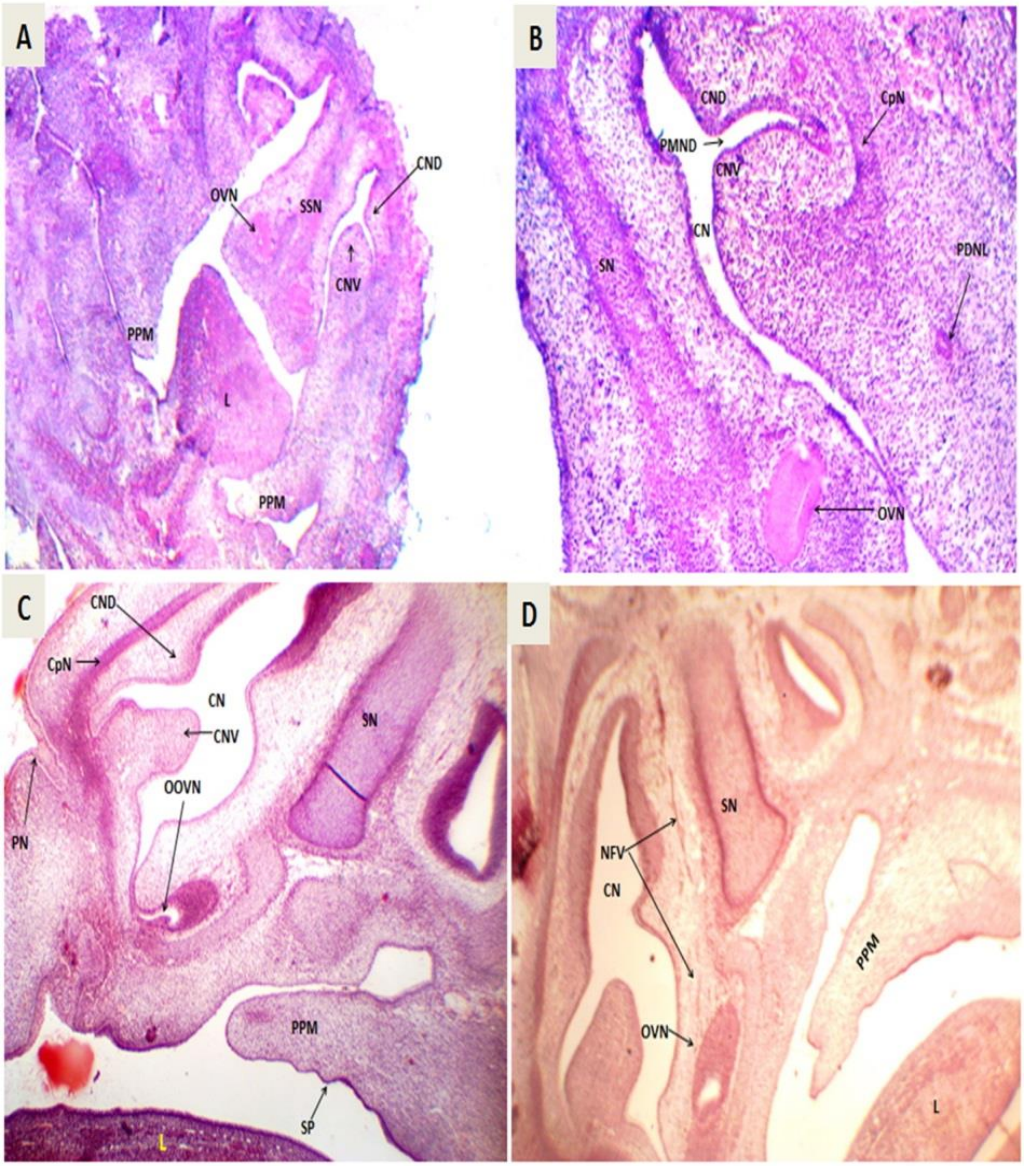

Figure 2: (A) A photomicrograph of C. S. of the head of the rabbit Embryo of 14 days old showing; the primordia of the secondary Septum nasi (SSN), primordia Concha nasalis ventralis (CNV), Concha nasalis dorsalis (CND), Processus palatinus medianus (PPM), primordia of Organum vomeronasale (OVN) and lingua (L) (H.E. stain 40X). **(B)** A photomicrograph of C. S. of the head of the rabbit Embryo of 14 days old showing; the primordia of the Septum nasi (SN), Cavum nasi (CN), primordia Concha nasalis ventralis (CNV), Concha nasalis dorsalis (CND), primordia of Ductus nasolacrimalis (PDNL), primordia of Organum vomeronasale (OVN), Primordial of Meatus nasi dorsalis (PMND) and Capsula nasalis primitivae (CpN). (H.E. stain 100X). **(C)** A photomicrograph of L. S. of the head of the rabbit Embryo of 16 days old showing; the primordia of Cavum nasi (CN), Septum nasi (SN), primordia Concha nasalis ventralis (CNV), Concha nasalis dorsalis (CND), Ostium of Organum vomeronasale (OOVN), primordia of Capsula nasalis primitivae (CpN). Processus palatinus medianus (PPM), primordia secondary palate (SP) and lingua (L). (H.E. stain 40X). **(D)** A photomicrograph of L. S. of the head of the rabbit Embryo of 16 days old showing; Neurofibra vomeronasale (NFV), Organum vomeronasale (OVN), Septum nasi (SN), Processus palatinus medianus (PPM), Cavum nasi (CN) and lingua (L). (Silver I. stain 40X)

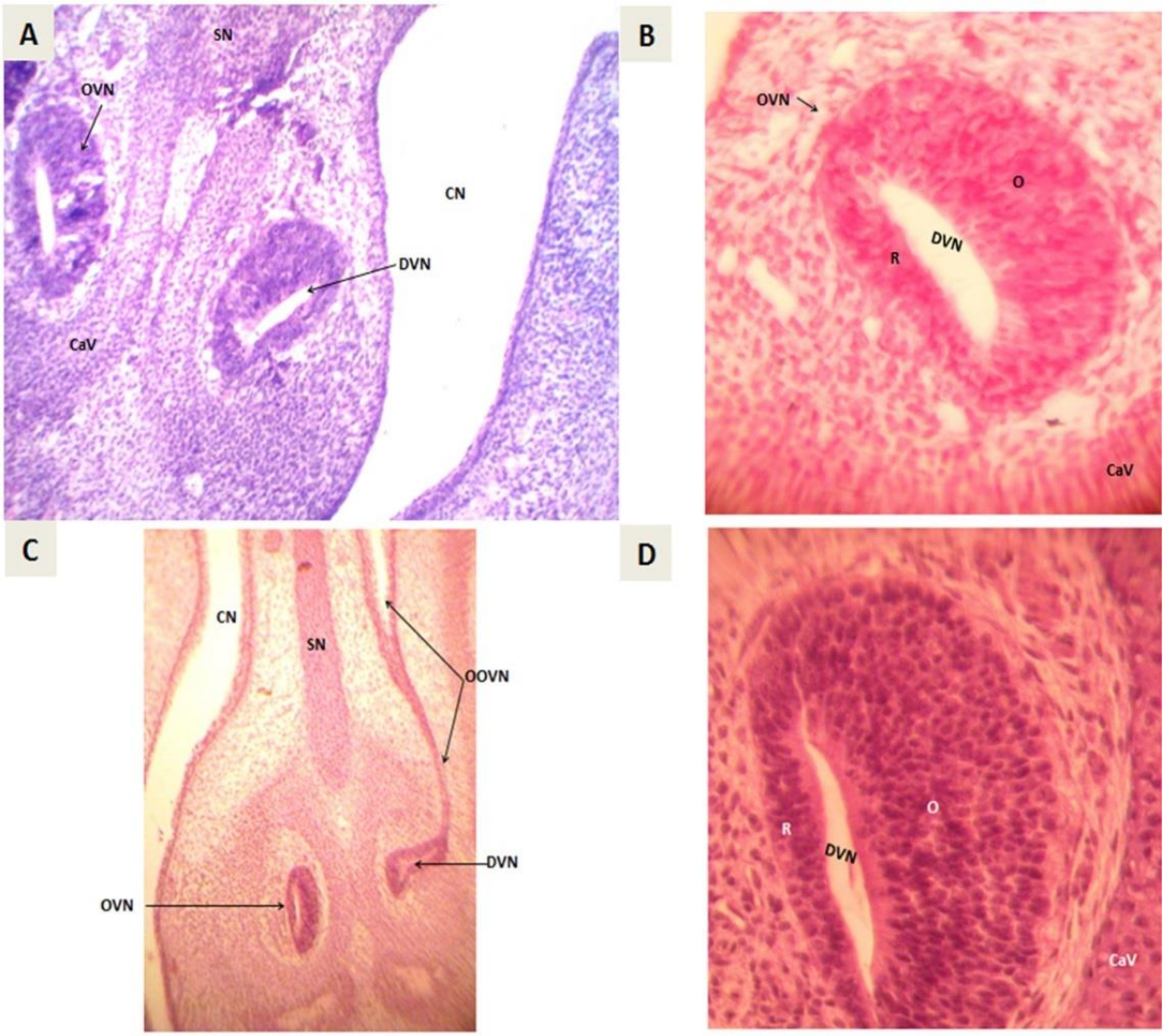

Figure 3: (A) A photomicrograph of C. S. of the head of the rabbit Embryo of 16 days old showing; Ductus vomeronasalis (DVN) slit like opening was lined by thin epithelium ventrolaterally and thick epithelium dorsomedial, Organum vomeronasale (OVN), Cartilago vomeronasalis (CaV), Septum nasi (SN), Cavum nasi (CN). (H.E. stain 100X). **(B)** A photomicrograph of C. S. of head of the rabbit Fetus of 18 days old showing; the lining Epithelium of Ductus vomeronaslis (DVN), Cartilago vomeronaslis (CaV), Organum vomeronasale (OVN). Was differentiated into thick Tunica mucosa olfactoria vomeronaslis(O) and thin Tunica mucosa respiratoria vomeronaslis(R). (H.E. stain 400X). **(C)** A photomicrograph of C. S. of head of the rabbit Fetus of 20 days old showing; The rostral opening Organum vomeronasale (OOVN) opened directly into the floor of Cavum nasi (CN), Septum nasi (SN), Ductus vomeronasalis (DVN). (H.E. stain 100X). **(D)** A photomicrograph of C. S. of head of the rabbit Fetus of 20 days old showing; lumen of Ductus vomeronaslis (DVN), a thick medialTunica mucosa olfactoria vomeronaslis(O) and thin lateral Tunica mucosa respiratoria vomeronaslis(R). Cartilago vomeronaslis (CaV), (H.E. stain 400X)

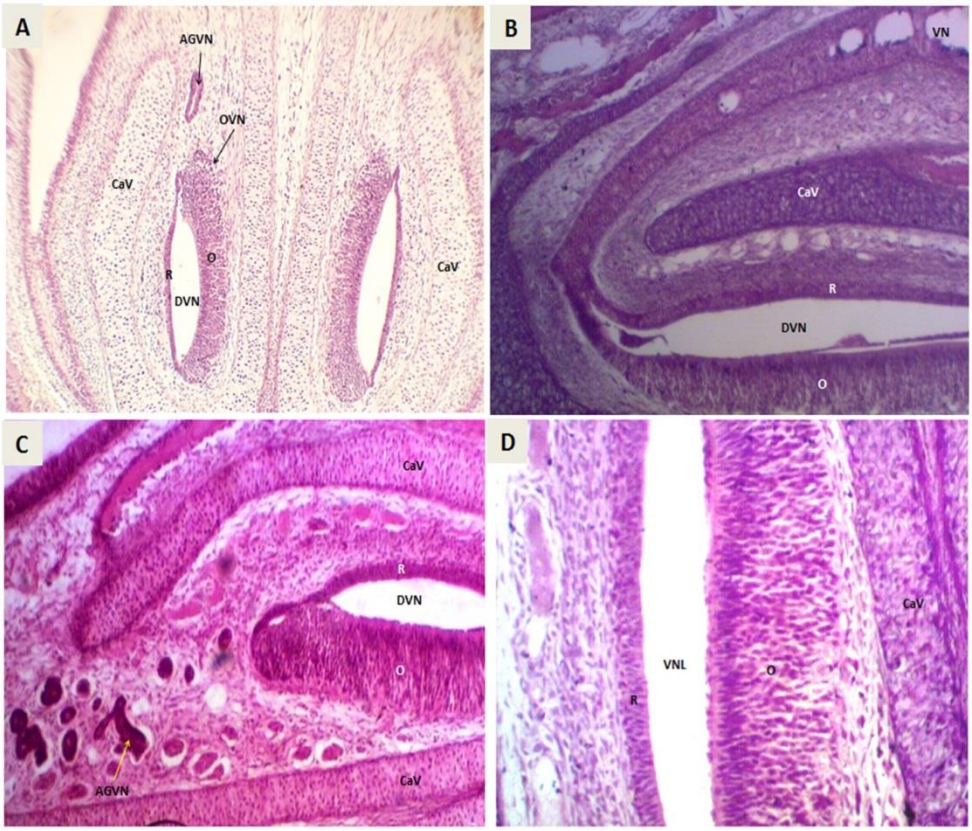

Figure 4: (A) A photomicrograph of C. S. of the head of the rabbit Fetus of 22 days old showing; lumen of Ductus vomeronaslis (DVN), a thick medialTunica mucosa olfactoria vomeronaslis(O) and thin lateral Tunica mucosa respiratoria vomeronaslis(R). Cartilago vomeronaslis (CaV), the Primordia Acinus glandula vomeronasalis (AGVN) were determined at the dorsal commissure of Organum vomeronasale (OVN). (H.E. stain 100X). **(B)** A photomicrograph of C. S. of the head of the rabbit Fetus of 28 days old showing; Ductus vomeronaslis (DVN), a thick medialTunica mucosa olfactoria vomeronaslis(O) and thin lateral Tunica mucosa respiratoria vomeronaslis(R). Cartilago vomeronaslis (CaV), was opened into the rostral of ventral part of Septum nasi, the mucous membrane was of stratified columnar type and the rostral opening of the duct blended directly with that of Vestibulum nasi (VN). (H.E. stain 400X). **(C)** A photomicrograph of C. S. of the head of the rabbit Fetus of 28 days old showing; Ductus vomeronaslis (DVN), Tunica mucosa olfactoria vomeronaslis(O),Tunica mucosa respiratoria vomeronaslis (R). Cartilago vomeronaslis (CaV) and Acinus glandula vomeronasalis (AGVN). (H.E. stain 400X). **(D)** A photomicrograph of C. S. of the head of the rabbit Fetus of 28 days old showing; of Ductus vomeronaslis (DVN), a thick medialTunica mucosa olfactoria vomeronaslis(O) and thin lateral Tunica mucosa respiratoria vomeronaslis(R). Cartilago vomeronaslis (CaV), (H.E. stain 400X).

Discussion

The present investigation showed that, the thickened epithelium of the nasal placode was invaginated to make the nasal pits (olfactory pits) at 11 days old of rabbit embryo. The primordia of VNO was performed at the medial aspect of the nasal pit as a thickening of the epithelium a result which came in agreement with, (6) in hamster, (7) in rabbit and (30) in rat whose mentioned that, the vomeronasal organ was embryologically derived from the olfactory placode. In human, early during the fifth week, the ectoderm in the upper one-third of each enlarging nasal sac became thickened and developed into the olfactory epithelium (19, 32). On the contrary in mammals (15) mentioned that the vomronasal organ originated from the medial wall of the olfactory pit shortly after the middle of the embryonic period.

In the present investigation, there was a clear invagination, the epithelium lined the pit was similar to that of the placode, and merged

steadily with the general ectoderm contiguous the outside opening, which majorities towards the mesenchyme and causes a slight recess on the olfactory purse at 11 days old of rabbit embryo. Later on, cellular bud grew dorsally, caudally, and to the midline on both sides and formed the primordia of the vomeronasal groove these results were in accordance with (32) in hamster whose compared between the vomeronasal sensory and the olfactory epithelia and noticed that, the both epithelia were divergently derived from the olfactory placodes.

At 13 days old embryo VNO appeared as arrangement of bilateral undifferentiating epithelial thickenings on rostroventral region of the nasal septum. The organ was enclosed via the immature vomeronasal cartilage, which appeared as but densely arranged in small amorphous cells. The latter cells were suggestive of their future dispositions. The medial and lateral sides of the nostrils showed the miniature of nasal cartilages. These results were in disagreement with (33) who stated that, the vomeronasal organ appear as two blind epithelial like tubes in the ventral aspect of the nasal septum at nasal vestibule at sixteenth prenatal day of rat. While our findings were in accordance with (7, 8) in rabbit, (9,34) in rat, (21) in goat, (19, 31, 35) in human, (36) in mammals and (37) in animals.

The present work revealed that, the (future) VNO was in contact with the nasal cavity at 13- 28 days old of the rabbit embryos and fetuses. This result could not met with the available literature in the rabbit or the other animals and human expect (16) in primates whose mentioned that, one exception occurred in the largest fetal Tarsius (25 mm crown–rump length), in which the vomeronasal organ communicated with the nasal cavity alone.

Conclusion

To the best of our knowledge, this may be the first description to the designing, categorize, define and demonstrate the normal explaining of the onset time of first appearance and origin as well as developmental changes of vomeronasal organ of the rabbit and its allied structures

Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

IMPACT OF DIETARY EICOSAPENTAENOIC AND DOCOSAHEXAENOIC FATTY ACIDS SUPPLEMENTATION ON INFLAMMATORY RESPONSE OF POST CALVING COWS DURING TRANSITION TO LACTATION

Shaimaa Elbaz[1], Eldsokey Nassef[2]*, Abdelnasser Bakr[2], Elsayed Hegazi[2], Abeer M. S. EL-Keredy[1]

[1]Department of Biochemistry, Nutritional deficiency diseases and Toxicology, Animal health Research Institute, Kafrelsheikh, Egypt, [2]Nutrition and Clinical Nutrition Department, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt

*Corresponding author, E-mail: dsokeynassef@yahoo.com

Abstract: The aim of current study was to determine the effect of dietary omega 3 fatty acids (FA) mainly eicosapentaenoic (EPA) and docosahexaenoic (DHA) on inflammatory response of post calving cows during transition to lactation. Twenty Holstein lactating cows were assigned into two groups (10 each). The two groups fed on the basal diet while one of them was supplemented with 70 g of omega-3 FA per cow. The supplement was added to the diet from the 1ST day of calving till the 60th days of lactation. Blood samples were taken 3 times weekly from the 2nd to the 4th week post calving. The blood samples were analyzed for glucose, insulin, non-esterified fatty acids (NEFA), beta hydroxyl butyric acid (BHBA), C- reactive protein (CRP), haptoglobin, albumin and globulin. Feeding omega-3 FAs significantly increased (P<0.05) blood glucose level by 17.35%, 28.23% and 24.73% than the control at weeks 1, 2, 3 of the experiment, respectively. BHBA was lower in cows fed omega-3 at 1st and 2nd week then became nearly similar to the control at the end of experiment. At 1st and 2nd week, cows fed omega-3 FAs had significantly lower serum CRP than those fed the control diet and became nearly similar to control at the end of the experiment. Serum, globulin was significantly higher in cows fed omega 3 FAs at 2nd and 3rd week by 81.1% and 51.2%, respectively. Omega 3 FA improved energy status of fresh cow by increasing serum insulin and glucose levels while decreasing NEFA and BHBA parameters. Also, feeding omega 3 FA improved humoral immunity through increasing serum globulin and reduced inflammatory response by lowering CRP post calving.

Key words: omega 3 fatty acids; lactating cow; inflammation; energy balance; serum protein

Introduction

The onset of lactation is one of the most critical period affecting dairy cows in which massive changes and more metabolic stresses for

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dairy cows (1). In this period there is high demand for energy for milk synthesis and secretion accompanied by changes in blood metabolites (2). The impaired glucose production that occurs in early lactating cows would lead to an increase in the breakdown of adipose tissue, elevated plasma NEFA and increased ketones production by the liver. After parturition by few several weeks there is more incidence of diseases and morbidity on many dairy cows with especially mastitis, metritis, milk fever, ketosis and fatty liver.

Postpartum dairy cow is suffering from systemic inflammatory state even with the absence of clinical disease (3). On the other hand, physiological changes that occurring in the fresh period after calving can affect cellular immune responses and modulate the influence of dietary fatty acids on immune functions. Also, the elevated NEFA leading to abnormal immune cell functions, increased inflammation and increased risk of metabolic and infectious diseases. The elevated plasma NEFA impaired cytokine production (4) and altered endothelial cell adhesion (5).

One of the different nutritional approach to decrease inflammation post calving is the use of omega 3 polyunsaturated fatty acids. It is thought that the n-3 fatty acids attenuate inflammatory response in lactating dairy cows (6) and eicosapentaenoic (EPA) and docosahexaenoic (DHA) have more immunomodulatory activities (7). (8, 9) reported that EPA and DHA performing anti-inflammatory functions via direct or indirect inhibitory mechanisms. A reduction of inflammatory cytokine production with feeding of EPA and DHA to dairy cows and therefore they have the ability to modulate the inflammatory response this was reported by (6). One effect of cytokines is to activate production of acute phase proteins, that primarily produced by the liver, this class of proteins includes haptoglobin, serum amyloid A and C-reactive protein. The importance of acute phase proteins in the response to infection is somewhat unclear, but they have gained wide spread acceptance as markers of inflammation. (10) stated that cell surfaces receptors were more directly characterized mediate anti-inflammatory effects of omega-3 fatty acids. An attenuated inflammatory response was observed by (11) due to increase intake of EPA and DHA fatty acids which resulted in increase the proportion of these FAs in the membrane phospholipids. (12) noticed an increased plasma glucose and decreased plasma ketones when feeding omega3 source compared to omega-6 sources in fresh cows in addition to anti-inflammatory effect of omega 3 due to increased phagocytic activity of circulating leukocytes. An increase in plasma glucose level was reported by (13) with feeding omega 3 polyunsaturated fatty acids. The improvement in metabolic profile with omega-3 fatty acids supplementation may be the key to beneficial effect on both immunity and inflammation. From the economic point of view, (14) reported that as the diet enriched in n-3 fatty acids, the nutrient expenditure would be reduced as omega-3 fatty acids attenuate the inflammatory responses.

This study aimed to evaluate the anti-inflammatory effect of EPA and DHA fatty acids in the post calving cows during transition to lactation. Also, their effects on energy balance were determined.

Materials and methods

Cows and dietary treatment

Twenty Holstein cows (10 multiparous and 10 primiparous) were assigned to two groups according to parity, body weight (BW) and previous milk yield (expected milk yield for primiparous cows). Cows averaged 680 kg of BW at the beginning of the experiment (post calving date). Cows had continuous access to fresh clean water. A basal postpartum TMR was formulated to meet or exceed (15) recommendation. Ingredients and nutrients composition are presented in Tables 1 and 2, respectively. Analysis of TMR on dry matter basis is presented in Table 3.

Cows were fed three times daily at 4 am, 11 am and 4 pm for *ad libitum* intake. Treated group was top-dressed on the TMR and hand mixed at the time of feeding 70 gm/head/day with omega 3 FA supplement (STRATA G113) which its composition was fish oil 90%, calcium 9.95% and BHT (antioxidant) 0.05%.

The supplement was added at a rate 40gm/cow at 4 am and 15 gm/cow for each feeding 11 am and 4 pm.

The typical analysis of fatty acids using gas chromatograph for STRATA G113 were: palmitic acid (16:0), 39.4%; palmitoleic acid (16:1), 6.8%; stearic acid (18:0), 9.9%; oleic acid (18:1), 18.8%; linoleic acid (18:2), 6.1%; linolenic acid (18:3), 2.2%; eicosapentaenoic acid (20:5), 10.3%; docosahexaenoic acid (22:6), 6.5%.

Experimental measures and samples analysis

Daily feed intake and refusal were recorded daily. Feed and refusal samples were collected daily, subsamples weekly, and composite at the end of experiment. TMR and feed refusal were immediately frozen.

The subsamples of TMR and feed refusal were dried at 60 ºC in hot air circulations oven (Heraeus UT 20 model, Germany). The dried samples of TMR, corn silage and other feed ingredients used were ground through 1mm screen of grinder (Cyclotec 1093 Foss, Sweden).

The ground samples were analyzed for final dry matter at 105 ºC for 3 hour, crude protein (Kjeltec system 2100-Foss, Sweeden), ether extract (Soxtec system 2045 Foss-Sweeden), and ash (Furnace6000, Thermolyne USA) according to (16). Neutral detergent fiber without using sodium sulfite, acid detergent fiber (ADF) (17) and lignin were determined by digesting the ADF residue in 72% sulfuric acid, (18). Fiber fractions were determined with fibertec M61020-Foss Sweden. Neutral detergent insoluble crude protein (NDICP) was determined after NDF extraction by analyzing the residue for crude protein. The NFC concentration was calculated using the following equation: NFC=100-(NDF%+CP%+EE%+Ash %).

The values of TDN and NEL were determined according to (15) based on nutrient analysis (CP, NDF, ADF, NFC, EE, Lignin, NDICP and ADICP). Total aflatoxins (B1, B2, G1 and G2) level was analyzed fluorometrically using VICAM series 4 USA according to (19).

Blood metabolites and hormones

Blood samples were collected from all animals at 11:00 am, 3 times weekly at the 2nd, 3rd and 4th weeks postpartum. Ten ml of blood was collected from coccygeal vessel and divided into 2 tubes for each cow. One tube contained K2EDTA for plasma separation and the other tube was without anticoagulant for serum separation. Plasma was separated immediately after the collection of blood by centrifugation at 3000 rpm for 10 minutes. The clear plasma was obtained using sterilized pipettes and kept at -20 ºC till analysis. Serum was separated by blood centrifugation at 3000 rpm for 15 minutes and then stored at -20 ºC till analysis.

Biochemical analysis

The collected plasma was analyzed for glucose according to (20) based on glucose oxidase enzyme activity. Plasma NEFA concentration was measured according to (21) using diagnostic reagent for quantitative *in vitro* determination of NEFA in plasma on photometric systems. Concentration of plasma BHBA was determined according to (22) using quantitative determination of B-hydroxy butyrate in plasma for *in vitro* diagnostic use Pointe Scientific, INC. Serum was analyzed for total protein according to (23) using quantitative method based on the intensity of the color formed which is proportional to the total protein concentration in the sample. Albumin was measured in serum according to (24) based on bromocresol green binding method using available commercial albumin kits. Globulin was calculated according to (25) by subtraction of albumin from total protein. Haptoglobin was measured according to (26) based on determination of serum hemoglobin binding capacity. C reactive protein (CRP) was measured in serum according to (27) based on Rete Nephelometry. Insulin was measured in serum according to (28) based on a solid phase enzyme-linked immunosorbent assay (ELISA).

Statistical analysis

The obtained data were analyzed using statistical software (SPSS Version 16). Group means were compared using analysis of T-independent-Samples T Test. Levene's test for equality of variances was used to indicate the

significance. The means were considered different at $P<0.05$.

Results

Results of blood insulin, glucose, NEFA and BHBA concentrations are shown in figures 1, 2, 3 and 4, respectively. Cows fed omega-3 FAs had significantly ($P<0.05$) higher blood insulin level (0.37 and 1.1 mIU/ml) than those fed the control diet (0.17 and 0.33 mIU/ml). This trend lasted to 2 weeks then declined to be non-significant at 3rd week of experiment (4th week post calving). Feeding omega-3 FAs increased blood glucose level (mg/dl) by 17.35%, 28.23% and 24.73% than the control at weeks 1, 2, 3 of the experiment, respectively. This was more significant at 2nd and 3rd weeks. Cows fed omega 3 FAs had significantly higher blood NEFA (mg/dl) at 1st week and then rapidly declined at 2nd week and became numerically lower but non-significant than the control at 3rd week of experiment. In contrast, BHBA (mg/dl) was lower in cows fed omega-3 at 1st and 2nd weeks then became nearly similar to the control at the end of experiment.

Results of CRP, haptoglobin, albumin and globulin concentration in serum are shown in figures 5, 6, 7 and 8, respectively. At 1st and 2nd weeks, cows fed omega-3 FAs had significantly ($P<0.05$) lower serum CRP (0.27 and 0.21 mg/L) than those fed the control diet (0.37 and 0.36 mg/L) and became nearly similar to the control at the end of the experiment. Cows fed omega 3 FAs had no significant difference between the control and the experimental cows on serum haptoglobin and serum albumin along the experiment. In contrast, serum globulin was significantly higher in cows fed omega 3 FAs at 2nd and 3rd week which increased by 81.1% and 51.2%, respectively, than the control. While at the 1st week serum globulin was nearly similar in between the control and the experimental cows.

Table 1: Ration ingredients used for early lactating cows during the experiment

Ingredients For each cow	DM%	DM Kg/day	As fed Kg/day
Corn silage	28.0	7.41	26.45
Wheat straw	92.0	0.39	0.43
Corn grain ground dry	88.0	4.61	5.24
Wheat bran	88.8	0.76	0.86
Soybean meal 46%	90.0	3.26	3.62
Soy Best	89.0	0.97	1.09
Megalac	97.0	0.45	0.47
Optigen	99.0	0.04	0.04
Calcium carbonate	99.5	0.20	0.20
Salt white	99.5	0.08	0.08
Sodium bicarbonate	99.5	0.21	0.21
Vitamin mineral premix*	99.5	0.06	0.06

Premix provides 5500 IU vitamin A; 500 IU vitamin D; 50 IU vitamin E; 100 mg zinc; 60 mg iron; 40 mg manganese; 20 mg copper; 0.6 mg iodine; 0.3 selenium and 0.25 mg cobalt per kg dry matter.

Table 2: Nutrients composition of the basal diet

Nutrient	DM	As fed
Dry matter (%)	100.00	47.99
Forage (%)	41.62	19.97
Crude protein (%)	17.98	8.63
Rumen undegradable protein (% of CP)	37.01	17.76
Rumen degradable protein (% of CP)	62.99	30.23
Soluble protein (% of CP)	27.89	13.38
Metabolizable energy (Mcal/kg)	2.60	1.25
Net energy for maintenance (Mcal/Kg)	1.69	0.81
Net energy for gain (Mcal/kg)	1.08	0.52
Acid detergent fiber (%)	19.25	9.24
Neutral detergent fiber (%)	30.17	14.48
Forage neutral detergent fiber (%)	23.39	11.22
Lignin (%NDF)	8.44	4.05
Lignin (%DM)	2.55	1.22
Non fiber carbohydrate (%)	38.75	18.60

Table 3: Analysis of total mixed ration on dry matter basis

Nutrients (%)	As fed basis	Dry matter basis
Dry Matter	46.47975%	100%
Moisture	53.52025%	0.00
Crude Protein	8.12%	17.47%
Neutral detergent fiber	16.8%	36.14%
Acid detergent fiber	10.1%	21.73%
Cellulose	8.61%	18.53%
Hemicellulose	6.7%	14.41%
Lignin	1.48%	3.19%
NDICP	1.44%	3.1%
ADICP	0.61%	1.31%
Crude Fat	1.48%	3.19%
Ash	3.81%	8.21%
Non fiber carbohydrate	16.27%	35%
Total digestible nutrients	32.48%	69.8865%
DE (Mcal/kg)	1.56	3.17
ME (Mcal/kg)	1.25	2.54
NEL (Mcal/kg)	0.79	1.6

ADICP, acid detergent insoluble crude protein; NDICP, neutral detergent insoluble crude protein. DE, digestible energy; ME, metabolizable energy; NEL, net energy for lactation

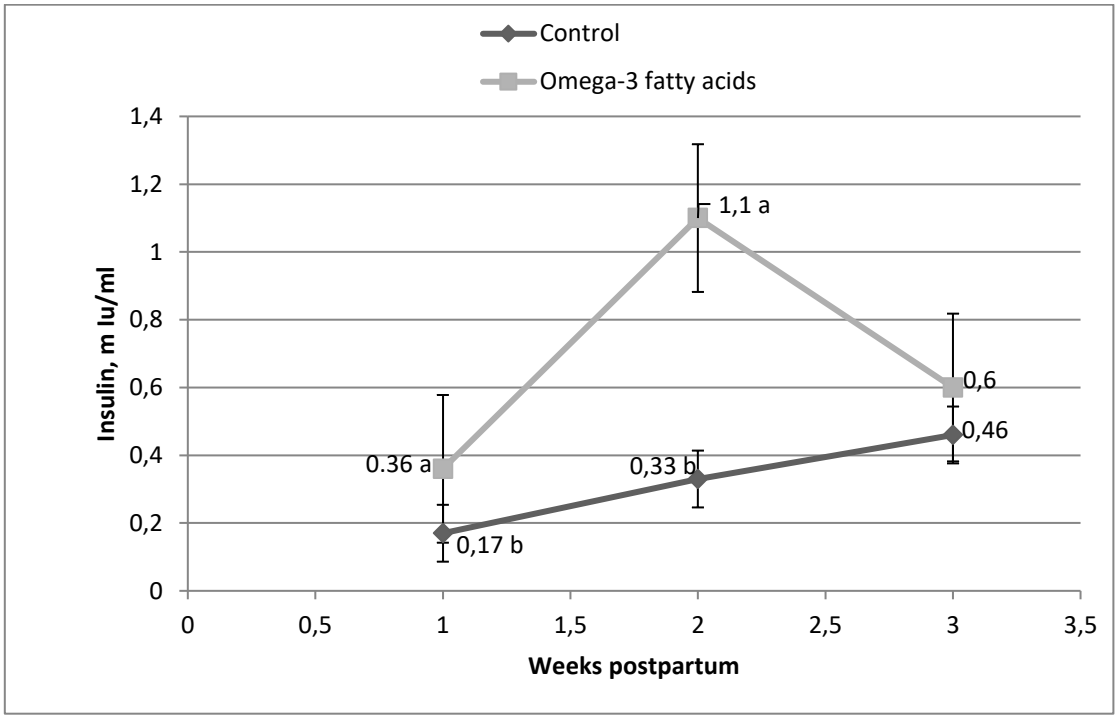

Figure 1: Influence of dietary omega-3 fatty acids on plasma concentrations of insulin in fresh lactating cows

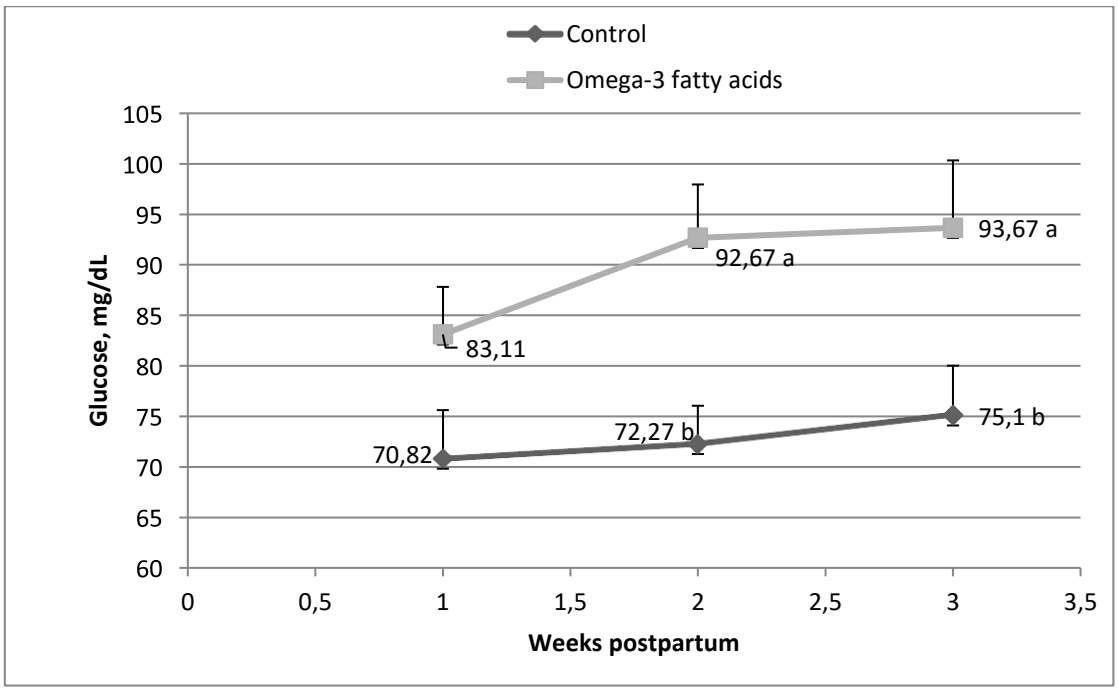

Figure 2: Influence of dietary omega-3 fatty acids on plasma concentrations of glucose in fresh lactating cows

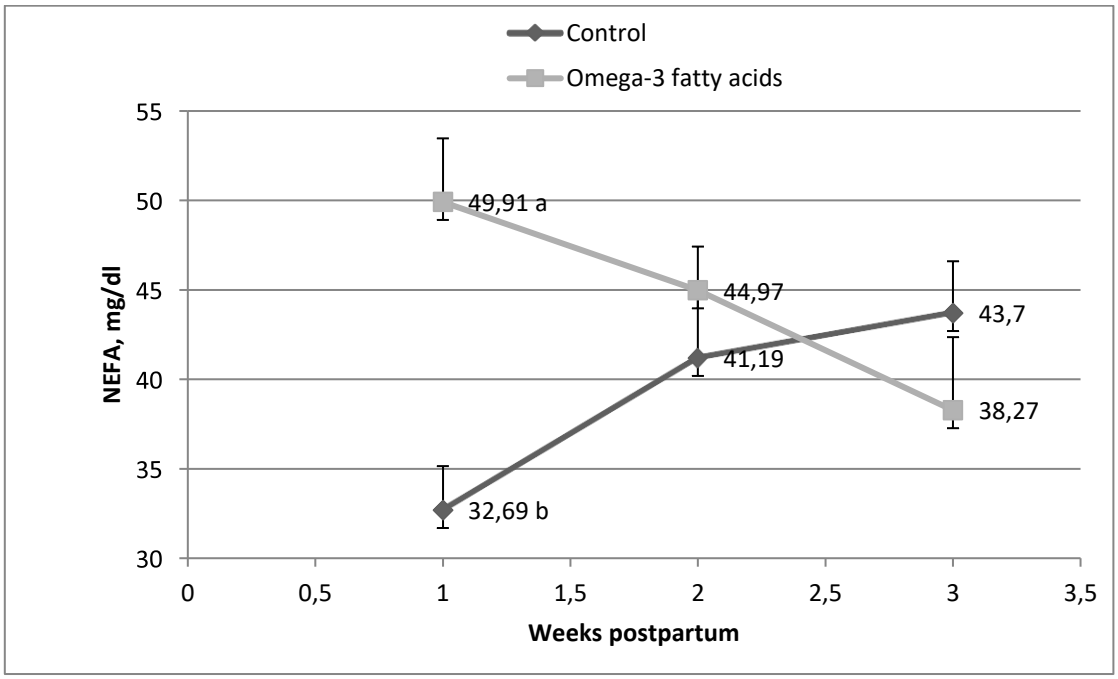

Figure 3: Influence of dietary omega-3 fatty acids on plasma non-esterified fatty acids (NEFA) concentration in fresh lactating cows

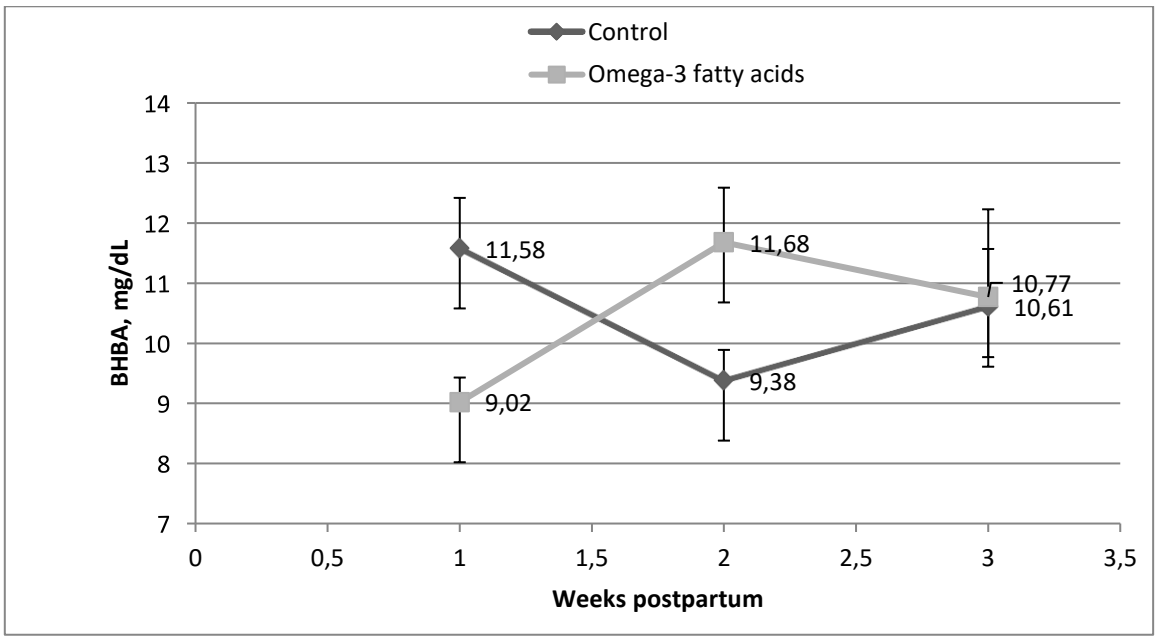

Figure 4: Influence of dietary omega-3 fatty acids on plasma beta hydroxyl butyric acid (BHBA) concentration in fresh lactating cows

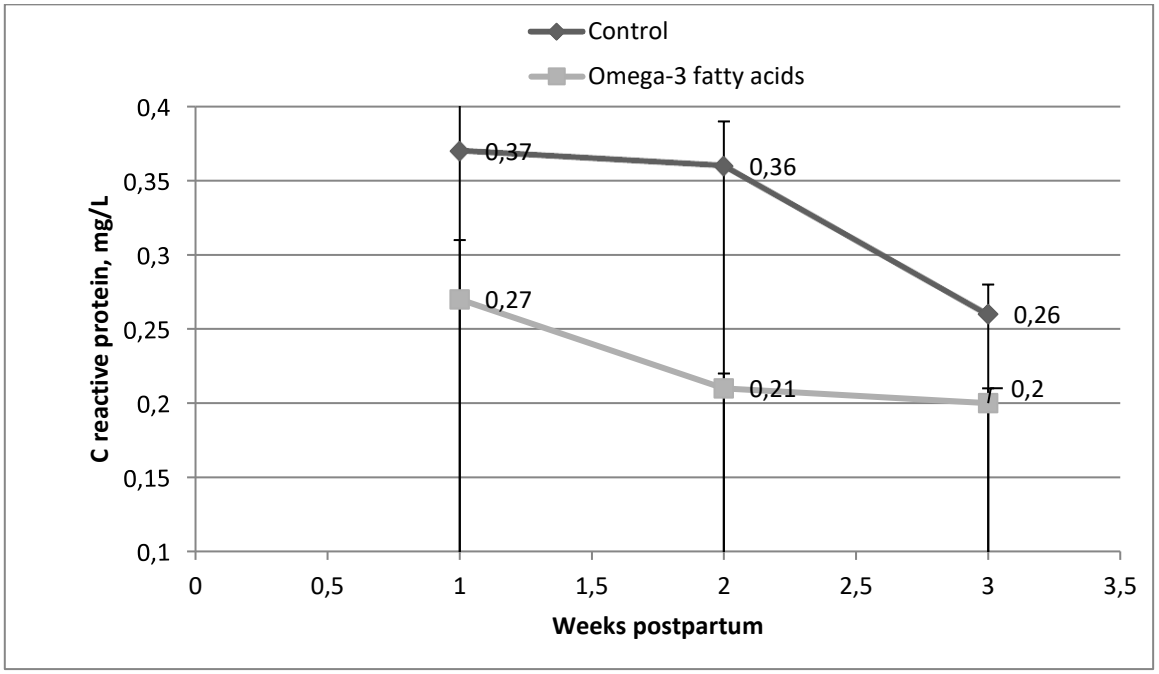

Figure 5: Influence of dietary omega-3 fatty acids on serum C reactive protein concentration in fresh lactating cows

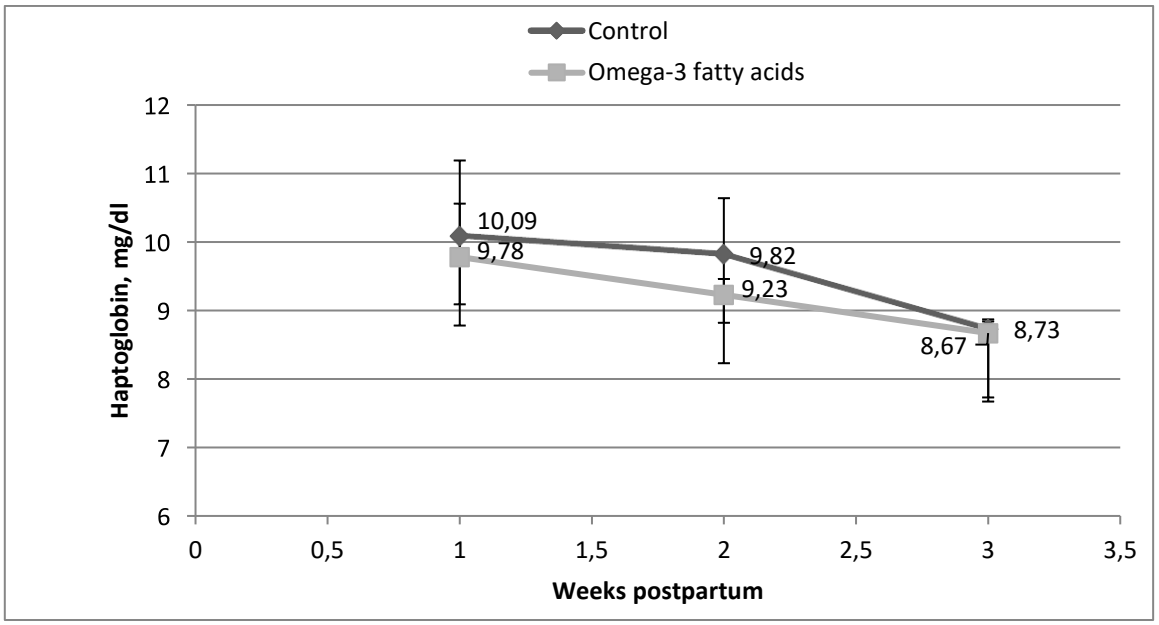

Figure 6: Influence of dietary omega-3 fatty acids on serum haptoglobin concentration in fresh lactating cows

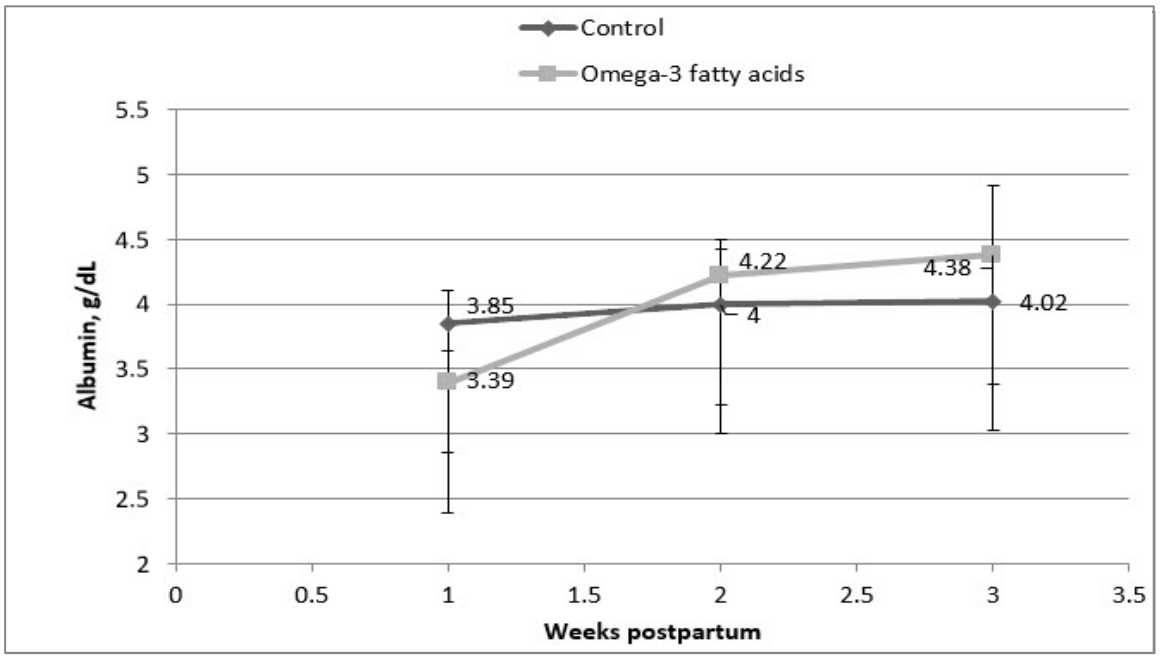

Figure 7: Influence of dietary omega-3 fatty acids on serum albumin concentration in fresh lactating cows

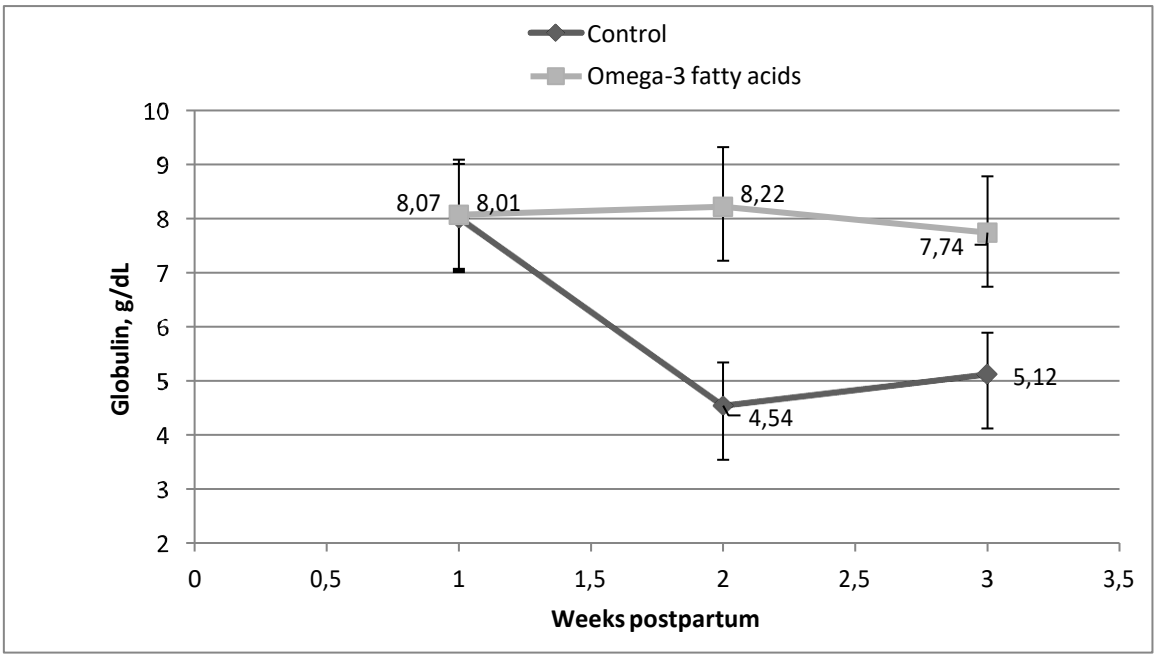

Figure 8: Influence of dietary omega-3 fatty acids on serum globulin concentration in fresh lactating cows

Discussion

Post calving cows suffering from many problems during the transition of these cows to lactation. In this period there is massive changes, more metabolic stresses for dairy cows and also more incidence of diseases and morbidity. Researchers reported that postpartum dairy cows suffering from systemic inflam-

matory state (29). It is thought that the fatty acids of n-3 family improving metabolic profile of dairy cows and also attenuate measure of inflammatory response in lactating cows and so we conducted the present study to investigate their effects on inflammation and energy balance post calving.

It is well known that insulin concentration reflecting the glucose availability to stimulate beta cells of pancreas to produce and release insulin. The concentration of blood glucose with omega-3 feeding increased during the 3 weeks of the experiment with significant difference in 2nd and 3rd weeks of the trial. But, no significant effect was detected in the 1st week of the trial between the control and the omega-3 groups. The concentration of insulin was significantly increased at 1st and 2nd weeks of the trial at P value 0.015 and 0.029 respectively, for the omega-3 group and also increased in the 3rd week but with no significant at P value 0.1. (30) reported that omega-3 of fish oil decreased insulin response to glucose in rats and this finding may explain why the plasma glucose concentration increased with feeding of omega-3 of fish oil and this greater concentration of plasma glucose for cow fed omega-3 was the result of a better energy status. The result of glucose was in agree with (31,32) and disagree with (33) that reported a decrease in plasma concentration of glucose with feeding fish oil and also with (34) that observed no change in blood glucose concentration with feeding of 5% fish meal and n-3 fatty acids to early lactating cows. The past using fish meal or fish oil as a source of long chain omega 3 fatty acids for ruminants might expose these fatty acids to saturation by rumen microbes which led to loss of its functional importance in the metabolism. In contrast, our study used EPA and DHA which were protected from rumen microbes, passed to abomasum in which were hydrolyzed and absorbed without change through the intestinal tract. So the difference might be due to the dose of EPA and DHA which arrived the metabolic pool without saturation.

The result of insulin in the current study is in agree with (34) and disagree with (35) that noticed a reduction in insulin concentration in blood of dairy cows when fed fish oil containing DHA. Most of the changes occurred in plasma concentration of NEFA in the early lactation as the result of changes in adipose tissue mobilization and energy status (36).

In our study, the plasma concentration of NEFA was higher (49.91±3.56 mg/dl) in the 1st week in the omega-3 group. Then it rapidly declined in 2nd (44.97±2.45 mg/dl) and 3rd (38.27±4.09 mg/dl) week. In contrast to the control group which was the lowest concentration in 1st week (32.69±2.46 mg/dl) and then increased till reached 43.70±2.90 mg/dl. This was reflecting sever mobilization of fat from adipose tissue and also increasing the effect of negative energy balance while the decline of NEFA concentration in omega-3 group that reached to 38.27±4.09 mg/dl reflecting an improvement in the energy status and metabolic profile.

The result in current study of NEFA concentration was in agree with (33) that noticed a decrease in NEFA concentration by 21 day postpartum with cows that fed fish oil, and also agreed with (37) that reported a high concentration of plasma NEFA in the 3rd week postpartum and then gradually decreased with omega-3 feeding. The result of plasma NEFA disagreed with (31) which reported a high plasma NEFA concentration with cows fed calcium soap of fatty acids and also stated that feeding them did not improve the energy status of early lactating cows. Also our study disagreed with (38) that reported high concentration of plasma NEFA in all groups that fed omega-3 compared to the control group. However, our findings agreed with (39) who observed a great increase in plasma NEFA concentration in the 2nd week post-partum and then decreased gradually. The differences among studies might be due to the type, dose and technology of protection of fatty acids used in the fat supplement.

BHBA is one of ketone bodies that produced by the liver as a result of incomplete oxidization of fat. Elevated BHBA above 14 mg/dl indicate ketosis that had impact on both cow health and milk yield. In the current study, BHBA in plasma was lower in cows fed omega-3 FA at 1st week at P-value 0.016 and then slight increase with no clinical ketosis at level 11.7

mg/dl. Then it decreased to become nearly the same to the control group at the end of experiment at P-value 0.576. In agreement, (37) and (40) also reported that cows fed flax seed were in positive energy balance that indicated by a lower BHBA, NEFA and high glucose concentration. In contrast, (33) stated that the concentration of BHBA in plasma in cows fed fish oil increased with the increasing days postpartum ($P<0.02$). The same result of the current study agreed with (12) who reported an increase in plasma concentration of glucose and decreased plasma ketones in fresh cows that fed flax seed (omega-3) compared to omega-6 and adding that the anti-inflammatory effect of omega-3 source resulted in increased phagocytic activity of leucocytes and thus omega-3 source could improve metabolic and immune function.

Acute phase proteins (APP) are a group of glycoproteins produced by liver in respond to an inflammatory stimulus, infection or trauma in animals (41). APP stimulate immunoglobulin production, tissue repair and prevent more injury (42). Results of APP including C-reactive protein (CRP) and haptoglobin (HP) in the current study are found in figures 5 and 6 respectively.

The mean of serum concentration of CRP (mg/l)was lower in cows supplemented with omega-3 in the 1st and 2nd weeks of experiment (0.27 and 0.21) respectively than the control group, and at the 3rd week of the trial there were no significant difference between control and omega-3 groups. The reduction in serum CRP in the 1st and 2nd weeks in the cows supplemented with omega-3 indicated that omega-3 has an anti-inflammatory effect. The same results was reported by (6) that noticed an anti-inflammatory effect when cows fed n-3 fatty acids from fish oil illustrated by decreased acute phase proteins.

The means of haptoglobin concentrations during the 3 weeks of experiment not significantly differ between control and omega-3 group and this in agree with (43) and dis agree with (44)that stated that haptoglobin was a sensitive marker of inflammation in cattle. A low levels of haptoglobin was documented by (6) when cows fed fish oil from 35 to 160 days postpartum. The difference of results in this study and other studies may be due to different level of addition of omega-3, different omega-3 sources or time of addition omega-3.

Blood serum proteins are significant indicators of animal health. There was no significant difference in the mean of serum globulin level in the 1st week of trial between control and experimental groups while in the 2nd and 3rd weeks there was a significant increase in the means of serum globulin in the cows supplemented with omega-3. These results indicate that omega-3 fatty acids are very important for humoral immunity. However, no difference in serum albumin was observed between control and experimental groups.

Further study will be conducted regarding with effect of omega 3 fatty acids on milk yield, persistency and reproductive performance of lactating cows. Also recording metabolic problems, cost of treatment and culling to evaluate economic importance of omega 3 fatty acids in lactating

Conclusion

Feeding omega-3 FA tended to increase blood glucose and insulin levels in lactating cows and decrease the plasma level of NEFA gradually in the blood from 2nd to 4th week postpartum. There was a significant decrease in serum C - reactive protein in the cows fed omega-3 FA. Also, omega 3 FA improved humoral immunity by increasing serum globulin. Finally feeding dairy cows with EPA and DHA fatty acids during transition to lactation minimized inflammatory response post calving and improved their energy status.

Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

PHENOTYPIC AND MOLECULAR IDENTIFICATION OF YEASTS ISOLATED FROM CULTURED TILAPIA (*OREOCHROMIS NILOTICUS*) IN KAFRELSHEIKH PROVINCE, EGYPT

Amgad A. Moawad[1]*, Ahmed I. Noor El Deen[2], Nadia B. Mahfouz[3], Salwa M. Helmy[1], Yasmen F. Hashem[4]

[1]Departments of Bacteriology, Mycology & Immunology Department, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt, [2]Department of Hydrobiology, Veterinary Division, National Research Center, Egypt, [3]Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt, [4]Veternarian in Directorate of Veterinary Medicine in Kafrelsheikh, Egypt

*Corresponding author, E-mail: Moawadamgad@gmail.com

Abstract: The present study aimed to identify yeasts by phenotypic, biochemical and genotypic methods. Three hundred live *Oreochromis niloticus* samples were collected from different locations at Kafrelsheikh Governorate, Egypt and during different seasons. Yeast isolates were identified according to their phenotypical characterization in combination with polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) method. Two hundred and twenty-six yeast isolates from eighty diseased and two hundred and twenty apparently healthy *O. niloticus* samples. Isolated yeasts were *Rhodotorula* spp. (18.14%), *Candida albicans* (19.47%), *Candida parapsilosis* (16.37%), *Candida guilliermondii* (14.6%), *Candida inconspicua* (8.85%), *Trichosporon asahii* (17.25%), *Geotrichum* spp. (2.66%) and unidentified *Ascus forming yeasts* (2.66%). Yeasts most frequently isolated from gills and skin. Phenotypic methods are valuable in identification of yeasts into genera but these methods take more time. Molecular methods through the amplification of (internal transcribed spacer) ITS1-5.8S-ITS2 regions of fungal rRNA, followed by RFLP-PCR using Msp1 restriction enzyme allowed simple, rapid, cost-effective, sensetive and accurate identification of the phenotypically identified yeasts.

Key words: *Oreochromis niloticus*; yeast; PCR; Msp1; PCR-RFLP

Introduction

Parasitic fungi are facultative saprophytic (1). Yeasts are identified according to the ability of sugars fermentation and assimilation of carbon and nitrogen compounds (2). Fungal reproduction is not simple and depends upon more than one method of propagation. Environmental conditions stimulate formation of special structures that can help in sexual and asexual reproduction by efficiently dispersing spores or spore-containing propagates (3). Unicellular reproduction can occur by budding, fission or by both processes, called bud fission. Yeasts that reproduce sexually are members of

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two large phyla of the fungi and have different modes of sporulation. After meiosis ascomycetous yeasts produce different shapes of ascospores inside an ascus that is not enclosed in a complex fruiting body or an ascocarp. Basidiomycetous yeasts show a wide variety in the basidium morphology, where the formation of the external meiotic spores (4). Phylogenetic and population genetic methods are used to identify species and pathogenic fungi and detect the modality of their reproduction in nature (5). Fish that infected by fungi was previously exposed to some other pathogens or stressors like bacterial, viral, parasitic diseases, poor water quality and trauma (6). Fungal can infect externally or internally and over the past decade fungi have received high attention all over the world (7). Many of fungi are considered opportunists because attacking fish under the effect of different stressors (8).

Conventional identification of pathogenic yeasts depends on the morphological and biochemical characters using tests that need more time. Also, the available commercial methods frequently fail to identify the less common pathogens or to differentiate between closely related species. Molecular methods used for the identification are rapid and sensitive alternatives to conventional identification for yeasts (9). RFLP method was relatively more accurate and reliable for the detection of various yeasts compared PCR (10).

The present study was conducted to isolate and identify yeast from freshwater fish by phenotypic methods and study reproductive behavior on different culture media, rice agar for asexual spores while carrot media for detection of sexual spores. Molecular identification of yeast by RFLP-PCR was further applied to confirm phenotypic results.

Materials and methods

A total of 300 live *O. niloticus* comprised 80 clinically diseased and 220 apparently healthy fish were collected from private fish farms at Kafrelsheikh province. The Body weights were ranged from 150±10 g. The collected fish were examined clinically with paying attention to the behaviors in the ponds, changes in color and respiratory manifestations with special care to the external lesions according to the methods described by (11). The samples were collected aseptically transferred alive to the provisional laboratory of department of microbiology in faculty of veterinary medicine, Kafrelsheikh University.

The live fish samples were transferred in a separate plastic bags one-third volume of water and provided with oxygen aeration by battery aerator (Beauty, Italy) and then subjected to mycological examination.

Media

Sabouraud's dextrose agar (SDA) (oxoid). It was used for isolation and preservation of fungi according to (13), Germ tube test (14) for detection of reproductive behavior, Rice agar media (15) for asexual reproduction of yeasts, Carrot agar (CA) (16) for induction of sexual sporulation of fungi, differential and selective chromogenic medium for the isolation and quick identification of *Candida* spp., Christensen's urea agar (Oxoid) for biochemical identification,

Isolation of yeast

The obtained samples were inoculated into duplicate plates of SDA with 500 mg of chloramphenicol dissolved in 3 ml ethanol 95% (that were added to the media after autoclaving). The inoculated plates were incubated at 30ºC–37ºC. A pure culture of each yeast colony was collected and the genus identified based on morphological characteristics (13).

RFLP-PCR

RFLP-PCR was carried out using primers (to amplify the ITS-1 and ITS-2 regions of yeast spp. using universal primers (internal transcribed spacer) ITS1 (5'-TCC GT AG-GTGAACCTGCCGG-3') and ITS4 (5'-TC CTCCGCTTATTCATATGC-3') and *Msp1* restriction enzyme for yeast spp. as previously described (17). Before RFLP-PCR, DNA extraction from yeast isolates was performed using DNA isolation kit (Jena Bioscience) and as previously described (18).

Results

Phenotypic identification of yeast isolates

Yeast isolates were identified according to microbiological characters (hyphae, pseudohyphae, chlamydospores, and blastospores) into genera. Two hundred and twenty-six yeast isolates that yielded from the samples were identified into 4 genera (*Candida*, *Rhodotorula*, *Trichosporon* and *Geotrichum*).

Identification of Candida species

One hundred and thirty-four isolates of *Candida* spp. were subjected for identification according to the following (Fig. 1):

A. Macromorphology on SDA

C. albicans colonies were characterized by creamy colored pasty colonies within 48-72hrs, germ tube positive, grew well on media containing cycloheximide with light green colonies on chromogenic candida agar, while *C. parapsillosis* colonies were white and smooth colonies on both SDA and chromogenic Candida agar with no growth on media containing cycloheximide. *C. guilliermondii* colonies were dull, white, creamy or yellow, flat smooth colonies, while on chromogenic agar, they appeared pale pink to purple. Other *Candida* spp had the same cultural characters on SDA but can't grow on media containing cycloheximide; with light pink colonies on Chromogenic candida agar and showed negative germ tube test.

B. Micromorphology on Rice agar media

Isolates could be identified into *C. albicans* according to Rice agar media which showed terminal chlamydospores, blastocondia and pseudohyphae. Pseudohyphae of *C. parapsillosis* were long and branched and carried clusters of elongated blastospores without chlamydospores. Other *Candida* spp. failed to produce pseudohyphae. *C. guilliermondii* appeared as short branched pseudohyphae bearing clusters of blastospores All *Candida* isolates were urease negative.

Identification of Trichosporon species

Thirty-nine isolates of *Trichosporon* isolated on SDA appeared firstly as smooth flat or wrinkled white to creamy colonies that turned waxy with central folds surrounded by wrinkled furrows. They grew well at 30^0C, 37^0C, and also on media containing cycloheximide. Microscopically, hyaline mycelium separated and fragmented into rectangular arthrospores appeared. On Rice agar media, *Trichosporon* appeared as septated hyphae, pseudohyphae, and arthrospores. *Trichosporon* species were urease positive.

Identification of Rhodotorula species

Forty-one isolates *Rhodotorula* were identified on SDA as *Rhodotorula* spp. (light pink flat colonies). It grows well at 30^0C with faint growth at 37^0C, no growth on media containing cycloheximide. Microscopic examination of Gram's stained films revealed large round yeast cells, on Rice agar media showed large round blastoconidia with no pseudohyphae. *Rhodotorula* spp. was urease positive.

Identification of Geotrichum species

Six isolates *Geotrichum* spp. were identified on SDA as they grew rapidly as white to creamy, flat and smooth, yeast-like colonies which turned into mold-like in old colonies, grew at 30^0C but not at 37^0C, or on media containing cycloheximide. Micromorphology, hyaline septated branched hyphae break up into short chains or individual arthroconidia appeared. No pseudohyphae or blastoconidia were formed, a character which differentiated it from *Trichosporon* species. Arthroconidia produced a hyphal extension from one corner producing a hockey stick appearance. *Geotrichum* spp. was urease negative.

Phenotypic identification of yeast isolates

Molecular identification of yeast isolates

Results obtained by preliminary conventional identification of yeast isolates combined with detection of characteristic asexual spore helping in identification of most of them into genera (*Candida, Trichosporon, Rhodotorulla*, *Geotrichum*), while using RFLP-PCR we found (*C. albicans, C. parapsilosis, C. guilliermondii, C. inconspicua, Trichosporon asahii*) (Figs. 2 and 3). Based on the results of RFLP-PCR we determined the incidence of yeast isolates.

The incidence of yeast spp. isolated from diseased and apparently healthy *O. niloticus* fishes was shown in Table 1. Incidence of yeast spp. isolated from different organs of *O. niloticus* fishes was shown in Table 2.

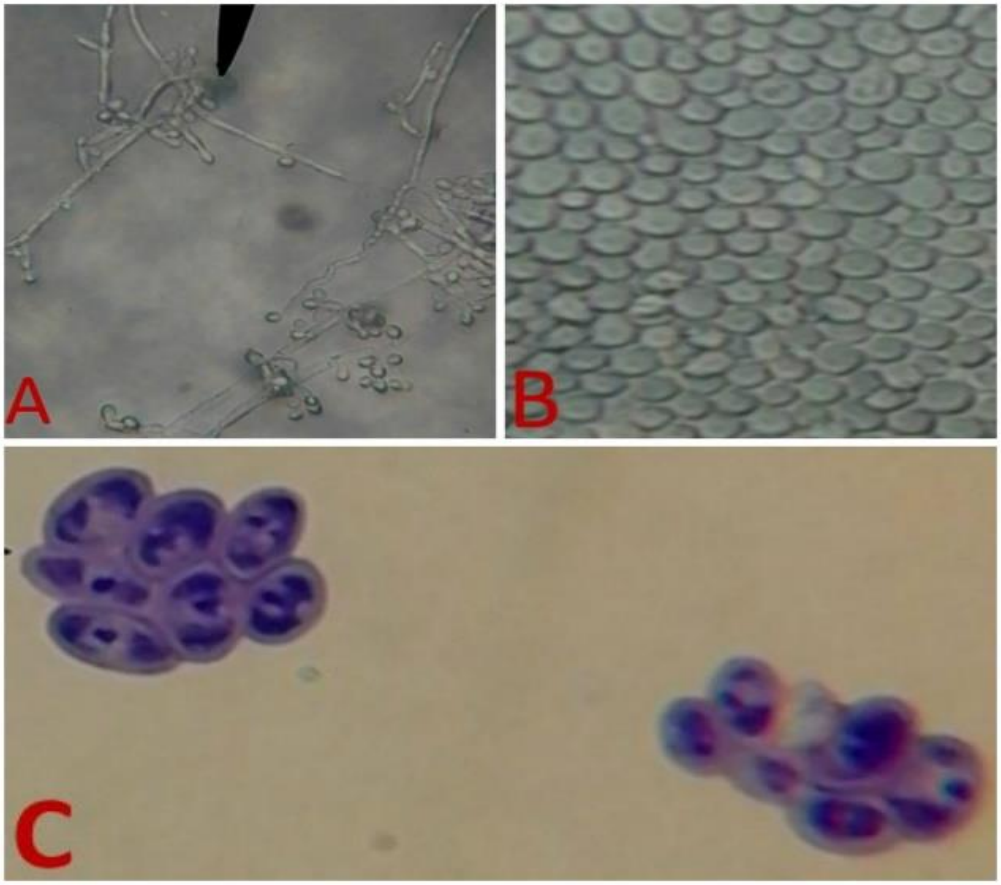

Figure 1: Microscopical identification of isolated yeasts. (A) *Candida albicans* showed on RAT blastspores, pseudohyphase and chlamydoconidia. (B) Yeast showed on RAT budding cells (blastspores without peudohyphae). (C) Ascospore forming yeast on carrot agar

Table 1: Incidence of yeast spp. isolated from diseased and apparently healthy *O. niloticus* fishes

Isolated yeast	No. of examined *O. niloticus*			
	No. of diseased (80)		No. of apparently healthy (220)	
	No.	%	No.	%
Rhodotorulla spp.	15	18.75%	30	13.63%
Candida albicans	16	20%	22	7.27%
Candida parapsilosis	5	6.25%	15	6.81%
Candida spp.	4	5%	11	5%
Candida guilliermondii	9	11.25%	15	6.81%
Trichosporon spp.	6	7.5%	18	8.18%
Ascus forming yeast	3	3.75%	7	3.18%

Table 2: Incidence of yeast spp. Isolated from different organs of *O. niloticus* fishes

Isolated Yeast	No. of isolates	%	Organs Skin		Gills		Liver		Kidney	
			No.	%	No	%	No.	%	No	%
Rhodotorulla spp.	41	18.14	12	29.3	22	53.7	2	4.9	5	12.2
C. albicans	44	19.47	13	29.5	15	34.1	10	22.7	6	13.6
C. parapsilosis	37	16.37	8	21.6	19	51.4	6	16.2	4	10.8
Candida spp.	20	8.85	4	20	10	50	4	20	2	10
Trichosporon spp.	39	17.25	21	53.8	9	23.1	9	23.1	0	0
Candida guilliermondii	33	14.6	0	0	18	54.5	15	45.5	0	0
Geotrichum spp.	6	2.66	2	33.3	3	50	1	16.7	0	0
Unidentified Ascus forming yeasts	6	2.66	2	33.3	3	50	1	16.7	0	0
Total	226	100	62		99		48		17	

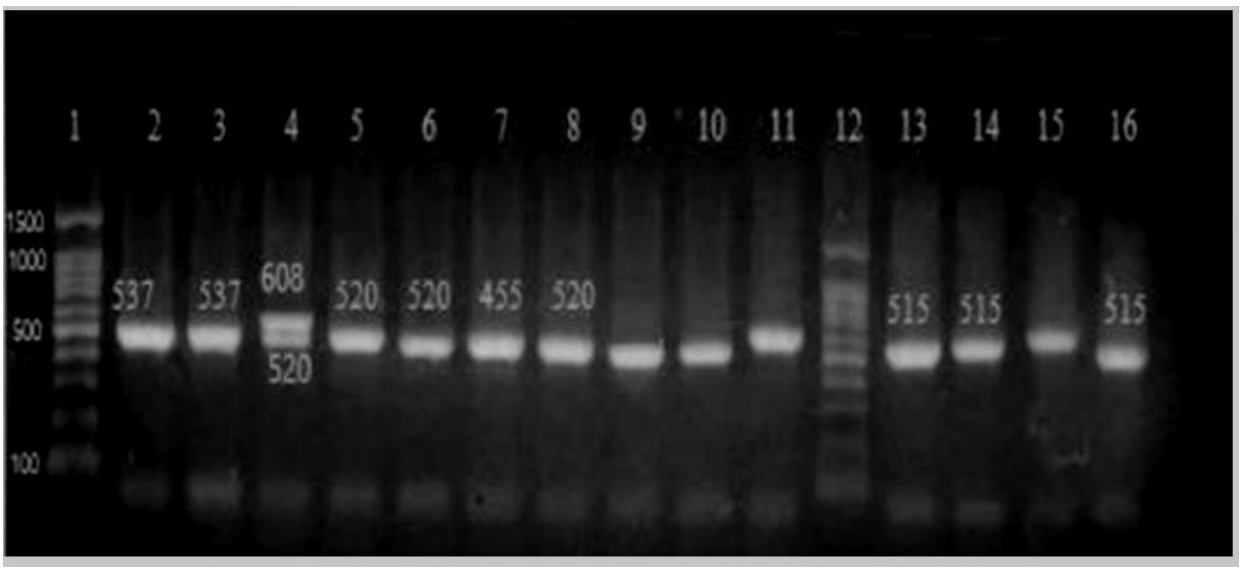

Figure 2: PCR product of yeast isolates, lane 1,12 – 100 bp ladder, lane2– *C. albicans*, lane3 – *C. albicans*, lane4 - *C. guilliermondii 608bp* and *C. parapsilosis 520bp*, lane5 – *C. parapsilosis* lane 6 - *C. parapsilosis*, lane 7 - *C. inconspicua*, lane 8 – *C. parapsilosis* lane 9 – Unidentified *Candida* spp. lane 10 – Unidentified *Candida* spp. lane 11 Unidentified *Candida* spp. ,Lane13 - *Tr. asahii* lane14 - *Tr. asahii*, lane15 –Unidentified *Trichosporon* spp. lane16 – *Tr. asahii*

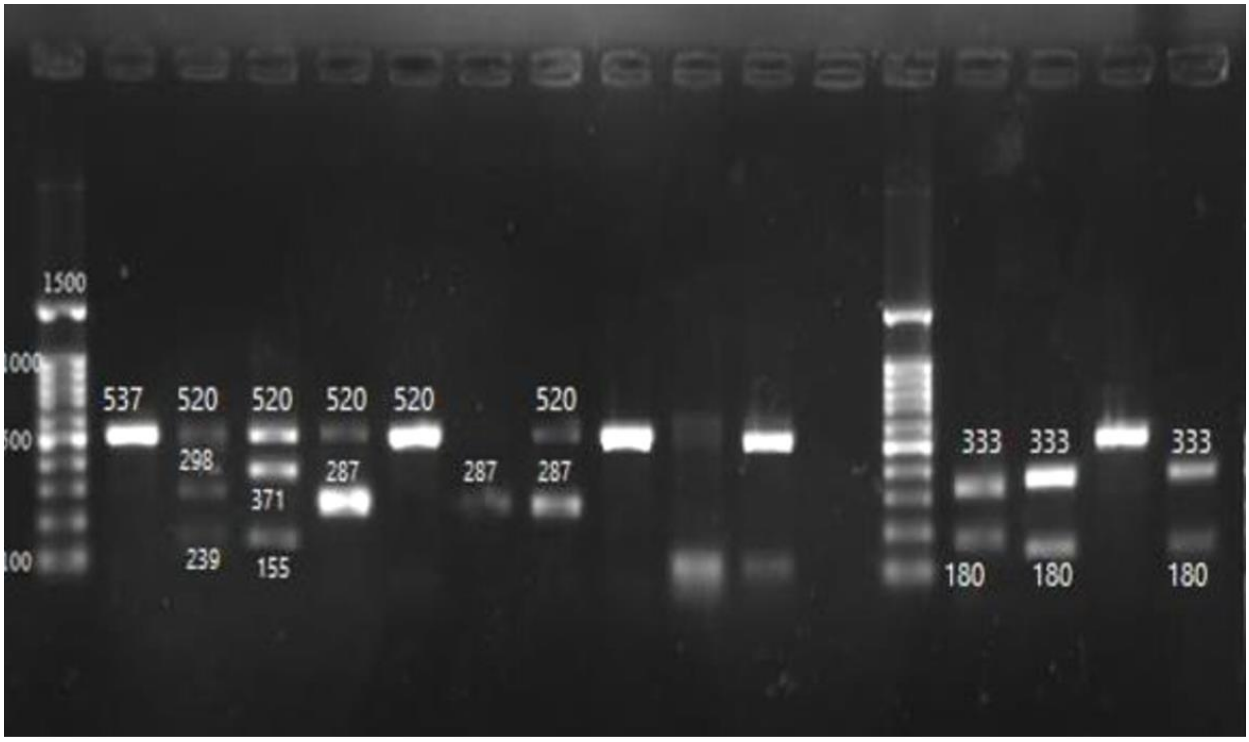

Figure 2: RFLP-PCR of yeast isolates by *MSP1* enzyme, lane 1,12 – 100 bp ladder, lane2– *C. albicans*, lane3 – *C. albicans and C. parapsilosis*, , lane4 - *C.parapsillosis and C. guillerimondi*, lane5 – *C. parapsillosis and C. inconspicua* lane 6 - *C. parapsilosis*, lane 7 - *C. inconspicua*, lane 8 – C. *C. parapsillosis and C. inconspicua* lane 9 – Unidentified *Candida* spp., lane 10 – Unidentified Candida *spp*, lane 11- Unidentified Candida spp. Lane13 - *Tr. asahii* lane14 -*Tr. asahii*, lane15 – Unidentified *Trichosporon* spp., lane16 – *Tr. Asahi*

Discussion

Yeasts play a key role in several ecosystem processes, establishing ecological relations with other organisms which have been identified as a part of wild caught as well as farm-raised fish microbiota and display an ability to interfere with the fish's nutrition and sanitary conditions (19). 226 yeast isolates out of 300 (75.33%) were isolated, with 5 genera: *Candida, Rhodotorula, Trichosporon, Geotrichum and unidentified ascus forming* yeast from *O. niloticus*. *Candida* spp. and were isolated at an incidence of 59.29% (134/226) and 18.14% (41/226), respectively. These results agreed with those recorded by (20) who isolated *Candida spp.* and *Rhodotorula* spp. at incidence rate of 65.12% and 28.84%, respectively.

C. albicans (19.47%) and *Rhodotorula* spp. (18.14%) were the highest yeast isolates collected from skin, gills, kidney, and liver and. These results coincided with (21) who found incidence of *C. albicans*, and *Rhodotorula* spp. at 35.9 % and 31.4%, respectively.

Trichosporon spp. was isolated with higher incidence (17.25%) and lower rate for *Geotrichum* spp. (2.66%) and *ascus forming unidentified* yeast (2.66%). Nearly similar results were recorded by (22). But (20) recorded that *Trichosporon* spp. (4.18%), and also *Geotrichum* spp. and *ascus forming unidentified* yeast in a low percent 0.46%. *C. guilliermondii* and *C. parapsilosis* were isolated in higher number that in agreement with (23) who found a rising of these pathogens but in freshwater environment.

In the present study, several species of genus Candida (such as *C. parasitosis, C. guilliermondii* and *C. inconspicua*) were mostly isolated from gills nearly by 50%. There is an evidence that exposure to environmental stressors can predispose fish to infection according to

(24). The polluted feeds, water and worker hands play important role in fish health (25). We observed that many yeast species that affect human have been isolated from freshwater environments. Terrestrial environments and wastewater may be the origin of many of these species (26).

Molecular approaches exhibit the potential to be a strong supplement for the phenotypic identification than the conventional methods (27). The most frequently used targets for yeast identification is the ITS region, which lies between the 18S and 28S rRNA gene and divided by 5.8S rRNA gene into ITS1 and ITS2 regions. In many studies the ITS2 showed considerable interspecies variability to identify 99.7% of yeasts and 100% of molds to species level, while ITS1 had identification accuracy of 96.8%–100% for yeasts and 100% for molds (28). Although ITS2 has been used in wide range for identification of fungi, in other study the ITS1 exhibit higher interspecies variability than ITS2 region (29). PCR-RFLP assays have been used to identify the isolated yeast species as was successfully done by (30). An advantage of this method is the stable and easy-to-read RFLP patterns. Unlike previous reports, this method involves only one or two DNA fragments. Also, it is easy and rapid to perform. In addition to time-saving restriction enzymes it takes few hours to be completed. It requires no complicated equipment except a traditional thermal cycler. Regarding that DNA sequencer may not be easily obtainable to most clinical laboratories, this molecular method is applicable for obvious identification and differentiation of different yeast species (31).

In the present study, yeast samples that was molecularlt identified as *Candida* spp. by universal primers became specifically identified as *C. inconspicua* by RFLP either in samples mixed with *C. parapsilosis* or alone. All examined isolates showed comparable RFLP patterns to their respective reference strains. Regarding to genus *Rhodotorula* is commonly observed in fish microbiota, as *Trichosporon* that in agreement with (32). Also, *Trichosporon asahii* is the most frequently isolated species in these infections that agreed with this study as PCR-RFLP assure the present results (33). The remaining unidentified *Candida spp* or unidentified ascus forming yeasts need for sequencing for accurate defining of yeast species.

Conclusion

Yeasts isolated from healthy and diseased *O. niloticus* included *Candida, Trichosporon, Rhodotorula, Geotrichum*. Phenotypic methods succeed in the identification of yeasts into genera. Molecular methods using RELP PCR confirm the phenotypic identification and specifically identify the unknown species. The RFLP method follows a common protocol that can be done for identification of yeasts in few hours. So, it can be used substitutional to the traditional methods.

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Original Research Article

PIVOTAL ROLE OF *Lactobacillus* STRAINS IN IMPROVEMENT OF SOFT CHEESE QUALITY AND INHIBITING THE GROWTH OF HARMFUL AND DANGEROUS BACTERIAL PATHOGENS

Yaser M. Hafez[1*], Azza M. Sobeih[2], Nehal M. Mansour[1, 2]

[1]EPCRS Excellence Center &Plant Pathology and Biotechnology Lab., Faculty of Agriculture, Kafrelsheikh University, 33516, Egypt, [2]Food Control Department, Faculty of Veterinary Medicine, Kafrelsheikh University, 33516, Egypt

*Corresponding author, E-mail: hafezyasser@gmail.com

Abstract: This study was carried out to determine the effect of addition of *Lactobacillus* bacteria to improve the soft cheese quality and inhibiting the microbial growth on cheese. *Lactobacillus acidophilus DSM 20079* and *Lactobacillus casei* ss. *casei DSM 20011* were studied *in vitro* for their probiotic properties through their antimicrobial activity. These strains were able to inhibit the growth of *Escherichia coli* O_{157} ATCC51659, *Staphylococcus aureus* and *Bacillus cereus*. Low salt soft cheese was manufactured, with acceptable organoleptic characters and prolonged shelf-life by using *L. acidophilus* and *L. casei* as bio preservative. The results cleared that, the addition of *L. acidophilus* or *L. casei* to the soft cheese improved the quality of the soft cheese through improvement of organoleptic characters of soft cheese such as appearance, body texture, flavour and extended soft cheese shelf-life through microbiological characteristics of manufactured cheese. The effect of the *L. acidophilus* is higher than the effect of *L. casei* than the control group. So we can use these strains as food preservative.

Key words: *Lactobacillus* spp.; cheese making; antibacterial activity

Introduction

Cheese is one of the essential milk products for human feed because it contains most of nutrients required for growth and body health (1). It considered as source of microbial contamination. It is commonly made from non pasteurized milk of poor bacteriological quality, under unhygienic conditions and final product kept uncovered. Thus, there is high threat of contamination with several pathogenic and spoilage microorganisms. Harmful pathogenic bacteria such as *Coliform, Escherichia coli, Staphylococcus* and *Salmonella* as well as yeasts and molds were recognized in Karish cheese which factory-made with traditional method (2, 3). These harmful microbes cause inflammation and allergic diseases (3). Consumers need natural and chemical-free cheese. Therefore, it became urgent to search for alternative methods for cheese bio-preservation.

Lactobacillus is energetic for recent food technologies, because of their probiotic potential to replace antibiotics (4). Lactic acid bacteria used as a starter for cheese conservation, improving the organoleptic properties, source of fermentation industry and control of food borne

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microbes (5, 6, 7). It enhanced the immune system and produce bacteriocins, organic acids, H_2O_2 as antimicrobial compounds as well as short chain fatty acids (8, 9).

According to our knowledge not enough research were conducted on soft cheese biopreservation in Egypt, therefore, the aim of this research was to study the antimicrobial activity of *Lactobacillus* strains against harmful bacteria such as *E. coli* O_{157},*S. aureus* and *B. cereus* as well as improving the hygienic quality of soft cheese.

Materials and methods

Bacterial Culture

Identified *Lactobacillus acidophilus DSM* 20079 and *Lactobacillus casei* ss. *casei DSM 20011* from Cairo-MIRCEN, Faculty of Agriculture, Ain-Shams University, Cairo, Egypt. Bacteria were cultured on MRS broth (9ml) media then, kept at 37°C for 24hours. After that, they were cultured in sterile skim-milk and incubated at 37°C for 24 hours to activate the bacterial strains and to increase the bacterial number to the target probiotic dose (10^7Colony forming unit/g) for soft cheese manufacture.

Antimicrobial activity of Lactobacillus strains

Antibacterial activity of *Lactobacillus* strains was evaluated by agar well diffusion assay **(10).**Culture media (15 ml), melted and tempered at 45ºC, were inoculated with *E. coli* O_{157},*S. aureus* and *B. cereus* at 10^6 CFU/ml then poured into Petri dishes that had wells which made by cork borer. Wells were filled with 100 µl of 48 hours-culture-supernatants of the *Lactobacillus* strains, then, incubated at 37 ºC for 24 hours. The bacterial activity seen by appearance of inhibition zone which measured by milli-meter ruler.

Cheese manufacturing

Soft cheese was made as by Mehanna and Rashed 1990 and El-Sheikh *et al.* 2001 (11, 12) with slight modification. Pasteurized half fat milk (12L, 1.5% fat,8.5% SNF) were heated to 40°C then, the total solid was standardized to 14% by adding 4% skimmed milk powder(<1.25% fat,< 32% protein and >53% lactose), Nacl 3% and CaCl2 0.02% also were added. The bulk volume of milk was divided into 3 groups (4 Kg each) after mixing and inoculated by the activated starter cultures the first group was considered as control, the second was inoculated with *L. acidophilus* culture and the third was inoculated with *L. casei*, then, 0.3g rennet was added to each group and incubated at 37°C until curd formation, the curd was kept to drain in previously sterilized stainless steal frames lined with cheese cloth. The produced cheese and their whey were packaged in pre-sterilized bottles and kept at4°C. The cheese was tested fresh (zero time) and at equal intervals of 7 days till the spoilage was appeared. The experiment was repeated in triplicates and average results for each group were tabulated.

Cheese characters evaluation

Organoleptic examination

Cheese samples were tested for appearance, texture, body, and flavor (13). The identical samples were labeled using random numbers and presented to the judges in random manner by 10 examiners.

Chemical examination

Cheese samples were tested for pH (14).

Microbiological examination

Samples were homogenized using sodium citrate (2%) and 10 fold serial dilutions were made (15). Then, they were tested for total *Coliform* count (15); *Lactobacilli* count (16); as well as mold and yeast count (17).

Statistical analysis

The statistical analysis was done using analysis of variance (ANOVA) to compare between the parameters studied among different treatments. Chi^2-test was used also, to determine the incidences of bacterial isolates between different treatments (18).

Results

Antibacterial effect of Lactobacillus strains

Lactobacillus acidophilus and *Lactobacillus casei* ss c*asei* inhibited the growth of harmful bacteria such as *Echerichia coli*O_{157}, *Staphylococcus aureus* and *Bacillus cereus* (Table 1,Fig. 1).

Positive impact of lactobacilli on soft cheese quality

Our results presented in table (2) cleared that, the *Lactobacillus acidophilus* and *Lactobacillus casei* improved the average appearance of the soft cheese and its body texture than the control group and the *Lactobacillus acidophilus* decreased the spoilage followed by the *Lactobacillus casei* than the control group. The results also, cleared that, the cheese still preserved its flavour in case of *Lactobacillus acidophilus* till 32 day from its storage and 30 day in case of *Lactobacillus casei* and in the control group the flavour still present till 14 day of preservation.

The overall score due to addition of *Lactobacillus acidophilus* and *casei* improved the score of cheese especially in *Lactobacillus acidophilus* followed by *Lactobacillus casei* than the control group.

Effect of Lactobacillus bacteria on pH level of soft cheese

The observed results in table (3), exhibited a significant differences ($P < 0.05$) on the effect of lactobacillus bacteria on pH level of soft cheese. *Lactobacillus acidophilus* improved the pH level till 30day of cheese storage, followed by *Lactobacillus casei* and both of them improved the pH level than the control group.

Effect of Lactobacillus level on the bacteriologic aspect of the soft cheese

Our results observed in table (4) cleared that, the level of *Lactobacillus acidophilus* increased gradually by storage time of soft cheese and reached its maximum level at 21 day of storage. By increasing the level of *Lactobacillus acidophilus* and *Lactobacillus casei* causes decreasing the level of coliforms bacterial growth but the effect of *Lactobacillus acidophilus* greater than the effect of *Lactobacillus casei*.

Effect of lactobacillus bacteria on mycological aspect of the soft cheese

Our results observed in Table (5), cleared that, there is a significant different of the effect of lactobacillus bacteria ($P < 0.05$) on the level of mould and yeast count. The results cleared that, the increasing level of *Lactobacillus* associated with decreasing level of moulds and yeast than the control group. The effect of *Lactobacillus acidophilus* higher than the effect of *Lactobacillus casei* in prevention of the growth of moulds and yeast.

Table 1: Inhibition zones (mm) caused by *Lactobacillus* strains in agar diffusion assay (n = 5)

Lactobacillus Species	(Diameter of inhibition zones in mm)		
	Escherichia coli O_{157}	*Bacillus cereus*	*Staphylococcus aureus*
Lactobacillus casei	2.6 ± 0.20^{B}	2.9 ± 0.10^{A}	2.75 ± 0.50^{AB}
Lactobacillus acidophilus	2.9 ± 0.3^{A}	2.57 ± 0.50^{B}	2.55 ± 0.20^{B}
Control	0	0	0

Means within the same row of different litters are significantly different at ($P < 0.05$)

Table 2: Effect of *Lactobacillus* on organoleptic characteristics of soft cheese.

Storage time	Average Appearance (10)			Body texture (60)			Flavor (30)			Overall score (100)		
	Control	*Lactobacillus casei*	*Lactobacillus acidophilic*	Control	*Lactobacillus casei*	*Lactobacillus acidophilic*	Control	*Lactobacillus casei*	*Lactobacillus acidophilus*	Control	*Lactobacillus casei*	*Lactobacillus acidophilus*
Zero time **	7	9	8	56	58	57	28	29	29	91	96	94
7 days	6	8	7.5	55	57	56.5	26	28	28	87	93	92
14 days	5	7	7	45	55	54	19	26	26	69	88	87
21 days	S	5	5	S	50	53	S	20	20	S	75	78
30 days	S	S	4	S	S	52	S	S	19	S	S	75
32 days	S	S	S	S	S	S	S	S	S	S	S	S
Chi^2	10.55**			11.22**			12.45**			9.24**		

S = spoiled ** = Significant at ($P < 0.01$)

Table 3: Effect of *Lactobacillus* on pH of the soft cheese**.**

Storage time	Average pH		
	Control	*Lactobacillus casei*	*Lactobacillus acidophilus*
Zero time	5.54 ± 0.04^{A}	$5,78\pm0.02^{A}$	5.64 ± 0.04^{A}
7 days	5.54 ± 0.05^{A}	5.78 ± 0.01^{A}	5.64 ± 0.04^{A}
14 days	5.45 ± 0.05^{A}	5.76 ± 0.03^{A}	5.54 ± 0.05^{A}
21 days	S	5.64 ± 0.04^{B}	5.50 ± 0.05^{B}
30 days	S	S	5.35 ± 0.03^{B}
32 days	S	S	S

S = spoiled. Means within the same row with different litters are significantly different at ($P < 0.05$)

Table 4: Effect of *Lactobacillus* on bacteriological aspect of the examined samples of soft cheese

Storage time	Average coliforms count(cfu/g)			Average Lactobacilli count (cfu/g)	
	Control	*Lactobacillus casei*	*Lactobacillus acidophilus*	*Lactobacillus casei*	*Lactobacillus acidophilus*
Zero time**	$9.9x10^{6}\pm9.0\ X10^{3}$ A	$4.5x10^{6}\pm1.50\ X10^{3}$C	$2.5x10^{5}\pm1.0\ X10^{3}$ D	$1.4x10^{7}\pm3.0\ X10^{3}$ B	$1.3x10^{7}\pm3.0\ X10^{3}$ B
7 days	$1.3x10^{8}\pm0.50X10^{3}$A	$2x10^{7}\pm 2.50\ X10^{3}$ B	$3x10^{5}\pm1.0\ X10^{3}$ C	$2x10^{7}\pm3.0\ X10^{3}$ B	$1.8x10^{7}\pm3.0\ X10^{3}$ B
14 days	$1.5x10^{8}\pm4.0\ X10^{3}$ A	$4x10^{6}\pm3.0\ X10^{3}$ C	$1x10^{5}\pm1.0\ X10^{3}$ D	$2.8x10^{7}\pm3.0\ X10^{3}$ B	$6.13x10^{7}\pm2.0\ X10^{3}$ B
21 days	S	$1x10^{3}\pm1.0\ X10^{3}$ D	$11x10^{2}\pm1.0\ X10^{3}$ D	$1.1x10^{7}\pm1.0\ X10^{3}$ D	$5.2x10^{7}\pm1.0\ X10^{3}$ D
30 days	S	S	$18x10^{2}\pm1.0\ X10^{3}$ B	S	$2.21x10^{7}\pm$A1.0 $X10^{3}$
32days	S	S	S	S	S

S= spoiled. Means within the same row of different litters are significantly different at $P < 0.05$

Table 5: Effect of *Lactobacillus* on mycological aspect of the soft cheese

Storage time	Average mould count (cfu/g)			Average yeast count (cfu/g)		
	Con-trol	*Lactobacilus casei*	*Lactobacillus aci-dophilus*	Control	*Lactobacillus casei*	*Lactobacillus aci-dophilus*
Zero time	<10D	<10D	<10D	$1.3x10^7$A	$4.80x10^6$B	$1.50x10^5$ C
7 days	<10D	<10D	<10D	$1x10^8$A	$2.5x10^7$B	$2.20x10^6$C
14 days	$2x10^1$D	<10D	<10D	$1.3x10^8$A	$2.40x10^7$B	$1.10x10^6$C
21 days	S	$1.50x10^1$C	<10D	S	$2.17x10^4$A	$1.18x10^4$B
30 days	S	S	$2x10^1$B	S	S	$1.06x10^3$A
32 days	S	S	S	S	S	S

S= spoiled, Means within the same row of different litters are significantly different at ($P < 0.05$)

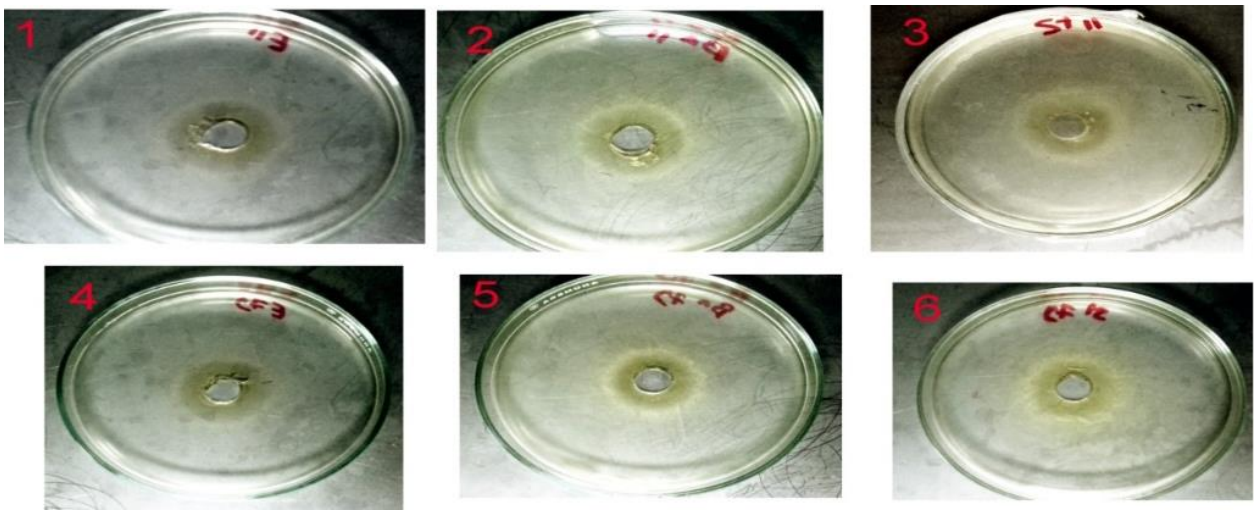

Figure 1: Antibacterial activity of *Lactobacillus casei* ss. *casei DSM 20011* against *(1) Escherichia coli* O_{157} *ATCC51659 (2) Bacillus cereus (3) Staphylococcus aureus* determined by appearance of inhibition zone(mm). Antibacterial activity of *Lactobacillus acidophilus DSM 20079* against *(4) Escherichia coli* O_{157} ATCC51659 *(5) Bacillus cereus (6) Staphylococcus aureus* determined by appearance of inhibition zone (mm)

Discussion

Influence of *Lactobacilli* on cheese quality showed that both of *Lactobacillus* strains were able to improve cheese appearance, body texture, flavour, than the control group. *Lactobacillus acidophilus* decreased the spoilage incidence than *Lactobacillus casei* than the control. These results attributed to the increasing pH level which prevents the cheese spoilage with improvement of sensory characters. *L. acidophilus* improved the pH level till 30 days of cheese storage, followed by *L. casei* as compared to control group. The effect of *Lactobacilli* level on the bacteriologic aspect of the soft cheese cleared that, the level of *L. acidophilus* increased gradually by storage time of soft cheese and reached to its maximum level at 21 day of storage. The obtained data from microbial quality assessment of collected soft cheese samples indicated that, their inferior hygienic quality with a great chance of being a cause of foodborne disease. According to the Egyptian Standard ES 1008-2000; 50 % of cheese samples are not agreed due to the high counts of

coliform as fecal contamination indicator mostly in cheese variety (19).

As a result of increasing the level of *L. acidophilus* and *L. casei*, the level of *Coliforms* bacterial growth was decreased however the effect of *L. acidophilus* greater than the effect of *L. casei*. These positive results attributed to the *Lactobacillus* ability to improve the pH level which prevents the growth of *coliforms* in soft cheese.

High yeast count regularly indicates disused hygienic measures during production and management (2). The effect of *Lactobacillus* bacteria on mycological side of soft cheese proved that, there is a significant difference effect of the bacteria ($P < 0.1$) on yeast and moulds count. The increasing level of *Lactobacilli* correlated with decreasing level of yeast and moulds compared with control group. Interestingly, the effect of *L. acidophilus* higher than *L. casei*e effect in prevention of moulds and yeast growth. *Lactobacilli* as biotherapeutic agents serve as natural food preservative through the antimicrobial bacterial activity (3). Both *Lactobacillus* strains were able to inhibit the growth of *Escherichia coli* O_{157}, *Staphylococcus aureus* and *Bacillus cereus* as is seen by appearance of inhibition zone. The obtained results were in supported with that results in which proved the antagonistic activity of *Lactobacillus* against *Staphylococcus* spp. and *Coliform* sp. (20, 21).

We were able to produce soft cheese with advanced procedure and reached the optimum hygienic conditions. Increased *Lactobacillus* counts might be relied on the absence of nutrients competition between *Lactobacillus* and other microorganisms, particularly in the individual culture treatments. *Lactobacillus* spp. compete with other microbes their metabolic end products (22). Molds absence as a result of treatments till the 21th day of cold storage, may translate the antimicrobial effect of those two promising strains which achieve the main aim of this study in better microbial quality and extended shelf life of produced cheese. These results agreed with (23) who stated that; *Lactobacillus* isolated from fermented foods produce organic acids and other antimicrobial agents, which are responsible for quality and palatability.

Analysis of chemical and texture analyses, where less protein/ dry matter % decreased hardness and compacted structure of the new products causing softness. Keeping organoleptic characteristics similar to conventional soft cheese with higher hygienic quality is encouraging. These results agreed with what reported that *Lactobacilli* are members of autochthonous non-starter lactic acid (24). The relation with the chemical properties where compact structure was a result of relatively higher protein/ dry matter % of control. The higher moisture content, the more soft cheese that coats the mouth during eating (25). Such characteristics improvements could increase consumer demand to the new cheese product.

Conclusion

It can be concluded that this research study cleared that, the two promising strains of *Lactobacillus acidophilus* and *Lactobacillus casei* ss. *casei* were able to inhibit the growth of harmful food pathogenic bacteria such as *Escherichia coli* O_{157}, *Staphylococcus aureus* and *Bacillus cereus*. This perhaps the first study which conducted on *Escherichia coli* O_{157} as one of the dangerous and fetal human bacteria in Egypt. In addition to that both of *Lactobacillus* strains improved the quality of the soft cheese through improving the pH value, improvement of organoleptic characters such as appearance, body texture and flavour as well as improving the total score level of the soft cheese microbiological characters.

The positive effect of the *Lactobacillus acidophilus* is higher than *Lactobacillus casei* effect than the control group.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

IMPACT OF L-CARNITINE SUPPLEMENTATION ON GROWTH OF BROILER CHICKEN THROUGH DETERMINATION OF CHANGES IN THE EXPRESSION OF CAT2, MYOD AND MYF5 GENES

Tarek K. Abouzed[1*], Doaa A. Dorghamm [1], Khaled A. Kahilo[1], Azza M. Elkattawy[1], Eldsokey Nassef [2], Hanan B. El-sawy[2]

[1]Department of Biochemistry, Faculty of Veterinary Medicine, Kafrelsheikh University, 33516, Egypt, [2]Nutrition and Clinical Nutrition Department, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt

*Corresponding author, E-mail: dosouk2000@yahoo.com

Abstract: This study explored the impact of supplementation of L- carnitine on the performance of broiler chicken, biochemical parameters and expression of cationic amino acid transporter (*CAT2*), myogenic determining factor (*MYOD*) and myogenic factor 5 (*MYF5*) genes. This study included two groups; the first one is the control group and the second one received L-carnitine at a dose 50 mg/kg/day in drinking water. After 35 day, the sera and muscle tissue were obtained for biochemical and real time polymerase chain reaction (qPCR) analysis. The obtained results revealed a significant increase in the body weight, total body weight gain and a significant decrease in food consumption, food conversion rate in L-carnitine-supplemented group as compared to the control group. Moreover, there was no significant changes in serum cholesterol, triacylglycerol, AST, ALT, total protein and uric acid levels which showed a slight decrease in L-carnitine-supplemented group in comparison with the control group. This study also revealed a significant up regulation for the expression of *CAT2*, *MYOD* and *MYF5* genes in muscles of broiler chicken-supplemented with L- carnitine as compared to the control one. The present study concluded that supplementation of L- carnitine to broiler chicken was useful to increase the total body weight gain and muscle mass and this beneficial effect was associated with up regulation of *CAT2*, *MYOD*, and *MYF5* genes.

Key words: L-carnitine; broiler chicken; growth traits; *CAT2; MYOD; MYF5*

Introduction

Poultry industry has assumed an imperative role in meeting the deficiency of animal protein in world. The poultry production, particularly broiler production, is the quickest way to increase the accessibility of high quality protein for human utilization (1, 2).The poultry production differs from animal production in a different ways. Broiler chicken production requires between fifty to sixty days, while the formation of red meats requires 9 to12 months. Additionally, chicken require around 3 kg of food (ration) to create 1 kg of meat, while animals should consume 7 kg of food to produce 1 kg of red meat (3).

The problem in the poultry production is containing excess body fat. There are many

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factors, for example diets and hereditary factors that increase the tendency to aggregate excess body fat. In this manner, improvement of poultry composition by other additives is consider a primary focal point of nutritional research (4).

Poultry food is fundamentally composed of soybean, maize and plant products which all have less carnitine content (5). The latter can be biosynthesized endogenously from methionine and lysine amino acids in the presence of folic acid and vitamin B3, B6, B12, and C, in addition to iron (6, 7). When diets are not provided with these two amino acids, the bird unable to form a sufficient amounts of carnitine (5).

Dietary L-carnitine addition induces the β-oxidation of unsaturated fats to produce adenosine triphosphate (ATP) energy and enhance energy utilization (8-10). L-carnitine has two main functions. The first is to encourage the transfer of long-chain fatty acids across the inner mitochondrial membrane, and the second is to encourage the expulsion of short and medium-chain fatty acids from the mitochondria that aggregate as a result of typical and unusual metabolism (11, 12). It also has an impact on fat metabolism and reduction of protein formation to form energy in chick's body ,subsequently a greater amount of energy will be oxidized and won't be decomposed in abdominal tissues (13).

Myogenic determining factor (MYOD) and myogenic factor 5 (MYF5) are a group of proteins belong to myogenic regulatory factors (MRFs). These basic helix–loop–helix (bHLH) transcription factors act consecutively in myogenic differentiation. MYOD is a key transcriptional factor for muscle differentiation and growth. MYF5 is the first myogenic regulatory protein expressed in the skeletal muscle lineage. In mammals, both MYOD and MYF5 are required for the formation of skeletal muscle (14). In chickens, MYOD and MYF5 are associated with post-hatch chicken myogenesis (15). CATs are primary amino acid transport system used by tissues to concentrate lysine, arginine and ornithine into cellular amino acid pools to be used in nitrogen metabolism (16).

This study was conducted to explore the potential effect of L- carnitine on the performance of broiler chicken, biochemical parameters and *CAT2*, *MYOD*, *MYF5* genes expression.

Materials and methods

This study was conducted in the chicken experimental house at Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt. A total number of 120 mixed sex Ross 308 broiler chicks at 1 day old age were obtained from a private poultry company at El-Gharbia Governorate, Egypt. All the chicks were immunized against both New Castle's disease on 7th and 18th days and Gumboro disease on 14th day. The experimental birds were offered feed and water *ad libitum* for 5 weeks.

Bird feeding

A starter diet was available for the first 21 days then changed to a grower diet till the end of the experiment (35 days). Diets were formulated to meet the supplement recommendations for broilers by the National Research Council (17) with some modification according to updated nutrients specification of broiler chickens (Tables 1&2).

Experimental design and treatment

One hundred and twenty chicks were allocated into two groups (60 chicks per group). Each group was subdivided into 6 replicates (10 chicks/replicate).The first group: received drinking water only and basal diet (without any additive) and was considered as the control group; and the second group received L-carnitine (sigma Aldrich company in drinking water at dose of 50 mg/kg/day (18).

Blood and tissue samples

At the end of experiment, chickens were subjected to 12 hours feed withdrawal and then blood samples were collected from the wing veins in clean and dry Eppendorf tubes. After coagulation and centrifugation at 3000 rpm for 15 minutes clear serum samples were collected. Sera immediately kept frozen at -20^{o}C until biochemical analysis. Birds were slaughtered after mild anesthesia and dissec-

tion of skin under complete aseptic condition. Then muscle specimen was excised from breast muscle and quickly dunked in liquid nitrogen, then frozen at -80ºC for RNA extraction.

Performance parameters

Body weight was recorded at the end of every week. Body weight gain was calculated weekly following this equation: body weight gain = final weight– start weight. Feed intake was recorded daily according to the following equation: Feed intake=feed allowed – feed refusal. Feed conversion ratio (FCR) was calculated according to the following equation: FCR = feed intake/body weight gain.

Biochemical analysis

Colorimetric estimation of glucose, cholesterol, triacyl glycerol, and uric acid was done as previously described (19). Serum total protein was evaluated by colorimetric procedure based on (20). Serum enzyme activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were spectrophotometrically measured as previously described (21).

Quantitative determination of CAT2, MYOD and MYF5 using real-time polymerase chain reaction (qPCR)

Breast muscle tissue was used to isolate total RNA using TRIZOL (GeneZOl, Lot≠:30117B07003, Genetix Biotech) following the manufacturer's protocol. The concentration of RNA was measured using a Nanodrop. cDNA was synthetized using HiSenScript^{TM} cDNA synthesis kit (Intron Biotechnology, cat.no.25014,). This was done by mixing 10 µl 2X RT reaction buffer, 1µl enzyme mix solution, 1µg of RNA and completed with RNas free water to 20 µl total volume. The mixture was incubated at 50 ºC for 30 min and 85ºC for 10 min. All PCR reactions were done by utilizing SYBR Green qPCR Master Mix (Enzynomics, cat. No. RT500) and specific primers (Table 3), at the following conditions: initial denaturation at 92 °C for 10 min, followed by 40 cycles of 15 s at 92 °C, 30 s at 60 °C and 30 s at 72 °C. The differences in gene expression among the different groups were estimated by using the $2^{-\triangle\triangle Ct}$ method (22), standardized to GAPDH and expressed as relative mRNA levels relative to the control. Melting curve analysis was performed to verify the correct product.

Statistical analysis

All data were presented as mean ± standard error (SE). Statistical analysis was conveyed utilizing the T-independent test (Levene′s test for equality of variances) to compare the significance among the groups. A value of $P < 0.05$ were considered to be significant.

Results

Effect of L- carnitine on performance parameters

Supplementation of L-carnitine group showed a significant increase in the final body weight as compared with control group .There were a significant increase in the body weight gain in the group received L-carnitine compared with control group. Feed intake in the control group was higher than the group treated with L-carnitine. However, feed conversion ratio was significantly lower in L-carnitine supplementation than group without supplementation (Table 4).

Effect of L- carnitine supplementation on biochemical parameters

As shown in (Table 5) the level of blood glucose, serum cholesterol, total proteins insignificantly change in chicken treated with L-carnitine relative to the control chicken. However, the levels of the remaining serum parameters (triacylglycerol, uric acid, ALT, and AST) showed an insignificant decrease as compared to the control group.

Effect of L- carnitine on CAT2, MYOD and MYF5 expression

As shown in figure 1, the L-carnitine supplementation significantly upregulated ($P<0.05$) the expression of *CAT2*, *MYOD* and *MYF5* in comparison with the control group.

Table 1: Ingredients composition of the diets (%)

Ingredients (%)	Starter diet	Grower diet
Yellow corn	53.55	58.2
Corn Gluten meal	6.9	6.0
Soybean meal, 44%	33.2	29.3
Soybean Oil	2.5	3
Di-calcium phosphate [1]	1.8	1.6
Ground Limestone [2]	1.2	1.0
Mineral and Vitamin premix[3]	0.3	0.3
Salt	0.3	0.3
DL- methionine[4]	0.1	0.1
Lysine [5]	0.1	0.15
Choline chloride	0.05	0.05
Total	100	100

[1] Dicalcium phosphate (contains 18% Phosphorus and 21% Calcium). [2] Limestone (contain 38 % calcium). [3] Premix provides vitamin A (12000 Iu), vitamin D (5000 Iu), vitamin E (50 mg), vitamin K3 (3 mg), vitamin B1 (3 mg), vitamin B2 (8 mg), nicotinic acid (30 mg), pantothenic acid (15 mg), vitamin B6 (4 mg), vitamin B12 (0.016 mg), folic acid (2 mg), biotin (0.2 mg), manganese (120 mg), zinc (100 mg), iron (40 mg), copper (16 mg), iodine (1.25 mg), selenium (0.3 mg) per 1 kg diet.[4] DL-Methionine (Produced by Evonic Co. and contains 99 % methionine).[5] Lysine = lysine hydrochloride (contains 70 % Lysine).

Table 2: Chemical composition of the diets.

	Starter diet	Grower diet
Moisture (%)	9	9
Dry Matter (%)	91	91
Crude protein (%)	23.01	21.20
Crude fat (%)	5.02	5.62
Crude fiber (%)	3.67	3.78
Ash (%)	6.23	5.59
Calcium (%)*	1.07	0.95
Available Phosphorus (%)*	0.49	0.45
Lysine (%)*	1.28	1.20
Methionine (%)*	0.56	0.52
Nitrogen free extract (%) **	53.07	54.81
ME (Kcal/kg) *	3043	3115
Calorie/protein ratio ***	132.3	146.9

* ME, lysine, methionine, calcium and phosphorus were calculated according to (17).** NFE= Nitrogen free extract was calculated by difference =100- (moisture% + CP% + EE % + CF% + ash %).*** Calorie/protein ratio = ME Kcal /CP

Table 3: Sequences of primers used in qPCR.

Gene	GenBank no.	Sequence
CAT2	BU492799	Forward CAACTGGAGAAAGAGGTCAGGAA
		Reverse AATTAGGCCATGAAGCAGACAAG
MYOD	NM_204214	Forward ATCACCAAATGACCCAAAGC
		Reverse GGGAACAGGGACTCCCTTCA
MYF5	NM_001030363	Forward TGAGGAACGCCATCAGGT
		Reverse GCGAGTCCGCCATCACAT
GAPDH	NM_204305	Forward ACATGGCATCCAAGGAGTGAG
		Reverse GGGGAGACAGAAGGGAACAGA

Table 4: Effect of L- carnitine supplementation on performance parameters

	Control	L. carnitine
Initial weight (g)	40.20±1.8	40.8±1.5
Final weight (g)	1689±3.5 [b]	1731±10.1 [a]
Body weight gain (g)	1648±2.6 [b]	1690±8.3 [a]
Average daily gain (g)	47.11±0.1 [b]	48.29±0.29 [a]
Cumulative feed intake/bird	3297.9±28.6	3235.9±25.2
FCR	2.0±0.01 [a]	1.91±0.01 [b]

Mean±SE (n=60/group) [a,b] Means in the same raw with different superscript are significantly different (P<0.05)

Table 5: Effect of L- carnitine supplementation on serum biochemical parameters

	Control	L- Carnitine
Glucose (mg/dl)	158±12.14	160.8±5.46
Cholesterol (mg/dl)	530±53.5	539.2±74
Triacylglycerol (mg/dl)	48.2±3.67	39.63±7.10
Uric acid (mg/dl)	3.32±0.30	3.2±0.4
Total protein(g/dl)	4.34±1.74	4.762±0.56
ALT(U/I)	24.5±6.11	16.25±4.95
AST(U/I)	347±18.52	247±15.55

Mean±SE (n=18/group) ALT (alanine transaminase), AST (Aspartate transaminase)

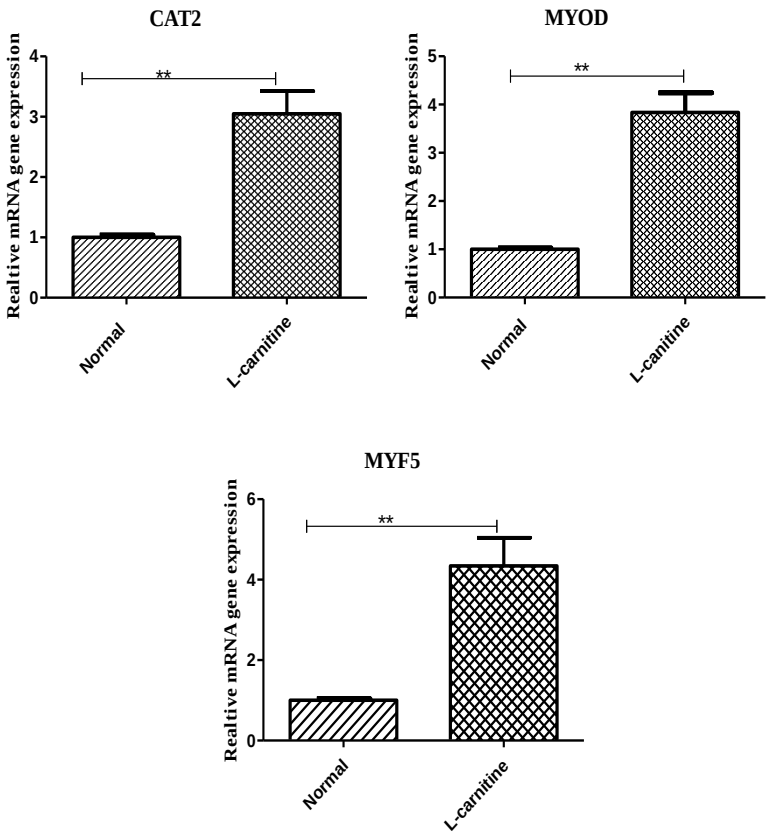

Figure 1: Quantitative real-time PCR analysis of the levels of expression of genes (A) cationic amino acid transporter (CAT 2),(B) myogenic determining factor (MYOD), (C) myogenic factor 5 (MYF5) *P <0.05 versus control group (n=18/group)

Discussion

L-carnitine supplementation has a positive effect on growth rate as revealed by higher growth rate, body weight gain and lower feed consumption or feed conversion rate. These result agree with different studies which reported that, addition of L-carnitine enhance growth rates and feed potency (23). The breast or thigh meat yield were increased, and abdominal fat content was decreased by supplementation of L-carnitine (10, 23). Likewise, L-carnitine incorporation to broiler feed enhanced body gain, improved FCR , yielded a lot of breast meat, and lowered abdominal fat depositions (13). Increased growth by addition of carnitine is also partly related to its amino-acid-sparing function in addition to its role in fat metabolism. Theoretically, the external addition of carnitine can lower the necessity for bio synthesis of carnitine from essential amino acid so stinting essential amino acid for different biological functions (24). L- carnitine can be used as a potential protecting agent against stress induced by high environmental temperatures in boilers (25).

In the contrary, some studies documented no change in growth rates or feed potency in L-carnitine-supplementation to broiler chickens (10, 26). Likewise, Leibetseder (27) reported that performance (BWG and FCR) and the content of abdominal fat of broilers weren't affected by dietary carnitine at a concentration of 200 mg/kg diet. These contradictory results may be attributed to variation in species, age, supplementary L-carnitine doses, dietary fat and protein intake. This clarification confirmed by Xu (10) who reported that L-carnitine at a dose of 25 mg/kg has no effects in daily body weight gain or feed conversion of birds. Whereas addition of L-carnitine at a dose higher than 25 mg/kg considerably exaggerated breast muscle yield and crude fat content of the muscles and lowered abdominal fat content.

The obtained results showed that supplementation of L-carnitine have no vital modification in blood glucose level. This is consistent with other publications which documented that orally administration of L-carnitine supplementation for 4 weeks did not effect of insulin sensitivity (28) or secretion (29).

This study revealed no significant change of cholesterol by L-carnitine supplementation and non-significant decrease Triacylglycerol, this result not consistent with Maccari (30) who documented that the significant lowered plasma triacylglycerol, cholesterol, phospholipids, nonesterified fatty acid and very low density lipoprotein concentrations (VLDL) by supplementation of L-carnitine to rats. Likewise, Amin (31) reported that adding of external L-carnitine made significant lower of serum TG, T-cholesterol. Additionally, L-carnitine makes vital reductions in total serum cholesterol in skeletal muscles of weighty rats (32). These contradictory results may due to variation in species, age, supplementary L-carnitine doses, dietary fat and protein intake.

Carnitine metabolism occurs mainly in the liver; therefore it is not surprising that carnitine metabolism is failed in patients and experimental animals with certain types of chronic liver disease. L-carnitine will have a therapeutic effect on certain type of liver diseases (33). Our study revealed insignificant decrease of AST, ALT , total protein. Amin (31) reported that administration of L-carnitine make a significant lowering influence in AST and ALT activity in obese rats. Malaguarnera (34) also reported that oral L-carnitine supplementation improve liver functions and histological findings in patients with nonalcoholic steatohepatitis. The present study revealed insignificant change in uric acid, this finding agrees with Rajasekar (35) who reported L-carnitine has beneficial effects on renal function.

The present study revealed upregulated expression of *MYOD*, *MYF5* by L- carnitine, and this agrees with Bailey (36) who reported that *MYOD*, *MYF5* and variety of MRFs initiate and maintain the expression of muscle-specific genes throughout embryogenesis and postnatal muscle growth. In rats, the loss of MRFs cause reduction in body size (37). This finding confirms that the expression of *MYOD*,

MYF is important for increase body weight. *CAT*s are the basic amino acid transport system utilized by tissues to concentrate lysine, arginine and ornithine into cellular amino acid pools for use in nitrogen metabolism (16), is harmonious with our finding that revealed upregulation of *CAT2* expression and this associated with increased body weight and growth.

Conclusion

The present study concluded that the supplementation of L-carnitine has appositive influence on growth performance through upregulation of CAT2, MYOD and MYF5 genes.

Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

PROTECTIVE EFFECT OF GLYCYRRHIZC ACID AGAINST CARBON TETRACHLORIDE-INDUCED LIVER FIBROSIS IN RATS: ROLE OF INTEGRIN SUBUNIT β LIKE 1 (ITG B L1)

Amal Ragab, Nasr Elsayed Nasr*, Khalid Kahilo

Biochemistry Department, Faculty of Veterinary Medicine Kafrelsheikh University

*Corresponding author, E-mail: nasr_157@yahoo.com

Abstract: Glycyrrhizc acid (GA) is one of the herbal plants with a proved hepato-protective effect. The current study was carried out to estimate the hepato-protective effect of GA against liver fibrosis and to disclose its mode of action. Thirty two male albino rats were randomly distributed into 4 groups (n=8), i.e., control group, GA group, CCl_4 group and CCl_4 + GA group. Liver enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), liver histopathology, malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CT) and gene expression of integrin subunit β like 1 (ITGBL1) were analyzed. The obtained data revealed that GA remarkably protected CCl_4-induced liver injury as reflected by reduced AST, ALT, GGT, and fibrosis compared with the CCl_4-only group. Moreover, GA significantly reduced the levels of MDA, as well as increased the activity of SOD and CT. Consequently, GA prevents CCl_4-induced fibrosis in rats. The prophylactic action of GA against liver fibrosis was mediated through its antioxidant and anti-inflammatory activities. Additionally, GA downregulated fibrogenic ITGBL1.

Key words: glycyrrhizc acid; ITGBL1; liver fibrosis; integrins

Introduction

Liver fibrotic diseases are generally resulted from chronic liver injury, leading to chronic inflammation and fibrosis with obliteration of the normal hepatic tissue construction and eventually loss of liver function (1). As a result of chronic liver diseases, cirrhosis is the sequelae of advanced liver fibrosis. Recently, it is reported that cirrhosis could be reversible in its early stages if the underlying liver diseases have been properly treated (2). However, advanced stages of cirrhosis are considered to be irreversible. Cirrhosis is believed to be the main source of a variety of serious complications, which lead to highly fatal systemic disorders (3). Therefore, cirrhosis the 8th deadliest disease and is responsible for high percent of mortality worldwide. Worryingly, the prevalence of cirrhosis is increasing in numerous nations, including well developed ones (4). Chronic liver diseases such as alcoholic liver disease, HCV, HBV, haemochromatosis and non-alcoholic fatty liver disease (NAFLD) are the most frequent causes of cirrhosis worldwide. Despite their lower incidence rate, a wide range of other diseases can end with cirrhosis (5). Unfortunately, there is a shortage of an effective remedy for liver fibrosis to date. Therefore, there

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is an urgent requisite for potent anti-fibrotic remedies.

Fibrogenesis is a highly complicated process employing a variety of different cells, including hepatocytes, hepatic stellate cells (HSCs) and immunocytes. The key cellular elements in development of liver fibrosis is the activation of HSC and their trans-differentiation into myofibroblast-like cells (6). HSC activation is mainly initiated by growth factors and fibrogenic cytokines released by activated epithelial cells (including hepatocytes and cholangiocytes) and inflammatory cells, which among them the TGFβ 1 is the key regulator (7).

Wide range genes take part in fibrogenesis through controlling the TGFβ signaling pathway, among which integrins have crucial regulatory role. Integrins are cellular receptors that consist of an α and a β subunit and form at least 24 different dimers that mediate cell-cell and cell-ECM interactions (8). Moreover, integrins also response to ECM-induced extracellular changes during pathological processes, initiating cellular responses, which manipulate ECM alteration (9). It has previously showed that integrins manage pivotal roles in fibrogenesis (7). Throughout biliary fibrosis development, integrin αvβ6 is highly upregulated in cholangiocytes and stimulate fibrogenesis through TGFβ 1 activation (10). Several genes and signaling pathways highly connected to fibrotic progression were discovered, among which integrin subunit β like 1 (ITGBL1) was recognized as a key factor (11). The pathological pathway of ITGBL1 were clearly demonstrated by using *in vitro* experiments, which revealed that ITGBL1 encourages HSC activation and the subsequent liver fibrosis by upregulating TGFβ1. These observations secure essential base for further research on liver fibrosis which may propose the discovery of new antifibrotic agents.

Through ancient eras, medicinal herbs have long been prescribed to prevent and treat liver diseases and have recently gained wider attention due to their availability, long term effectivity and benign side effects (12). Generally, hepatoprotective effects of medicinal herbs is conducted via mechanisms including hindering fibrogenesis, defeating tumorigenesis, eradicating viruses, and suppressing oxidative tissue damage (13). *Glycyrrhizae Radix et Rhizoma*, also known as licorice root, is commonly consumed to treat viral hepatitis (14). Licorice root main constituents include glycyrrhizc acid (GA), β-sitosterol, flavonoids, and hydroxycoumarins. GA improves CCL4-induced liver damages by down-regulating proinflammatory mediators (15), as well as its antioxidative action via upregulation of catalase and glutathione-S-transferases (16).

The current study aimed to define the underlying mechanism of anti-fibrotic action of GA on the sub-molecular level by investigating the effects of GA on ITGBL1 binding activity, one of the major controllers of fibrosis, in the CCl4 rat model of liver cirrhosis.

Materials and methods

Animals and ethics statement

This research was approved by the Ethics Committee of faculty of veterinary medicine, Kafrelsheikh University.

Experimental design

Thirty male albino rats weighing 180-200 g were recruited after 7 days for adaptation to the animal house circumstances (12-hour light/dark cycle). Water and food were supplied *ad libitum*. The rats were arbitrarily distributed to 4 groups: the control group (n = 8), the CCl_4 group (n = 8), the CCl_4 + GA (n = 8) and the GA 150 mg/kg BW group (n = 8). The liver fibrosis was induced by intraperitoneal (i.p.) injection with CCl_4 mixed with olive oil as vehicle in 1:1 ratio (0.2 mL/100 g BW) twice weekly for 2 weeks followed by i.p. injection of reduced dose (0.1 mL/100 g BW) twice weekly for 6 weeks as described by Constandinou (17). GA was given by oral gavage once daily for 8 weeks.

Three days after the last CCl_4 injection, rats were sacrificed, and blood samples were taken in the plain tubes and EDTA tubes. Samples in plain tubes were left to clot then centrifuged at 3,000 *g*, 4°C for 15 min, to separate serum. The serum samples were stored at -20°C until analyzed. The liver was immediately excised from

each animal, washed by saline and divided to 2 parts: one part was prepared for histopathological examination, while the other part was preserved frozen at -80°C for both oxidative stress and genetic analysis.

Biochemical analysis

Activities of liver enzymes ALT, AST, GGT, levels of total protein, albumin and total bilirubin in plasma were measured using commercial kits (Spinreact, Spain) according to the manufacturer's directions. Hepatic SOD, CT and MDA were determined in the hepatic tissue homogenate using (Biodiagnostic, Egypt) kits following the manufacturer's guideline and as previously described (18, 19).

RNA extraction and real time PCR

RNA was extracted out by using Trizol (Invitrogen Co., Carlsbad, CA, USA) and real time PCR performed following the manufacturer's instructions using M-MLV reverse transcriptase (Takara Shuzo Co., Ltd, Japan) and real time PCR Master Mix (SYBR Green) Kit (Toyobo Co., Ltd. Japan). The sequences of ITGBL1 primers were forward 5'TTTGTGAGAAAGGATGGTTTGGT3' and reverse 5'TGCTTTGTTCTTCGGTCATATTA CA3'. GAPDH was used as an internal control. The PCR conditions were 95°C for 10 min, and then 40 cycles of 95°C for 20 s, 54°C for 30 s and 72°C for 30s. Each experiment was carried out thrice in triplicate. The fold-change in mRNA of target gene relative to that of GAPDH was calculated according to previously described (20).

Histopathological examination

Sections of liver tissue 3 μm thickness were obtained from each animal under investigation and fixed in 10 % neutral buffered formalin, then dehydrated in ascending concentration of ethyl alcohol (70: 100%) followed by staining according to standard protocol of Hematoxylin an Eosin stain (H&E) as described by Bancroft e al., (21)

Statistical Analysis

Statistical analysis was carried out using Graphpad prismV5 software package. Results were obtained as means ± standard deviations (SD). Statistical analysis was performed using one way analysis of variance (ANOVA) comparisons. Values showing $p < 0.05$ was considered as statistically significant.

Results

Biochemical parameters

Serum ALT, AST, and GGT activities were determined as indicators of liver damage. As displayed in table 1, significant high levels in the activities of these marker enzymes were recorded in CCl_4-intoxicated rats. In contrast, GA supplement significantly reversed those enzyme activities.

Histopathological Findings

The liver of control animal (Fig.1A) showed normal hexagonal shape lobule with centrally located blood vessels (central vein) while the peripheral area revealed normal portal areas which contained hepatic artery , portal vein and bile duct. Hepatocytes (large round to polygonal cell with eosinophilic cytoplasm and vesicular nucleus) mostly arranged in radiating manner around the central vein. Similarly, animal treated with GA were with normal limits (Fig.1B). Animals treated with CCl_4 (Fig.1C) showed remarkable hepatic distortion associated with centro-lobular hepatic vacuolation and necrosis. Obvious periportal hepatic fibrosis accompanied with noticeable periportal, interlobular and intralobular proliferating fibrous connective tissue (arrowhead) that given the nodular appearance of the hepatic tissue. While diseased animal treated with GA showed distinct decrease of hepatic necrosis and fibrosis (Fig.1D).

Effect of GA treatment on ITGB1 expression

The obtained qPCR results revealed a significant ($P \leq 0.05$) upregulation of the fibrosis marker *ITGB1* gene in liver of CCl_4-intoxicated rats as compared to the control group (Table 1). This elevated expression was significantly downregulated following treatment by GA, but still higher than that in the control group. Additionally, no significant difference was noticed between the two control groups (G1, G2).

Table 1: Effect of GA on biochemical parameters.

	Normal	GA	CCl_4	GA + CCl_4
AST (IU\L)	62.8 ± 3.58 [c]	64.63 ± 3.18 [c]	346.16 ± 11.15 [a]	222.20 ± 8.93 [b]
ALT (IU\L)	59.7 ± 1.82 [c]	63.10 ± 3.95 [c]	253.23 ± 22.65 [a]	118.31 ± 7.56 [b]
GGT(IU \ L)	67.5 ± 1.77 [c]	67.44 ± 3.96 [c]	114.26 ± 4.54 [a]	90.18 ± 3.26 [b]
Total protein (g\dl)	6.54 ± 0.12 [a]	6.01 ± 0.16 [a]	3.03 ± 0.24 [c]	4.41 ± 0.17 [b]
Albumin (g\dl)	4.27 ± 0.20 [a]	4.02 ± 0.26 [a]	2.20 ± 0.20 [c]	2.92 ± 0.10 [b]
Total bilirubin (mg\dl)	0.91 ± 0.06 [c]	0.90 ± 0.06 [c]	1.56 ± 0.05 [a]	1.15 ± 0.04 [b]
SOD (u\g tissue)	13.03 ± 0.57 [a]	13.16 ± 0.52 [a]	4.56 ± 0.46 [c]	7.19 ± 0.33 [b]
Catalase (u\g tissue)	41.43 ± 1.94 [a]	39.78 ± 1.65 [a]	20.25 ± 0.92 [c]	27.14 ± 0.93 [b]
MDA (nmol\g tissue)	4.00 ± 0.36 [c]	4.40 ± 0.49 [c]	10.51 ± 0.63 [a]	7.84 ± 0.56 [b]
ITGB1 gene (fold change)	1.00 ±0.09 [c]	1.51±0.24 [c]	11.16 ± 0.43 [a]	5.78 ± 0.29 [b]

Data were presented as mean ± SEM. Means carrying different superscript letters are significantly different at P≤0.05.

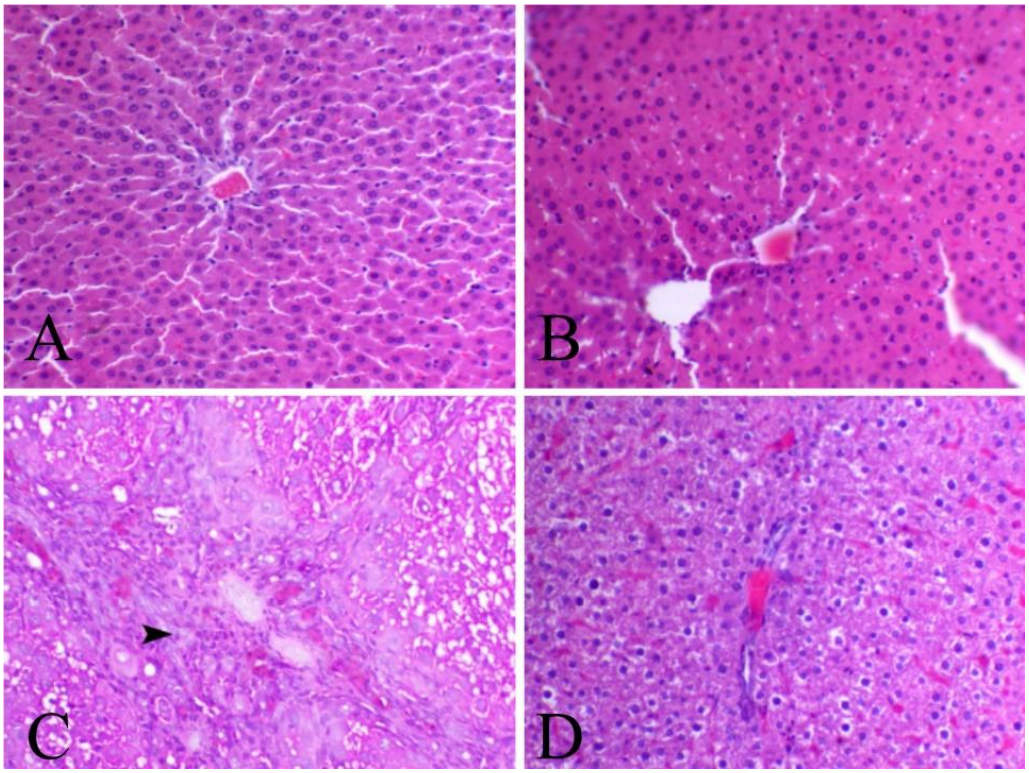

Figure 1: Liver sections of control (A), GA (B), CCL4 (C), and GA+CCL4 (D) groups. H&E, X200

Discussion

Inflammation is considered the native defense system of the body against harmful factors with playing a vital role in healing the potential injury. Consistently, liver is highly related to inflammation being highly vascular and vulnerable to serious hepatic injurious factors including toxin metabolites, viruses, fat rich diet and excessive alcohol intake. Prolonged exposure to those agents leads to chronic hepatitis accompanied by fibrosis and subsequent cirrhosis with loss of liver function (22). Accordingly, liver fibrosis in the current study was induced by repeated doses of CCl_4 which is the most popular procedure between the liver research laboratories. However, it differs from one laboratory to another in terms of CCl_4 dose, route of administration, treatment duration and the expected changes to be studied.

Communally, CCl4 mediated liver fibrosis proceeds with elevated serum AST and triglycerides along with liver atrophy (23). Furthermore, these alterations are accompanied by a significant low value of serum albumin indicating advanced loss of hepatic function during extended fibrogenesis. These observations appear consistent with our recorded albumin levels in CCl4 intoxicated group supported by histopathological lesions.

Cytochrome P450 superfamily of monooxygenases process CCl4 to the trichloromethyl radical (CCl_3)(24). Subsequently, this radical damages the key cellular metabolic pathways resulting in altered lipid metabolism (fatty degeneration and steatosis) and decreased protein quantities. Moreover, CCl_3 interacts with hepatocytic DNA leading to mutations and the development of HCC. Further oxygenation of CCl_3 results in the formation of trichloromethylperoxy radicals (CCl_3OO^*) initiating lipid peroxidation by breakdown of polyunsaturated fatty acids with reduction of membrane permeability of the plasma membrane extended to mitochondrial and endoplasmic reticulum membrane ended by cellular death. The cellular death eventually develops as zonal or focal necrosis with destruction of normal hepatic tissue construction (22). Biochemically, this severe OS leads to exhaustion of antioxidant activity of SOD and release of high levels of MDA in agreement with our results of the same group. Inappropriately, Fibrosis develops as a healing course in response to inflammation and OS (25) , and can finally progress into HCC (26). Prolonged stimuli of liver injury leads to failure of the regenerative response and substitution of hepatocytes with massive ECM (27) formed mainly by Hepatic stellate cells (HSC) (28).

Interestingly, integrins direct the development of fibrosis regulating inflammation, and by transforming hepatocytes injury into stimulus of matrix-producing mesenchymal cells [HSC/myofibroblasts (MFB)]. The expression of integrins by wide range of cells engaged in liver fibrosis course, as well as their ability to interact with growth factors and other signaling molecules render the concept of targeting integrins an interesting tactic for antifibrotic therapy.

There is no typical treatment plan for hepatic fibrosis, however prophylaxis against liver injury, including minimizing of fat consumption and toxin exposure or administration of an efficient viral hepatitis treatment can resist fibrosis. Surprisingly, no efficient anti-fibrotic drugs have yet to be developed although substantial progress has been made in exploring the pathogenesis of hepatic fibrosis over the last two decades. Medicinal herbs and their bioactive ingredients and extracts could prevent liver fibrosis by two means: through suppression of HSC activity and via inhibition of ECM expansion. HSCs activation are initiated when gene expression and phenotype changes render the inactive cells responsive to other cytokines and stimuli (29).

Oral traditional Chinese herbal medicine has long been used as a non-invasive therapy. The therapeutic mechanisms of herbal medicines and their active compounds have been gradually uncovered and interpreted through *in vivo* studies. Recent studies have provided a greater understanding of the molecular mechanisms and new therapeutic approaches for liver fibrosis, but it still requires an efficacious remedy. We believe that herbal medicines are sufficiently worthy as potential therapy agents for liver fibrosis if more profound studies about the underlying mechanisms of herbal medicines with improved methodological quality are undertaken.

GA can prevent CCl_4- related liver fibrosis effectively. This is evidenced in restoring the nearly normal hepatic lobule in GA+CCl_4 animals. It could happen by less HSC proliferation, thus reduced levels of collagen, hyaluronic acid (HA), and laminin (LN). In an animal model, GA reduced the death rate of acetaminophen intoxicated mice via inhibition of acetaminophen-induced hepatotoxicity, and reduced the number and area of GGT positive foci, thus reserving liver function and preventing HCC from development (30). GA showed a highly effective chemopreventive action agent against lead acetate hepatic induced oxidative stress in rats because it chelates lead (31), which support the current data obtained from improved oxidative stress markers in the sera of the same group. In

the molecular level, GA supplement accompanied by suppression of the profibrotic gene ITGBL1, which has a crucial role in management of ECM and the activity of HSC. Up to the published data, it is the first time uncover the role of GA in correlation to integrins during its anti-fibrotic effect. However in concanavalin A- (ConA-) mediated mouse model, GA attenuated ConA-induced hepatitis and fibrosis pgrowth in livers via supression of CD4+ T cell proliferation in response to ConA via the Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and phosphoinositide 3-kinase (PI3K)/AKT pathways(32).

Conclusion

Wide attention should be given to integrins as they are infirmly connected to the development of liver fibrosis. Integrins targeting therefore represents an interesting concept of therapeutic strategy, particularly because experimental data recommend potent efficiency of such trend. However, more investigations are needed to identify potentials to specifically antagonize integrins (including specific integrin antagonists, or small nonpeptidic molecules) to stop or reverse fibrosis and avoid detrimental effect of such inhibition.

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Original Research Article

HEMOTOXIC AND GENOTOXIC EFFECTS OF LEAD ACETATE AND CHLORPYRIFOSE ON FRESHWATER CAT FISH *(Calarias gariepinus)*

Shireen S. Abd El-satar[1], Nasr E. Nasr[2*], Khaled A. Khailo[2], Hossam E. Sayour[3]

[1]Biochemistry Department, Animal Health Research Institute, Kafrelsheikh, Egypt, [2]Biochemistry Department, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt, [3]Biomedical Chemistry Unit, Department of chemistry, Animal Health Research Institute, Dokki, Giza, Egypt

*Corresponding author, E-mail: nasr_157@yahoo.com

Abstract: This study was conducted to evaluate hemotoxicity and genotoxicity induced by lead acetate and chlorpyrifos in catfish (Clarias gariepinus) as a model for checking genotoxic pollutants in aquatic surroundings. Lead acetate was added by a dose of 24.4 mg/L (20% of the LC50) daily, chlorpyrifos was added by a dose of 1.65 mg/L (1/10 LC50) daily. Fish were kept in standard condition in which water temperature (25°C± 2); pH (7.6 ± 0.4) and dissolved oxygen (5.4±0.4 mg/L). Blood and liver were sampled after 4 weeks. The result revealed a significant reduction of RBCs count, Hb, hematocrit in fish exposed to lead and chlorpyrifos. Furthermore, MDA level and catalase activity showed significant increase and decrease, respectively in groups exposed to lead acetate and chlorpyrifos in comparison with the control fish. Toxic effect of lead acetate and chlorpyrifos confirmed by histopathological changes in liver sections which showed marked hepatic vacuolation and parenchymal hemorrhage. DNA damage detected by comet assay also revealed a significance increase in tail length, tail DNA% and tail moment at sublethal concentration of lead acetate and chlorpyrifos. This study concluded that lead acetate and chlorpyrifos have hemotoxic and genotoxic effect probably through, at least in part, induction of reactive oxygen species and chlorpyrifos has more hemotoxic and genotoxic effect than lead acetate.

Key words: catfish; lead acetate; chlorpyrifos; DNA damage

Introduction

Pollution of the aquatic surroundings is a serious and growing problem, caused by increasing variety and quantity of industrial, agricultural and business chemicals (1-3). Many water pollutants are capable of inducing oxidative stress on fish (4) which consequently led to cellular and tissues damage in fish (5). Heavy metals and pesticides are poisonous to animals just as individuals (6). Heavy metals are considered as the most important pollutants, because of their notable impacts on the aquatic system equilibrium, bioaccumulation in life forms, long term persistence and ability to aggregate in sediments and water (7). Heavy metals, like mercury, cadmium, copper, lead and zinc are the most vital poisons which influence aquatic environment and fish. They are extremely unsafe for the health of fish. These metals can ef-

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fectively affect the vital operations and reproduction of fish; weaken the immune system, and lead to pathological changes (8)

Lead (Pb) is a prevalent, cumulative and insidious environmental waste product that induces a broad range of physiological, biochemical and behavioral dysfunctions (9-11). With the increasing prevalence of lead pollution in the aquatic surroundings, the potential danger of lead to aquatic animals must be evaluated (12). Studies have demonstrated that lead can induce neurological, gastrointestinal, reproductive, circulatory, immunological, histopathological and histochemical effects in the animals and human (13, 14). One of the best known impacts of lead is its interference with synthesis of heme (15). High Pb concentration in soft tissues inactivate alpha-amino levulinic acid dehydratase (ALAD) enzyme which associated with rise of alpha -aminolevulinic acid (ALA) level (16). Increase of ALA level leads to production of reactive oxygen species (17)

Pesticides are used to control pests, insects, aquatic weeds, plant diseases, and Aquatic snails which transfer schistosomiasis. Pesticides have been observed to be very harmful not only to fish but also to the other organisms. (18). Widespread of pesticides and their improper use cause changes in the aquatic medium. Observing of different effects is wanted for good pesticides control (19). Two main types of pesticides; organophosphates and organochlorines, cause serious harmful impact on the aquatic system and their residues present in water are toxic to aquatic organisms (20, 21). One such organophosphate, is chlorpyrifos (CPF) [O, O-diethyl-O-(3,5,6 -trichloro-2-pyridyl) phosphorothionate] which is broadly utilized for the management of domestic and agricultural pests. CPF utilized additionally for over 10 years to control foliar insects (arthropoda) that influence farming yields, and subterranean termites (22). Oxidative stress which induced by pesticide has been a focal point of toxicological research for the last decade as a potential mechanism of toxicity (23, 24) . Toxicity of reactive oxygen species counteracted by antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), and glutathione reductase (GR) (25). Antioxidants protect the cells and tissues from oxidative damage under normal conditions (26). The antioxidants in fish could be considered as biomarkers of exposure to aquatic contaminants (27). Exposure to pollutants causes several biochemical and physiological responses which may be adaptive or lead to toxicity. Thus, it is important that pollutant effects be detected and interpreted in biochemical terms, to show mechanisms of pollutant action, and possible ways to prevent there bad effects (28).

African catfish (*Clarias gariepinus*) is very important fish because of its high growth rate, high consumer acceptability and high resistance to bad water quality and low oxygen (29). It is omnivorous feeder and a general scavenger with a marked tendency to feed on benthic organisms and this make it in continuous exposure to different pollutants which concentrated in the sediments. Biomagnification of pollutants throuth eating fish which already accumulate chemicals make this fish accumulate pollutants in greater amounts than other herbivorous fish (30). Catfish considered as an excellent model for toxicological studies and has been used in fundamental research (31, 32). Since fish are important as proteins and lipids sources for humans and domestic animals, so health of them is very important for human beings (18). Therefore, this study was conducted to indicate the hemotoxic and genotoxic effect of both chlorpyrifose and lead acetate on African catfish

Materials and methods

Chemicals

Lead acetate trihydrate (Mol.Wt. 379.33 g/mol, product No. 316512, purity 95%)and other chemicals used in this study were purchased from (Sigma–Aldrich), chlorpyrifos was obtained as a commercial product (Pyrifos EL NASR 48% EC) from (El Nasr Pharmaceutical Company, Cairo-Egypt).

Experimental fish

Catfish (*Clarias gariepinus*) of both sexes, average weight 150 were purchased from fish

farm in Kafrelsheikh, Egypt. Fish was transported in large plastic water containers and maintained in the glass aquaria in the laboratory of faculty of veterinary medicine, Kafrelsheikh University. Fish left without any treatment for two week for acclimation before starting the experiment. Fish were kept in standard condition during the period of acclimation and the experiment in which water temperature is 25°C± 2, pH is 7.6 ± 0.4 and dissolved oxygen (5.4±0.4 mg/L). Water was supplied with oxygen by air pump, fish kept in 12/12hour light and dark, and were fed on commercial high protein diet (30 %) at ratio of 3% of body weight/day. The water was changed every 2 days to prevent the accumulation of fecal matter and to maintain the pollutants concentration.

Experimental design and treatment

A total of 120 fresh water catfish were divided into three groups, each group has two replicates (20 fish replicate), kept in (100×50×30 cm) glass aquaria contain 100L tap water (dechlorinated); the first group (G1) was considered as control group, the second group (G2) was exposed to lead acetate by a dose of 24.4 mg/L (20% of the LC_{50}) (33) and the third group(G3) was exposed to chlorpyrifos by a dose of 1.65 mg/L (1/10 LC_{50}) (34), for 4 weeks, the water was changed every 2 days to avoid the accumulation of fecal matter and to maintain the toxicant concentration.

Blood and tissue samples

By the end of the experimental period at 4th week, whole blood was collected from caudal blood vessels in clean and dry Eppendorf tubes containing EDTA as anticoagulant, other blood samples were collected without anticoagulant and left at room temperature to coagulate then centrifuged at 3000 rpm for 15 min and clear sera were separated and kept in -20 ^{o}C until biochemical analysis. Fish were then killed by spinal rupture; the liver was rapidly excised and divided into three parts: the first part was kept in sterile Eppendorf tube, was immediately immersed in liquid nitrogen and then kept in -20 ^{o}C for anti-oxidant determination, the second part about (1g) was kept in PBS in sterile plastic tubes then was stored in -20 ^{o}C for comet assay analysis and the third part was stored in 10% formalin for histopathological analysis.

Hematological analysis

Total red blood cell (RBCs $10^6/\mu l$**)** counts were determined by the Natt & Herrick (35) method (NH) using a Newbauer hemocytometer. The microhematocrit (PCV%) was estimated by the method of Hesser (36). Hemoglobin (Hb g/dl) values were assessed calorimetrically according to method of Wintrobe and Greer (37) by determining the formation of cyanomethemoglobin.

Biochemical analysis

Malondialdehyde was spectrophotometrically measured in serum based on (38) at wave length of 534 nm, catalase activity was determined in liver homogenates , briefly obtained as follow (homogenize 1 gm. of liver tissue in 5 ml of cold phosphate buffer saline PH:7.4 ,centrifuge at 4,000rpm/ 15 minute at 4^{o}C, then used supernatant for assay) according to Aebi (39).

Histopathological analysis

Fixed liver samples were dehydrated in ascending grades of ethanol, then embedded in paraffin wax, cut into sections of 5 µm thickness. Sections were stained using Mayer's hematoxylin and eosin (H&E) stains according to the method described by Bancroft (40) and were examined under a light microscope. Hepatic histological changes photographed by computer system with a digital camera (Nikon digital camera, Japan).

DNA damage detection

Comet assay (single cell gel electrophoresis, SCGE) was used to detect any prospective damage for DNA after treatments. DNA strand breaks and alkali labile sites detected by measuring the migration of damaged DNA from immobilized nuclear DNA. The comet assay was performed according to the protocol described by Singh et al., (41) and the calculations were done as previously described (42).

Statistical analysis

Data were analyzed by one way (ANOVA), followed by Bonferroni's Multiple Comparison Test to compare the significant differences between different groups. All the data were expressed as mean ±SE. A value of $P < 0.05$ was considered to be significant.

Results

Hematological parameters

The hematological parameters are listed in Table (1). Exposure to lead acetate and chlorpyrifos led to a significant reduction in RBCs count as compared to the control fish. Furthermore, lead acetate and chlorpyrifos exposed group illustrated a significant decrease in Hb concentration (in comparison with the control group. Also, there was a significant reduction in PCV% in group exposed to lead acetate and chlorpyrifos when compared with the control group.

Effect of lead acetate and chlorpyrifos on MDA level and catalase activity

Exposure to lead acetate and chlorpyrifos led to a significant increase in serum MDA level and a significant decrease in hepatic catalase activity as compared with the control fish a (Fig. 1). Furthermore, chlorpyrifos led to a higher increased MDA level than lead acetate.

Effect of lead acetate and chlorpyrifos on DNA (comet assay)

DNA damage in liver of lead acetate, chlorpyrifos-intoxicated fish were detected by comet assay. The results of comet assay were shown in Fig. (2) and Table (2). Fish exposed to lead acetate showed asignificant ($P<0.05$) increase in DNA damage as revealed by increase in tail length, tail DNA% and tail moment. Fish exposed to chlorpyrifos showed a significant ($P<0.05$) increase DNA damage with higher damage in chlorpyrifos than lead acetate groups as compared to the control group (G1).

Histopathological changes

Normal fish (Fig. 3A) showed normal hepatocytes separated with blood sinusoids, while liver of lead acetate -intoxicated fish (Fig. 3B) showed hepatic vacuolation, parenchymal haemorrahge and focal leukocytic infiltration. Furthermore, liver of chlorpyrifos - intoxicated fish (Fig.3C) showed hepatic vacuolation and hepatic necrosis.

Table 1: Effect of lead and chlorpyrifos on hematological parameters in cat fish

Groups	RBCs(10^6/µl)	PCV%	Hb(g/dl)
Control	3.90±0.06[a]	39.06±0.6 [a]	9.26±0.07[a]
Lead acetate	3.10±0.06 [b]	31.07±0.61[b]	7.76±0.08[b]
Chlorpyrifos	2.91±0.06[c]	29.10±0.07[c]	7.27±0.05[b]

Mean±SE (n=5/group) [a,b] Means in the same raw with different superscript are significantly different ($P<0.05$)

Table 2: Comet assay parameters obtained by image analysis in all groups

Group	Tailed %	Untailed %	Tails length (µm)	Tail DNA%	Tail moment
Normal	2	98	1.53±0.16 [c]	1.64	2.51
Lead acetate	19	81	6.13±0.49 [b]	5.48	33.59
Chlorpyrifos	29	71	9.34±0.70 [a]	8.02	74.91

Different superscript letters in the same column of tail length showed significance difference at $P< 0.05$

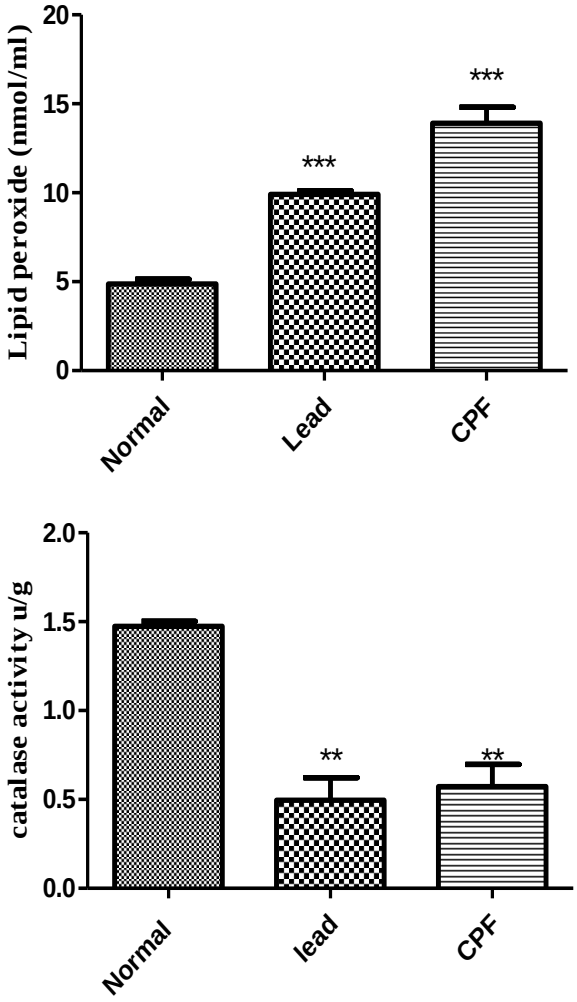

Figure 1: Showing effect of lead acetate and Chlorpyrifos on MDA and catalase activity. Data expressed Mean±SE. (n=10/group). *P <0.05 versus control group

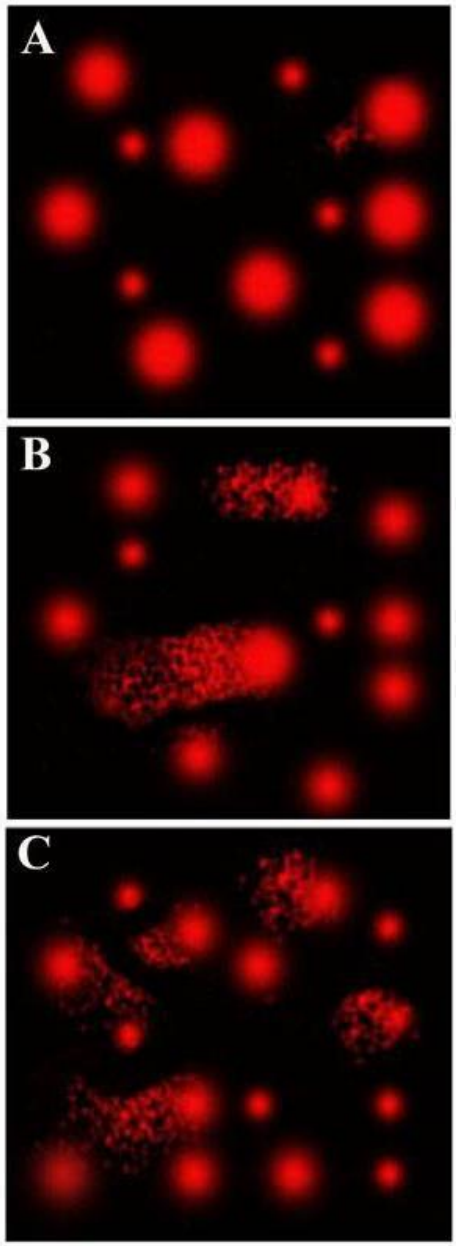

Figure 2: Showing DNA damage (comet assay) in normal fish (A), lead acetate -intoxicated fish (B) and Chlorpyrifos - intoxicated fish (C)

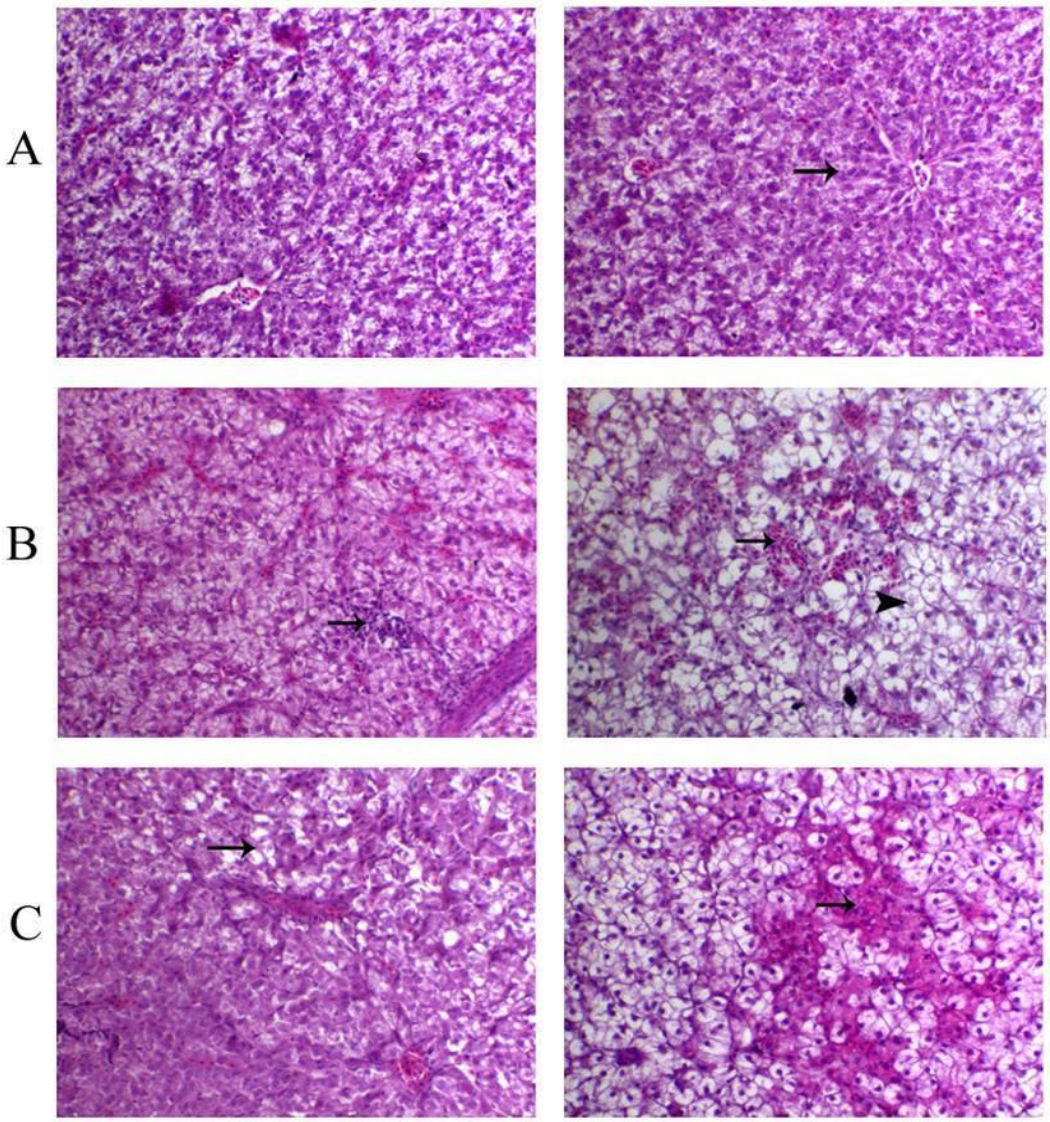

Figure 3: Showing Hematoxylin and eosin (H&E) stains of liver normal fish (A) ,lead acetate -intoxicated fish (B) and Chlorpyrifos - intoxicated fish (C)

Discussion

Because of over population and unintended civilization, many aquatic ecosystems are facing complicate problems of contamination (43). A few contaminates, for example detergents, household waste pesticides, insecticides, bacteria, parasites, metals, salts, acids, and other chemicals have been found in many aquatic surroundings (44). African catfish (*Clarias gariepinus)* is an excellent model for aquaculture, fundamental research, and environmental contamination studies (45, 46), So the present study try to illustrate, hemotoxicity and genotoxicity of lead and chlorpyrifos as pollutant on the *Clarias gariepinus* fish.

The result of present study revealed a significant decrease of RBCs count, Hb concentration and PCV values in lead acetate -intoxicated fish comparison with control fish. This was in harmony with (33) who reported that sub-chronic exposure of *C. gariepinus* to lead decreased RBCs count. Changes in Hb concentration and PCV values, were also compatible with

Adeyemo (47), who reported significant decreases in Hb, RBCs count and PCV values in *C. gariepinus* exposed to lead nitrate. Lead causes damage of hemopoietic organs , structural damage of RBC membranes resulting in hemolysis, impairment in hemoglobin synthesis, stress-related release of RBCs from the spleen and hypoxia (48). Lead causes early mortality of mature red blood cells and inhibition of hemoglobin synthesis through inactivation of RBC alpha-amino levulinic acid dehydratase (17). Also, our results revealed that RBCs count, hemoglobin concentration and hematocrit a significantly less in CPF -intoxicated fish in comparison with the control group. This result was compatible with (34) who reported that exposure of freshwater fish *Clarias batrachus* to chlorpyrifos led to significant decrease in RBCs and Hb indicating a condition of erythropenia and hemolysis. This may be attributed to inhibition of erythropoiesis, hemoformation, osmoregulatory dysfunction or due to increased rate of RBCs destruction in hematopoietic organ by chlorpyrifos (49). Indeed, chlorpyrifos more toxic than lead acetate.

Contaminants can possibly initiate oxidative stress in fish through creation of free radicals and reactive oxygen species (ROS) which lead to an imbalance between intracellular ROS levels and antioxidant defense (50, 51). ROS cause damage to proteins, lipids, carbohydrates and nucleic acids (52). This damage may alter cell functions, eventually leading to cell death (53). Our results showed that serum MDA was significantly increased and catalase activity was significantly decreased in fish exposed to lead as compared to the control fish. Similarly, other studies also reported that toxic action caused by lead might be due to its ability to produce ROS which cause oxidative damage in several tissues by increasing lipid peroxidation through Fenton reaction (54, 55). We also found a similar change following addition of CPF. Our study revealed that serum MDA was significantly increased and catalase activity was significantly decreased in tissue of CPF-exposed fish. Similar results obtained by Kaur and Jindal (56) who reported that CPF was very toxic to *Ctenopharyngodon idellus* even at very low concentration. Its administration increases MDA level and affected adversely the antioxidative defense system in different organs of the fish. This study indicated chlorpyrifos more toxic than lead acetate.

Histopathological examination of liver tissue is important marker for exposure to environmental stressors or pollutants, as the liver is important organ of detoxification (57). This study revealed that liver tissue in fish exposed to lead acetate showed hepatic vacuolation, parenchymal haemorrahge and focal leukocytic infiltration, which agreed with Rubio, et al., (58) who reported that lead cause inhibition of mono amino oxidase and acetylcholine esterase, to cause pathological changes in tissue and organs. Additionally, CPF also resulted in vacuolation and hepatic necrosis in hepatic tissue of cat fish, and this was in consistent with Deb and Das (59) who reported that common carp exposed to CPF revealed different degree of hydropic degeneration, vacuolization, pyknotic nuclei, and fatty infiltration in their liver tissue.. Although every metal have distinctive mechanism of toxicity however there are some common mechanism such as mimicry, adduct formation with DNA or protein and oxidative damage. Generation of ROS is caused by heavy metals in their ionic forms leading to oxidative changes in DNA, causing aberrant gene expression and carcinogenesis (60). The present study revealed induction of DNA damage by lead and CPF. In support, other studies also reported exposure to lead cause genotoxic effects, such as chromosome aberration, mutation, DNA breakage and DNA synthesis inhibition (61, 62). This result may due to lead can mimic the essential elements such as magnesium, iron, calcium and zinc, increased the production of inaccurate nucleotides in which it implicated as a co- carcinogen and effect on DNA repairing mechanisms (63). Similarly, Yin, et al., (64) reported that chlorpyrifos caused genotoxicity in RBCs and liver cells of Chinese toad (*Bufo gargarizans*) when the tadpoles were exposed to the sub lethal dose of chlorpyrifos. Indeed DNA damage was higher in chlorpyrifos than lead acetate.

Conclusion

This study illustrated that lead acetate and chlorpyrifos have hemotoxicity through decrease RBCs count, Hb concentration, and hematocrit, generate ROS and has genotoxicity through induction of DNA damage in cat fish after exposure to sub-lethal concentrations. Moreover chlorpyrifos is more toxic than Lead acetate

Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

MOLECULAR DETECTION OF invA AND hilA VIRULENT GENES IN SALMONELLA SEROVARS ISOLATED FROM FRESH WATER FISH

Sarah A. Allam[1]*, Nader Y. Mostafa[1], Ghada A. K. Kirrella[1], Nesreen Z. Eleiwa[2], Mohammed A. El-Magd[3]

[1]Food Control Department, Faculty of Veterinary Medicine, Kafrelsheikh University, 33516, Egypt, [2]Animal Health Research Institute, Dokki, Giza, Egypt, [3]Anatomy Department, Faculty of Veterinary Medicine, Kafrelsheikh University

*Corresponding Author: sara.alam9912@yahoo.com

Abstract: The present study was conducted to monitor the prevalence of salmonellae in fresh water fish in Gharbia governorate, Egypt. A total of 120 random samples of fresh water fish (*Tilapia niloticus*, *Mugil cephalous* and *Clarias lazera*, 40 of each) were analyzed bacteriologically for the presence of salmonella pathogens and were further identified using biochemical and serological tests. Positive samples were confirmed by polymerase chain reaction (PCR) through detection of common virulent genes invasion A (*invA*) and hyper-invasive locus (*hilA*). The obtained biochemical and serological results revealed presence of seven different serotypes including *S. typhimurium* which was the most frequent one followed by *S. enteriditis, S. infantis , S. virchow*, *S. heidelberg, S. wingrove* and *S. tsevie* with a prevalence of 33.3%, 23%, 12.8%, 12.8%, 10.25%, 5% and 2.5%, respectively. The obtained molecular results revealed presence of the two virulent genes in all the detected serovars with a total prevalence rate of 100% for *invA* gene and 66.6% for *hila* gene. These results reflected a high prevalence of salmonella species in fresh water fish which act as a potential source of food borne infection and constitutes a major concern for public health.

Key words: fresh water fish; salmonella; serotypes; invA; hilA; PCR

Introduction

Fish are considered as an excellent cheap source of high quality protein with very low cholesterol levels and large quantities of polyunsaturated fatty acids that cannot be synthesized by human metabolism (1, 2). However, fish are also considered as a major carrier for food borne pathogens as its natural habitat is extremely susceptible to pollution from domestic, industrial and agricultural discharges (3). Bacterial diseases in fish cause a serious threat not only to aquaculture industry but also on human health. Thus, it is not surprising to find that 12% of the food poisoning outbreaks associated with fish consumption are caused by bacteria including salmonella (4).

Salmonella, as a member of family enterobacteriacea, is a Gram negative rod shaped bacteria that contains more than 2500 serovars. Salmonella, which is considered as a common water-borne pathogen, apparently presents in the tissues of normal fish and represents no clinical signs. It can easily reach water through many

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routes, especially through sewage discharges, and can concentrate in fish tissues. It also can reach fish outside water through fish handling without following strict hygienic measures. When these contaminated fish were eaten raw or with quick inappropriate heating, the consumers would suffer from enteritis and typhoid fever. Despite extensive public health measures over the past century, salmonella remains the second leading cause of food-borne diseases worldwide (5). Accordingly, providing a safe, high quality and acceptable food to a consumer is considered a major goal for food processing industry. This can be applied by prompt and accurate isolation and characterization of salmonella and other food-borne pathogens which are the cornerstone required for proper control of food-borne diseases. This can be achieved using molecular identification of these pathogens by polymerase chain reaction (PCR) technique. The latter, is highly sensitive and more accurate method for bacterial isolation and identification, especially for salmonella sp. (6). Thus, our study was planned to identify the prevalence of salmonella species in fresh water fish in Gharbia Governorate, Egypt and to detect the common virulent genes of salmonella serovars using PCR technique.

Materials and methods

Isolation of salmonellae

A total of 120 random samples of fresh water fish including *Tilapia niloticus*, *Mugil cephalous* and *Clarias lazera* (40 of each) were collected from different markets in Gharbia governorate, Egypt. Samples were placed separately in sterile plastic bags in an ice box and transported immediately to the laboratory for bacteriological examination. According to ISO 6579 (2002) method [11]: 25 g of back muscles of each sample of fresh water fish were transferred to a homogenizer flask containing 225 ml of sterile buffered peptone (1%) and homogenized for 1 min, then incubated at 37 °C for 18 h. According to ISO 6579 (2002) method: 1 ml of each homogenate was inoculated separately into a tube of 9 ml selenite-f- broth and incubated at 37°C for 18 h or 0.1 ml was transferred to 9 ml of Rappaport - Vassiliadis Soya broth (RVS) and incubated at 42°C for 24 h. A loopful from selective enriched broth was streaked onto plates of Xylose Lysine Deoxycholate (XLD) and incubated at 37 °C for 24 h. Typical pink colonies with or without black center were picked up for further identification.

Identification of salmonellae

For microscopical identification, films from suspected colonies were prepared, fixed and stained with Gram stain showing Gram negative rod-shaped, motile bacilli that indicates salmonella pathogen. For biochemical identification, according to ISO 6579 (2002) method: purified isolates were examined by different biochemical reaction based on oxidase, urea hydrolysis, H2s production on tripple sugar iron, indole, methyl red, vogus-proskaur and citrate utilization tests. For biochemical identification, salmonella isolates were serotyped according to Kauffman (1974) method (7) for the determination of somatic (O) and flagellar (H) antigens using salmonella antiserum (DENKA SEIKEN Co., Japan).

Molecular detection of salmonella virulent genes by multiplex PCR

Bacterial DNA was extracted using QIA amp extraction kit following manufacturer's protocol and as previously described (8). Multiplex PCR was applied to amplify salmonella virulence genes *invA* and *hila* using specific primers as shown in table 1. PCR reaction mixture (25 µl) contained 5 µl of bacterial DNA, 2.5 µl of 10x Master mix (containing 1.25 U of Taq DNA polymerase, 1.5 mM MgCl2 and 2 µl 10mM dNTP mix), 1 µl of each primer (10 pmol) 15.5 µl DNase free water. The PCR cycling protocol was applied as following: an initial denaturation at 94°C for 60 sec, followed by 35 cycles of denaturation at 94°C for 60 sec, annealing at 64°C for 30 sec and extension at 72°C for 30 sec, followed by a final extension at 72°C for 7 min. Finally, 5 µl of each amplicon was electrophoresed in 1.5 % agarose gel, stained with ethidium bromide and visualized and captured on UV transilluminator. A 100 bp DNA ladder was used as a marker for PCR products

Results

The prevalence of salmonella species

The total prevalence rate of isolated salmonella was 32.5% (39/120) in all fish with highest incidence rate in Mugil species (47.5%, 19/39) followed by *Clarias lazera* (30%, 12/39) and *Tilapia niloticus* (20%, 8/30).

Serotyping

Salmonella isolates (n = 39) were serotyped using "O" and "H" antisera and the results showed 7 different salmonella serotypes: *S. typhimurium, S. enteritidis, S. infantis, S. virchow, S.Heidelberg, S. wingrove and S. tsevie* with a total percentage of 33.3%, 23%, 12.8%, 12.8%, 10.25%, 5% and 2.5%, respectively. The prevalence of each serotype in each fish type was shown in Table 2.

Incidence of virulence genes

The incidence of *invA* and *hilA* virulent genes among the 7 salmonella serotypes as detected by multiplex PCR revealed prevalence of *invA* gene in all salmonella serotypes with a percentage of 100%, hilA gene in only 66.6% of all serotypes (Table 3 and Fig. 1).

Table 1: The primer sequence of salmonellae used for PCR

Target gene	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
invA (F)	5′ GTGAAATTATCGCCACGTTCGGGCA ′3	284	(9)
invA (R)	5′ TCATCGCACCGTCAAAGGAACC ′3		
hilA (F)	5′ CTGCCGCAGTGTTAAGGATA ′3	497	(10)
hilA (R)	5′ CTGTCGCCTTAATCGCATGT ′3		

Table 2: Serotyping of salmonellae isolated from the examined samples of fresh water fishes

Serotypes	Fish species			Group	Antigenic structure	
	Tilapia niloticus	Mugil cephalus	Clarias lazera			
	No (%)	No (%)	No (%)		O	H
S. typhimurium	3(7.5)	6(15)	4(10)	B	1,4,5,12	i :1,2
S. infantis	1(2.5)	1(2.5)	3(7.5)	C1	6,7	r :1,5
S. virchow	2(5)	1(2.5)	2(5)	C1	6,7,14	r :1,2
S. enteritiditis	2(5)	5(12.5)	2(5)	D1	1,9,12	g,m
S. heidelberg	0	3(7.5)	1(2.5)	B	1,4,5,12	r :1,2
S. tsevie	0	1(2.5)	0	B	4,5	i:e,n,z15
S. wingrove	0	2(5)	0	C2	8,20	c:1,2
Total (120)	8(20)	19 (47.5)	12(30)			

Table 3: Incidence of virulence genes of different salmonella strains isolated from the examined samples of fish.

Salmonella serovars	No. of isolates	invA	hila
		No (%)	No (%)
S. typhimurium	4	4 (100)	3 (75)
S. infantis	4	4 (100)	2 (50)
S. virchow	4	4 (100)	2 (50)
S. enteritidis	3	3 (100)	3 (100)
S. heidelberg	1	1 (100)	1(100)
S. tsevie	1	1 (100)	1(100)
S. wingrove	1	1 (100)	0

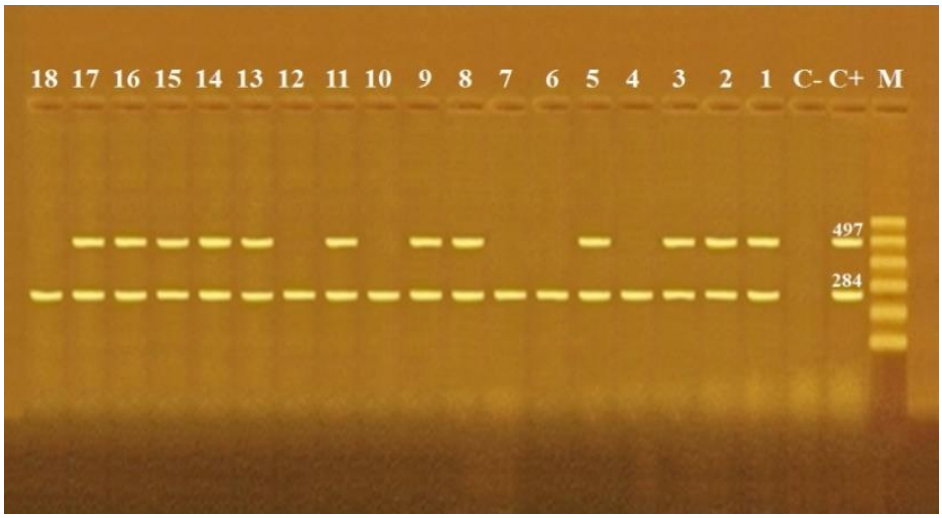

Figure 1: Agarose gel electrophoresis of multiplex PCR of *invA* (284 bp) and *hilA* (497 bp) virulence genes for characterization of salmonella species. Lane M: 100 bp ladder; lane C+: Control positive strain for invA and hilA genes; lane C-: Control negative; lanes 1-18 isolated samples.

Discussion

In the present study, we found high incidence of salmonella pathogen among different fish species with highest rate in *Mugil cephalous* (47.5%), followed by *Clarias lazera* (30%), and then *Tilapia niloticus* (20%). Higher incidence in *Mugil cephalous* may be due to its feeding nature as omnivorous bottom feeders swim over sandy-muddy bottom were they greases the pond detritus, organic matter and sediments which are usually of a high bacterial count (11) and salmonella can survive (54 days in water and up to 119 days in sediment) (12). This also may be due to the higher exposure of mugil species to contamination during their long transportation at high temperature from far cities as the point of production (farms) is so far from the point of distribution (fish markets) in Gharbia governorate. On the other hand, tilapia mainly inhabited shallow streams which are usually of a low bacterial count as a result of higher speed of water flow which prevent exceeding contamination with microorganisms, indicating the possibility of occurring cross contamination during offloading, landing and marketing of tilapia fish besides applying of poor hygienic practice via using of unsanitary water, ice and containers (13). And is not surprising in cat fish species since they mostly bred in area near from sewage which are usually related to fecal contamination of the water. Collectively, our findings are in consistence with (14) who reported that the variation in salmonella percentage in different types of fish could be attributed to fish species, environments, and methods of catch, extend of handling during transportation, distribution and storage. Our results partially agreed with (15) who also isolated different salmonella serotypes from fresh water fish (tilapia, mugil and catfish) from another locality in Egypt (Zagazig). However, this latter study showed a slight different in prevalence order, with highest rate in mugil samples (26.66%), followed by *Tilapia niloticus* (20%) then cat fish (13.3%). This variations may be due to variety of factors such as geographical distribution, applying of poor personal hygine of fish handlers during sorting and selling, the degree of utensils contamination, the bacterial load of ice used for fish preservation and the time of sampling (16).

The results obtained from serological identification of the examined salmonella isolates revealed presence of 7 different salmonella serovars which are *S. typhimurium, S. enteritidis, S. infantis , S. virchow , S.Heidelberg , S. wingrove* and *S. tsevie* with a percentage of 33.3%, 23%, 12.8%, 12.8%, 10.25%, 5% and 2.5%, respectively. These results agreed with (17) who reported that *Salmonella typhimurium* was the most frequently serovar isolated from

Nile tilapia from Winam Gulf of lake victoria, kenia with a percentage of 14.3% regarding other salmonella serovars indicating its ability for survival and adaptation in the aquatic environment. This also was in consistence with WHO, (18) which reported that *Salmonella typhimurium* is the most common salmonella serotype isolated from cases of food poisoning and represents about 50-60% of such cases. Many of these incidents have been linked to consumption of raw or improperly cooked fish and fish products.

It has been found that salmonella has a large number of genes which implicated in its virulence. Many of these virulent genes are chromosomal genes located on the pathogenicity islands referred to as salmonella pathogenicity island (spI) which is a part of type 3 secretion systems (T3SS) and encoded genes like *invA*, and *hilA* allowing some salmonella species to invade the host cells and induce infection. Our obtained PCR results revealed that *invA* gene was present in all the isolates with a percentage of 100%. Meanwhile, *hila* gene was present only in 66.6% of all the isolates. Other studies have reported similar results (19-22) indicating that *invA* gene can be used as a specific target for detection of salmonella as they are widely distributed among salmonella serovars reflecting the potential virulence of these isolates. These finding play a great role in determining the severity of the microbe since *invA* gene was found to be present and functional in most if not all salmonella serotypes responsible for the invasion of the cells of the intestinal epithelium allowing salmonella pathogen to enter and survive inside the eukaryotic cells with subsequent diseases in variety of hosts. In this regard, it has been used in determining the presence and metabolic activity of salmonella species (23). Meanwhile, *hilA* gene is required for regulation of type 3 secretion apparatus genes which secret proteins that are related to cell invasion [13]. And has been used in detecting of S.enterica serovars from fecal samples according to [19]. In recent studies, it has been used as a diagnostic target to validate alternative methods for food analysis and food-borne pathogen detection instead of the standard method of ISO 6579, 2004 which are time consuming and can take up to 4-6 days for confirmative results.

Conclusion

Presence of salmonella as enteropathogens in fresh water fish can reflects the poor hygienic conditions under which fish have been captured, handled and transported. The main finding in our study was the high prevalence of salmonella pathogen with a diversity of serotypes in fresh water fish in gharbia governorate creating a high level of attention towards the hazards of presence of these zoonotic pathogens in marketed fish and their bad impact on public health. so, our study recommends a monitoring and surveillance programs to be adopted against the food borne-salmonella by applying a strict hygienic measures under the supervision of the food controlling authorities in order to control the keeping quality of the locally consumed fish and to protect the consumer from the risk of food poisoning associated with consumption of contaminated fish with such dangerous pathogens.

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Original Research Article

GROWTH AND ECONOMIC PERFORMANCE OF USING DRIED TOMATO POMACE FOR MALLARD DUCKS

Mohamed A. Omar[1], Tamer M. Abdel-hamid[2], Sara Esam[3], Annam E. Omar

[1,3]Veterinary Economics and Farm Management, Animal Wealth Development, Faculty of Veterinary Medicine, Zagazig University. [2]Animal Breeding and Production, Animal Wealth Development, Faculty of Veterinary Medicine, Zagazig University. [4]Animal Nutrition and Clinical Nutrition Department, Faculty of Veterinary Medicine, Zagazig University.

*Corresponding author, E-mail: omarkafay@yahoo.com

Abstract: This study was aimed to economic evaluate the effect of Dried Tomato Pomace (DTP) on growth performance, biochemical profiles and economic efficiency of Mallard Ducks from 1 days old until 72 days (age of marketing). A total of 240 unsexed one – day old, Mallard ducklings were used in this study and were randomly and independently allocated to the four dietary treatments, each containing 60 ducks and divided in to four groups, T1 (control), T2 (10 % DTP), T3 (15% DTP) and T4 (20% DTP). The diets were formulated in mash form fortified with vitamin and mineral premix and chemical analysis were applied for starter and finisher rations. Moreover, feed and water were provided for *ad-libitum* consumption. The results showed that, ducks fed 20 % DTP had higher live body weight with a coincident significant increase of the feed intake ($P < 0.05$). In addition, total cholesterol, Triglycerides, high density lipoprotein (HDL) are decreased significantly ($P < 0.05$) meanwhile low density lipoprotein (LDL), total protein profiles non-significant ($P > 0.05$). In terms of economic analysis, T4 (20% DTP) diet revealed the lowest cost per Kg live weight of ducks 77.57 L.E / duck, along with the highest economic return 91.38 L.E per duck and net profit 13.81 L.E per duck. In conclusion, Mallard duck can efficiently utilize diet containing high DTP (20%) diets, consequently, it can be used to optimize their growth performance and maintain the maximum economic return.

Key words: Mallard duck; dried tomato pomace; growth performance; economics efficiency

Introduction

The poultry sector is considered the fast growing and flexible of all live stock sectors and Egyptian poultry industry has improved and become occupied a place of glory among the livestock enterprises as it is providing a great part of increasing demand for animal protein. Moreover, it is characterized by rapid monetary turnover, short production cycle and higher return on investment (1). The feed plays remarkable role in poultry production by sharing about 70% of total production costs. So, great efforts have been made to improve feed utilization and conversion to meat to minimize the feeding cost by feeding a well-balanced diet and supplementing diet with various feed additives (2, 3).

Tomato (*Lycopersicon esculentum*) is one of the most popular vegetables used as salad in

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food preparation and as juice, soup, ketchup or paste. Commercial processing of tomato produce large amount of waste at various stages. Tomato pomace (TP) is mixture of tomato skin, pulp and crushed seed that remain after the processing of tomato for juice, paste and ketchup (4). From 1000 kg of fresh tomatoes, 100 to 300 kg wet tomato pulp are produced and normally disposed of being sold as animal feedstuffs (5), and its nutritional value is highly dependent on the tomato cultivars, growing conditions, degree of drying and processing method (6).

Some by-products originated from food industry processing are utilized in animal feeding. However, the utilization of certain products is not generally applied in animal nutrition. One of these by-products is the peels and seeds of tomato called in common (tomato pomace) remaining usually from the processing of tomato. In Egypt, about 550.000 to 660.000 tons of tomato by-products are yearly produced from canning industry. Unfortunately, a great part of it is lost without utilization. These by-products remain from the squeeze of tomato; is rich in protein, energy and crude fiber (7, 8). Wet tomato pulp can be further dried to approximately 900 g/kg DM and, because of its chemical composition, which possesses nutritional value, can provide the poultry industry with an alternative feedstuff (9).

The objective of this study was to economically evaluate substitution the corn with Dried Tomato Pomace (DTP) for mallard duck by study their effect on the productive and economic performance traits that included final body weight, feed conversion rate, feed efficiency, total costs, total returns and finally net returns. Also biochemical profiles of total proteins and lipids were measured for determine the effect of DTP on the quality of the meat for Mallard duck.

Materials and methods

This work was carried out during the period from January 2017 till March 2017.

A total number of 240, unsexed one – day old, Mallard ducklings were used in this study. All ducks were weighed individually and distributed randomly among 4 treatments each treatment include 60 ducks were nearly similar in initial average body weights.

Data collection

Rations were formulated to contain approximately the same crude protein level, and energy (kcal/ kg). Four treatments were used: - T1 (controlled), corn – soy diet fed for group (1). T2, 10% Tomato Pomace replaces corn fed for group (2). T3, 15% Tomato Pomace replaces corn fed for group (3). T4, 20% Tomato Pomace replaces corn fed for group (4). Four experimental starter diets (from 1day to 42 days) and finisher diets (from 42 day to76days) were fed to ducklings and chemical analysis of experimental diets (10)

Growth performance measurements

Through the experiment, the following measurements were recorded:

Live body weight (LBW)

Ducks were weighted in each blocks every 7 days during experimental period. Total individual live weights in each blocks were divided by the number of ducks in the blocks to obtain the average live body weight.

Feed intake

Ducks in each treatment were provided with a weighed amount of feed every 1 week, the residual were obtained at the end of the 1 week of age and the amount consumed was calculated by the difference. The average amount of feed consumed per duck = amount of feed consumed per ducks / number of duck consuming feed.

Feed conversion rate (FCR)

Feed conversion rate = (feed intake per kilograms in week / body weight gain per kilograms in week) (11).

Feed efficiency (FE)

The feed efficiency is weekly or for total experimental period and calculated as follow: Feed efficiency = (Gain in live body weight in this period / Feed intake in certain period) (12,13).

Blood parameters

On a random basis, blood samples were collected from 5 ducks per group at slaughtering

and lipid profiles including triglycerides (TG) (14), total cholesterol (TC), high density lipoprotein (HDL) (15), low density lipoprotein (LDL) (16) were determined. Also total plasma proteins (TP) (17), albumin (ALB) (18), globulin (GLB) (19), were determined calorimetrically using the commercial kits (ELITech SEEPPIM S.A.S. Zone industrielle - 61500 France).

Economic analysis

At the end of experiment, the following indices were calculated per each groups of duck: Total fixed costs (TFC), total costs (TC) (15,20). Total return (NR), net profit (NP) and economic efficiency (EE)(11, 12, 21, 22, 23, 24, 25, 26).

Statistical Analysis

Data handling and statistical analysis was carried out at the Dept. of Animal Wealth Development, Faculty of Vet. Medicine, Zagazig University. Analysis was done using SPSS/PCT, (Statistical Package for Social Sciences version 22.0) (IBM Corp., Armonk, NY, USA) software Results were reported in means ± SEM (Standard Error of Mean). The value of $P < 0.05$ was used to indicate statistical significance. The statistical method was ANOVA test (one way analysis of variance) to test the differences in productive and economic efficiency parameters of ducks according to different experimental diets. The Duncan multiple range test are also used (27, 28).

Results

Growth performance

The effects of different levels of dietary DTP on final body weight, Total feed intake, FCR and FE were showed in Table 3. And results showed that the final body weight was significant ($P < 0.05$) where the highest in T4 and the lowest was in T1.also the total feed intake was significant ($P < 0.05$) different in different groups the highest was in T3. Meanwhile the feed efficiency and the feed conversion ratio are high with significant in T1 and T3 respectively.

Biochemical analysis

The different protein profiles are analyzed in table (4) where the total protein, globulin and A/G ratio are non-significant at ($P > 0.05$). Meanwhile the total albumin are high significant at ($P < 0.05$) for different groups where the value are high in control groups (5.66) and in T2, T3 and T4 the values were 5.27, 5.36 and 5.45 respectively. Table 5 shows the analysis for different lipid profiles, the mean values for total cholesterol, high density lipoprotein (HDL) , low density lipoprotein(LDL) and triglycerides all are significant at ($P < 0.05$).

Economical analysis

The different economic measures are shown in table 6 that shown highly significant ($P < 0.05$) for total variable costs (LE/ duck), total costs (LE/ duck), total returns (LE/ duck) and net profit (LE/ duck) for different groups and shown that T4 is the highest in terms of net returns and control group (T1) is the lowest in that term of net returns. Also this table shown that total fixed costs (LE/ duck) are non-significant at ($P > 0.05$) for all groups.

The efficiency measures for economic analysis are shown in table 7 that includes percent of total returns to total costs, percent of total returns to total variable costs, percent of net profit to the total variable costs and finally percent of net profit to the total costs. All are significant at ($P < 0.05$).

Discussion

Considering our main interest was to establish a bio-economic optimum for feed formulation with DTP that meet the nutritional requirements of mallard duck and maintain maximum performance with least cost as well.

Growth Performance

The final body weight was highest in T4 with significant effect that indicate that the high percent of DTP (20%) results in increasing the body weight gain and this results are consistent with those of (4) who reported that DTP increase feed performance and final body weight.

Table 1: Chemical composition (%) of experimental diets used in the starter stage from 1 weeks

to 7 weeksItem %	Experimental diets for each 100 kg diet			
	Control	10%Tomato pomace	15%Tomato pomace	20%Tomato pomace
ME, Kcal/Kg	2891	2890	2883	2872
CP, %	22.41	22.25	22.24	22.22
EE, %	4.38	4.64	4.76	4.90
CF, %	3.5	5.4	6.43	7.29
Ca, %	0.92	0.92	0.92	0.92
Lysine, %	1.13	1.12	1.11	1.11
Methoinine, %	0.4	0.4	0.4	0.4
Available ph.%	0.58	0.58	0.58	0.58

ME: Meatabolizale Energy CP : Crude Protein CF: Crude Fiber Ca : Calcium

Table 2: Chemical composition (%) of experimental diets used in the finisher stage from 7 weeks to 10 weeks

Item %	Experimental diets for each 100 kg deit			
	Control	10% Tomato pomace	15% Tomato pomace	20% Tomato pomace
ME, Kcal/Kg	3000	2994	2987	2981
CP, %	19.83	19.76	19.74	19.71
EE, %	6.8	7.07	7.21	7.34
CF, %	3.37	5.37	6.37	7.43
Ca, %	0.95	0.95	0.95	0.95
Lysine, %	1.18	1.17	1.16	1.15
Methoinine, %	0.50	0.49	0.48	0.48
Available ph.%	0.45	0.45	0.45	0.45

ME: Meatabolizale Energy CP : Crude Protein CF: Crude Fiber Ca : Calcium

Table 3: Economic parameters of Mallard duck performance that affected by dietary treatments

Items	Different treatments that supplied with dried tomato pomace			
	Treatment 1 (T1)	Treatment 2 (T2)	Treatment 3 (T3)	Treatment 4 (T4)
Number of ducks	60	60	60	60
Initial body weight (g)	57.53±0.18^{a}	57.37±0.18^{a}	57.46±0.20^{a}	57.36±0.19^{a}
Final body weight (g)	4020.93± 5.78^{c}	4108.11± 8.42^{b}	4146.68±3.44^{a}	4154.03±3.03^{a}
Total Feed intake (g)	8082.73± 6.18^{c}	8513.60± 54.17^{b}	8786..27± 40.84^{a}	8716.85± 39.18^{a}
Feed Conversion rate (FCR)	2.01± 0.003^{c}	2.07± 0.10^{b}	2.11± 0.009^{a}	2.09± 0.009^{a}
Feed Efficiency (FE)	0.49± 0.008^{a}	0.48± 0.002^{b}	0.47± 0.002^{c}	0.47± 0.002^{c}

Means within the same row in each category carrying different litters are significant at ($P \leq 0.05$)

Table 4: Effect of experimental diets on protein profiles of Mallard ducks (Mean ± SE)

Groups	No.	Treatment 1 (T1)	Treatment 2 (T2)	Treatment 3 (T3)	Treatment 4 (T4)
Total protein (g/dl)	5	5.66±0.05[a]	5.27±0.03[a]	5.36±0.07[a]	5.45±0.01[a]
Albumin (g/dl)	5	2.49±0.03[a]	2.16±0.03[b]	2.17±0.05[b]	2.27±0.05[b]
Globulin (g/dl)	5	3.17±0.07[a]	3.10±0.04[a]	3.19±0.03[a]	3.18±0.06[a]
A/G ratio	5	0.78±0.01[a]	0.69±0.01[a]	0.68±0.02[a]	0.71±0.01[a]

Means carrying different superscripts in the same row are sig. different at (P<0.05)

Table 5: Effect of experimental diets on Lipid profiles of Mallard ducks (Mean ± SE)

Groups	No.	Treatment 1 (T1)	Treatment 2 (T2)	Treatment 3 (T3)	Treatment 4 (T4)
Total cholesterol (mg/dl)	5	196.6± 1.36[a]	172.2±1.04[b]	172.9±0.81[b]	173.6±0.82[b]
LDL-cholesterol (mg/dl)	5	77.21± 1.04[b]	77.16±0.43[b]	77.76±1.07[a]	77.85±0.87[a]
HDL-cholesterol (mg/dl)	5	83.52±0.90[a]	66.93±2.91[b]	66.00±0.79[b]	67.65±0.75[b]
Triglycerides (mg/dl)	5	218.69±2.29[a]	200.08±3.15[b]	198.92±1.61[b]	201.84±1.52[b]

Means carrying different superscripts in the same row are sig. different at (P<0.05)

Table 6: Economic parameters of duck performance that affected by dietary treatments

Items	Different treatments that supplied with dried tomato pomace			
	Treatment 1 (T1)	Treatment 2 (T2)	Treatment 3 (T3)	Treatment 4 (T4)
Number of ducks	60	60	60	60
Total Variable cost (LE/Duck)	70.04±0.15[a]	67.78±0.21[b]	67.04±0.14[c]	66.06±0.15[c]
Total Fixed cost (LE/Duck)	11.65±0.11[a]	11.65± 0.12[a]	11.64±0.11[a]	11.51±0.10[a]
Total cost (LE/Duck)	81.69± 0.19[a]	79.43± 0.24[b]	78.69± 0.18[c]	77.57± 0.17[c]
Total Returns (LE/Duck)	88.46± 0.12[c]	90.37± 0.18[b]	91.22±0.07[a]	91.38± 0.06[a]
Net Profit (LE/Duck)	6.76± 0.20[c]	10.94±0.37[b]	12.53± 0.20[a]	13.81±0.20[a]

Means within the same row in each category carrying different litters are significant at (P ≤ 0.05)

Table 7: Economic efficiency of Mallard duck performance that affected by dietary treatments

Items	Different treatments that supplied with dried tomato pomace			
	Treatment 1 (T1)	Treatment 2 (T2)	Treatment 3 (T3)	Treatment 4 (T4)
Number of ducks	60	60	60	60
Total Return / total cost (%)	1.08 ±0.002^{c}	1.13±0.005^{b}	1.15±0.002^{a}	1.17±0.002^{a}
Total Return / total variable cost (%)	1.26± 0.002^{c}	1.33± 0.006^{b}	1.36±0.003^{a}	1.37±0.003 ^{a}
Net Return / Total variable cost (%)	0.09± 0.003^{c}	0.16± 0.005^{b}	0.18± 0.003^{a}	0.20± 0.003^{a}
Net Returns / Total cost (%)	0.08± 0.002^{c}	0.13± 0.005^{b}	0.15± 0.002^{a}	0.17± 0.002^{a}

Means within the same row in each category carrying different litters are significant at ($P \leq 0.05$)

The total feed intake (g/duck), feed conversion ratio and feed efficiency are the highest in T4 and T3 that results are agreement with (7) who found that dried tomato pomace can be used in broiler chicken diets up to 20 %. However, in general it seems dried tomato pomace can be used in poultry diets as a feed ingredient any level and results in increasing total feed intake, FE and FCR. These results were in contrary with (29) who reported that increased TP level (10, 20%) in both starter and finisher broiler chicken diets resulted lower live weight ($P < 0.01$). However there was no significant difference between control and 5% DTP supplemented diet groups.

Biochemical analysis

The total protein, globulin and A/G ratio are non-significant at ($P > 0.05$). The observed result was agreed with (30) who recorded that total protein, globulin and albumin: globulin ratio levels of broilers not affected by different levels of DTP.

Table 5 shows the analysis for different lipid profiles, the result revealed significant ($P<0.05$) decrease in total cholesterol, triglycerides, HDL and LDL levels. The total cholesterol of control group was 196.6 mg/dl while that of the groups fed diet contained 5%, 10% and 20% DTP were 172.2, 172.9, and 173.6 mg/dl respectively. The triglycerides of control group was 281.69 mg/dl while that of the groups fed diet contained 5%, 10% and 20% DTP were 200.08, 198.92, and 201.84 mg/dl respectively. Also the HD of control group was 83.52 mg/dl and that T2, T3 and T4 were 66.93, 66.00 and 67.65 mg/dl respectively. The LDL level of control group was 77.21 mg/dl and for T2, T3 and T4 were 77.16, 77.76 and 77.85mg/dl respectively

The observed result was agreed with (30) who recorded that the serum cholesterol content, LDL and HDL of poultry fed on diet contained 8, 16% DTP were lower as compared with other groups ($P<0.05$). In the same line (31) reported that tocopherols and tocotrienols in DTP lowers serum cholesterol by supperessing the posttranscriptional action of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)reductase, the rate –limiting enzyme in the mevalonate pathway of endogenous cholesterol synthesis by the liver

Economical analysis

Table (6): showed non significant difference ($P > 0.05$) among all groups for the total fixed cost where at the control groups it was 11.65

LE/duck and for T2,T3 and T4 total fixed costs were 11.65, 11.64 and 11.51 LE/duck. The total variable costs are significant difference ($P < 0.05$) among all groups where the large total variable costs was at T1 (70.04 LE/duck) and the lowest one was at T4 (66.06LE/duck).

Also in Table (6): showed significant difference ($P < 0.05$) among all groups for the total returns where at the control groups it was 88.46 LE/duck and for T2,T3 and T4 the total returns were 90.37 91.22, and 91.38 LE/duck respectively. This results indicates the significance using Tomato pomace at 20% for the group four more than 10% and 15%. And the highly total returns in this group may be due to the high final growth weight in this group.

Table (6): showed significant difference ($P< 0.05$) among all groups for the net profit where at the control groups it was 6.76 LE/duck and for T2,T3 and T4 the for the net profit were 10.94, 12.53, and 13.81 LE/duck respectively. This results indicates the significance difference among all groups in the net profit and as showed the higher body weight gain in T4 are due to the high feed intake and consequently high total returns and finally higher net profit.

Table (5): showed significant difference ($P< 0.05$) between all groups for the different economic efficiency measures. The percent of total returns to the total cost for the control groups it was 1.08 and for T2,T3 and T4 were 1.13, 1.15 and 1.17 respectively.

Meanwhile The percent of total returns to the total variable cost for T4 groups was 1.37 and for T1, T2 ,and T3 were 1.26, 1.33 and 1.36 respectively. Also in table (5), The percent of net returns to the total cost is higher in T4 group (0.17) and for T1, T2 ,and T3 were 0.08, 0.13 and 0.15 respectively. These economic results agree with (32,20).

Conclusion

Using of Tomato pomace as a percentage of corn has no side effect on the final growth weight of the mallard ducks and through this research work the 20% is more economic than 15% and 10% so we concluded that using the tomato pomace with percentage of 20% of the corn to the ration of the mallard duck and suggested that new research are needed to study the effect of percentage more than 20% as percentage of corn in ration of mallard duck.

Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

GROWTH AND ECONOMIC PERFORMANCE, SOME HEMATOLOGICAL, BIOCHEMICAL AND OXIDATIVE STRESS PARAMETERS, AND CARCASS TRAITS OF SUMMER STRESSED CALIFORNIAN AND CROSSBRED RABBITS AS AFFECTED BY DIETARY SUPPLEMENTATION OF FORMIC ACID

Tamer M. Abdel-hamid[1], Mohamed A. Omar[2]

[1]Animal Breeding and Production, Animal Wealth Development, Faculty of Veterinary Medicine, Zagazig University, Egypt, [2]Veterinary Economics and Farm Management, Animal Wealth Development, Faculty of Veterinary Medicine, Zagazig University

*Corresponding author, E-mail: Omarkafafy@yahoo.com

Abstract: The aim of present study was to examine the effect dietary supplementation of formic acid on growth performance, some heamatological, biochemical and oxidative stress parameters as well as carcass traits of summer stressed Californian (CAL) and Californian × Rex (CAL × RX) crossbred rabbits. A total of 60 rabbits 4 weeks old were allotted into 2 × 3 completely randomized design (two genotypes; CAL and CAL × RX and three dietary treatment; 0, 0.3 and 0.5% formic acid).Rabbits were subjected to a temperature of 32±1°C during the experimental period, they were slaughtered at 10 weeks of age and blood samples were collected at slaughtering. Rabbits fed diet with 0.5% formic acid had the higher final body weight (P<0.001), body weight gain (BWG) (P<0.001), average daily gain (ADG) (P<0.001) and the lowest feed to gain ratio (P<0.001) compared with those fed diet with 0.3% formic acid and the control rabbits. Rabbits fed diet supplemented with 0.3% formic acid had higher significant total protein (P<0.001) and globulin (P<0.001) than the control rabbits whilst, the highest significant red blood cell (RBCs), white blood cell (WBCs) count and catalase activity have been recorded in 0.5% formic acid group. Carcass traits significantly affected (P<0.05) by dietary supplementation of formic acid were; hot and reference carcass weights, and dressing-out, skin, stomach, intestine, liver, hind part proportions. The effect of genotype × dietary supplement of formic acid was significant for average daily feed intake (ADFI), (P=0.028), cholesterol (P=0.001), Hb (P=0.002), haematocrit percentage (P=0.006) glutathione peroxidase (P=0.026), and dressing-out% (P=0.028). Net profit for CAL and CAL × RX were 8.68 and 10.34 (LE/rabbit) and for 0, 0.3 and 0.5% formic acid were 4.87, 10.11 and 13.72 LE/rabbit. In conclusion, Formic acid can be used as a dietary supplement at 0.5% of diet to improve economic efficiency, growth traits and health, immune parameters in the blood and improved the antioxidant status without any harmful effect on carcass traits.

Key words: formic acid; blood; oxidative stress; carcass; gross income

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Introduction

Rabbits can be used as a good alternative source of animal protein for humans in the developing countries (1). They characterized by high reproductive output and can digested high fibers diet such as forages and agriculture by-product to produce high quality meat (2) These meat high in protein and low in fat (3). The problem of bacterial antibiotic resistance arises from use of antibiotics as a feed additive attracts a public health concern (4, 5). This fact encourages the European Union to ban the use of antibiotic as a growth promoters since 2006 (6). So other safe, natural, efficient and economic feed additives that exert the same purpose becomes very interesting.

Organic acids can be used as replacement for antibiotics in rabbits' diet (7). There are many benefits of using organic acids as a feed additives such as antifungal property, antibacterial activity against anaerobic pathogens (8), reduced mortality caused by gastrointestinal tract disease (9-11) and stimulate gastrointestinal mucosal growth (12). Also (13) observed that dietary treatment with 0.5% acetic acid or 0.5% lactic acid had a positive effect on body weight gain and feed conversion ratio. In addition to, plasma cholesterol and total lipids have been lowered in summer stressed growing New Zealand White rabbits. (14,15) found that rabbits fed diet supplemented with a blend of microencapsulated formic and citric acids 0.2% in the finishing period (56-77 d) had higher significant ADG compared with control. The diet supplementation with organic acids had no effect on carcass traits (13-18) detected that inclusion of organic acids, butyric acid, calcium formate or its salts in the diet had no effect on internal organs and skin weights, but hot carcass weight and dressing-out percentage were significantly affected.

The objective of this study was to study the effect dietary supplementation with formic acid on growth performance, haematological, biochemical and oxidative stress parameters as well as carcass traits in rabbit of different genotype exposed to heat stress. In addition to this, to evaluate the economics of formic acid inclusion in rabbits' feed.

Materials and methods

Animals, diet and management

A total of 60 CAL and CAL × RX of equal sex rabbits about 4 weeks old at the start of this investigation nearly homogenous with average initial body weight of 434.33±17.43 have been subjected to 2 × 3 completely randomized design (two genotypes; 30 of each CAL and CAL × RX, and threes dietary treatments; 20 of each 0, 0.3 and 0.5% of diet formic acid) and ten replicates. The ingredients of the basal diet was firstly prepared then divided into three equal parts. The formic acid was added to the last two parts at 0.3 and 0.5% of the diet.

The proximate chemical composition of the basal diets were determined according to AOAC (19). The experimental rabbits were ear tagged, housed in well ventilated house in flat deck arranged metal cages (two rabbit of the same genetic group per cage; one male and one female), the dimensions of the cage were (45 × 45 × 35 cm). The manure was regulatory disposed on a daily bases and a metal feeder and a drip nipple drinker have been provided for each cage. Ration and water were offered for *ad libitum* consumption. This experiment has been performed during summer months (July and August) of Egypt and inside temperature has been maintained to be averaged 32±1°C. A fourteen hours of day light has been maintained. The fattening period was 6 weeks (from 4 to 10 weeks of age).

Growth performance

The initial and the final body weights were recorded on individual bases while feed intake were recorded on cage bases and the feed to gain ratio were calculated.

Carcass traits

All the experimental rabbits were slaughtered at 10 weeks of age in the same day and all the slaughtered procedures follow the world rabbit science association recommendations (20). The rabbits were fasted to about 12 hours. Firstly, the live weights were recorded, then rabbits were stunned and the two jugulars were served. Blood, skin including distal paws and

tail, intestine, stomach were weighed, the remaining was the hot carcass, then chilled in ventilated room at 4°C for 24 hour and then head, liver, heart, lungs, esophagus, trachea, thymus gland, and kidney free of perirenal fat were removed as reference carcass. Finally, the reference carcass was divided into three anatomical parts (fore, mid and hind parts).

Blood haematological, biochemical and oxidative stress parameters while slaughtering rabbits two blood samples per rabbit have been collected, on ethylenediaminetetra-acetic acid (EDTA) for haematological studies and one without anticoagulants for plasma separation for biochemical and oxidative stress parameters. Red blood cell count (RBC), white blood cell count (WBC), h aemoglobin (Hb) concentration and haematocrit% were performed as previously described (21-23). For serum separation the blood samples were centrifuges 3000g for 15 min at a temperature of 15-24°C and the samples were stored at -20°C until they were assayed. Total proteins, albumin, triglycerides and cholesterol were determined photometrically by using commercial kits and as previously described (24). The serum level of glutathione peroxidase, superoxide dismutase, catalase activity and lipid peroxidase were measured as previously detailed (25, 26).

Statistical analysis

A general linear mode (GLM) of (27) was utilized to analyze the data of growth performance, blood parameters, carcass traits and economic efficiency parameters. The following model used:

$$Y_{ijk} = \mu + G_i + T_j + (GT)_{ij} + e_{ijk}$$

Where Y_{ijk} is an observation on *n*th rabbit of each trait, μ is the overall mean, G_i is the fixed effect of *i*th breed (*i* = 1and 2, i.e. CAL and CAL × RX), $T_j$is the fixed effect of dietary treatment with formic acid (*j* = 1, 2 and 3, i.e. 0, 0.3 and 0.5% of diet), GT_{ij} is the effect of interaction between genotype and dietary treatment with formic acid level and e_{ijk} is the random residual effect. The comparisons between means were performed using (28) at $P<0.05$.

Economic parameters

The following indices were calculated per each groups of rabbits: Total fixed costs (TFC), total costs (TC)(30,31). Total return (NR), net profit (NP) and economic efficiency (EE)(29-32).

Results

Growth performance

CAL rabbits had heavier final body weight than CAL × RX (P=0.009) (Table 1).Rabbits fed diet with 0.5% formic acid had the highest significant final body weight (P<0.001), BWG (P<0.001), ADG (P<0.001) and the lowest feed to gain ratio (P<0.001) compared with those fed diet with 0.3% formic acid and the control rabbits (Table 1). The effect of genotype × dietary supplementation was non-significant on all growth traits except for ADFI (Table 1).

Blood heamatological, biochemical and oxidative stress parameters

The CAL genotype had higher significant serum triglycerides concentration (P=0.003) than CAL × RX, however, CAL × RX rabbit had higher significant WBC count (P<0.001) and catalase activity (P<0.001) than their CAL counterparts (Table 2). Regardless the rabbits' genetic group, those fed diet with 0.3% formic acid had higher significant total protein (P<0.001) and globulin (P<0.001) than the control rabbits. In contrast, they had lower significant albumin/globulin ratio (P<0.001) than the 0% formic acid group (Table 3). The control rabbits had the highest significant albumin (P<0.001) albumin/globulin ratio, whilst they recorded the lowest significant total protein, globulin, RBC (P<0.001), WBC count (P<0.001) and catalase activity compared with those fed diet supplemented with 0.3% and 0.5% formic acid. The highest significant RBC, WBC count and catalase activity have been determined in 0.5% formic acid group (Table 2).

The only blood parameters showed a significant genotype × dietary supplementation with formic acid were; cholesterol (P=0.001), Hb (P=0.002), haematocrit percentage (P=0.006) and Glutathione peroxidase (P=0.026) (Table

3). CAL rabbits supplemented with 0.3% formic acid had the highest cholesterol and Glutathione peroxidase concentration whereas, the same genetic group fed diet with 0.5% formic acid had the highest Hb concentration. Rabbits of CAL × RX genetic group had received 0.5% acid had depicted the highest haematocrit percentage.

Carcass traits

Heavier hot and reference carcasses (P=0.001and P=0.003) have been recorded in CAL rabbits compared with CAL × RX (Table 4). Rabbits supplemented with 0.5% formic acid had the highest significant hot and reference carcass weights (P=0.001), and hind part percentage (P=0.018). However, those fed diet supplemented with 0.3% formic acid had the highest stomach (P=0.001), intestine (P<0.001), liver (P=0.005) and periscapular fat percentages (P=0.008). On the other hand, the control rabbits had the highest skin% (P=0.016) (Table 4). The effect of genotype × dietary supplementation was non-significant on all carcass traits with the exception of dressing out % (P=0.028) (Table 4).

Economic parameters

Significant genotype × dietary treatment interaction with formic acid had been detected on total feed intake and total feed cost per rabbit (P<0.05), but if the fixed effects of dietary formic acid level and breed considered separately, it is cleared that rabbits fed on diet with 0.5% formic acid revealed lower total feed intake and total feed cost per rabbit than either control or 0.3% formic acid rabbit groups, but not significant. The effect of genotype × dietary treatment interaction on total feed intake and total feed cost per rabbit was non-significant (P>0.05) and different economic measures are presented in (Table 5). Where Net profit for CAL and CAL × RX were 8.68 and 10.34 (LE/rabbit) and for 0, 0.3 and 0.5% formic acid were 4.87, 10.11 and 13.72 LE/rabbit

Table 1: Effects of genotype and formic acid on growth performance of rabbits

Variable	Genotype (G)			[1]Dietary treatment with formic acid (T)				P-value		
	CAL	CAL × RX	sem	0%	0.3%	0.5%	sem	G	T	G × T
Rabbits, No.	30	30		20	20	20				
Initial body weight, g	415.12	453.53	25.26	408.78	446.29	447.91	30.93	0.287	0.602	0.987
Final body weight, g	1718.03	1676.80	10.82	1528.74^{c}	1716.43^{b}	1847.09^{a}	13.26	0.009	<0.001	0.180
Body weight gain, g	1276.69	1250.05	27.85	1113.22^{c}	1275.13^{b}	1401.76^{a}	34.11	0.502	<0.001	0.753
Average daily gain, g/d	30.39	29.764	0.66	26.50^{c}	30.36^{b}	33.37^{a}	0.81	0.502	<0.001	0.754
Average daily feed intake, g/d	118.62	117.29	2.05	119.66	118.28	115.928	2.51	0.650	0.573	0.028
Feed to gain ratio	4.077	4.01	0.104	4.58^{a}	4.02^{b}	3.531^{c}	0.12	0.673	<0.001	0.862

Means within the same row within the dietary treatment category not sharing the same superscript letter were significantly different at P< 0.05. [1]Dietary treatment with formic acid at 0, 0.3 and 0.5% of diet; CAL = Californian; CAL × RX = Californian × Rex; G × T = genotype × dietary treatment interaction; sem = standard error of mean

Table 2: Some blood biochemical, haematological and oxidative stress parameters of Californian and Californian × Rex rabbits at 10 weeks of age as affected by formic acid. Results of significant genotype × dietary treatment interaction were summarized in Table 4

Variable	Genotype (G)			[1]Dietary treatment with formic acid (T)				P-value		
	CAL	CAL × RX	sem	0%	0.3%	0.5%	sem	G	T	G × T
Rabbits, No.	30	30		20	20	20				
Total protein (g/dl)	5.70	5.67	0.045	5.30^{b}	5.93^{a}	5.82^{a}	0.055	0.626	<0.001	0.355
Albumin (g/dl)	3.48	3.46	0.028	3.61^{a}	3.42^{b}	3.38^{b}	0.034	0.534	<0.001	0.075
Globulin (g/dl)	2.21	2.21	0.048	1.68^{b}	2.51^{a}	2.43^{a}	0.059	0.922	<0.001	0.482
Albumin / Globulin ratio	1.67	1.62	0.055	2.16^{a}	1.37^{b}	1.41^{b}	0.067	0.549	<0.001	0.391
Triglycerides (mg/dl)	16.78	16.73	0.013	16.73	16.76	16.75	0.017	0.003	0.378	0.901
RBC_S ($\times 10^6$ /mm^3)	4.04	4.07	0.059	3.79^{b}	4.12^{a}	4.26^{a}	0.072	0.709	<0.001	0.555
White blood cells ($\times 10^9$/L)	8.95	9.59	0.11	6.96^{c}	9.14^{b}	11.70^{a}	0.13	<0.001	<0.001	0.485
Lipid peroxidase (nmol/mol x10^6)	15.27	15.30	0.15	15.13	15.40	15.34	0.19	0.880	0.577	0.199
Superoxide dismutase (m/mg)	84.62	85.73	1.69	85.95	86.17	83.41	1.98	0.629	0.554	0.749
Catalase (mg/g)	393.74	578.14	3.21	428.13^{c}	484.09^{b}	545.61^{a}	3.93	<0.001	<0.001	0.228

Means within the same row within the dietary treatment category not sharing the same superscript letter were significantly different at $P< 0.05$. RBC_S = red blood cell count ($\times 10^6$/mm^3);[1]dietary treatment with formic acid at 0, 0.3 and 0.5% of diet CAL = Californian; CAL × RX = Californian × Rex; G × T = genotype × dietary treatment interaction; sem = standard error of mean

Table 3: The effects due to interactions between genotype and formic acid on some blood biochemical, haematological and oxidative stress parameters at 10 weeks of age

Variable	CAL			CAL × RX			sem	*P*-value		
	[1]Control	[2]0.3%	[3]0.5%	Control	0.3%	0.5%		G	T	G ×T
Cholesterol (mmol/L)	0.867^{c}	0.962^{a}	0.910^{b}	0.926^{b}	0.940^{ab}	0.937^{ab}	0.010	0.012	<0.001	0.001
Hb (g/dL)	11.283^{a}	10.901^{b}	11.476^{a}	10.908^{b}	11.354^{a}	11.252^{a}	0.117	0.612	0.051	0.002
Haematocrit percentage	29.36^{b}	30.11^{a}	30.04^{a}	30.11^{a}	29.86^{a}	30.20^{a}	0.148	0.079	0.037	0.006
GP (mg protein)	110.002^{ab}	120.531^{a}	98.028^{ab}	103.461^{ab}	95.453^{b}	112.881^{ab}	7.156	0.343	0.939	0.026

Means within the same row not sharing the same superscript letter were significantly different at $P< 0.05$. CAL = Californian; CAL × RX = Californian × Rex; [1, 2, 3]dietary treatment with formic acid at 0, 0.3 and 0.5% of diet; Hb (g/dL) = haemoglobin concentration measured by gram in deciliter; G × T = genotype × dietary treatment interaction; sem = standard error of mean. GP = Glutathione peroxidase. Number of rabbits per group is 10 (5 males and 5 females).

Table 4: The carcass traits of Californian and Californian × Rex rabbits at 10 weeks of age as affected by formic acid level

Variable	Genotype (G)		sem	[1]Dietary treatment with formic acid (T)			sem	*P*-value		
	CAL	CAL × RX		0%	0.3%	0.5%		G	T	G ×T
Rabbits, No.	30	30		20	20	20				
Live body weight, g	1718.03	1676.80	10.82	1528.74^{c}	1716.43^{b}	1847.09^{a}	13.26	0.009	<0.001	0.180
Hot carcass, g	1106.65	1036.40	11.66	945.85^{c}	1056.32^{b}	1212.40^{a}	14.28	0.001	0.001	0.674
[2]Reference carcass, g	881.74	846.35	7.94	757.41^{c}	882.71^{b}	952.02^{a}	9.73	0.003	0.001	0.221
[3]Dressing-out, %	51.23	50.45	0.82	49.54	51.41	51.56	1.01	0.152	0.005	0.028
% of Live weight										
Blood	3.07	3.00	0.107	3.06	3.16	2.89	0.131	0.665	0.336	0.821
Skin	17.71	17.46	0.32	18.46^{a}	17.52^{ab}	16.77^{b}	0.39	0.599	0.016	0.264
Stomach	5.67	5.43	0.167	5.77^{a}	6.01^{a}	4.88^{b}	0.20	0.314	0.001	0.264
Intestine	10.50	10.49	0.23	10.65^{a}	11.26^{a}	9.59^{b}	0.28	0.974	<0.001	0.945
% of hot carcass										
Liver	4.59	4.76	0.18	4.13^{b}	5.21^{a}	4.70^{ab}	0.22	0.509	0.005	0.966
Heat & lung	1.62	1.65	0.04	1.56	1.72	1.62	0.05	0.727	0.087	0.667
Kidney	1.06	1.04	0.03	1.04	1.08	1.02	0.04	0.689	0.610	0.629
Head	10.45	10.49	0.13	10.65	10.42	10.33	0.16	0.835	0.368	0.464
% of reference carcass										
PSF	0.44	0.44	0.01	0.41^{b}	0.50^{a}	0.42^{b}	0.02	0.936	0.008	0.856
PRF	2.18	2.19	0.06	2.10	2.28	2.17	0.08	0.924	0.326	0.437
Fore part	37.14	37.29	0.42	37.03	37.40	37.21	0.51	0.793	0.879	0.699
Mid part	21.21	21.32	0.39	21.91	21.16	20.73	0.47	0.844	0.223	0.893
Hind part	38.59	38.24	0.25	38.06^{b}	38.01^{b}	39.17^{a}	0.31	0.347	0.018	0.815

Means within the same row within the dietary treatment category not sharing the same letter were significantly different at $P< 0.05$. CAL = Californian; CAL × RX = Californian × Rex; [1]dietary treatment with formic acid at 0, 0.3 and 0.5% of diet; G × T = genotype × dietary treatment interaction;[2]reference carcass = chilled carcass minus head, liver, heart, lungs, esophagus, trachea, thymus gland, and kidney free of perirenal fat;[3]calculated in relation to reference carcass; PSF = periscapular fat; PRF = Perirenal fat.

Table 5: Effects of genotype and formic acid level on some economic parameters and efficiency for Californian and Californian × Rex rabbits

Variable	Genetic group (G)			[1]Dietary treatment with formic acid (T)				*P*-value		
	CAL	CAL × RX	sem	0%	0.3%	0.5%	sem	G	T	G ×T
Rabbits, No.	30	30		20	20	20				
Total feed intake per rabbit, g	4982.13	4926.36	86.36	5025.69	4968.09	4868.95	105.78	0.650	0.573	0.028
[2]Total feed cost per rabbit, LE	22.42	22.16	0.38	22.61	22.35	21.91	0.47	0.650	0.573	0.028
[3]Price per rabbit, LE	54.97	53.65	0.34	48.92^{c}	54.92^{b}	59.10^{a}	0.42	0.009	<0.001	0.180
[4]Total Returns	56.01	54.50	0.25	50.20^{c}	56.30^{b}	60.30^{a}	0.78	0.002	<0.001	0.170
[5]Total variable cost	40.42	39.16	0.23	40.33	41.19	41.58	0.47	0.530	0.523	0.122
[6]Total fixed costs	5.00	5.00	0.14	5.00	5.00	5.00	0.02	0.11	0.321	0.180
Total costs(LE/rabbit)	47.42	44.16	0.22	45.33	46.19	46.58	0.33	0.221	0.273	0.128
Net profit (LE/rabbit)	8.68	10.34	0.03	4.87^{c}	10.11^{b}	13.72^{a}	0.22	0.03	0.001	0.008
Net Profit/ total costs	0.140	0.234	0.001	0.107	0.218	0.294	0.02	0.12	0.101	0.11
Net Profit / total returns	0.154	0.189	0.021	0.097^{c}	0.179^{b}	0.227^{a}	0.002	0.001	0.002	0.028

Means within the same row within the dietary treatment category not sharing the same superscript letter were significantly different at $P< 0.05$. [1]Dietary treatment with formic acid at 0, 0.3 and 0.5% of diet; CAL = Californian; CAL × RX = Californian × Rex; LE = Egyptian pound; [2]calculated as total feed intake per rabbit multiplied by price per kg of feed (4.5 LE); [3]calculated as final body weight multiplied by price per kg of live rabbit sold (32 LE); G × T = genotype × dietary treatment interaction; sem = standard error of mean.[4] Calculated as price of rabbit plus price of litter. [5]Calculated as price of rabbit and all medicaments (LE/ rabbit). [6] Calculated as rent, employments and other fixed costs (LE/ rabbit).

Discussion

The improvement in body weights, BWG, and ADG and the worseness in the feed to gain ratios after supplementation with organic acids (formic acid) has been seen in this study are in agreement with those reported previously (33,34). The first authors found feed intake to decrease and consequently, improvement feed conversion with the supplementation of diet with citric acid from 0.5% and up to 2.0% whereas, the second author recorded improvement of body weight gains with 0.5% fumeric, citric, and malic acids. However, no response has been recorded on weight gain, but feed intake was lowered and thus the feed conversion was improved in rabbits fed citric acid at g/kg diet (35). Dietary treatment with 0.5% acetic acid or 0.5% lactic acid had a positive effect on body weight gain and feed conversion ratio of summer stressed growing New Zealand White rabbits (13). Similarly, positive effects of organic acids on growth traits have been reported by (36). The addition of 1.5% fumaric acid to rabbits' diet improved daily gain and feed efficiency (37,34).

This positive effect of formic acid on growth performance could be attributable to the unfavorable environment to pathogens in the cecum and ileum that the formic acid exert by lowering the ph. Also, it acts as substrates in the intermediary metabolites, increase gastric protein digestion and availability of some elements that complex with (17).

There is no doubt that the average final body weights had been recorded in the present study were below the average expected for these animal and for these ages and this could be attributed to the higher temperature to which the rabbits were exposed and this previously documented (38,39). Genetic group × dietary treatment was non-significant on final body weight (40).In a study on New Zealand × Californian mixed-sex rabbits, rabbits fed diet supplemented with a blend of microencapsulated formic and citric acids 0.2% in the finishing period (56-77 d) had higher significant (P=0.019) ADG (48.0 g) compared with control (43.9 g) (8) Rabbits fed diet with organic acids at 1.0 g/kg depicted higher significant body weights at 14 weeks of age and daily body weight gains

at 7-14 weeks age interval and lower significant feed conversion ratios compared with control.

Blood heamatological, biochemical and oxidative stress parameters

The significant differences between purebred CAL and CAL× RX crossbred rabbits in the serum level of triglycerides are in accordance with the findings of (41). Whereas, other authors reported that genetic group to have a non-significant effect on blood haematological and biochemical parameters (40, 36).

In agreement with (13) they found that dietary supplementation with organic acid had a significant effect on serum globulin and cholesterol concentration. However, (13) observed that dietary supplementation of 0.5% acetic acid or 0.5% lactic acid had a non-significant effects on plasma total protein, albumin, globulin and total lipids, but they significantly lowered plasma cholesterol concentration compared with Control rabbits. The dietary treatment of rabbits with organic acids at 1.0 g/kg had no effect on blood biochemical parameters. Also, blood biochemical parameters not significantly differed in rabbits fed diet supplemented with mixture of formic and propionic acids and those on the basal diet only (16,41).

The exposure of rabbits to high temperature leads to stress on these rabbits, leading to increased free radicals and lipid oxidation and these free radicals can lead to irreversible damage to cells if not treated (42).Antioxidant enzymes have an important role in the animals in getting rid of free oxygen radicals that are released as a result of heat stress (43). The rabbits fed diet supplemented with Mucuna Pruriens leaf meal had higher and significant glutathione peroxidase and catalase in the blood these enzymes help these rabbit to cope with stress-induced oxidative destruction (1)

Carcass traits

The higher significant hot and reference carcass weights of CAL than that of CAL × RX are consistent with those reported by (38), they detected significant differences among genetic groups for the majority carcass traits. Also, the commercial and the reference carcass weights were higher in purebred rabbits than crossbreds at 30°C.

The higher periscapular in rabbits fed on diet with 0.3% formic acid collaborate those reported previously (13) who found higher abdominal fat in rabbits supplemented with 0.5% lactic acid than control but not significant. The diet supplementation with organic acids had no effect on carcass traits (13,16,17).Inclusion of organic acids, butyric acid, calcium formate or its salts in the diet had no effect on internal organs and skin weights, but hot carcass weight and dressing out percentage were significantly affected.

Any contrasting results with previous studies regarding the effect of organic acid on carcass traits could be explained by different experimental animal, methods of calculations, slaughter age and/or experimental procedures. The non-significant effect of genetic group × dietary treatment on carcass traits are in agreement with those reported by (40).

Economic parameters

The results showed significant effect of formic acid at 0.5% where the total returns CAL and CAL × RX were 56.01 and 54.5 (LE/rabbit) and for 0, 0.3 and 0.5% formic acid were 50.2, 56.3 and 60.30 LE/rabbit respectively. Meanwhile Net profit for CAL and CAL × RX were 8.68 and 10.34 (LE/rabbit) and for 0, 0.3 and 0.5% formic acid were 4.87, 10.11 and 13.72 LE/rabbit on the side of economic efficiency measurements the Net profit/ total returns were significant at $p \leq 0.05$ for CAL and CAL × RX were 0.15 and 0.18 and for 0, 0.3 and 0.5% formic acid were 0.09 , 0.17 and 0.22 that indicated the economic importance of using formic acid at 0.5% for rabbits.

Conclusion

The dietary supplementation with formic acid at 0.5% of diet has been associated with an improvement in growth traits, health, immunity and antioxidant blood parameters of summer stressed CAL and CAL × RX rabbits without any negative effect on carcass traits. In addition to, the economic efficiency was found to be higher in rabbits fed diet with 0.5% formic acid.

Significant genotype × dietary supplementation with formic acid interactions have been detected for ADFI, cholesterol, Hb, haematocrit percentage, glutathione peroxidase, dressing-out% ,total feed intake, total feed cost, net profit per rabbit and net profit/ total return.

Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

THE ROLE OF L. METHIONINE, L. CARNITINE, CHOLINE AND/OR SILYMARIN IN HEPATOPROTECTION AGAINST PARACETAMOL INTOXICATION AND OXIDATIVE STRESS IN BROILERS

Mustafa A. Aziz[1], Abu Elnasr A. Zahra[1], Zaghloul A. Kheder[2], Hend M. Fikry[2]*

[1]Department of Pharmacology, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt, [2]Animal Health Research Institute, Mansoura Provinical Lab, Egypt

*Corresponding author, E-mail: hendfikry999@gmail.com

Abstract: Paracetamol (Acetaminophen) was used for long time in poultry as an antipyretic drug and as a growth stimulator. However, high doses of paracetamol cause unpleasant side effects such as hepatorenal toxicity as mirrored by depletion of glutathione reserve, increase lipid peroxidation and increase liver enzymes or even sudden death with lethal doses. Therefore, this study was designated to evaluate the ameliorative effect of L. methionine, L. carnitine, choline and/or silymarin on the hepatotoxic effect induced by high doses of paracetamol. The study was applied on 80 chicks (from 1 till 33 days old) in special cages divided into 8 groups each one contained 10 chicks. The 1st group was used as a control, while the next 2-5 groups were supplemented with L. methionine, L. carnitine, choline and silymarin, respectively with doses as recommended by NRC, the 5th group was supplemented with silymarin (1 g/kg diet), the 6th group was supplemented with a mixture of the 4 supplements, the 7th group (hepatic intoxicated group) was given paracetamol (650 mg/kg diet for 7 days), and the 8th group was administrated paracetamol with a mixture of the 4 supplements. Serum samples were collected to determine levels of lipid profile [triglycerides (TG) and total cholesterol (TC)], liver damage enzymes [alanine transaminase (ALT), aspartate transferase (AST)], lipid peroxidation marker malondialdehyde (MDA), and activity of antioxidant enzymes [glutathione reductase (GR), superoxide dismutase (SOD)]. The obtained results revealed that there was a significant improvement in all measured serum biochemical parameters and final body weight gain in the combined group (4 supplements + paracetamol) as compared to the paracetamol group. These data conclude that supplementation of poultry diets with L. methionine, L. carnitine, choline and silymarin can improve the negative effect of paracetamol through increasing the body weight gain and antioxidant activity of glutathione and superoxide dismutase and decreasing malondialdehyde, liver enzymes, cholesterol and triglycerides.

Key words: L. methionine; choline; L. carnitine; silymarin; hepatoprotection; broilers

Introduction

Paracetamol was used for long time as an antipyretic drug and as a growth stimulator. There was unpleasant side effects of paracetamol with

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high doses as hepatorenal damage (1). Toxic effect of paracetamol is caused by its toxic metabolite N-acetyl-P-benzoquinone imine which is normally conjugated with glutathione in liver and converted into mercapturic acid which is not toxic and excreted by kidney. High doses of paracetamol cause toxic metabolite accumulation which leads to depletion of glutathione reserve , increases liver enzymes levels in blood, lipid peroxidation and consequently leads to hepatic necrosis (2).

There are ongoing trends of improving poultry performance and productivity in ways that are different from antibiotics (3). Supplementing poultry diets with different amino acids improve health status of productive animals especially in organic poultry systems (4). Rapid growth, higher feed intake, lower feed conversion ratio and higher final body weight could be recently achieved by many natural supplements to poultry diets (5).

Methionine is an essential sulphur containing amino acid which is important to the health and growth of broilers either when given in natural or synthetic form (6). Poultry performance and body weight are affected by methionine supply but not by the methionine source. While, deficiency of methionine in poultry diets increased the redness value of post mortem tissue (7). High methionine supplemented poultry diet positively affects broiler growth performance and meat quality (8). On the other hand, broiler fed methionine supplemented diet for days then slaughtered and processed showed high meat quality as fresh smell and fresh red color during storage (9).

Choline is an important amino acid in poultry nutrition due to its stimulatory effect on growth. Higher choline concentration in poultry diets results in higher growth rate and average daily feed intake (10). Another study by Saunderson and Mackinlay (11) evaluated the effect of methionine and choline supplementation to poultry diets and found that low methionine level significantly decreased the growth rate and cystathionine beta-synthase enzyme acivity, while high choline level increased significantly choline oxidase enzyme activity.

L. carnitine is the main abdominal fat lowering supplementer in poultry diets beside its growth enhancement effect (12). The same results were obtained by Leibetseder (13) who found that feeding chickens with 500 mg L. carnitine lead to a reduction in the abdominal fat and serum and yolk cholesterol levels and feed conversion ratio but with increased body weight gain and poultry performance. L. carnitine lowers subcutaneous fat deposition through reducing fat metabolism enzymes activity such as glucouse -6- phosphate dehydrogenase, malic dehydrogenase, isocitrate dehydrogenase and lipo protein lipase (14).

It is well known that silymarin has a prominent hepatocyte protective effect in hepatic intoxication caused by ochratoxin A or paracetamol, via its anti-oxidative and anti-apoptotic effect as well as through increasing superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity and decreasing lipid peroxidation product malondialdehyde (15). Moreover, it has a performance enhancement effect as revealed by increasing daily feed intake and final weight gain (16).

This study was planned to investigate the potential ameliorative effect of L. methionine, choline, L. carnitine and silymarin on liver intoxication induced by paracetamol high doses through measuring serum AST, ALT, lipid peroxidation product (malondialdehyde), cholesterol and triglycerides levels, SOD and GPx activity. The effect of these 4 supplements on growth performance was also investigated.

Materials and methods

The experimental protocol was approved by the Animal Care and Use Committee of Faculty of Veterinary Medicine, Kafrelsheikh University.

A total number of 80 mixed sex Ross 308 broiler chicks at 1 day old age were obtained from a private poultry company at Kafrelsheikh Governorate, Egypt. All the chicks were immunized against both New Castle's disease on 7^{th} and 18^{th} days and Gumboro disease on 14^{th} day. The experimental birds were offered feed and water *ad libitum* for 33 days. A starter diet was available for the first 21 days then changed

to a grower diet till the end of the experiment (33 days). Diets were formulated to meet the supplement recommendations for broilers by the National Research Council with some modification according to updated nutrients specification of broiler chickens.

Chicks were divided into 8 equal groups. The 1st group was used as a control, while the next 2-5 groups were supplemented with L. methionine, L. carnitine, choline and silymarin, respectively with doses as recommended by NRC, the 5th group was supplemented with silymarin (1 g/kg diet), the 6th group was supplemented with a mixture of the 4 supplements, the 7th group (hepatic intoxicated group) was given paracetamol (650 mg/kg diet for 7 days, orally by gastric tube), and the 8th group was administrated paracetamol with a mixture of the 4 supplements. The doses were given each first 3 days of each week till 33 days age.

Body weight was recorded at the end of every week. Final body weight gain was calculated following this equation: final body weight gain = final weight– start weight.

Blood samples were collected from wing vein. Serum was prepared as previously described (17) and was used to determine SOD, GR, MDA, cholesterol, triglycerides, ALT and AST levels using commercially available kits and as previously described (18-20).

Data were analyzed using One Way ANOVA test with post Hoc Tukey test was used to compare between groups using IBM SPSS software package version 20.0. Quantitative data were described using mean ± standard error. Significance of the obtained results was judged at $p \leq 0.05$.

Results and discussion

Effect of the 4 supplements on final body weight gain

The present study showed that paracetamol group showed a significant decrease in the final body weight gain as compared to the control group. While L. methionine, L. carnitine, choline and the 4 supplements combined groups showed a significant increase in the final body weight compared to the control group. Moreover, chicken administrated the 4 supplements and paracetamol showed a significant increase in final body weight compared to the paracetamol group without statistical change relative to the control group (Table 1). This means that these 4 supplements had the ability to improve the reduced body weight induced by paracetamol. In consistence with our findings, Jahanian and Khalifeh-Gholi (21) also reported that supplementation of broiler diets with methionine at NRC recommendation levels Ross 308 broiler chicks led to increasing feed conversion ratio and final body weight.

Effect of the 4 supplements on triglycerides and total cholesterol

The present study reported that paracetamol group showed a significant increase ($p<0.001$) in TG and TC levels as compared to the control group (Table 1). L. methionine, choline, L. carnitine and combined groups showed a significant decrease in TG and TC levels, with best effect for combined group, as compared to the control group. Moreover, chicken administrated the 4 supplements and paracetamol showed a significant decrease ($p<0.002$) in TG and TC levels as compared to the paracetamol group without statistical change relative to the control group (Table 1). This means that these 4 supplements had the ability to reduce the elevated TG and TC levels triggered by paracetamol. In agreement, Jahanian and Ashnagar (22) reported that chicks fed diet supplemented with choline and L. carnitine had a decreased feed conversion ratio and leg fat content and total plasma lipid level. Similarly, Khajali and Khajali (23) also found a decreasing in total plasma cholesterol and abdominal fat deposition in chicks fed diet supplemented with 200 mg /kg of L. carnitine.

Effect of the 4 supplements on ALT and AST

The paracetamol group showed a significant increase ($p<0.001$) in ALT and AST levels as compared to the control group. While chicks fed on diet supplemented with the 4 supplements each alone or in combination, with best effect for combined group, showed a significant decrease level of these two liver enzymes as compared to the control group. Moreover,

chicken administrated the 4 supplements and paracetamol showed a significant decrease ($p<0.001$) in ALT and AST as compared to the paracetamol group without statistical change relative to the control group (Table 2). This indicates that these 4 supplements had the ability to decrease the elevated AST and ALT elevated by paracetamol. In support, Tedesco, et al. (24) also reported a similar anti-hepatotoxic effect for silymarin (600 mg/kg) as revealed by reduction in AST and ALT level in chicks. Additionally Selvan et al. (25) reported that broiler diets deficient in choline resulted in elevation of liver enzymes and liver histopathology showed many abnormalities and fatty liver.

Table 1: Effect of the 4 supplements on final body weight gain and lipid profile parameter

	Final body weight gain (g)	Triglyceride (µg/dl)	Cholesterol (µg/dl)
Control	1802.0± 39.6 [b]	17.04± 0.77 [b]	31.56± 2.29 [b]
L. methionine	1971.0± 29.7 [a]	13.88± 0.48 [c]	24.92± 2.05 [c]
Choline	1946.0± 59.4 [a]	12.54± 1.24 [c]	23.72 ±3.73 [c]
L. carnitine	1932.6± 24.6 [a]	12.82± 0.94 [c]	25.34± 2.31 [c]
Silymarin	1924.0± 22.2 [a]	16.32± 0.78 [b]	28.08± 3.45 [b]
4 supplements	1994.0± 50.3 [a]	12.10± 1.68 [c]	20.44± 2.33
Paracetamol	1578.0± 25.9 [c]	21.08± 0.86 [a]	41.92± 2.69 [a]
4 supplements + paracetamol	1741.0 ±46.7 [b]	16.66± 0.79 [b]	31.02 ±2.18 [b]

Data are presented as mean ± SEM. Means carrying different superscript letters are significantly different at $p \leq 0.05$

Table 2: Effect of the 4 supplements on liver damage enzymes and oxidant/antioxidant status

	ALT (U/L)	AST (U/L)	GR (U/L)	SOD (U/L)	MDA (nm/ml)
Control	36.58± 3.39 [b]	132.81± 3.37 [b]	51.86± 1.7 [b]	65.30± 1.63 [b]	6.24± 0.75 [b]
L. methionine	28.68± 4.16 [c]	118.28± 5.54 [c]	61.54± 2.8 [a]	69.90± 1.88 [a]	4.84± 0.36 [c]
Choline	28.16± 3.72 [c]	117.32 ±5.18 [c]	59.08± 2.03 [a]	72.48± 2.85 [a]	6.06± 0.27 [b]
L. carnitine	27.14± 4.74 [c]	113.26± 5.04 [c]	58.26±2.89 [a]	69.90± 3.15 [a]	6.18± 0.36 [b]
Silymarin	28.68± 4.46 [c]	118.82± 3.80 [c]	59.12± 3.21 [a]	70.32± 2.09 [a]	6.08± 0.30 [b]
4 supplements	16.12± 4.27 [d]	83.96± 4.89 [d]	61.5 ±3.11 [a]	71.98± 3.29 [a]	5.16 ±0.65 [c]
Paracetamol	69.78± 3.42 [a]	174.74± 5.8 [a]	40.34 ±3.16 [c]	47.56± 6.80 [c]	8.96 ±0.69 [a]
4 supplements + paracetamol	40.46± 4.43 [b]	135.04± 4.73 [b]	49.0± 1.8 [b]	62.08± 4.79 [b]	6.72 ±0.48 [b]

Data are presented as mean ± SEM. Means carrying different superscript letters are significantly different at $p \leq 0.05$

Effect of the 4 supplements on glutathione reductase and superoxide dismutase

Chick fed diet supplemented with paracetamol exhibited a significant decrease in serum level of glutathione reductase (GR) and superoxide dismutase (SOD) as compared to the control group (Table 2). While L. methionine, choline, silymarin and combined groups showed a significant increase in these two enzymes, with best effect for combined group, as compared to the control group. Moreover, chicken administrated the 4 supplements and paracetamol showed a significant increase in GR and SOD as compared to the paracetamol group without statistical change relative to the control group. This indicates that these 4 supplements had the ability to increase the activity of antioxidant enzymes that was increased by paracetamol. Consistent with these results**,** Zhang, et al. (26) denoted that increasing methionine level above NRC recommendation for 500 male broiler chickens from one day old to 26 days of age led to increasing total glutathione and reduced glutathione in blood and breast muscle. Also, Jankowski, et al. (27) reported that increasing methionine level in broiler diets resulted in a significant increase in glutathione concentration and decreasing both malondialdehyde and plasma triglycerides levels. Furthermore, Ruan, et al. (28) reported that 120 one day old broilers supplemented with methionine deficient diet resulted in decreasing SOD, catalase and glutathione peroxidase. Similarly, Wang, et al. (29) reported that adding L. carnitine at a dose of 100 mg/kg to broilers diet decreased MDA level in heart tissue, triglycerides content and increased SOD and GR.

Effect of the 4 supplements on malondialdehyde

The paracetamol group showed a significant increase in malondialdehyde (MDA) level as compared to the control group. Only L. methionine and combined groups showed a significant decrease in MDA relative to the control group. Moreover, chicken administrated the 4 supplements and paracetamol showed a significant decrease in MDA as compared to the paracetamol group without statistical change relative to the control group (Table 2). In agreement, Park, et al. (30) also reported that increasing methionine level during first 28 days of age of chicks resulted in decreasing plasma MDA and increasing total plasma glutathione level. In support, Mohammadi, et al. (31) also recorded that methionine supplementation in broilers at 1-42 days of age at a concentration of 80 mg/kg of diet decreased plasma MDA level, reduced abdominal fat and plasma cholesterol level.

Conclusion

The present study reported that supplementation of poultry diets with L. methionine, L. carnitine, choline and silymarin improved chicken final body weight gain and enhance the negative impact of paracetamol through increasing the activity of glutathione reductase and superoxide dismutase and decreasing malondialdehyde, liver enzymes, cholesterol and triglycerides.

Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

CHARACTERIZATION OF CLASS 1 INTEGRONS AND SOME ANTIMICROBIAL RESISTANCE GENES IN *Salmonella* SPECIES ISOLATED FROM POULTRY IN EGYPT

Safaa M. Shabana[1]*, Salwa M. Helmy[2], Abd El-Halem M. Hegazy[3]

[1]Department of Microbiology, Animal Health Research Institute, Kafr El Sheikh branch, Agriculture research center, Giza, Egypt, [2]Department of Bacteriology, Mycology and immunology, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt, [3]Department of Poultry Disease, Animal Health Research Institute, Kafr El Sheikh branch, Agriculture research center, Giza, Egypt

*Corresponding author, E-mail: sofy.shabana@yahoo.com

Abstract: *Salmonella enterica* includes serotypes that were implicated as a food borne pathogens crucially affecting public health and the economic organization. This study was directed to isolate and identify of *Salmonella* strains from 222 different species and ages of poultry (broiler, chick, ducks, pigeon, quails) from Kafr El Sheikh governorate. The Salmonella isolation rate was (4.5%) as (0.9%) from apparently healthy, (3.6%) from diseased birds. The outer membrane protein F gene was used as promising tool for detection of Genus *Salmonella*, after that four isolates were identified serologically as two *Salmonella enterica* serovar Enteritidis and two *Salmonella enterica* serovar Typhimurium. The resistance pattern of positive Salmonella isolates showed multidrug resistance phenotypes and *qnrS* for quinolone resistant genes was recorded in one isolate while *bla*TEM for β-lactam resistant isolates, *aacC* for aminoglycosides were recorded in all four *Salmonella* isolates using PCR technique. Also, Class 1 integrons detected with a percentage of (100%) in examined isolates. Sequencing of the class 1 integrons cassettes showed genes encoding resistance specified to streptothricin acetyltransferase (*sat*) gene, aminoglycoside acetyltransferase (*aac3-Id*) and aminoglycoside adenyltransferase (*aadA7*). Class 1 integrons harbored gene encoding domains unfunction protein (*duf*) in one *S. enterica* serovar Typhimurium isolate. This study spotlights the significant role of the drug–resistance genes and Class 1 integrons in *Salmonella* as zoonotically important pathogens of public health importance.

Key words: poultry; *Salmonella*; drug- resistance genes; integrons gene cassettes

Introduction

Salmonella include approximately 2500 serovars. *Salmonella enterica* represents the most of the *Salmonella* serovars and *Salmonella enterica* serovar Enteriditis was the most popular serovar with a zoonotic effect, then *Salmonella enterica* serovar Typhimurium (1). Globally, *Salmonella enterica* subsp. *Enterica* included serotypes that have economically and public health significantly effects (2). The most non-typhoidal salmonellosis (NTS) cases related to consuming of contaminated animal

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origin foods, especially fowl, meat and in some cases vegetables (3). Poultry considered an important reservoir of many zoonotically important pathogens, such as Salmonella, which acted as a prime importance (4).

The pore-forming proteins of Salmonella and other Gram negative bacteria outer membrane (OM) called porins (5). Among OMPs (outer membrane proteins), the outer membrane protein F (*ompF*) and outer membrane protein C (*ompC*) were the most types porins that represented 2% of the total porins, and *ompF* was the most ideal structural and functional characterization porin protein (6). Also, the *ompF* gene was used as a promising tool for detection of Salmonellae where it could discriminate genus Salmonella from other non-Salmonella organisms in clinical samples (7).

Multidrug resistant (MDR) non-typhoidal *Salmonella* (NTS) might be transmitted from the poultry to human through the food series, whilst the antimicrobial resistance (AMR) could be carried among bacteria throughout the resistance genes associated with integrons and another mobile genetic elements as plasmids and transposons (8). Avian Salmonella showed resistance against many antimicrobial groups such as β-lactam, aminoglycosides and quinolones (9).

Salmonella species associated with *qnr* genes were isolated from the poultry field might cause a harmful effect on the public health because these could be transmitted to humans via poultry products or by contact with poultry and could rapidly increase fluoroquinolone resistance in various bacterial species through the transfer of plasmids harboring *qnr* genes. (10). The resistance to aminoglycosides as Gentamicin could confer using the aminoglycoside acetyltransferase (*aac*) genes which were detected in numerous isolates of Salmonella (11).

The class 1 integrons played a character in the presence of AMR in *Salmonella enterica* which might isolate from broilers, meat and hogs products (12). Class 1 integrons, the most communal integron located on Salmonella genomic island 1 (SGI 1), was found in various Salmonella serovars, including S. enterica serovar Typhimurium; *S. enterica* serovar Newport and S. enterica serovar Oslo. (13).

The classes of integrons which might be recovered from GenBank were nine, but the first four categories had been sustained only. Cass 1 integrons was widely distributed among the family Enterobacteriaceae organisms (14). These integrons include two conserved segments (5′ CS and 3′ CS) separated by a variable region that normally comprises one or more gene cassettes. Integrons encompass three important parts: an integrase gene (*IntI1*); an adjacent attachment site (attI1) and a promoter region (PC) (15). An open reading frame (ORFs) where a specific site containing a modular structure called gene cassettes (16). The collection of gene cassettes (up to nearly half a dozen) had related the integrons with MDR (17).

This study highlighted the importance of the strains of Salmonella, which isolated from different species and age poultry as zoonotically important pathogen, some antimicrobial drug resistance genes of Salmonella species and class 1 integrons gene cassettes in this public health importance organism.

Materials and methods

Collection of samples and isolates characterization

In this work, which was conducted from April 2017 to April 2018, a total of 222 apparently healthy (56) and diseased (166) from different species and ages of poultry (Broiler, chick, ducks, pigeon, quails) from the Kafr El Sheikh governorate. The internal organs (liver, spleen, gall bladder, ileocaecal tonsil, yolk sac), cloacal swabs and the pooled samples. Samples were transferred to the laboratory in an ice tank within 2hours for bacteriological isolation and identification (18), then confirmed biochemically by the API 20E system.

Serological identification

Biochemically suspected isolates were serotyped according to Kauffman (19) at Serological unit in Institute of Animal Health Research,Giza, Egypt.

Identification of genus Salmonella using ompF gene

Programming of PCR to amplify *ompF* gene was used as promising tool for detection of genus *Salmonella* was done according to Tatavarthy and Cannons (20) using oligonucleotide primers in Table 1.

Antimicrobial susceptibility

The susceptibility test of samples were done as Finegold and Martin, (21). A total of 11 antimicrobial discs was used for sensitivity (Oxoid) were Amoxicillin–clavulanic acid (AMC), 30 µg; Cefotaxime (CTX), 30 µg; Ceftazidime (CAZ), 30 µg; Chloramphenicol (C), 30 µg; Ciprofloxacin (CIP), 5 µg; Gentamicin (CN), 10 µg; Nalidixic acid (NA),30 µg; Spectinomycin (SH), 10 µg; Colistin (CT), 10 µg; Norfloxacin (NOR), 10 µg and Doxycycline (DO), 30 µg. Interpretation as resistant, moderately susceptible or susceptible as recorded in the Clinical and Laboratory Standards Institute CLSI (22).

Molecular analysis of antimicrobial resistance genes

The DNA extraction was done using QIAamp DNA Mini Kit (Catalogue no. 51304) according to manufactures' guidelines. The primer sequences for detection of *aacC* gene (encoded for aminoglycoside resistance) (23), *qnrS* gene (encoded for quinolones resistance) (24), *blaTEM* gene (encoded for β-lactams resistance) (25) and class 1 integrons gene cassettes (26) (Table 1).

Sequencing screen for class 1 integrons gene cassettes

QIAquick kit. (Qiagen Inc. Valencia, CA): It was used for purification of the PCR product from 1.5 % agarose gels. Applied Biosystems 3130 automated DNA Sequencer *(ABI, 3130, USA)*. Identification similarity of nucleotide and amino acid sequences between *Salmonella* strains and other Enterobacteriacae recorded in GenBank was done using (National Center for Biotechnology Information "NCBI"). Using the BioEdit sequence alignment editor for comparisons of the nucleotide sequences (27). Phylogenetic analysis was done using ClustalW (http://www.ebi.ac.uk/clustalw/).

Results

The incidence of Salmonellae from different samples

The obtained results of *Salmonella* isolation revealed that 10 (4.5%) were positive for *Salmonella* identified biochemically out of 222 examined birds, The isolation rates from chicken, ducks and quails were 8 of 156 samples (5.1%), 1 of 35 samples (3.2%) and 1 of 2 samples (50%), respectively, while could no isolation of *Salmonella* from chick and pigeon samples. The positive biochemically *Salmonella* isolates from different samples represented in 4 out of 100 (4%), 2 out of 51 (3.9%), 2 out of 36 (5.5%), 1 out of 19 (5.2%) and 1 out of 2 (50 %) from cloacal swabs, pooled samples, liver, gall bladder and yolk sac samples respectively, while the isolation from the spleen and ileocecal tonsil samples was negative for Salmonellae (Table 2).

Serotyping of Salmonella isolates

Four isolates from ten biochemically positive suspected *Salmonella* isolates were classified under two different serotypes, including two *Salmonella enterica* serovar Enteritidis were isolated from cloacal swab of chicken and duck and *Salmonella enterica* serovar Typhimurium isolated from the quail yolk sac and chicken liver samples.

Antimicrobial susceptibility

Salmonella isolates showed resistance to Gentamycin, Ciprofloxacin, Doxycycline, Spectinomycin and Colistin with (50%), however, showed sensitive to Ceftazidime with (100%), followed by Cefotaxime by (75%), amoxicillin clavulinic acid, Nalidixic acid, Chloramphenicol and Norofloxacine with (50%) (Table 3). Two non-typhoidal *Salmonella* isolates showed multidrug resistant (MDR) phenotypes to five different antibiotic classes (Table 3).

Detection of genus Salmonella using OmpF gene by PCR

All examined *Salmonella* isolates was positive at 519 bp of *ompF* using the PCR technique with a percentage of (100%) (Fig. 1).

Antimicrobial resistance encoding genes

The phenotypic antimicrobial resistant *Salmonella* isolates was analyzed by PCR technique to key out some resistance coding genes. The positive percentage of *qnrS* gene for quinolone resistant was (25%), where *bla*TEM for β-lactam resistant gene, *aacC* for aminoglycosides resistant gene and Class 1 integrons were (100%) (Fig. 1).

Class 1 integrons sequencing of the variable amplicons showed the gene cassettes containing streptothricin acetyltransferase (*sat*) gene encoding resistance against Streptothricin (an early aminoglycoside) in two *Salmonella* serovars isolated in the current work, but aminoglycoside acetyltransferase (*aac(3)-Id*) and aminoglycoside adenyltransferase (*aadA*7) genes which encoding resistance against Gentamycin and to streptomycin and spectinomycin, respectively in isolate of *S. enterica* serovar Typhimurium only. One *S. enterica* serovar Typhimurium isolate Class 1 integrons harbored gene encoding domains of unknown function protein (*duf*).

Table 1: Oligonucleotide primers used for detection of *ompF* , antimicrobial resistance coding genes (*aacC, qnrS* and *bla*TEM) and class 1 integrons cassettes

Gene	Primer sequence (5'-3')	Length of amplified product	Reference
ompF	Forward- CCTGGCAGCGGTGATCC Reverse- TGGTGTAACCTACGCCATC	519 bp	Tatavarthy and Cannons,(20)
aacC	Forward- GGCGCGATCAACGAATTTATCCGA Reverse- CCATTCGATGCCGAAGGAAACGAT	448 bp	Lynne et al., (23)
qnrS	Forward- ACGACATTCGTCAACTGCAA Reverse- TAAATTGGCACCCTGTAGGC	417 bp	Robicsek et al., (24)
*bla*TEM	Forward- ATCAGCAATAAACCAGC Reverse- CCCCGAAGAACGTTTTC	516 bp	Colom et al., (25)
class 1 integron cassettes	Forward- GGC ATC CAA GCA GCA AG Reverse- AAAG CAG ACT TGA CCT GA	Variable	Sow et al., (26)

Table 2: The incidence of Salmonellae isolated from different organs and identified biochemically

Poultry species	Organs samples							Samples No.	positive samples	
	Cloacal swab	*Pooled samples	liver	Spleen	Gall bladder	Yolk sac	iliocecal tonsil		No.	%
Chicken	67	34	29	7	17	-	2	156	8	5.1%
Chicks	6	5	2	2	-	1	3	19	-	0%
Duck	19	10	4	-	2	-	-	35	1	3.2%
Pigeon	8	2	-	-	-	-	-	10	-	0%
Quails	-	-	1	-	-	1	-	2	1	50%
Total	100 (4%)	51 (3.5%)	36 (5.5%)	9 (0%)	19 (5.2%)	2 (50%)	5 (0%)	222	10	4.5%

*Pooled samples from different organs of poultry submitted to Kafr El Sheikh lab. For examination

Table 3: Antimicrobial resistance patterns, resistance genes and class 1 integron profiles of *Salmonella* serotypes in this study

NO	Serovars (source of isolates)	Resistance pattern	**MDR isolates N (%)	Resistance genes	Integron amplicon size (bp)	Genes cassettes	Accession numbers
1	S. *enterica* serovar Enteritidis (duck)	CT	-	*bla*TEM, *aac*C	+	-	-
2	*S. enterica* serovar Enteritidis (chicken)	CN, DO, C, CT, CIP	+	*bla*TEM, *aac*C	600	*sat*	MK335377
3	*S. enterica* serovar Typhimurium (quail)	SH	-	*bla*TEM, *aac*C	800	*duf* gene	MK359461
4	*S. enterica* serovar Typhimurium (chicken)	CN, NOR, DO, AMC, CIP, SH	+	*bla*TEM, *aacC*, *qnr*S	650	*sat*	MK349001
					1600	*aac3-Id,aadA7*	MK349002
					1800	*aadA7*	MK359462
	Total		2(50%)				

**Multidrug resistant (MDR) *Salmonella* isolates were 2(50%) to five different antibiotic classes

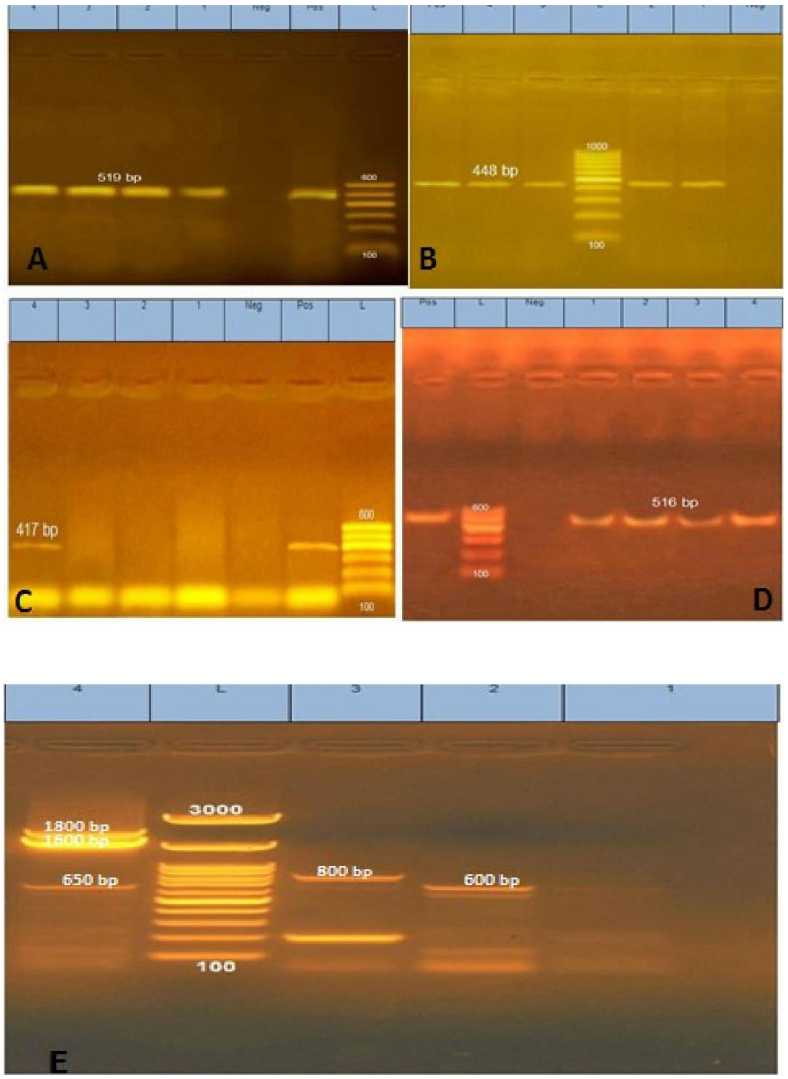

Figure 1: PCR amplification of the different genes in this study; "Pos" stands for positive control, "Neg": Negative control; L: 100 bp DNA ladder; Lane (1, 2, 3, 4) examined *Salmonella* isolates. A. *ompF* gene(519 bp). Resistance associated genes, B. *aacC* gene (448bp). C. *qnr*S gene (417bp). D. *bla*TEM gene (516 bp). E. Class 1 integrons at variable sizes in *Salmonella* isolates

Discussion

Salmonella represents a critical problem to livestock in countries where not interest to the control measures or in those where the environmental conditions help in the development of these microorganisms (28).

In the present study, *Salmonella* spp. were isolated and identified from different species and ages of poultry and molecular characterized for many important antimicrobial resistance genes and class 1 integrons of *Salmonella* species.

The results indicated that 10 (4.5%) isolates out of 222 examined bird suspected to be *Salmonella* isolates from 166 diseased birds and 56 apparently healthy birds with the percentage of (3.6%) and (0.9%), respectively by phenotypic and biochemical characterization that agree with report in Egypt where 4.4% were positive for *Salmonella* isolated from poultry farms (7), but higher than those of *Salmonella* isolation from small poultry farms with (1.6%) in California (29), and lower than (8.65%) of *Salmonella* isolated from poultry (30) .

The consequence of isolation appears to be high from the diseased bird than apparently healthy bird 8vs 2, although the samples were gathered up from each of diseased and apparently healthy birds together. These variations in the overall prevalence of *Salmonella* may be related to several factors such as environment, hygienic conditions of the farm and health status of the examined bird (31, 32) which leading to the bird become weaker and therefore are easily infected by *Salmonella*. Similarly, *Salmonella* was isolated from apparently healthy chickens lower than from diseased chickens in Shanghai and in Egypt (32, 33).

Currently, the isolation percentages from chicken, ducks and quails were 8 of 156 samples (5.1%), 1 of 35 samples (3.2%) and 1 of 2 samples (50%), respectively, were positive for *Salmonella* strains while the chick and pigeon samples were negative for Salmonellae which are not compatible with (7.25%) *Salmonella* incidence from chickens and (15.55%) of pigeons (30) and also with percentage (6%) in ducks in Egypt (34).

The high *Salmonella* isolation rate of liver and gallbladder samples 2 of 36 samples (5.5%), 1 out of 19 samples (5.2%), respectively agrees with the highest rate of *Salmonella* isolation from liver samples (35, 36). All spleen and ceca samples were negative for Salmonellae that agree with another study on *Salmonella* was not isolated from spleen samples (37) but also, disagree with those isolated the highest *Salmonella* percentage from spleen samples in Egypt (38).

It is common knowledge that the cloacal swab is considered a particular signal of incessant intestinal colonization in poultry, but its diagnostic accuracy is minimized where the *Salmonella* infected birds are intermittent shedding via feces (39).

In present the study the four isolated Salmonellae were classified under two different serovars, *Salmonella enterica* serovar Enteritidis and Typhimurium with a percentage of 2 of 4 (50%) for each.

The difference between the results of serological and bacteriological examination to identify *Salmonella* assigned to *Salmonella* give identical colony morphology on S.S agar and biochemical reactions with the other members of the family Enterobacteriaceae and this difference consistent with the opinion of there are problems in the biochemical identification reactions (40). Similarly, there were differences in the identification of *Salmonella* spp. as used conventional techniques was (10.5%), the API 20E system was (9%) and by serotyping was (7.8%) (35).

The serological identification result referred to an isolation of two serotypes, *Salmonella enterica* serovar Typhimurium and Enteritidis, similar that reported in central Ethiopia (41) and Egypt (42) where they isolated only *Salmonella enterica* serovar Enteritidis and Typhimurium, but disagrees with a previous study on *S. enterica* serovar Enteritidis isolated from commercial layer farms (43).

The phenotype antimicrobial resistance result was resistant to (CN), (CIP), (DO), (SH) and (CT) with a percentage (50%). Moreover, the isolates were sensitive to (CAZ) with (100%), followed by (CTX) with (75%) then

(AMC), (NAL), (C) and (NOR) with (50%). These results concur with study reported that the resistance to Gentamycin was observed in (39.58%) (44) and those reported that the resistance to Tetracycline, Ciprofloxacin and Spectinomycin was (51.9%), (48.7%) and (34.4%), respectively (45), but disagree with those reported that Gentamycin inhibited to all *Salmonella* strains and resistance of Ciprofloxacin with a percentage (10%) (46).

The high sensitivity to Ceftazidime (100%), followed by Cefotaxime (75%) in the present work agrees with the previous reports described a low Cephalosporin resistance prevalence of *S. enterica* serovar Enteritidis in Kohat and Egypt (47, 48). Two non-typhoidal *Salmonella* isolates (50%) showed multidrug resistant (MDR) phenotypes to at least five various antibiotic types which similar with another study reported that the multidrug resistant *Salmonella* isolates represented 55% in Malaysia (49).

The outer membrane protein F (*ompF*) gene detected in the examined isolates in this current study with a percentage of (100%) using the PCR technique. The *ompF* gene considers a good tool for fast identification of *Salmonella*, so *ompF* mutation or loss might lead to mistakes in the identification analyze of *Salmonella* strains (20). Similarly, using the *ompF* gene as a tool for detection of *Salmonella* genus in Egyptian poultry farms (7).

Poultry acts as a carrier of multidrug resistant *Salmonella* and this no related to resistance genes presence, so other acquiring resistance mechanisms might be present (50). The detection result of resistance coding genes (*bla*TEM and *aacC*) was (100%) and this disagrees with a previous report detected *bla*TEM in *S. enterica* serovar Typhimurium isolates only in Japan (51), also with another report detected *aacC* gene with (30%) of *Salmonella* isolates in broiler in Egypt (52). The result of the current study similar to another study detected the *bla*TEM gene with (93.3%) in *Salmonella* isolates obtained from commercial layers in Egypt (31). The *qnrS* gene, a gene quinolone resistant was reported in the present work with the percentage of (25%) that parallel with the result of another study in Egypt (31).

The differences in phenotypic-genetic antibiotic resistance results recorded in this study of *Salmonella* isolates was also registered in other reports (53), and was usually mentioned to either existence of resistance alternative mechanisms or defect in the resistant genes expression .

The result of Class 1 integrons detection was (100%) of this work, similarly, the result of Class 1 integrons detection of *Salmonella* isolated from Egypt (54).

The *sat* gene was detected within class 1 integrons of *S. enterica* serovar Enteritidis (chicken, 600bp) and *S. enterica* serovar Typhimurium (chicken, 650bp) in this investigation was preceding identity in *S.* Typhimurium (KT449570) in Egypt (54), *S.* Choleraesuis (EU834941) in southern Taiwan (55), other family Enterobacteriaceae organisms class 1 integrons as, *Shigella sonnei* from western Ireland (AY090896) (56), *E.coli* plasmid (CP022735) (57), and other bacteria as, in *Vibrio alginolyticus* plasmid (KU160531) (58). The *aac (3)-Id* and *aadA7* genes had been identified in class 1 integrons gene cassettes of (*S.* Typhimurium, chicken) showed a preceding identity in class1 integrons of *S.* Derby (KT427378), *S. enterica* (KT581256) in Egypt (54).

In the current investigation, the detection of *sat*, *aac (3)-Id* and *aadA7* genes within class1 integrons of isolated *Salmonella* may be related to the extensive using the aminoglycoside antibiotics group in poultry farms.

The domains of unknown function protein (*duf*) gene was identified in class 1 integrons gene cassettes of (*S.* Typhimurium, quail) in the current work, which difficult to decide its function due to lack of its protein sequences identity with interpreted biochemical function. The *duf* gene represents more than (20%) of all protein domains (59).

The class 1 integrons cassettes sequencing of the two isolated *Salmonella* serovars in this current investigation were documented into the GenBank with accession numbers (MK335377); (MK349001); (MK349002), (MK359462) and (MK MK359461).

Conclusion

Poultry acts as the important reservoir of many zoonotically important pathogens, such as *Salmonella* and detection of resistance genes related to significant antimicrobial drugs which used in the medical establishments. Integrons cassettes carrying antimicrobial resistance genes in *Salmonella* have an important role in the spreading of AMR so, the strategy used to control of using of antimicrobial drugs against this organism as well as other emerging pathogens of public health importance should be improved.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

THE ROLE OF THYMOQUINONE IN AMELIORATING THE HEPATOXIC EFFECT OF DIAZINON IN MALE RATS

Emad W. Ghazy[1], AbdAllah A. Mokh[1], Doaa H. Abdelhady[1], Wael M. Goda[2], Eman M. Hashem[1*]

[1]Department of Clinical Pathology, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt,
[2]Department of clinical Pathology, Faculty of Veterinary Medicine, Damanhur University, Egypt

[*]Corresponding author, E-mail: eman.amr123.eh@gmail.com

Abstract: Diazinon (DZN) is one of most dangerous hepatotoxic organophosphorous insecticides used in veterinary practices which induces oxidative stress. The present study aimed to evaluate the ameliorative role of thymoquinone (TQ) in diazinon toxicity. Forty nine male albino rats and were divided into seven groups (n =7 for each). The first group (c-ve group) orally received saline daily all over the experiment (8 weeks). The second group (c+ve1) orally received DZN (15 mg/kg body weight, b-w) for the first four weeks and saline for the next four weeks. The third group (c+ve2) orally received saline for the first four weeks and DZN (15 mg /kg b-w) for the following four weeks. The fourth group (DZN-TQ 10 mg group) received DZN (15 mg/kg b-w) daily for the first four weeks then TQ (10 mg/kg b-w) for the next four weeks. The fifth group (TQ 10 mg-DZN) orally treated with TQ (10 mg/kg b-w) and DZN (15 mg/kg b-w) for the next for weeks. The sixth group (DZN-TQ 5 mg group) received DZN (15 mg/kg b-w) for first four weeks and TQ (5 mg/kg b-w) for the following four weeks 5 mg. The seventh group treated by TQ (5 mg/kg b-w) daily for the first four weeks and DZN (15 mg/kg b-w) for the following four weeks (TQ 5 mg-DZN group). DZN intoxicated groups showed macrocytic hypochromic anemia and serum biochemical alteration related to liver injury, including elevation of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and a significant decrease in total protein and albumin. Furthermore, these groups also exhibited a significant increase in liver tissue malondialdehyde (MDA), significant decrease in total antioxidant capacity (TAC) and catalase (CAT) activities and over-expression of the two apoptotic hepatic genes *Bax* and caspase 3. Administration of DZN also resulted in hepatic vacuolation, necrosis and congestion of hepatic sinusoids. Thymoquinone ameliorated the most deleterious effect of diazinon on hematological, biochemical, antioxidant, molecular and histopathological parameters in a dose dependent manner and a prophylactic strategy is better than therapeutic one.

Key words: diazinon; thymoquinone; oxidative stress; hepatotoxicity

Introduction

Organophosphorous are still used as pesticides in agriculture, industry and in animal care as insecticides to control external parasites on

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animals all over the world. However, they are the most toxic pesticides to all animal species and human (1). The improper use of these pesticides is dangerous on human being and environment (2, 3). Organophosphorus insecticide residues are detectable in grains, crops, soil and some food products. Diazinon (DZN) considered as one of the most important organophosphorous compounds used in agriculture practices to control external parasites and flying insects of food crops and ornamental plants (4). It is also used to control the external parasites in veterinary practices. It was found that the main mechanism of action of DZN is inhibition of acetyl choline esterase enzyme (5). Furthermore DZN increased the level of malondialdehyde and lipid peroxidation biomarker in male rat (6).

Nowadays, the interest for using natural products for pharmacological purposes was increased. As this natural products gaining acceptance from the public, due to their useful effect on treatment of many disease without side effects which caused by many chemical products (7-9). *Nigella sativa* commonly known as black seed and black cumin. The essential oil and fixed oil of *Nigella sativa* showed powerful action in treatment of respiratory and gastrointestinal diseases (10). Furthermore, *N. sativa* has anti-oxidant, anti-inflammatory and hepatoprotective activities (11). Thymoquinone (TQ) is the most potent active constituent in the volatile oil of seeds of *N. sativa*. The possible mechanisms of TQ action including suppression of protein kinase B activation (Akt/PKB) by dephosphorylation (12), induction of apoptosis in cancerous cells by increasing apoptotic genes expression and decreasing anti-apoptotic gene expression (13, 14), induction of cytokines production (15) and activation of anti-oxidant enzymes (16). As result of these different beneficial effects of thymoquinone, TQ has various therapeutic effects such as anti-inflammatory effect (17), immune modulatory effect (18), free radical scavenger, antioxidant effect (19), anti-carcinogenic effect (20), apoptotic effect (21) and hepatoprotective effect by inhibiting lipid peroxidation (22). For this reasons, the present study aimed to assess the role of thymoquinone in alleviating the harmful effect of diazinon toxicity in male albino rats.

Materials and methods

Chemicals

Ectodat 60 EC, commercial formulation containing 60% of active ingredient (diazinon), was purchased from Kemet Company which imported the product from Jorden Company ATI. Thymoquinone was obtained from Sigma-Aldrich Company (product of china origin), CAS 490-9-5C10H12O2 MW 164.201 g/mol.

Animals and treatments

Forty nine male albino rats, purchased from Animal House Colony of Alexandria Center institute, weighting average 70±5 g/rat. The rats were kept in well ventilated animal house with a controlled dark –light cycle (12 h light – 12 h dark) and constant temperature (26±2 ºC) food and water were provided *ad libitum*.

Rats were allowed to accommodate two weeks before the start of the experiment. They were randomly divided into seven groups (n=7 for each group). The first group (c-ve group) orally received saline daily all over the experiment (8 weeks). The second group (c+ve1) orally received DZN (15 mg/kg body weight, b-w) (23) daily for the first four weeks and saline for the next four weeks. The third group (c+ve2) orally received saline for the first four weeks and DZN (15 mg/kg b-w) for the following four weeks. The fourth group (DZN-TQ 10 mg) received DZN (15 mg/kg b-w) for first four weeks then TQ (10 mg/kg b-w) (24) for the next four weeks. The fifth group (TQ 10 mg-DZN) orally treated by TQ (10 mg/kg b-w) daily and DZN (15 mg/kg b-w) for the next four weeks. The sixth group (DZN-TQ 5 mg) received DZN (15 mg/kg b-w) daily and TQ (5 mg/kg b-w) (20) for the following four weeks. The seventh group (TQ 5 mg-DZN) treated by TQ (5 mg/kg b-w) for first four weeks then treated daily by DZN (15 mg/kg b-w) for the next four weeks.

Twenty four hours, after the end of the experiment, the blood samples were taken from retro-orbital venous plexus under effect of Di Ethyl Ether anesthesia and taken in aliquots

with and without anti-coagulant for hematological and biochemical analysis, respectively. Blood samples without anticoagulant were left at room temperature to clot, then were centrifuged at 3000 rpm for 15 min and the obtained were collected for biochemical analysis**.** Then rats were sacrificed by decapitation. The liver of each animal was rapidly removed, trimmed from excess tissues and washed by saline and distilled water. The liver samples divided into three parts; the first part used to preparation of tissue homogenate for determination antioxidant activities. The second part was cut into slices of about 0.1 g and directly put in liquid nitrogen then stored at -80˚C for molecular analysis. The last part was kept in 10% formalin to be used for histopathological examination. Animal rearing, handling and all experimental designs were approved by the Research Ethical Committee of faculty of veterinary medicine, Kafrelsheikh University, Egypt.

Hematological examinations

Aliquot containing EDTA (1mg/ml) were used for determination of complete blood count (RBC, WBC, MCH, MCHC, PCV, platelets count, and differential leukocytic count) by using Sysmex-Xp 300 automatic hematological analyzer (25).

Biochemical analysis

Serum levels of liver damage marker (ALT and AST) were determined as previously described (26). Determination of total protein and albumin were determined according to (27) and (28) methods respectively using Genway spectrophotometer instrument**.** Determination of glucose was done by (29) method**.**

Determination of oxidative stress/antioxidants

The dissected tissues were washed by sodium phosphate buffered saline (50 mmol/l) with EDTA (0.1 mmol/l) in ice to remove any RBCs and clots. The tissues were homogenized in 5 ml cold buffer /g of tissue then were centrifuged at 2000 rpm for 20 minutes. The resulting supernatant was collected and preserved in 80˚C for estimation of MDA (30), catalase (CAT) (31) and total antioxidant capacity TAC (32).

Histopathological examination

Tissue preparation and histopathological examination were performed as previously described (33)**.**

Molecular investigation

RNA extraction from hepatic tissue was applied using QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH) GmbH) following manufacture protocol. A 25 µl mixture containing 12.5 µl of the 2x Quantitect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 0.25 µl of Revert Aid Reverse Transcriptase (200 U/µL) (Thermo Fisher), 0.5 µl of each primer of 20 pmol concentration (Table 1), 8.25 µl of water, and 3 µl of RNA template was put in a Stratagene MX3005P real time PCR machine. Amplification curves and Ct values were determined by the Stratagene MX3005P software. To estimate the variation in gene expression, the Ct of each sample was compared with that of the positive control group according to the $2^{-\Delta\Delta ct}$ method (34).

Statistical analysis

For data analysis, Graph pad prism statistical package version 5.0 for windows was used. Differences in values were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple range test. All data expressed as mean ±SEM, and the level of significance were cited at $p \leq 0.05$.

Results

Hematological findings

Data explored in table (2) showed a significant ()$p \leq 0.05$ decrease in hematological parameters (RBCs, HGB and MCHC) and an insignificant decrease in (HCT, MCH and PLT) in DZN treated group (c+ve1). DZN c+ve2 group showed marked but not a significant decrease in hematological parameters (RBCs, HGB and PLT) and a significant decrease in HCT, MCH and MCHC. Significant ($p \leq 0.05$) increase in MCV and WBCs was noticed in

DZN treated groups as compared with the non-treated group (c-ve).

DZN-TQ 10 mg group showed insignificant decrease in WBCs and a significant ($p \leq 0.05$) increase in hematological parameters (RBCs, HCT, MCH and MCHC), similarly a marked increase in PLT but still in significant was also observed in DZN-TQ 10 mg group as compared with the DZN treated group (c+ve1). TQ prophylactic group (TQ 10 mg-DZN) showed marked increase in hematological parameters (RBCs, HCT, HGB, MCHC and PLT). Meanwhile, insignificant decrease in WBCs count was observed in this group as compared to the c+ve2 group. TQ therapeutic group (DZN-TQ 5 mg) showed a significant increase in RBCs and MCHC and a marked increase but still insignificant in HCT, HGB, MCH and PLT as compared with the c+ve1 group. TQ prophylactic group (TQ 5 mg-DZN) showed a marked increase in hematological parameters (HCT, HGB, MCH, MCHC and PLT) and a marked increase but still insignificant in RBCs as compared with the c+ve2 group.

Serum biochemical parameters

The harmful effect of diazinon toxicity and the useful effect of TQ on some biochemical parameters illustrated in table (2). Significant increase in serum level of (ALT and AST) in DZN treated groups (c+ve1 and c+ve2) as compared with non-treated group (c-ve). In contrast to this result, there was a significant decrease in total protein and albumin in DZN treated groups in comparison with c-ve group. Significant increase of glucose level in DZN treated groups as compared with c-ve group.

DZN-TQ 10 mg showed a significant decrease in serum level of ALT, AST, glucose and a significant increase in TP and albumin as compared with c+ve1 group. In TQ prophylactic group (TQ 10 mg-DZN) showed marked decrease in serum ALT, AST, glucose and a significant increase in TP and albumin as compared with c+ve2group. TQ therapeutic group (DZN-TQ 5 mg) showed a significant decrease in ALT, AST and glucose. Meanwhile, significant increase in TP and albumin as compared with DZN treated group (c+ve1). TQ prophylactic group (TQ 5 mg-DZN), showed a significant decrease in serum biochemical parameters (ALT, AST and glucose) as compared with DZN treated group (c+ve2).

Antioxidants biomarkers on liver tissues

The antioxidant effect of TQ on DZN toxicity was shown in (Fig.2). DZN toxic groups (c+ve1 and c+ve2) illustrated significant decrease in TAC, Catalase activities and significant increase in MDA as compared with c-ve group. TQ therapeutic group (DZN-TQ 10 mg) showed a significant increase in TAC, Catalase activities and significant decrease in MDA as compared by c+ve1. TQ prophylactic group (TQ 10 mg-DZN) showed a significant increase in TAC, Catalase activities and a significant decrease in tissue MDA as compared with (c+ve2). TQ therapeutic group (DZN-TQ 5 mg) showed marked decrease of MDA but still insignificant and a significant increase in TAC and Catalase activities as compared with c+ve1 group. TQ prophylactic group (TQ 5 mg-DZN) showed significant decrease in MDA and a significant increase in TAC, Catalase activities and as compared with c+ve2. Significant increase in TAC and CAT activities in TQ10 mg groups (prophylactic and therapeutic groups) as compared with TQ 5 mg groups. TQ prophylactic groups (TQ 10 mg-DZN and TQ 5 mg-DZN) showed a significant decrease in MDA as compared by TQ therapeutic groups (DZN-TQ 10 mg and DZN-TQ 5 mg).

Role of thymoquinone in ameliorating the histopathological alteration induced by diazinon

Data explored in (Fig.2) showed the histopathological alteration caused by DZN. Normal hepatocytes arranged in cords was observed in non-treated group (Fig.2A). DZN intoxicated group (c+ve1) showed marked congestion of hepatic sinusoids associated with atrophy of hepatocytes (Fig.2B), focal hepatic necrosis (Fig.2Cm D). DZN intoxicated group (c+ve2) showed marked periportal inflammatory cells infiltration (Fig.2E), diffuse hepatic vacuolation (Fig.2F) and focal hepatic necrosis

(Fig.2J). TQ reversed histopathological changes in liver tissues caused by DZN. (DZN-TQ 10 mg) showed mild degree of granular hepatic vacuolation (Fig.2H) as compared with (c+ve1) group. (TQ 10 mg-DZN) group showed mild degree of centrolobular vacuolation (Fig.2K) as compared with c+ve2 group. TQ therapeutic group (DZN-TQ 5 mg) showed periorbital inflammatory cells infilteration (Fig.2M), mild to moderate degree of granular hepatic vacuolation (Fig.2N) as compared with c+ve1. TQ prophylactic group (TQ 5 mg-DZN) showed mild to moderate degree of granular vacuolation (Fig.2O) as compared with c+ve2.

Effects of thymoquinone on Bax and caspase3 gene expression in liver of DZN intoxicated rats

The expression of caspase3 and *Bax* genes were significantly increased in liver of DZN intoxicated groups (c+ve1 and c+ve2) as compared to non-treated group (c-ve) as showed in Fig.3. TQ therapeutic group (DZN-TQ 10 mg) illustrated a significant decrease in the levels of caspase3 and *Bax* gene expression compared with DZN intoxicated group (c+ve1). The mRNA levels of caspase3 and *Bax* were significantly downregulated in TQ prophylactic group (TQ 10 mg-DZN) as compared with the c+ve2 group. The gene expression of caspase3 and *Bax* were significantly attenuated in TQ therapeutic group (DZN-TQ 5 mg) as compared with c+ve1 one. TQ prophylactic group (TQ 5 mg-DZN) showed a significant decrease of caspase3 and *Bax* genes expression as compared with the c+ve2 group. The caspase and *Bax* genes expression was significantly decrease in (TQ 10 mg) groups as compared by (TQ 5 mg) groups.

Table 1: Primers used in qPCR

Gene	Primer sequence (5'……..3')	references
β-actin	F:TCCTCCTGAGCGCAAGTACTCT R:GCTCAGTAACAGTCCGCCTAGAA	(Banni et al., 2010)
BAX	F:CACCAGCTCTGAACAGATCATGA R:TCAGCCCATCTTCTTCCAGATGGT	(Kinouchi, 2003)
Caspase3	F:AGTTGGACCCACCTTGTGAG R:AGTCTGCAGCTCCTCCACAT	(Shi et al., 2009)

Table 2: Hematological and biochemical parameters of control and treated groups

Parameters	c-ve	c+ve1	c+ve2	DZN-TQ10mg	TQ10mg-DZN	DZN-TQ5mg	TQ5mg-DZN
RBCs($\times10^6$/μL)	7.6±0.22^{a}	5.8±0.16^{b}	6.4±0.5^{ab}	6.85±0.3^{a}	7.09±0.1^{a}	7.17±0.14^{a}	6.7±0.1^{ab}
HCT (%)	44.2±1.66^{a}	40.5±0.9^{ab}	40.1±0.9^{b}	43.34±0.39^{a}	45.2±0.56^{a}	41.2±0.84^{ab}	43.2±0.56^{a}
HGB(g/dl)	15.9±0.4^{a}	11.8±0.4^{b}	12.8±0.4^{ab}	15.2±0.39^{a}	15.1±0.13^{a}	14.4±0.4^{ab}	15.8±0.12^{a}
MCV(FL)	57.1±0.4^{bc}	71.3±1.3^{a}	66.5±1.6^{a}	58.1±0.44^{b}	59.3±1.07^{ab}	61±0.12^{ab}	59.3±1.07^{ab}
MCH(pg.)	21±0.48^{ab}	20.4±0.3^{ab}	19.6±0.3^{b}	22.1±0.1^{a}	21±0.12^{ab}	20.08±0.14^{ab}	23.5±0.12^{a}
MCHC(g/dl)	35.9±0.3^{a}	29.7±1.3^{b}	31.9±0.8^{b}	35±0.06^{a}	34.6±0.06^{a}	34.9±0.12^{a}	34.6±0.06^{a}
PLT($\times10^3$/μl)	1080±174^{a}	741±63.6^{ab}	710±28.4^{ab}	1004.3±127^{ab}	1133.6±36^{a}	993±54^{ab}	1077±116^{a}
WBCs($\times10^3$/μl)	10.5±1.7^{b}	24.7±4.5^{a}	22.3±4.7^{a}	12.1±0.4^{ab}	12.1±1.3^{ab}	11.9±0.6^{ab}	14.5±0.14^{ab}
ALT(U/l)	14.6±6.69^{cd}	92.9±0.97^{a}	73±4.6^{a}	41±1.4^{b}	43.o3±5.6^{bc}	43±1.6^{b}	39.5±2.2^{b}
AST(U/l)	36±8.1^{bc}	94±11.24^{a}	101±23^{a}	52.5±2.6^{b}	47.16±1.9^{bc}	65.7±0.8^{b}	57.8±8^{b}
TP(g/dl)	8.4±0.24^{a}	5.7±0.088^{c}	6.12±0.10^{c}	7.32±0.26^{b}	7.3±0.080^{b}	7.38±0.27^{b}	5.9±0.134^{c}
Albumin(g/dl)	4±0.1^{a}	3.9±0.13^{b}	3.3±0.05^{b}	4.1±0.05^{a}	4±0.01^{a}	4.15±0.05^{a}	3.7±0.05^{ab}
Glucose(mg/dl)	102±4.8^{c}	145±7.6^{a}	163±1.5^{a}	121±1.3^{b}	116±1.67^{b}	128±0.2^{b}	117.6±3.28^{b}

Notes; the data are expressed as ±SE (n=7). Different upper case letters within a row indicate different mean value ($p\leq0.05$). TQ, thymoquinone; DZN, diazinon; RBCs, red blood cells; HCT, hematocrit; HGB, hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelets; WBCs, white blood cells, ALT, alanine aminotransferase; AST, aspartate aminotransferase; TP, total protein.

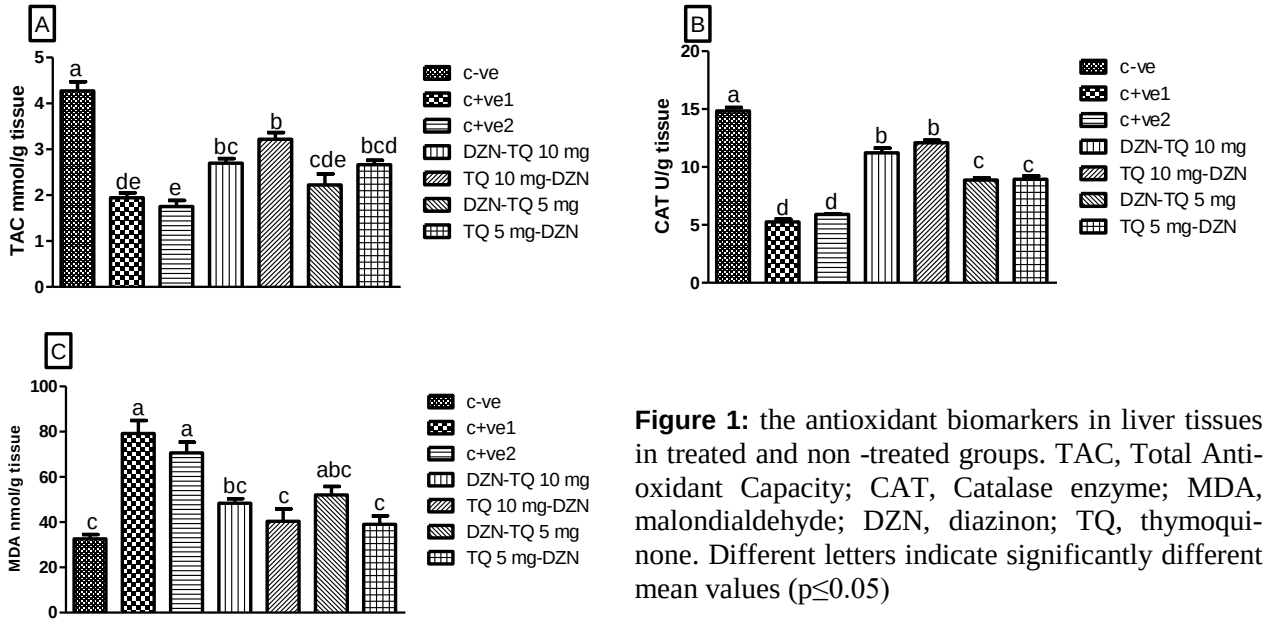

Figure 1: the antioxidant biomarkers in liver tissues in treated and non -treated groups. TAC, Total Anti-oxidant Capacity; CAT, Catalase enzyme; MDA, malondialdehyde; DZN, diazinon; TQ, thymoqui-none. Different letters indicate significantly different mean values ($p \leq 0.05$)

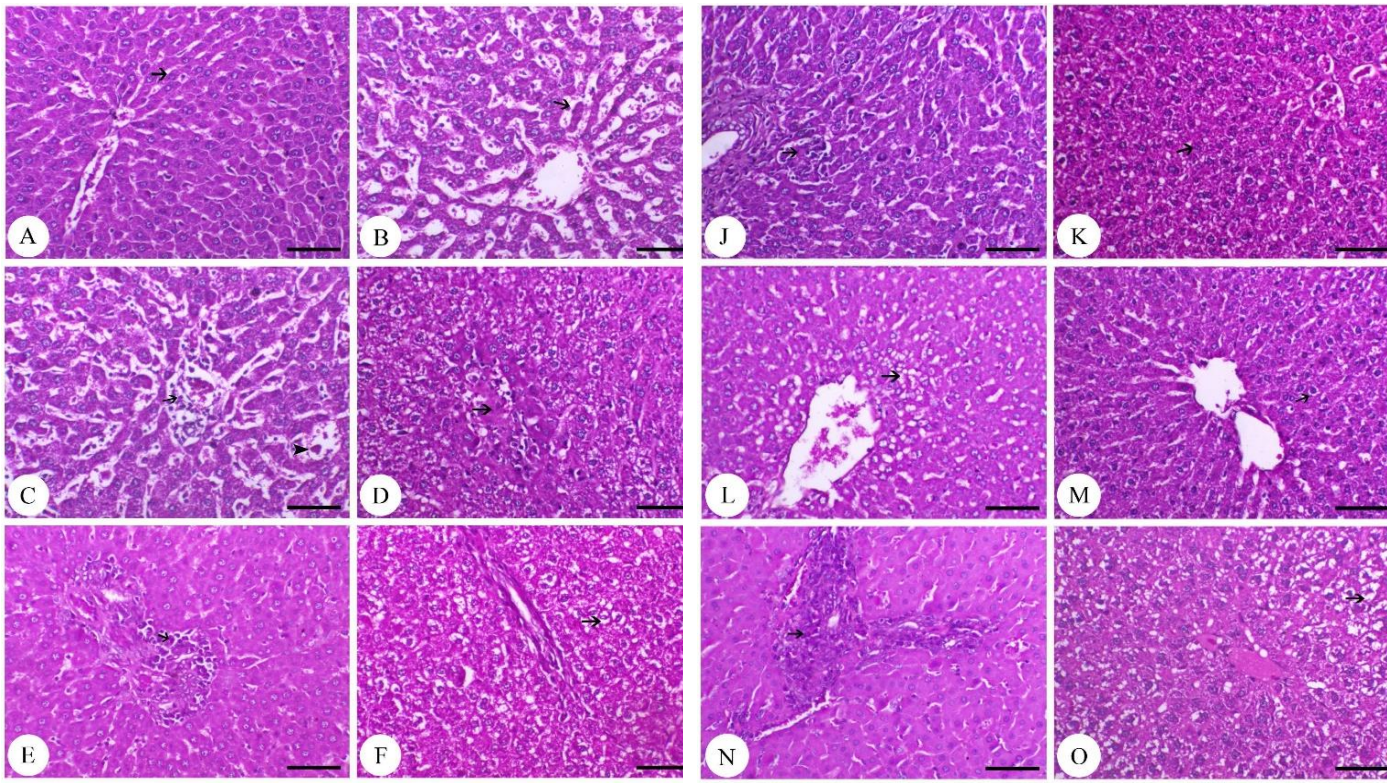

Figure 2: A; Liver of rat of non-treated group showing normal hepatocytes arranged in cords (arrow). B; Liver of animal from (c+ve1) showing marked congestion of the hepatic sinusoids (arrow). C; Liver of animal from (c+ve1) showing focal hepatic necrosis (arrowhead). D; Liver of animal from (c+ve1) showing focal hepatic necrosis (arrow). E; Liver of animal from (c+ve2) group showing marked periportal inflammatory cells infiltration with presence of few eosinophils (arrow). F; Liver of animal from (c+ve2) group showing diffuse vacuolation of hepatocytes (arrow). J; Liver of normal animal (c+ve2) showing focal hepatic necrosis (arrow). K; Liver of normal animal (DZN-TQ 10 mg) group showing mild degree of granular hepatic vacuolation (arrow). L; Liver of normal animal (TQ 10 mg-DZN) group showing mild degree of centrolobular vacuolation (arrow). M; Liver of normal animal (DZN-TQ 5 mg) group showing periportal inflammatory cells infiltration (arrow). N; Liver of normal animal (DZN-TQ 5 mg) group showing mild to moderate degree of granular vacuolation (arrow). O; Liver of normal animal (TQ 5 mg-DZN) group showing mild to moderate degree of granular hepatic vacuolation (arrow). H&E, bar= 100 µm

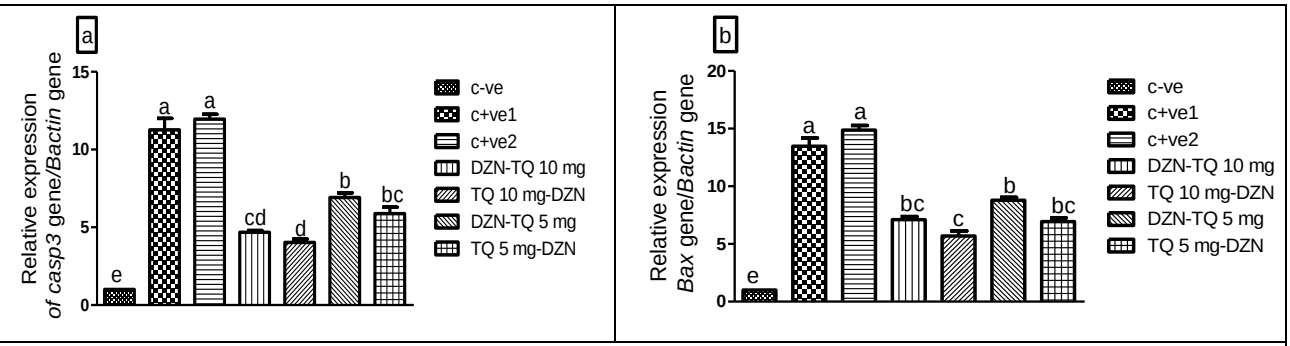

Figure 3: Caspase 3 and *Bax* gene expresion in treated and non-treated groups. Data are presented as mean (fold change) ± SEM (n =5). Column carrying different superscript letters are sigbificantly different at P≤ 0.05

Discussion

The present study investigated the effect of two different doses of TQ as prophylactic and therapeutic on the toxic effects of DZN organophosphorous insecticide through investigation of hematological, biochemical, lipid peroxidation, antioxidant biomarker, molecular and histopathological alterations. The results of the present study revealed a significant reduction in hemogram (RBCs, HGB, MCH, MCHC and PLT) and increase in MCV and total leucocytic count (TLC) in DZN intoxicated group. These data agree with (35, 36) and indicate the presence of macrocytic hypochromic anemia. DZN can cause destruction of RBCs (37) as it increase production reactive oxgen species (ROS) which have adverse effect on membrane and macromolucules of erthrocytes as it induces osmotic fragility, decreases cellular deformability and damage cell membrane (38). The development of macrocytic hypochromic anemia may be due to either interference of DZN with HGB biosynthesies or decrease life span of erthrocytes (39). (40) reported that the decrease in RBCs count and HGB biosynthesies due to effect of DZN on bone marrow tissues. The increase in MCV and decrease in MCHC suggested that hemolytic or haemorragic anemia. The increase in WBCs count in DZN intoxicated group aggree with (36). As a result of tissue injury caused by DZN, increase level of tissue malondiadehyde consequently activation of immune system (41) and calling the inflamatory cells and this resulted in an increasing in total leucocytic count. The reduction of PLT may be due to the effect of free radicals on bone marrow which decreased PLT production or depressed thrombopoiesis (42).

The enhancement in hepatocellular injuries could be refered to the production of ROS which accelerate lipid peroxidation and increase production of toxic aldyhde. The exhaustion of antioxidant defense mechanism may led to damage of hepatocytes and release of interacellular enzymes like ALT and AST enzymes. These results run parellel with (36). These suggestion is supported by liver histopathological finding which include diffuse hepatic vacuolation, mononucler cell infilteration and hepatic necrosis. As a result of toxic damage of DZN on liver tissues, the liver can not perform the function of synthesis of different types of protiens, this finding was confirmed by decrease serum level of total protien and albumin (43).

(44) approved that DZN led to increase in serum glucose level as illustrated in this study. The increment in serum glucose could be attributed to oxidative stress caused by DZN which stimulate gluconeogenesis and glycogenolysis (45).

Diazinon enhances MDA production which is considered as one of most critical indicator of oxidative stress and lipid peroxidation (46). (6) reported that diazinon enhances oxidative stress leads to production of free radicals and alteration of free radical scavengers enzymes

(antioxidants enzymes) in different tissues. In the present study there was an increase in the level of MDA, a decrease in TAC and catalase activities in DZN treated group. These data agree with (47, 48).

DZN causes toxic injury to hepatocytes and increase number of apopototic cells within hepatic tissues (6)**.** The current study showed DZN increased expression of *Bax* and caspase 3 genes. The harmful effect of DZN on mitochondria through increase its membrane permeability and ROS production. Bax is a proapoptotic gene which has an important role in release of cytochrome c which subsequently activates procaspase 9 and activation of intrinsic apopototic pathway. Procaspase 9 (the intiator) activates streams of caspases including caspase 3 (49). caspase 3 responsible for cleavation of specific target protien which lead to apoptosis (increase number of apopoyotic cells).

The current study demonstrated that TQ by different doses (5 mg and 10 mg) and as either prophylactic or therapeutic ameloriated the toxic effect of DZN. This may be due to TQ antioxidants activities and free radical scavanging properities that protect against oxidative damage (50). TQ alleviated the toxic effect of DZN on hemogram. These results run parallel with those obtained by (51). TQ counteract the increase in TLC caused by DZN may be due to either supression of cell mediated immune response (52) or its anti inflamatory properties (11). Administration of TQ alleviated the toxic effect of diazinon on liver tissues. TQ reduced serum ALT, AST and increased TP and albumin because of its hepatoprotective properities (22, 53) as thymoquinone stimulate the antioxidant scavenging enzymes system (i.e catalase, glutathione, superoxide dismutase). This effect is supported by histopathological changes caused by TQ as mild to moderate degree of hepatic vacuolation. In this study TQ make reduction in glucose level and this reduction may be due to inhibition of hepatic gluconeogenesis (54). (55) reported that TQ reduced hepatic antioxidants enzymes activities (i.e TAC and catalase) and this agree with this study. The stimulatory effect of TQ on hepatic antioxidant enzymes activities with hepatic lipid peroxidation reduction could be attributed to its free radical scavenging abilities and its ability to compansate the depleted antioxidant enzymes (56). Our study showed that TQ significantly attenuated hepatic apopotosis. As TQ supresses the release of cytochrome c which is a key of intrinsic apoptotic pathway, subsequently TQ reduced apoptosis and this indicated by molecular investigation which illusterated significant decrease in Caspase 3 and *Bax* gene expression (57) and histopathological finding which confirmed by marked decrease in apoptotic cells. These results showed a significant decrease in *Bax* and caspase 3 gene expression in a prophylactic and therapeutic (TQ 10 mg) groups as compared with (TQ 5 mg) groups (58)

Conclusion

TQ has prophylactic and therapeutic abilities to countract the deterious effects of DZN due to free radical scavenging and antioxidant properities of TQ. The degree of development is dose dependant and aprophylactic supplementation is better than therapeutic one.

Conflict of interest

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Original Research Article

ECOLOGY OF *Staphylococcus aureus* AND ITS ANTIBIOTIC RESISTANCE GENES IN DAIRY FARMS: CONTRIBUTING FACTORS AND PUBLIC HEALTH IMPLICATIONS

Walid Elmonir[1]*, Hader Essa[1], Wael F. El-Tras[2]

[1]Hygiene and Preventive Medicine (Zoonoses) Department, Faculty of Veterinary Medicine, Kafrelsheikh University, Kafrelsheikh 33516, Egypt, [2]Faculty of Aquatic and Fisheries Sciences (Zoonoses), Kafrelsheikh University, Egypt

*Corresponding author, E-mail: walid.elmonir@gmail.com

Abstract: Dairy farms are major sources for zoonotic *Staphylococcus aureus* pathogens and their antibiotic resistance genes. This study was conducted to assess critical sources and factors related to dissemination of *S. aureus* and its resistance genes within dairy farms in Egypt. In addition, workers' knowledge, attitudes and practices (KAPs) was evaluated. A total of 102 pooled samples were collected from 3 medium-scale dairy farms in Egypt. *S. aureus* was detected in 72.5% of the examined samples: lactating cows (72.9%), workers (81.5%), barns environment (88.9%), milking equipment (40%), and bulk tank milk (100%). Cows (udder milk and nostril), workers (hand skin and nostril), barns, open-sides parlor, and lack of acid rinse were associated with *S. aureus* contamination of milking equipment (P= 0.004 - 0.04). Methicillin resistant *S. aureus* (MRSA, *mecA*+), vancomycin-resistant *S. aureus* (VRSA, *vanA*+) and methicillin-vancomycin-resistant *S. aureus* (MVRSA, *mecA*+-*vanA*+) represented 27.5%, 5% and 12.5% of *S. aureus* isolates, respectively. This is the first report of MVRSA in dairy farms in Egypt. For workers KAPs, 48.7% didn't know milk-borne zoonoses, while their high risk practices included consumption of raw milk (52.2%), lack of hand wash (48.7%), and willing to work with sore throat (82.6%). This study highlights the critical sources of *S. aureus* pathogens and their antibiotic resistance genes in dairy farms. This will help in reforming biosecurity plans in dairy farms; an urgent demand for consumers safety in Egypt.

Key words: *Staphylococcus aureus* ecology; dairy farms; antibiotic resistance genes; public health implications

Introduction

Staphylococcus aureus is the third most recorded etiology of food-borne diseases worldwide (1), and it is one of the top causes of mastitis in dairy animals (2). Dairy farms are major reservoirs of *S. aureus* pathogens. The pathogen can circulate within the farm through cow, workers, farm environment and may occasionally pass to bulk tank milk posing a public health risk for consumers (1,3). Another major public health concern is the continuous evolving of antibiotic resistant strains of *S. aureus*. Methicillin resistant *S. aureus* (MRSA) is a global public health hazard. Reports of dairy farms associated MRSA are rising worldwide

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(1, 4,5). Misuse and overuse of antimicrobials in dairy farms for therapy (e.g. clinical mastitis) or prevention (e.g. dry cow) may evolve resistance mechanisms in *S. aureus* and fasten the emergence of multi-drug resistant strains as MRSA. The ecology of MRSA in dairy farms is complex and the exchange of strains in-between cows, workers and farm environment was recorded (5). This continuous inter-sources transaction helps in persistence of MRSA infection in the farm, which may pose an animal health risk for dairy animals and a zoonotic risk for workers. Occupation exposure to MRSA may be increased by inadequate awareness and unhygienic practices as raw milk consumption, inadequate use of personal protective equipment (PPE) or hand washing (6). Dairy farm associated MRSA threat may extend to public health if these strain gain access to bulk milk or spread environmentally through contaminated air, water, or manure to population in close proximity to dairy farms (2).

In last few years, a new resistance genotype assigned as vancomycin resistant *S. aureus* (VRSA) has emerged when a MRSA isolate gained *vanA* gene and expressed resistance to vancomycin in a clinical case (7).Humans' clinical reports of VRSA infection are expanding in the Middle East, particularly in Egypt (8). However, dairy farm associated VRSA was never recorded in Egypt and also very limited data are available worldwide (9).

Identifying critical sources of *S. aureus* propagation in dairy farms will guide implementation of preventive strategies to improve both animal health and consumers' safety. Therefore, the objectives of the study were to evaluate the role of lactating cows, workers and barns environment as potential critical sources of *S. aureus* pathogens in dairy farms, to assess the factors contributing to their dissemination to milking equipment, and to determine the frequency and diversity of their antibiotic resistance genotypes. Knowledge, attitudes and practices (KAPs) of dairy workers were also recorded.

Materials and methods

Dairy farms

The study was conducted in 3 medium-scale dairy farms (A, B and C) located in Kafrelzayat district (30°49'14" N and 30°48'57" E), Gharbia Governorate, Egypt. Numbers of lactating cows were 63, 90 and 122 for A, B and C farms, respectively. Lactating cow barns in the three farms were open yards with cow sheds and soil bedding. Parlors in the three farms were pipeline milking machines. In farms A and B, the parlors were open-sides with only half length walls surrounding the parlor, while in farm C the parlor was closed with windows and gates. Teat dipping was conducted in farms B and C. Cleaning of milking equipment was conducted by hot alkaline detergent wash in farms A and B, while two-step sequential alkaline detergent wash followed by acid rinse was conducted in farm C. In farm A, milk was sold raw to consumers, while in farms B and C the milk was sold to processing plants.

Samples collection

A total 102 pooled samples were collected from cows, workers, barns, milking equipment and bulk tank milk in the 3 farms during the period between October 2016 and February 2017.

Cows, workers and barn environment: all samples were collected according to Roberson et al. (10). For cows, 16 pooled samples were collected in each farm: 5 samples of composite udder milk (25 cows) and 5 samples of nasal swabs (25 cows), 3 samples (15 cows) of udder skin and 3 samples (15 cows) feces samples. For workers, 9 pooled samples were collected per farm: 3 samples (15 workers) for each of hands skin swabs, nasal swabs and stool samples. For barns environment: 3 pooled samples were collected per farm: 1 sample (500-1000 g or ml) for each of bedding, feed and water.

Milking equipment and BTM: samples were collected according to Lee et al. (3). Five pooled samples (20 teat cups) of milking equipment and one pooled sample (500 ml) of BTM were collected per each farm. All samples were transported in ice-box to the laboratory within 2 hours to be processed.

Detection and molecular analysis of S. aureus

All samples were cultured on Baired Parker Agar (Oxoid, Hampshire, U.K.) supplemented with Egg Yolk Tellurite (50mL/L) (Oxoid, Hampshire, U.K.) and incubated at 37ºC for 24-48 hours. Identification based on biochemical and tube coagulase tests was conducted as previously described (11).

DNA extraction from overnight broth cultures was conducted using QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Detection of *mecA* and *vanA* genes as molecular determinants of MRSA and VRSA, respectively was conducted using multiplex PCR according to Amghalia et al. (12). The primers *mecA* (F): 5′AAAATCGATGGTAAAGGTTGGC′3 and *mecA* (R): 5′AGTTCTGCAGTACCGGATTTGC′3 were used to amplify 533 bp of mecA gene. The primers *vanA* (F): 5′CATGAATAGAATAAAAGTTGCAATA′3 and *vanA* (R): 5′CCCCTTTAACGCTAATACGATCAA′3 were used to amplify 1030 bp of vanA gene. The reaction mixture contained 12.5 µl EmeraldAmp Max PCR Master mix (Takara Bio, Kusatsu, Japan), 1 µl (10 pmol) of each primer, 3 µl of DNA template (~ 50 ng) and water up to 25 µl reaction volume. Cycling conditions started with an initial denaturation step at 94°C for 5 min, 10 cycles of amplification (94°C for 30 sec; 64°C for 30 sec; 72°C for 45 sec), then followed by another 25 cycles of amplification (94°C for 45 sec; 50°C for 45 sec; 72°C for 1 min), and ending with a final extension at 72°C for 10 min. PCR products were electrophoresed in 2% agarose gel with ethidium bromide and photographed under UV illumination (Fig. 1).

Knowledge, attitudes and practices (KAPs) questionnaire

KAPs of farm workers (23 workers) were assessed using a pre-tested semi-structured questionnaire. The questionnaire recorded knowledge of milk-borne zoonoses and practices regarding milk consumption, personal hygiene (hand washing), using PPE, and working with illness (sore throat or diarrhea).

Statistical analysis

Factors association with milking equipment contamination with *S. aureus* was estimated using Fisher's Exact test and Pearson's R correlation coefficient on SPSS v19 (SPSS Inc. 2010). Significant association was recorded at $P<0.05$.

Ethical approval

All research details were explained to dairy farm owners and workers. Their written consents were obtained.

Results and discussion

High rate of S. aureus dissemination in diverse sources within dairy farms will eventually reside in bulk tank milk and consequently passes to consumers as public health hazards.

Prevalence of S. aureus in Farm level and in bulk tank milk (BTM)

The prevalence of S. aureus at farm level ranged from 67.6 to 76.5% with overall prevalence of 72.5% (Table 1). These rates were higher than other reports from dairy farms in Brazil (6.6%) and Ethiopia (19.6%) by Lee et al. (3) and Ayele et al. (6), respectively. Examined BTM samples from all farms harbored S. aureus (100%). Lower rate (21.7%) were reported in Brazil (3), however our finding lined with that of Haran et al. (1) who detected S. aureus in BTM samples from 84% of the examined dairy farms in USA.

Critical points for S. aureus dissemination in dairy farms

Dairy cows, workers, barns environment, and milking equipment are critical points for dissemination of S. aureus within dairy farms.

Dairy cows

Cows are primary reservoirs of S. aureus in dairy farms. The overall prevalence of S. aureus in Cows' samples was 72.9% (Table 1). This high rate was in line with findings of Jørgensen et al. (13), who reported S. aureus in 90.1% of examined cows in a small-scale dairy farm in Norway. *S. aureus* was detected in udder milk (86.7%), nostrils (80%), feces

(55.6%), and udder skin (55.6%) samples (Table 1). Prevalence of S. aureus in udder milk in this study was higher than other reports (5.5 – 36.4%) from Norway (13), Brazil (3), and Ethiopia (6). Nostrils carriage rate was higher than another report (3.2%) in Turkey (14). Fecal prevalence rate in this study was higher than previous reports (1.6 - 12%) in Greece (15) and USA (10). Udder skin rate was higher than that reported (8.4%) in USA (10) but lower than that reported (90.1%) in Norway (13). There was no association between cows' samples (udder milk, nostrils, feces or udder skin). In contrast, Piccinini et al. (16) suggested a persistent association between udder skin and intra-mammary infection in dairy cows. However, our finding agreed with Zadoks et al. (17), who reported minor role of teat skin infection in intra-mammary carriage of *S. aureus*.

Table 1: Frequency distribution of *S. aureus* pathogens and their association with milking equipment contamination in examined dairy farms

Variables		Farm A	Farm B	Farm C	Total	*P*-value
Cows	Udder Milk	100	80	80	86.7	0.01
	Nostril	80	60	100	80	0.03
	Udder skin	66.7	0	100	55.6	0.4
	Feces	33.3	66.7	66.7	55.6	0.4
	Subtotal	75	56.3	87.5	72.9	0.02
Workers	Nostril	66.7	100	100	88.9	0.02
	Hand skin	100	100	100	100	0.004
	Stool	0	66.7	100	55.6	0.4
	Subtotal	55.6	88.9	100	81.5	0.01
Barns		100	100	66.7	88.9	0.02
Milking equipment		40	80	0	40	-
Bulk milk		100	100	100	100	-
Total		67.6	73.5	76.5	72.5	-

*All frequency results are represented as percent

Table 2: Parlor design and practices associated with milking equipment contamination with *S. aureus* in this study

Variable	Categories	Farms			Percent	*P*-value	
		A	B	C		F	R
Parlor design	Open-sides	1	1	0	66.7	0.04	+0.02
	Close-sides	0	0	1	33.3		-0.02
Parlors practices	Teat dip	1	1	0	66.7	0.7	1
	No Teat dip	1	0	0	33.3		1
	>Once/day cleaning[1]	0	1	1	66.7	0.7	1
	Once/day cleaning	1	0	0	33.3		1
	Acid rinse	0	0	1	33.3	0.04	-0.02
	No acid rinse	1	1	0	66.7		+0.02

[1]: cleaning of milking equipment after milking cycle. F: Fisher's Exact test R: Pearson's R correlation coefficient

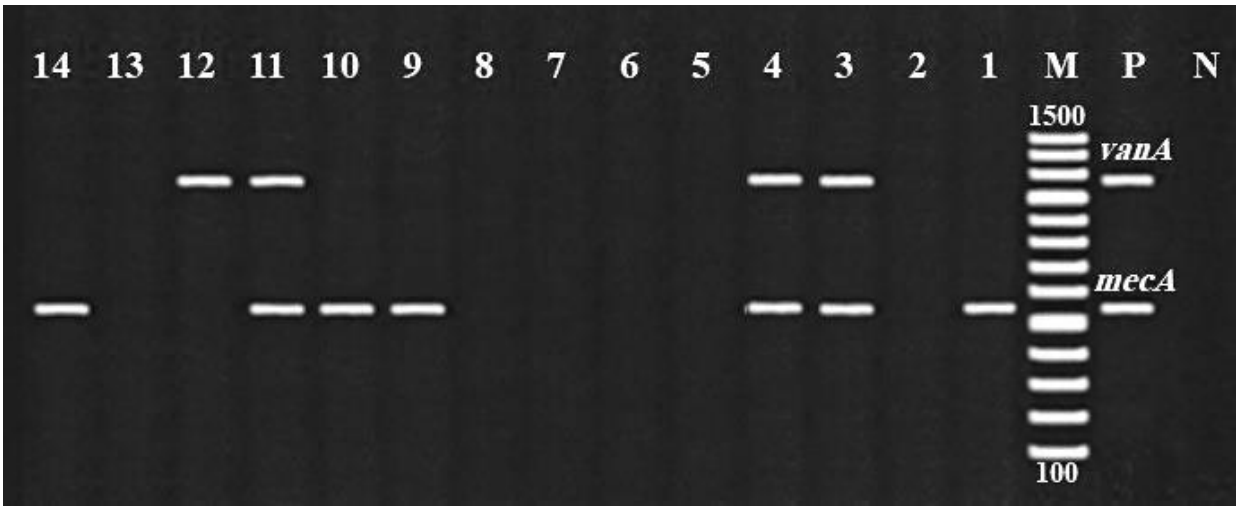

Figure 1: Molecular detection of antibiotic resistance genes (*mecA* and *vanA*) among *S. aureus* pathogens isolated from different samples in farm A. *mecA* gene: 533 bp, *vanA* gene: 1030 bp, Lanes 1-6: Cows' isolates, Lane 7: Milking equipment's isolates, Lanes 8-10: Barns' isolates, Lanes 11-14: Workers' isolates, M: 100 bp DNA marker, P: Positive control, and N: Negative control

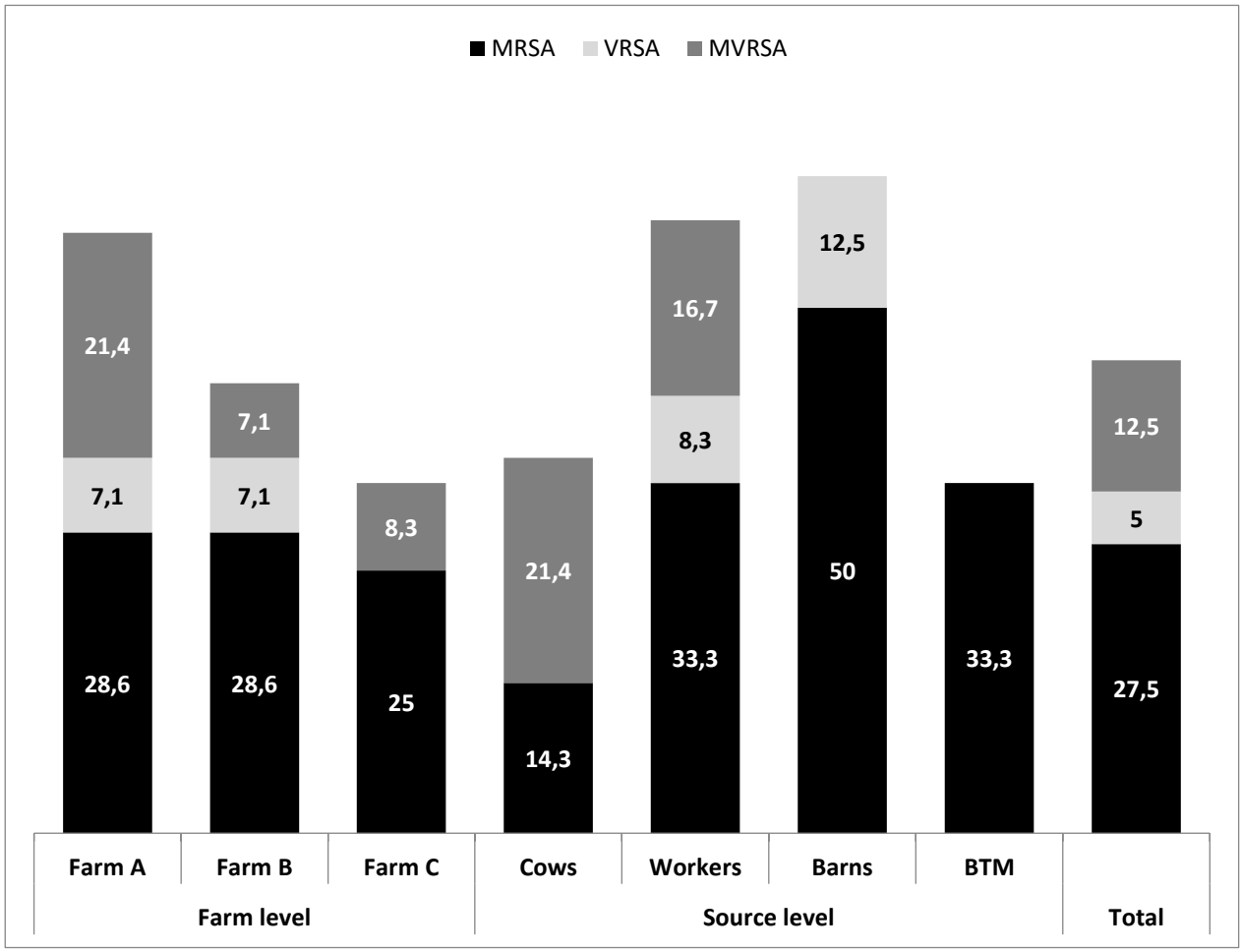

Figure 2: Frequency distribution of antibiotic resistance genotypes among *S. aureus* pathogens isolated from examined farms. MRSA: Methicillin resistant *S. aureus* (*mecA*+), VRSA: Vancomycin-resistant *S. aureus* (*vanA*+) and MVRSA: Methicillin-vancomycin-resistant *S. aureus* (*mecA*+ -*vanA*+). BTM: Bulk tank milk. All frequency results are represented as percent

Farm workers

Farm worker are at risk of occupational infection with livestock associated *S. aureus* infection. In addition, they may play a critical role in dissemination of infection to cows, equipments, farm environment, and milk (3, 10, 17). The overall prevalence of S. aureus in workers' samples was 81.5%, which was higher than a report (27%) in USA (10). S. aureus was detected in nostrils (88.9%), hand skin (100%), and stool (55.6%) samples from workers in this study (Table 1). Nostrils prevalence was higher than a report (29.3%) in Turkey (14). Lower rates for hand skin (3.3 - 32%) were reported in Brazil (3) and Ethiopia (6). Intestinal colonization and stool carriage of *S. aureus* in humans were previously reported (18). There was a significant association (P= 0.04) between hand skin and fecal carriage of *S. aureus*. Same association was recorded in USA (18). This could be attributed to lack of adequate personnel hygiene of examined workers as improper hand washing. No other association was recorded between workers samples.

Barns environment

Barns environment may act as a vehicle for S. aureus transmission to cows, workers, farm equipment and BTM (10, 13). *S. aureus* was detected in 88.9% of the examined barn samples (Table 1), which was higher than other reports (1-20%) in USA (10) and Norway (13).

Milking equipment

Milking equipment are critical vehicles for dissemination of S. aureus between individual dairy cows as well as from barns, cows and worker to milk (3, 17). In the present study, S. aureus was isolated from 40% of milking equipment (Table 1). This was higher than other reports (2.1-11.1%) in USA (10), Brazil (3) and Ethiopia (6).

High rates of *S. aureus* detection in various sources in examined dairy farms highlight their role as critical points for S. aureus dissemination and may also refer to unsanitary dairy farm practices. This is an alarming threat for both dairy animals and public health. Differences in the prevalence rates may be due to variations in the farm sanitary conditions, animal breeds, animal health status (e.g. subclinical mastitis), sampling strategies, seasons and geographical locations.

Factors contributing to milking equipment contamination with S. aureus in dairy farms

Carriage of *S. aureus* via cows, workers and barns was significantly associated with milking equipment contamination (P= 0.01 - 0.02) (Table 2). Nostrils (cows and workers), udder milk (cows) and hand skin (workers) were the sources that contributed significantly to milking equipment contamination (P= 0.004 - 0.03) (Table 2). Our finding agreed with Zadoks et al. (17) who confirmed S. aureus transmission from hand skin and udder milk to the milking equipment.

Open-sides parlors design positively associated with milking equipment contamination (P= 0.04) (Table 2). This could be linked to the significant effect of contaminated barns environment. With open-sides parlors walls, air drafts can introduce infection from contaminated barns to parlors during and in between milking cycles.

Lacking of acid rinse was significantly associated with milking equipment contamination (P= 0.04) (Table 2). This finding agreed with Elmoslemany et al. (19) who reported the positive association between inadequate acid rinse and milk contamination within dairy farms. Acid rinse removes milk stones, which could act as niches for microbial growth within milking equipment (19). Also acid has antibacterial activity against broad range of bacteria (20). Both mechanisms may explain the significant effect of acid rinse in reducing *S. aureus* contamination of milking equipment.

Teat dip was not associated with milking equipment contamination in this study (Table 1). However it may be contributed to the elimination of teat skin role in contaminating milking equipment as recorded in this study (P= 0. 4) (Table 1).

Frequency distribution of S. aureus antibiotic resistance genotypes in dairy farms

Almost half (45%) of the *S. aureus* isolates carried at least 1 antibiotic resistance gene. MRSA (mecA+), VRSA (vanA+), and MVRSA (mecA+, vanA+) resistance genotypes represented 27.5%, 5%, and 12.5% of *S. aureus* isolates respectively (Fig. 2). MRSA isolates were detected in cows, workers, barns, and BTM. VRSA isolates were from workers and barns, while MVRSA isolates were from cows and workers. Hence, workers were the only source that harbored the 3 resistance genotypes in the examined farms. In agreement with our findings, MRSA isolates were detected in cows, workers, and environment of dairy farms in Korea (4) and Italy (5). VRSA isolates were reported in cows with mastitis in China (9). However, as far as we know, this the first report of MVRSA in dairy farms in Egypt. Detection of MVRSA isolates in dairy farms is alarming and their emergence requires further investigation. MRSA acquiring vanA gene by plasmid transfer was previously reported in human clinical cases (21). Same mechanism of gene transfer could explain the emergence of MVRSA in this study. High rate of intersources transmission and mixing of *S. aureus* isolates within same farm may facilitate this gene transfer. The detection of the 3 genotypes in 2 farms (A and B) and the findings of Locatelli et al. (5), who reported MRSA genotypes exchange between cows, workers and environment within same dairy farm, support our hypothesis. Yet, further investigation is required to elucidate the ecology and molecular bases of MVRSA emergence in dairy farms.

Knowledge, attitudes and practices of farm workers

Among respondents, 47.8% lacked awareness regarding milk-borne zoonoses, which was lower than that (87%) reported in Ethiopia (6). Raw milk consumption was reported by 52.2% of the workers. This was higher than another report (35%) of raw milk consumption by dairy workers in Ethiopia (6). None of workers used PPE (gloves or masks) during work. Around half (47.8%) of workers don't wash their hands, which was lower than that reported in Ethiopia where none of the workers (100%) wash their hands (6). Finally, 82.6% and 73.9% of the worker would work with sore throat and diarrhea, respectively. Lack of awareness and unhygienic practices (raw milk consumption, lack of hand wash and PPE use) may pose an occupational zoonotic threat for the workers in these farms. In addition the unhygienic practices and willing to work with illness may contribute to the significant role of workers in disseminating S. aureus contamination in examined farms.

Conclusion

This study records high dissemination rate of *S. aureuspathogens* and their antibiotic resistance genes in dairy farms in Egypt, which may impact the health of dairy products consumers in Egypt. The study also highlights the critical points and practices associated with S. aureus dissemination in dairy farms, which will help in improving biosecurity planning and application in dairy farms in Egypt.

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Original Research Article

ANTIMICROBIAL PHENOTYPES OF GEOGRAPHICALLY MATCHED *Staphylococcus aureus* ISOLATED FROM BUFFALO'S MILK AND CLINICAL HUMAN CASES IN EGYPT: POTENTIAL ZOONOTIC RISKS

Walid Elmonir[1*], Hader Aglan[1], Ehab K.Elmahallawy[2], Wael F. El-Tras[3]

[1]Hygiene and Preventive Medicine (Zoonoses) Department, Faculty of Veterinary Medicine, Kafrelsheikh University, Kafrelsheikh 33516, Egypt, [2]ZoonosesDepartment, Faculty of Veterinary Medicine, Sohag University, Sohag82511 Egypt, [3]Faculty of Aquatic and Fisheries Sciences (Zoonoses), Kafrelsheikh University, Kafrelsheikh33516, Egypt

*Corresponding author: walid.elmonir@gmail.com

Abstract: Global emergence of antibiotic-resistant food-borne pathogens is a major public health problem. This study aimed to determine the potential zoonotic risk of *Staphylococcus aureus* in buffalo's milk in Egypt. A total of 102 raw buffalo's milk samples and 51 human nasal swabs were collected at Kafrelsheikh city, Egypt. All samples were examined for occurrence of *S. aureus*. Detected isolates were characterised based on *DNase* activity, *mecA* gene acquisition, and antibiotic resistance patterns. *S. aureus* was detected in 33.3% of buffalo's milk samples and29.4% of human nasal swabs. Multiple drug-resistant *S.aureus* (MDRSA) represented 88.2% and 90% of buffalo and human *S. aureus* isolates, respectively. Buffalo and human *S. aureus* isolates showed highest resistance rate for erythromycin (100%), and lowest resistance rate for gentamicin (22.2%). Interestingly, there was no significant difference in resistance patterns between methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) (P<0.46 - 0.97). One-third of the detected *S. aureus* phenotypes (5/15, 33.3%) were identical between buffalo and human isolates. Moreover, there was no significant difference in antibiotic resistance patterns between buffalo and human isolates (P<0.1 - 0.97).This study highlights the potential public health risk of MDRSA transmission via buffalo's milk.

Key words: buffalo's milk, *S. aureus*, *DNase*, *mecA*, antibiogram, zoonotic risk

Introduction

The last few years have witnessed an alarming increase in the reports of antibiotic-resistant pathogens in human and veterinary practices. Special concern is given to the possibility of food contamination with multiple drug-resistant (MDR) bacteria or bacteria carrying antibiotic-resistant genes (1,2). Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are β-lactam-resistant *S. aureus* that can easily acquire resistance to a wide spectrum of other antibiotics (3). Thus, the high rate of dissemination of these pathogens worldwide has created an additional infection control problem in both human and veterinary medicine (4). Multiple drug-resistant *S. aureus* (MDRSA) strains, including MRSA strains, have been isolated from bovine milk and different dairy products worldwide (5-7). Bovine MRSA strains have

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emerged as zoonotic organisms based on several lines of evidence showing the possibility of direct transmission of MRSA between bovines and humans (4, 8).

In Egypt, buffalo's milk and its derived dairy products are more popular than those derived from cows owing to the higher fat content, whiter colour and creamier flavour of buffalo's milk than of cow's milk (9). Buffalo's milk is the second most produced milk in Egypt, with over2 million tons produced annually (10), making Egypt the fourth largest buffalo's milk producer worldwide (10). Despite this important aspect, there is not sufficient data on the role of Buffalo's milk in maintaining the epidemiological foci of MDRSA at the national level. Therefore, the aim of this study was to elucidate the potential public health risk of *S. aureus* in buffalo's milk by reporting the prevalence and antibiotic resistance patterns of *S. aureus* isolates from buffalo's milk used for consumption by residents of Kafrelsheikh, Egypt. Furthermore, the phenotypes of buffalo *S. aureus* isolates were compared with those isolated from geographically matched human clinical cases to evaluate the potential zoonotic risk of buffalo's milk-borne *S. aureus* isolates.

Materials and methods

Sampling

Buffalo's milk and human nasal samples were collected from Kafrelsheikh city, the capital of Kafrelsheikh Governorate, which is located in the northern region of the Nile Delta, Lower Egypt (31°06′42″N 30°56′45″E). Buffalo milk used for consumption in the study region was bought from either markets or livestock smallholders. A total of 102 milk samples (100 ml per sample) were collected from Kafrelsheikh between September and December 2016. The buffalo's milk samples were divided as follows: half of the samples (51 samples) were bought from various markets at different localities in the city, while the other half were collected directly from buffaloes owned by smallholders. Potential heat treatment of market milk samples was evaluated by the *peroxidase* (Storch) test (11), and only raw samples were included in this study. Milk samples from buffaloes were collected under aseptic conditions as composite milk samples from the 4 quarters. All sampled buffaloes were apparently healthy, and the collected milk samples showed no physical or organoleptic abnormalities. During the same study period, a total of 51 nasal swabs were collected from outpatients at Kafrelsheikh Chest Hospital. All human samples were collected by the medical staff of the hospital.

Isolation and identification of S. aureus

Isolation of *S. aureus* were conducted according to the guidelines of the Food and Drug Administration (12). Suspected *S. aureus* colonies were identified based on the following criteria: grape-like clusters of gram-positive cocci by Gram staining and yellow colonies on mannitol salt agar (Oxoid, Hampshire, U.K.), beta haemolysis on sheep blood agar (Oxoid, Hampshire, U.K.), and firm coagulation in the tube coagulase test using rabbit plasma (12).

Phenotyping of S. aureus isolates

From the 49 detected *S. aureus* isolates, 27 (17 buffalo and 10 human) isolates were chosen for phenotyping based on biochemical profiles, *mecA* gene acquisition, and antibiotic resistance patterns.

For biochemical profiles, the following tests were used (13): *DNase*, *catalase*, *oxidase*, Voges-Proskauer, growth in 10% NaCl, arginine *decarboxylase*, esculin hydrolysis, and sugar fermentation (lactose, maltose, ribose, arabinose, sorbitol, and raffinose). All *S. aureus* isolates showed the same biochemical profile except for the *DNase* test results; *DNase* activity was used as a marker for the biochemical profiles. For molecular detection of the *mecA* gene, *S. aureus* cultures incubated in broth overnight were used for DNA extraction by the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Amplification of the *mecA* gene was conducted according to Murakami *et al*. (14) using the following primers: forward *mecA1*-5' AAAATCGATGGTAAAGGTTGG 3' reverse *mecA2*-5'AGTTCTGCAGTACCGGATTTG

3'. Five μL of DNA extracted from *S. aureus*-culture was added to a PCR mixture that contained 1 μL (20 pmol) of each primer, 12.5 μL of DreamTaq PCR Master Mix (Thermo Fisher Scientific, Waltham, USA) and distilled water up to the 25 μL reaction volume. PCR was performed in a Mastercycler (Eppendorf, Hamburg, Germany) using the following conditions: 95°C for 3 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min and a final extension at 72°C for 10 min. Positive control (*mecA*+ *S. aureus* isolate) was kindly provided by Prof. Mohamed Hassan, Prof. of Food Hygiene, Faculty of Veterinary Medicine, Benha University, Egypt. PCR products were examined by gel electrophoresis and UV illumination (Fig. 1).

For antibiotic resistance patterns, a standard disk diffusion assay was used for antibiotic sensitivity testing according to the guidelines of the Clinical and Laboratory Standards Institute (15). The antibiotic disks (Oxoid, Hampshire, U.K.) used in this study are listed in table 2. *S. aureus* isolates resistant to cefoxitin were considered resistant to all β-lactams and were designated as MRSA. *S. aureus* isolates sensitive to cefoxitin were designated as methicillin-sensitive *S. aureus* (MSSA). MSSA isolates were further tested using ampicillin (10 μg) disks. *S. aureus* isolates that showed resistance to ampicillin were tested by nitrocefin discs (Thermo Scientific, Lenexa, USA) for β-lactamase activity.

Statistical analysis

The potential difference in antibiotic resistance patterns among the chosen 27 *S. aureus* isolates was assessed using a univariate logistic regression model to compare MRSA versus MSSA isolates or buffalo versus human isolates as the response variables. The model was built for all tested antibiotics with the exception of erythromycin and ampicillin. These antibiotics were removed because all 27 isolates were resistant to erythromycin and because ampicillin was used only for MSSA isolates. The statistical analyses were carried out using SAS 9.2 (SAS Institute Inc., 2008). Statistical significance was considered at $P<0.05$.

Ethical approval

The research details and methods were approved by the Ethics Committee of the Hygiene and Preventive Medicine Department, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt. For human samples, all nasal samples were collected by the medical staff of Kafrelsheikh Chest Hospital. The research details and risks of participation were explained to the participating outpatients, and written consent was obtained.

Results and discussion

In the present study, *S. aureus* was isolated from 33.3% of the examined buffalo's milk samples (table 1), which is similar to the results of a previous study (33.3%) in Turkey (5). However, our result was higher than another report (17.5%) in Iran (16). *S. aureus* was detected in 29.4% of nasal swabs from outpatients at Kafrelsheikh Chest Hospital (table 1). Likewise, Ungureanu *et al*. (17) isolated *S. aureus* from 35.38% of nasal exudates collected from hospitalized patients and outpatients in Romania.

Bovine milk could act as a vehicle for MRSA transmission to humans (4, 8). Acquisition of the *mecA* gene defines MRSA isolates (4).A total of 35.5% of *S. aureus* isolates from buffalo's milk were MRSA (table 1). This value was higher than a report (9.2%) in Turkey (5). However, a higher rate of MRSA (56.1%) was reported in Uganda (6). The relatively high rates of MRSA reported in this study may be attributed to unhygienic milking and rearing of buffaloes in Egypt. The majority of buffalo populations in Egypt are household reared, and their milk is sold in informal markets (7). Manual milking and unhygienic milking equipment, milk storage conditions, and milk transportation are common practices in this production system. These practices may contribute to the high contamination rate observed in this study. MRSA represented 40% of human *S. aureus* isolates, which was higher than that in other studies (18.5–30%) reported elsewhere (17,18).

MDR to more than 3 classes of antibiotics was observed in 88.2% and 90% of buffalo and

human *S. aureus* isolates, respectively (table 1). In agreement with our findings, high rate (95.5%) of MDRSA were previously reported in bovine milk in Egypt (7). In contrast, other studies reported high sensitivity of bovine milk *S. aureus* isolates to several antibiotics in Brazil (19) and in Pakistan (20). The majority (90%) of the human *S. aureus* isolates were MDRSA (table 1). This value was higher than that in a study in Romania (18.75 - 45.45%) by Ungureanu et al. (17) but comparable to that in another report in Iran (61 - 93%) (18).

All MRSA isolates in this study were cefoxitin-resistant (table 2). This result implies a broad-spectrum resistance to all ß-lactams, with the exception of anti-MRSA cephalosporins (15). Interestingly, 47.1% of the MSSA isolates were resistant to ampicillin (table 2). Ampicillin-resistant MSSA is also resistant to penicillinase-labile ß-lactams (15). Both buffalo and human MSSA isolates showed ampicillin resistance at rates of 45.5% and 50%, respectively (table 2). In line with our findings, several pre vious studies reported resistance of bovine MSSA (2, 5), and human MSSA (17, 18) to penicillinase-labile ß-lactams at variable rates. MRSA isolates harboured the *mecA* gene, which mediates resistance to ß-lactams by encoding penicillin-binding protein 2a (PBP2a). On the other hand, MSSA isolates in this study lacked the *mecA* gene but produced the *ß-lactamase* enzyme (100%); the *ßlactamase* enzyme is encoded by the *blaZ* gene (21). In agreement with this finding, Yokoyama *et al*. (21) attributed ß-lactam resistance in MSSA to the production of the *ß-lactamase* enzyme. They also reported a higher prevalence of the *blaZ* gene among MSSA isolates than among MRSA isolates (21).

All buffalo and human *S. aureus* isolates were resistant to erythromycin (100%) (table 2). High rates of erythromycin resistance among bovine milk *S. aureus* isolates (50.5 – 77.3%) were also reported by Pamuk *et al*. (5) and Elmonir *et al*.(7). In contrast, AiresdeSousa *et al*. (19) and Asiimwe *et al*.(6) reported much lower erythromycin resistance rates (3.3 – 6.5%). Similarly, high rates of erythromycin resistance (61.3 - 93%) were shown in human *S. aureus* isolates in other studies (17, 18).

In addition, buffalo and human *S. aureus* isolates showed high resistance rates (70.4 - 88.9%) for kanamycin, sulphamethoxazole/trimethoprim, and tetracycline (table 2). Previous reports recorded comparable resistances of bovine and human *S. aureus* isolates for kanamycin, sulphamethoxazole/trimethoprim, and tetracycline (6, 7, 17, 18). However, other reports showed high efficacy of sulphamethoxazole/trimethoprim and tetracyclines on bovine *S. aureus* isolates (6, 19, 20). All *S. aureus* isolates in this study showed low resistance for ciprofloxacin (33.3%) and gentamicin (22.2%) (Table 2). High efficacy of ciprofloxacin was also reported elsewhere for bovine isolates (6, 20) and for human isolates (17). In contrast, Rahimi *et al*. (18)reported high resistance (95%) of human MRSA isolates for ciprofloxacin. Gentamicin is used in combination with vancomycin or cephalosporins as an alternative therapeutic choice for severe MRSA infections that show reduced susceptibility to vancomycin or daptomycin (22, 23). Hence, resistance to gentamicin limits treatment options and raises public health concerns. A total of 11.8% and 40% of buffalo and human isolates, respectively, showed resistance to gentamicin (table 2). In line with our findings, several studies reported resistance of bovine *S. aureus* isolates to gentamicin at rates ranging from 10% to 64.3% (5, 7, 20, 24).However, the findings of this study disagree with those of other studies that showed high rates of gentamicin efficacy (6, 19). High rates (18.5 – 59%) of gentamicin resistance among human *S. aureus* isolates were previously reported (17, 18), which agrees with our findings. The discrepancies in the present results compared with the results from various previous studies could be attributed to multiple factors, including differences in national policies for antibiotic administration to animals, animal production systems, sanitary measures of animal rearing and animal byproduct marketing, personal hygiene, and sampling methods as well as demographic and regional differences.

Table 1: Frequency distribution of *S.* aureus isolated from buffalo's milk and human nasal swabs in this study

Isolates	Buffaloes			Humans	Total
	Market Milk	Household Milk	Total Milk	Nasal swabs	
S. aureus	16/51 (31.4)	18/51 (35.3)	34/102 (33.3)	15/51 (29.4)	49/153 (32.03)
MRSA	3/8 (37.5)	3/9 (33.3)	6/17 (35.3)	4/10 (40)	10/27 (37.04)
MSSA	5/8 (62.5)	6/9 (66.7)	11/17 (64.7)	6/10 (60)	17/27 (62.96)
MDRSA	7/8 (87.5)	8/9 (88.9)	15/17 (88.2)	9/10 (90)	24/27 (88.9)
MDR-MRSA	3/3 (100)	3/3 (100)	6/6 (100)	3/4 (75)	9/10 (90)
MDR-MSSA	4/5 (80)	5/6 (83.3)	9/11 (81.8)	6/6 (100)	15/17 (88.2)

Brackets: Percent; MRSA: Methicillin-resistant *S. aureus*; MSSA: Methicillin-sensitive *S. aureus*; MDRSA: multiple drugs resistant *S. aureus*

Table 2: Antibiotic resistance diversity of *S. aureus* isolates detected in this study

Antimicrobial agent		Species		Methicillin resistance		Total No. (%)
		Buffalo No. (%)	Human No. (%)	MRSA No. (%)	MSSA No. (%)	
Cephems	FOX (30µg)	6 (35.3)	4 (40)	10 (100)	0 (0)	10 (37.04)
Penicillins	AMP (10µg)	11 (64.7)	7 (70)	10 (100)	8 (47.1)	18 (66.7)
Aminoglycosides	CN (10µg)	2 (11.8)	4 (40)	3 (30)	3 (17.6)	6 (22.2)
	K (30µg)	15 (88.2)	9 (90)	9 (90)	15 (88.2)	24 (88.9)
Macrolides	E (15µg)	17 (100)	10 (100)	10 (100)	17 (100)	27 (100)
Fluoroquinolones	CIP (5µg)	4 (23.5)	5 (50)	4 (40)	5 (29.4)	9 (33.3)
Nitrofurantoins	F (300µg)	5 (29.4)	5 (50)	4 (40)	6 (35.3)	10 (37.04)
Tetracyclines	TE (30µg)	12 (70.5)	7 (70)	7 (70)	12 (70.6)	19 (70.4)
Phenicols	C (30µg)	9 (52.9)	6 (60)	6 (60)	9 (52.9)	15 (55.6)
Sulfonamides	SXT (25µg)	15 (88.2)	9 (90)	9 (90)	15 (88.2)	24 (88.9)

FOX: cefoxitin; AMP: ampicillin; CN: gentamicin; K: kanamycin; E: erythromycin; CIP: ciprofloxacin; F: nitrofurantoin; TE: tetracycline; C: chloramphenicol; SXT: sulfamethoxazole/trimethoprim

The main difference between MRSA and MSSA is the spectrum of antibiotic resistance. Previous studies showed that the majority of MRSA strains are multi-resistant to β-lactams and to a wide spectrum of other antibiotics, while MSSA strains show much lower rates of resistance to multiple antibiotics in both human and bovine infections (3, 17, 24). Unlike these studies, our study showed no significant difference (P<0.46 - 0.97) in resistance patterns between MRSA and MSSA isolates for 7 classes of antibiotics (table 3). This study recorded unprecedentedly high rate (81.8%) of MDR among MSSA isolates from buffaloes (Table 1). Reports of MDR-MSSA in bovine milk are mostly from developing countries (2, 7). This fact could be attributed to unhygienic milk production and misuse of antibiotic therapy in veterinary practices in these countries. This evidence also highlights the role of buffaloes in the carriage and dissemination of MDR-MSSA,

Table 3: Univariate regression model for the association of antibiotic resistance among MRSA vs. MSSA and Buffalo vs. Human *S. aureus* isolates

Variable	MRSA vs. MSSA				Buffalo vs. Humans			
	C.	OR	*P*<	95% CI	C.	OR	*P*<	95% CI
K	MRSA MSSA	- 0.83	- 0.89	- 0.1 - 10.6	Buffalo Human	- 1.2	- 0.88	- 0.1 - 15.20
SXT	MRSA MSSA	- 0.83	- 0.88	- 0.1 - 10.6	Buffalo Human	- 1.2	- 0.88	- 0.1 - 15.20
TE	MRSA MSSA	- 1.03	- 0.97	- 0.19 - 5.68	Buffalo Human	- 0.97	- 0.97	- 0.18 - 5.37
C	MRSA MSSA	- 0.75	- 0.72	- 0.15 - 3.65	Buffalo Human	- 1.33	- 0.72	- 0.27 - 6.0
F	MRSA MSSA	- 0.82	- 0.80	- 0.16 - 4.10	Buffalo Human	- 2.4	- 0.3	- 0.48 - 12.13
CIP	MRSA MSSA	- 0.63	- 0.57	- 0.12 - 3.22	Buffalo Human	- 3.25	- 0.17	- 0.61 - 17.28
CN	MRSA MSSA	- 0.5	- 0.46	- 0.08 - 3.15	Buffalo Human	- 5.0	- 0.10	- 0.72 - 34.92

C.: Categories; OR: Odd ratio; CI: Confidence interval

which raises concerns regarding potential hazards to the animal industry and safety of milk in these countries, including Egypt. MSSA is characterized by higher fitness and shorter generation time than MRSA; hence, MSSA causes a higher number of cases than MRSA does (3). If such strains attain MDR, which hinders the treatment and eases the dissemination of these pathogens, a serious public health hazard is predictable. Klein *et al*. (25) reported an increase in hospitalization costs associated with MSSA-related infections relative to MRSA-related infections between 2010 and 2014 in the USA. They hypothesized that ineffective treatment approaches for MSSA may be one of the reasons for this shift (25). We may expect a further increase in the burden of MSSA infections at the human-animal interface with the potential emergence of MDR-MSSA.

Zoonotic transmission of *S. aureus* between bovines and humans is well documented (4, 8). Fifteen phenotypes of *S. aureus* were determined in this study (table 4). Approximately one-third of these phenotypes (5/15, 33.3%) were shared by 8/10 (80%) and 6/17 (35.3%) of the human and buffalo isolates, respectively. Additionally, none of the tested antibiotics showed a significant difference between buffalo and human isolates by univariate analysis ($P<0.1 - 0.97$), (table 3). In contrast, Jayaweera and Kumbukgolla (2) reported significantly higher odds of resistance to gentamicin and ciprofloxacin for animal *S. aureus* isolates than for human isolates; however, they included isolates from other species (e.g., poultry and pigs) in their analysis, which may explain the contradiction with our findings. The high phenotypic similarity of human isolates with buffalo isolates, especially in terms of antibiotic resistance patterns, highlights the potentially high rate of zoonotic interspecies transmission between humans and buffaloes and emphasizes the role of buffalo's milk as a vehicle of MDRSA for humans in the study region.

Table 4: Phenotyping of isolated *S. aureus* isolates from buffalo's milk and human nasal swabs samples

P*	*Dnase*	*mecA*	Antibiogram profile	Source	No.
P1	+	+	FOX, E, K, SXT, TE, C, F, CIP, CN	Human	2
				Buffalo	1
P2	-		FOX, E, K, SXT, TE, C, F, CIP	Human	1
P3	+		FOX, E, K, SXT, TE, C	Buffalo	2
P4	+		FOX, E, K, SXT, TE	Buffalo	1
P5	+		FOX, E, K, SXT	Buffalo	2
P6	+		FOX, E	Human	1
Total MRSA					10
P7	+	-	AMP, E, K, SXT, TE, C, F, CIP, CN	Human	2
				Buffalo	1
P8	+		AMP, E, K, SXT, TE, C, F, CIP	Buffalo	2
P9	+		AMP, E, K, SXT, TE, C, F	Buffalo	1
P10	+		AMP, E, K, SXT, TE, C	Human	1
				Buffalo	1
P11	+		E, K, SXT, TE, C	Buffalo	1
P12	+		E, K, SXT, TE	Human	1
				Buffalo	2
P13	+		E, K, SXT	Human	2
				Buffalo	1
P14	+		E	Buffalo	1
P15	-		E	Buffalo	1
Total MSSA					17

P: Phenotype

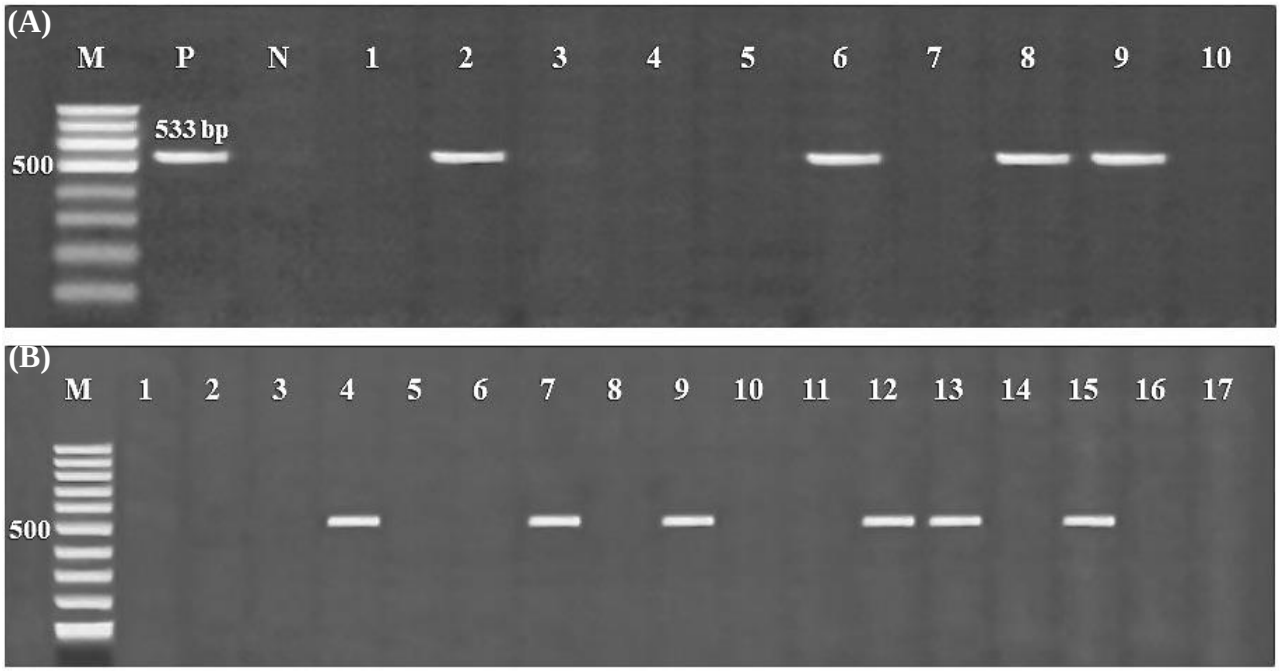

Figure 1: Molecular detection of *Methicillin* resistance gene (*mecA*) among *S. aureus* pathogens isolated from Buffalo's milk and human nasal swabs. (A) Human nasal swabs isolates. (B) Buffalo's milk isolates. M: 100 bp DNA marker, P: Positive control, and N: Negative control.

Conclusion

Our study highlights the possibly high risk of MDRSA dissemination to humans via buffalo's milk in the study region. The high rates of buffalo's milk-borne MDR-MRSA and MDR-MSSA need further investigation to explore resistance mechanisms and risk factors that contribute to the emergence of these pathogens in the study region. This study also highlights the genuine need for hygienic production and marketing of buffalo's milk, restriction and supervision of antibiotic therapy in veterinary practices, and public awareness about the potential risks of raw buffalo's milk consumption and processing.

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Conflict of interest

None declared.

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Original Research Article

EFFECT OF SUPPLEMENTATION OF OMEGA-3 FATTY ACIDS ON BLOOD PARAMETERS AND SEMEN QUALITY OF FRIESIAN BULLS

Mohamed A. Abu El-Hamd[1]*, Abd Salam M. Metwally[2], Mohamed M. Hegazy[1], Zahya R. Ghallab[2], Ola A. Elateeqy[2]

[1]Animal Production Research Institute, Agricultural Research Center, Dokki, Egypt, [2]Department of Animal Production, Faculty of Agriculture**,** Kafrelsheikh University, 33516, Egypt

*Corresponding author E-mail: abuelhamd68@yahoo.com

Abstract: The aim of this work was to evaluate the effects of flaxseed oil supplementation as a source of omega-3 fatty acids (OFA) on some blood parameters, semen quality and testosterone level in male Frisian bull. A total of 30 Friesian bulls (14.2 ± 0.16 month of age and 265±15 kg body weight) were divided into three equal groups according to age and live body weight. Bulls in the 1st group (G1) were supplemented with 2% dray matter intake (DMI) flaxseed oil as a source of OFA, while those in 2nd group were supplemented with 4% DMI flaxseed oil as a source of OTA at 14 to 21 months of age (G2). Bulls in the 3rd group were fed a basal diet and considered as a control group (G3). The obtained results revealed that OFA administrated animals had a significant (P< 0.05) inducing effect on serum levels of total protein, globulin, glucose, high density lipoprotein (HDL) and triglyceride compared to the control group. No significant changes were noticed in albumin levels among the three groups. However, the levels of total cholesterol, urea and low density lipoprotein (LDL**)** were significantly lower in bulls received OFA than the control group. Omega-3 fatty acids treatment has a significant positive effect on the semen characteristics and lower abnormality in G2 and G3 than in G1. G2 and G3 also showed a significant higher intact spermatozoa cell membrane than in G3 by about 9.68 and 10.84%, respectively. Omega-3 treatment significantly increased blood testosterone levels to be 23,78% in G2 and 31.82% in G3 higher than that of the control. In conclusion, dietary supplementation with omega-3 in male Frisian ration improved semen quality and reproductive potentiality as well as testosterone level.

Key words: Frisian bull; omega-3 fatty acids; testosterone and semen quality

Introduction

Bull fertility has a high economic importance in cattle artificial fertilization (AI) industry as semen high quality is crucial for success-ful AI. Flaxseed oils are an excellent as a source of α- linolenic acid, a member of the omega-3 fatty acids (OFA) (1). The fraction of flaxseed oil is approximately 0.55 omega-3α-linolenicacid (2). OFAs are able to lower the risks of some diseases (1, 3). Alpha linolenic acid, an essential OFA, is a precursor of eicosapentaenoic acid (EPA), which in turn is a precursor for the formation of eicosanoids. Eicosanoids are hormone-like compounds that play an essential role in immunity faction. Some

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study reported that EPA can elongate further to docosahexanoic acid (DHA), an OFA that is essential for cell membrane integrity and brain health (4).

Prostaglandins (PG) may play an important role in reproductive performance, especially semen quality (5). Arachidonic acid is a subsequent production of PG and is involved on synthesis of the steroid hormone (6). Total sperm number (7) and sperm motility (8) were improved following administration of fish oil to boars. On the other hand, semen characteristics were negatively affected after treatment with omega-6 in humans (9). High concentrations of polyunsaturated fatty acid (PUFA) in sperm membranes may improve semen quality after supplementation of long-chain omega-3 (5).

The aim of this work was to study the effect of OFA on some blood parameters, testosterone level and semen quality in Friesian bull.

Materials and methods

The experiment was performed at Sakha Experimental Station, Kafr-Elsheikh Governorate located in the Northern part of the Nile delta, at Animal Production Research Institute (APRI), Agricultural Research Center, Ministry of Agriculture, Egypt.

Animals

A total number of 30 Friesian bulls averaging 14.2 ± 0.16 month of age and 265±15 kg body weight were used in this study. The bulls were randomly classified into three groups (10 each), according to their body wieght and age. Bulls in the 1st group (G1) were supplemented with 2% DMI flaxseed oil as a source of OFA, while the bulls in the 2nd group were supplemented with 4% DMI flaxseed oil (G2). Bulls in the 3rd group were fed a basal diet and considered as a control group (G3). All bulls were judged as free of diseases and physical defects genitalia. The experimental animals were kept freely under semi-open sheds and were fed according to the recommendations of Animal Production Research Institute (APRI, 10) throughout the experimental period.

Feeding system and management

The concentrate feed mixture (CFM) was used in bull feeding in all groups. It was composed of 37.5% yellow corn, 20% soybean meal, 15% corn gluten, 22.5% wheat bran, 3% molasses, 0.5% premix (one kg of premix contained 3.3×10^6 IU vit. A; 3.3 g vit. E; 3.3×10^6 IU vit. D3; 0.33 g vit. K; 0.33 g vit. B1; 1.33 g vit. B2; 6.67 g vit. B5; 0.50 g vit. B6; 3.3 g vit. B12; 3.3 g vit. pantothenic acid; 0.33 g folic acid; 16.67mg Biotin; 166.67 g Cholin; 1g Copper; 10 g Iron; 13.3g Mn; 15 g Zn; 0.1 g iodin; 0.03 g Se and carrier $CaCO_3$ to 1kg) and 1.5% common salt.

Bulls in all groups were fed equal amounts of diet containing CFM, rice straw and fresh berseem (during winter season) or berseem hay (during summer season) according to the recommendation of the APRI, (10). Allowances for growing dairy bulls based on live body weight. Chemical analysis of representative monthly samples of foodstuffs was analyzed for CP, CF, EE, NFE and ash on DM basis according to the official methods of the A.O.A.C (11). Chemical composition of CFM, rice straw, fresh berseem and berseem hay used in feeding bulls in both groups is shown in Table (1).

Experimental procedures

Throughout the experimental period, semen samples were collected on all animals twice weekly using an artificial vagina at 18 months of age bulls at time of collection up to 21 months of age. After semen collection, each ejaculate was evaluated for volume, sperm concentration ($x10^6$/ml) mass motility (%), live sperm (%), sperm abnormality (%) and sperm concentration/ejaculate ($x10^6$/ml). As well as, sperm cell concentration was directly evaluated according to Barth (12).

The integrity of plasma membrane for the fresh spermatozoa was examined using hypo-osmotic swelling (HOS) test (13). The HOS solution at a concentration of 100 mOsm/kg was prepared by mixing 0.49 g of sodium citrate and 0.9 g fructose with 100 ml distilled water. In brief, 250 µl of diluted semen were added to

1ml of the pre-warmed HOS solution and incubated for 60 min at 37°C. A volume of 5µl from each sample was put on clean and warm, microscope slide and were examined at x400. Spermatozoa were counted 200 per sample and the indicative of intact plasma membrane were also determined.

Blood sampling

Blood samples were monthly collected during the experimental period in clean test tubes via the jugular vein from all the experimental bulls and were centrifuged at 3000 rpm for 10 minutes. The obtained serum was keep at -20^{o}C until determination of testosterone concentration.

Testosterone assay

Total and free serum testosterone assay was conducted by radio immune assay method (RIA) using Pontex 335 kit (I^{125}). Total testosterone included free testosterone and that bound to sex steroid binding globulin hormone, albumin, corticosteroid binding globulin (CBG). The standard curve of testosterone ranged between 0.1 and 25.6 ng/ml.

Biochemical assays

Serum biochemical parameters (total protein, globulin, glucose, cholesterol, triglyceride, HDL and LDL) were done using commercial kits (Diagnostic System Laboratories, Inc., USA) and as previously described (14-16).

Statistical analysis

Statistical analyses of data were carried out applying the package of Snedecor and Cochran (17). A factorial design (3 groups x ages) was used and the statistical model was:

$$Y_{ijk} = U + A_i + B_j + AB_{ij} + e_{ijk}.$$

Where:

Y_{ijk} = Observed values;
U = Overall mean;
A_i = group;
B_j = age;
AB_{ij} = Interaction due group x age;
e_{ijk} = Random error

The significant differences among means were tested using Duncan Multiple Range Test. Correlation analysis was carried out using computer programmer of SAS system. The percentage values of semen characteristics were tested by arcsine transformation so the means were presented after recalculated from the transformed values to percentages.

Results and discussion

Serum biochemical parameters in blood

Flaxseed oil is essential polyunsaturated fatty acids work as constituent of many enzymes which involved of majority of metabolic pathways also was important for metabolism of protein and growth of organ and immunity response. In the current study, flaxseed oil (as a source of OFA) supplementation to Friesian bulls improved the serum content of total protein and lipid profile and immunity markers (Table 2). The addition of flaxseed oil to the bulls ration, significantly ($P<0.05$) increased serum HDL, albumin and globulin, and decreased the LDL, cholesterol, TG and blood urea as compared to the control. These results agree with those of other studies where flaxseed oil was reported to reduce total lipids concentration in calves' blood serum (18-22).

Some studies suggested different ways by which PUFA can alter cholesterol concentration (23, 24). The synthesis of cholesterol is known to be increased with high PUFA intake (23, 24). However, in the current study, the lowering of serum cholesterol with supplementation of flaxseed oil as source of PUFAs could be attributed to the upregulation of LDL receptors (25) and/or the cholesterol redistribution between tissue pools and plasma (23). Flaxseed oil had high percentage of a-linolenic acid about 55% of oil's total fatty acids (26, 27). The diets which are rich in OFA decrease aggregation of platelet also, blood triglycerides and levels of cholesterol, blood clots formation, also, show both antithrombotic and anti-inflammatory effects (28, 29).

Table 1: Chemical analysis of different feedstuffs (on dry matter basis) used in feeding bulls

Item	Chemical composition (%) (on DM basis)			
	CFM	Rice Straw	Fresh Berseem	Berseem Hay
Dry matter, DM	90.42	89.24	15.26	88.23
Organic matter, OM	90.24	83.22	86.15	88.58
Crude protein, CP	16.04	1.59	14.71	14.41
Crude fiber, CF	10.96	37.21	24.9	24.67
Other extract, EE	4.91	1.47	2.90	6.04
Nitrogen free extract, NFE	56.38	42.85	43.64	43.16
Ash	9.76	16.78	13.85	11.42

Table 2: Concentration of biochemical parameters in serum as affected by flaxseed oil supplementation

Item	Experimental group			±MSE
	G1	G2	G3	
Total protein (g/100 ml)	7.68^{a}	7.55^{a}	7.23^{b}	0.03
Albumin (g/100 ml)	3.62	3.67	3.51	0.02
Globulin (g/100 ml)	4.06^{a}	3.89^{ab}	3.72^{b}	0.01
Glucose (mg/100ml)	69.87^{a}	71.03^{a}	61.66^{b}	2.54
Total cholesterol (mg/100ml)	166.2^{b}	163.1^{b}	187.3^{a}	3.12
High density lipoprotein (mg/100ml)	99.50^{b}	102.4^{a}	77.39^{c}	3.80
Low density lipoprotein (mg/100ml)	67.33^{b}	61.39^{b}	93.99^{a}	4.62
Triglyceride (mg/100ml)	37.03^{a}	38.01^{a}	28.9^{b}	0.85
Urea-N (mg/dl)	26.45^{b}	24.75^{c}	31.56^{a}	2.12

G1and G2: Bulls received flaxseed oil 2 and 4%/kg DMI, respectively. G3: Control
Data in the raw followed by different letters are significant at P<0.05.

Table 3: Semen quality in Friesian bulls as affected by flaxseed oil supplementation

Item	Experimental group			±MSE
	G1	G2	G3	
Ejaculate volume (ml)	4.13^{a}	4.24^{a}	3.74^{b}	0.03
Sperm cell concentration (x 10^{6}/ml)	1.372^{a}	1.444^{a}	1.138^{b}	0.02
Mass motility (%)	75.76^{a}	79.17^{a}	68.06^{b}	0.01
Live sperm (%)	77.00^{a}	81.17^{a}	71.17^{b}	1.85
Sperm abnormality (%)	10.06^{b}	9.39^{b}	13.67^{a}	3.12
Sperm cell concentration per ejaculate (x10^{6}/ml)	5.776^{a}	6.240^{a}	4.261^{b}	0.32
Hypo-osmotic swelling test (%)	53.94^{a}	54.51^{a}	49.18^{b}	1.24

G1and G2: Bulls received flaxseed oil 2 and 4%/kg DMI, respectively. G3: Control
Data in the raw followed by different letters are significant at P<0.05.

Table 4: Concentration of testosterone hormone(ng/ml) in blood serum as affected by flaxseed oil supplementation

Time (month)	Experimental group				Overall means
	G1	G2	G3	±MSE	
15	1.507	1.960	1.189	0.12	1.552^{c}
16	2.340	2.633	2.328	0.10	2.434^{ab}
17	2.474	2.263	2.023	0.12	2.253^{b}
18	2.323	2.339	1.949	0.11	2.204^{b}
19	2.541	3.084	2.082	0.11	2.569^{a}
20	2.732	2.860	1.753	0.15	2.448^{a}
21	2.736	2.860	2.121	0.14	2.572^{a}
Overall means	2.774^{a}	2.954^{a}	2.241^{b}	0.13	

G1and G2: Cows received flaxseed oil 2 and 4%/kg DMI, respectively. G3: Control
Data in the raw followed by different letters are significant at P<0.05.

Semen quality

In the current study, flaxseed oil as a source of omega-3 fatty acids treatment significantly ($P<0.05$) increased ejaculate volume, sperm cell concentrations, mass motility (%), live sperm (%) and sperm cell concentrations per ejaculate ($1x10^6$ ml) in G2 and G3 than in G (Table 3). Moreover, sperm abnormality (%) was significantly lower in treated groups than in the control group by 26.41% in G1 and 31.31% in G2, respectively. This improvement in all semen characters could be attributed to flaxseed oil supplementation with its high content of linoleic and linolenic acids as good antioxidants. Supplementation of flaxseed oil also produced a great improvement of all semen characters of rams (31). Moreover, different ratios of omega-3/omiga-6 PUFA were reported to improve semen characteristic by elevating omega-3/omega-6 PUFA that increased sperm concentration and motility and to reduce the deformity rate of the sperm (7). In the rats diet appropriate ratio of omega-3/omega-6 PUFA improved semen quality and changes in hormone metabolism due to improving reproductive performance (32). The progressive motility was reported to be higher in frozen-thawed semen in the flaxseed oil treated group than in the fish oil group (33). Dietary supplemented with PUFA improved reproductive performance, development of testis, spermatogenesis, sperm of motility and viability in fresh or freeze semen sample in ruminant (34).

In the HOS test, incubation of sperm in hypo-osmotic media is necessary to estimate the plasma membrane covering the principle piece (35, 36). In the current study HOS test show that flaxseed oil supplementation to bulls significantly ($P<0.05$) increased the resistance of the sperm covering membrane to the hypo osmotic challenge in both G1 and G2 compared to the control G3 by about 9.68 and 10.84%, respectively. The incorporation of DHA may be increased with Omega-3 treatment in the principle piece, facilitating sperm membrane stability against hypo-osmotic media (37).

The improvement of semen quality may be related to the supplementation with flaxseed oil with its PUFA the important molecules that serve as a source of energy and are critical components of the physical and functional structure of cells (38). Addition of OFA to animals diets improved sperm characteristics (39), increased sperm density and concentration per ejaculate (40, 41).

Testosterone concentrations

Testosterone is the key player in spermatogenesis and development reproductive tract in male (49). In this study, Friesian bulls serum testosterone concentrations were significantly increased by flaxseed oil treatments in G2 and G1 by 31.82 and 23.78% as compared to bulls in control (G3) (Table 4). The results agree with some study stated that omega-3 and 6 PUF may affect metabolism of important reproductive hormones. Testosterone concentration significantly increase in bulls supplemented with omega-3 may be due to the adequate amount of unsaturated fatty acids such as linoleic and linolenic acids. These unsaturated fatty acids especially lenolenic could be converted or involved in the synthesis of cholesterol which is considered the precursor materials for testosterone synthesis (43). It was indicated that spermatogenesis and steroidogenesis in the avian testis are increased with the omega-3 diets and this improvement dependent on the increase levels of FSH, LH and testosterone. However, O'Donnell *et al.* (45) reported that the concentrations of reproductive hormones and testosterone were positively higher related to presence of some important fatty acids and quality and morphology of sperm.

Conclusion

Dietary supplementation with omega-3 in male Frisian ration improved semen quality, testosterone level, lipid profile and immune function. Therefore the addition of flaxseed oil as a source of omega-3 is recommended to improve male animals semen quality and reproductively.

Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

PRODUCTION OF CHITOSAN FROM SHRIMP SHELLS BY MICROWAVE TECHNIQUE AND ITS USE IN MINCED BEEF PRESERVATION

Neama A. Omara, Essam M. Elsebaie*, H. E. Kassab, A. A. Salama

Department of Food Technology, Faculty of Agricultural, Kafrelsheikh University, P. B. 33516, Kafrelsheikh, Egypt

*Corresponding author, E-mail: essam.ahmed@agr.kfs.edu.eg; http://orcid.org/0000-0002-8507-0694

Abstract: This work was undertaken to figure out the optimum conditions for transformation of chitin to chitosan via microwave and to determine chitosan physical properties, antioxidant and antimicrobial activities to evaluate the possibility of using such by-products in preparing some functional foods. The obtained results revealed that deacetylation degree (DA) 93 % attained by chitin remediation with 50% aqueous sodium hydoxide sol at 1:25 solid:liquid ratio for 10 min into a microwave using a power 800W. Purified chitosan was characterized for molecular weight (4200 KDa), solubility (94%), water holding capacity (505%), oil holding capacity (321%) as well as intrinsic viscosity (13.2 dl/g). Samples of meat containing chitosan (0.02%) have the minimal experiment into the microbiological count, e.g. total count, yeasts and moulds. The anti-microbial effectiveness of chitosan was frequently higher than that of sorbistat-K or their blend by adding 0.01% chitosan and the same percent from sorbistat-K. Sensual attributes of samples contained chitosan, i.e. odour, texture and colour, were greater than other samples (sorbistat-K and control treatments). Though, chitosan nominated like a potent, eco-friendly and naturalistic substitution to reserve meat and protect its comprehensive quality.

Key words: chitosan; microwave; antioxidant activity; natural preservative

Introduction

Artificial anti-oxidative substances like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were utilized to hold up oxidation of lipids in meat (1). Nonetheless, contemporary attention over their utilization has generated a necessity and induced research for alternate antioxidants, predominately from naturalistic origin. The usage of naturalistic preservatives with meat products increases the storage shelf life is a hopeful technology as numerous vegetative materials posse antimicrobial and antioxidant characteristics (2). Functional substances supplement into meat products might upgrade the health and nutritional attributes and extension their storage shelf-life (3).

The meat industry is rottenly suffering from many critical challenges related to well-being and hygienic of the final product (4). Microbial pathogens transference to the products can be carried out during processing steps or from outer parts of the carcass (5). The removing of contamination or disinfection of the carcass

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body is hard to be realized by the use of a co-administered antimicrobial agent. In addition, commonly used antimicrobial and preservative substances possess artificial and alchemical properties, offering a lot of harmful effects and prospect hazards to consumer health (5).

Shrimp considered as one of the world's leading fishery products, especially in Egypt (6). The product is mainly exported under freezing conditions, passing through the shells and heads removing process (7). The economic value of raw shrimp shell substances and heads is little and they are considered as biological wastes or sold to factories of animal feeds (8). In the processing of shrimp industry, shrimp produce a large amount of biological waste, accounting for 45-55% of the raw weight of shrimp (9). Also, these biological wastes are able to be utilized in producing high-added value substances like chitosan and chitin. Chitosan, consisted of D-glucosamine units linked with β-linkage, is a cationic amino-polysaccharide which produced by partially deacetylation of chitin (10). If the acetyl- glucosamine groups proportions are higher (above 50%), it is named chitin, however if the proportion is lower than 50%, the ingredient is called chitosan (11, 12). The conventional methods for chitin extraction from crustaceans are chemical processes which involve the use of strong acid for demineralization and strong base for deproteination. However, these methods reduce the quality and increase the costs and environmental problems (13, 14). Chitosan can be characterized in terms of its quality, intrinsic properties (purity, molecular weight, viscosity, and degree of deacetylation) and physical forms (15).

In modern decades, microwave radiation has received considerable attention for its ability to speed up the reaction duration as compared to else procedures which transfer heat by conventional heating procedures, and can also perform heat transfer more uniformly in food compositions (16, 17). Therefore, the aim of the present study was to figure out the optimum conditions to get chitosan from chitin via microwave and to evaluate its physical properties, antioxidant and antimicrobial activities to determine the possibility of using this chitosan as preservatives in minced beef.

Materials and methods

Materials and preparation of samples

All chemical used in this study of HPLC grade (99.9% purity) were obtained from Sigma company of chemicals and drugs, St. Louis, MO, USA. Wastes of shrimp (shells and heads) have been acquired from local restaurant in Kafr El-sheikh governorate, Egypt. The wastes were dried in an electric oven then were ground up to pass through 20 mesh screen sieve, the yielded powder samples were mixed, and stored in polyethylene bags, then kept at room temperature until use.

Chemical composition

Moisture, crude protein, ether extract, ash, crude fiber contents of investigated samples (shrimp waste, crude chitin and chitosan) were performed using the methods given in the (18).

Isolation of chitin

Various chitin extraction stages were carried out according to Synowiecki and Al-Khateeb (19). Demineralization process was operated by mixing 4 g shrimp waste with 40 ml hydrochloric acid solution 2% (v / v) for 12 hrs at 30 ° C, and the mixture was centrifuged for 15 min at 4000 rpm, then the precipitate twice treated by distilled water. Deporteinization of the precipitate was operated by adding sodium hydroxide solution 4% (w / v) to precipitate at liquid to solid ratio (10:1 v/w) for 12 hrs at 30 ° C, and separation of different insoluble alkaline fractions by centrifugation for 15 min at 4000 rpm, then was twice treated with distilled water and was dried overnight at 40° C. The product obtained after drying was marked as pure chitin.

Chitosan production via microwave route

The various stages for chitosan production have been done as briefed via Sahu, et al. [13]. Chitosan was prepared from isolated chitin using various concentrations of sodium hydroxide aqueous solution (NaOH 30%, NaOH 40% and NaOH 50 %) at several sample to solvent ratios

(1:10, 1:15, 1:20 and 1:25) at different microwave power (800W, 900W and 1000W) for various irradiation durations (5, 10, 15 and 20 minutes). chitosan yield recovered from each step was determined.

Chemical, physical and functional attributes for chitosan

Extracted and commercial chitosan deacetylation grade was measured using the method of Qin et al., (20). Molecular weight and solubility of extracted and commercial chitosan were determined according to the method of (21). Water binding capacity and oil binding capacity of extracted chitosan sample were measured using the method of (22).

Chitosan usage to preserve minced beef

Fresh clean meat specimens were treated with the disinfectant sodium hypochlorite (100 g meat piece was soaked into 10 ppm aqueous solution of sodium hypochlorite for 60 min followed by twice washes in distilled water). Pieces of meat were finely minced, blended and split to four groups. The first group (G1) untreated samples, G2 treated with 0.02% chitosan, G3 treated with 0.02% sorbistat-K, and G4 treated with a mixture of 0.01% chitosan and 0.01% sorbistat-K. All samples were stored at 5ºC for seven days.

Microbiological analyses

Total count of aerobic microorganisms and fungi and yeasts were carried out at zero time and the end of storage period in triplicate as previously described (23).

Sensory assessment

After storage period, twenty semi-trained panelists from food technology department, kafrelshiekh University evaluated texture, color, and odour according to the method described by Pohlman, et al. (4).

Statistical analysis

All data (except phenolic content) were done in triplicate independent analyses and expressed as mean ± standard deviation. Data of antibacterial activity were analyzed using one-way ANOVA according to (24) procedure. The significance level was set at $p \leq 0.01$.

Results and discussion

Chemical composition of shrimp waste

Shrimp wastes were chemically analyzed for their contents of moisture, crude protein, ether extract, ash, crude fiber, total carbohydrates and the results were tabulated in Table (1). The data in Table (1) indicate that shrimp waste is considered a good source of protein, crude fiber and total carbohydrates which are important from the nutritional point of view. The results showed that the moisture, crude protein, ether extract, ash, crude fiber and total carbohydrate contents were 74.38, 11.55, 6.47, 32.21, 7.81 and 41.96%, respectively. These results were in harmony with those of (25, 26). Also, the data in the same table revealed that chitin content of shrimp wastes was 36.43% (on dry weight bases). According to the pervious results, shrimp wastes considered as a good source for chitin which can be modified to chitosan.

Production of chitosan by microwave

Effect of sodium hydroxide concentration (%) on chitosan yield

Table (2) shows the effect of using different concentrations (%) of sodium hydroxide on the chitosan yield. The modification process by microwave heating was carried out using different concentrations of sodium hydroxide (30, 40 and 50%) at 800W microwave power for 5 min and solid : liquid ratio (1:10). It could be observed that, there was an increment in chitosan yield as a function for increasing sodium hydroxide concentration. The highest chitosan yield (53.2%) was obtained with NaOH concentration (50%). These results were in harmony with those of (17).

Effect of solid : liquid ratio on chitosan yield

In this experiment, chitin modification process was carried out at 800W microwave power for 5 min where, sodium hydroxide (50%) was used as a solvent. The effect of solid: solvent ratio on chitosan yield was studied and the results were presented in Table (2). The results

revealed that chitosan yield was significantly increased with increasing solid: liquid ratio from 1:10 to 1:20. Meanwhile, chitosan yield was decreased when solid: liquid ratio reached up 1:20. The decrement in chitosan yield after reaching the optimum condition of solid: liquid ratio may be due to the degradation effect of alkali solution on chitosan (27). From such results, it could be noticed that, the highest chitosan yield was obtained at 1:20 of sample: solvent ratio .Where at this ratio, the percent of chitosan yield was 67.4%. These results are almost in agreement with those reported by (17).

Effect of microwave power on chitosan yield

In this experiment, chitin modification process was carried out using sodium hydroxide (50%) as a solvent at 1:25 solid: liquid ratio for 5 min. The effect of microwave power on chitosan yield was studied and the results were presented in Table (2). The results revealed that, chitosan decreased with increasing microwave power from 800 to 1000. From such results, it could be noticed that, the highest chitosan yield was obtained at microwave power (800 W). At this microwave power; the percent of chitosan yield was 67.4%. These results are in agreement with those found by (17). The obtained results may be due to thermal degradation of chitosan as a function to microwave power (28).

Effect of using different modification periods on chitosan yield

In this experiment, the modification process of chitin was carried out using 800 W as microwave power, sodium hydroxide (50%) as a solvent at 1:25 solid: liquid ratio for different periods (5, 10 and 15 min) and the results are illustrated in Table (2). The results indicated that, chitosan yield (%) increased gradually with extending the modification time from 5 to 10 min. This means that, the optimum period for chitin modification to chitosan was 10 min. These results are in agreement with those found by (17). The previous results may be explained by (29) who reported that, prolonging the modification time after reaching the optimum time leads to degradation of chitosan and lost into the reaction solvent.

Gross chemical composition of chitosan

Table (3) show that, the total carbohydrates content of chitosan prepared from shrimp shell wastes was 89.11%. It had low percentages of crude protein 0.98%, ether extract 1.30%, ash 0.31% and 2.21% crude fiber, respectively. Similar results were found by (30, 31). A high quality grade of chitosan should have less than 1% of ash content (32).

Physicochemical and functional properties of chitosan

The produced chitosan had a degree of deacetylation and molecular weight higher than commercial chitosan (Table 4). The deacetylation degree and molecular weight are important parameters for chitosan, as they affect its functional properties (33). The degree of deacetylation of chitosan is important for its application in the industry. Thus, certain researchers (34) suggested that the term chitosan should be used when the degree of deacetylation is above 70%. Solubility, water holding capacity, oil holding capacity and intrinsic viscosity values of the produced chitosan were 94, 5.05, 3.21 and 10.13, respectively. Moreover, produced chitosan has water holding capacity and oil holding capacity compared with commercial chitosan. Our results were in the same line with those obtained by (31, 32).

Using chitosan in minced beef preservation

Microbiological criteria

The influence of adding chitosan and sorbistat-K into minced meat, on the amount of microorganisms, is presented in Figure 1. It was evidenced that the addition of 0.02% chitosan had the strongest effect for lowering the microbial count toward all examined microbial species. The combination of chitosan and sorbistat-K, at percentage of 0.01% from each, was the second strongest supplement, whereas the application of sorbistat-K was the weakest treatment to reduce microbial count. The total

Table 1: Proximate chemical composition of shrimp waste (on dry weight basis)

	Shrimp wastes
Moisture	74.38
Crude protein (N x 6.25)	11.55
Ether extract	6.47
Ash	32.21
Crude fiber	7.81
Total carbohydrates	41.96
Chitin	36.43

Total carbohydrates were calculated by difference

Table 2: Effect of process variables on chitosan producing by microwave from shrimp waste

Type of experiment	Sodium hydroxide Concentration (%)	Ratio (sample/solvent)	Time (min)	Microwave power (W)	Chitosan yield (%)
Sodium hydroxide concentration					
	30	1:10	5	800	52.40
	40	1:10	5	800	52.80
	50	1:10	5	800	53.20
Sample/solvent ratio					
	50	1:10	5	800	53.20
	50	1:15	5	800	60.40
	50	1:20	5	800	67.40
	50	1:25	5	800	63.10
Microwave power					
	50	1:20	5	800	67.40
	50	1:20	5	900	60.40
	50	1:20	5	1000	54.80
Time					
	50	1:20	5	800	67.40
	50	1:20	10	800	69.83
	50	1:20	15	800	61.09

Table 3: Gross chemical composition Isolated chitosan

Samples / Components %	chitosan
Moisture	8.30
Crude protein (N x 6.25)	0.98
Ether extract	1.3
Ash	0.31
Crude fiber	2.21
Total carbohydrates	89.11

Table 4: Physicochemical properties of prepared chitosan

Components % \ Samples	Produced chitosan	Commercial chitosan
Degree of deacetylation	93	85
Molecular weight (k Daltons)	4200	300
Solubility	94	99
Water holding capacity	5.05	3.12
Oil holding capacity	3.21	1.56
Intrinsic viscosity	10.13	15.92

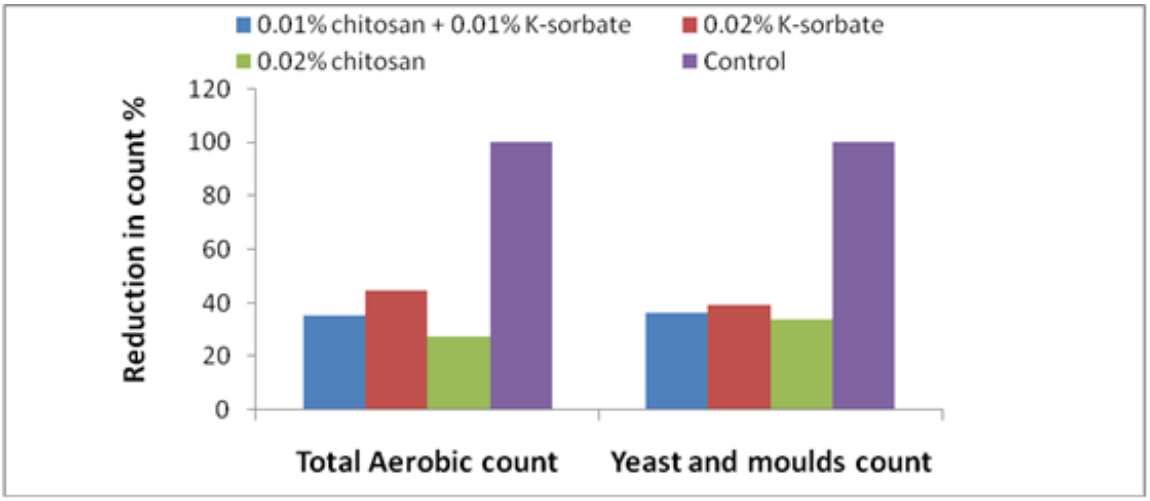

Figure 1: Effect of treating minced beef with chitosan and potassium sorbate on microbial count

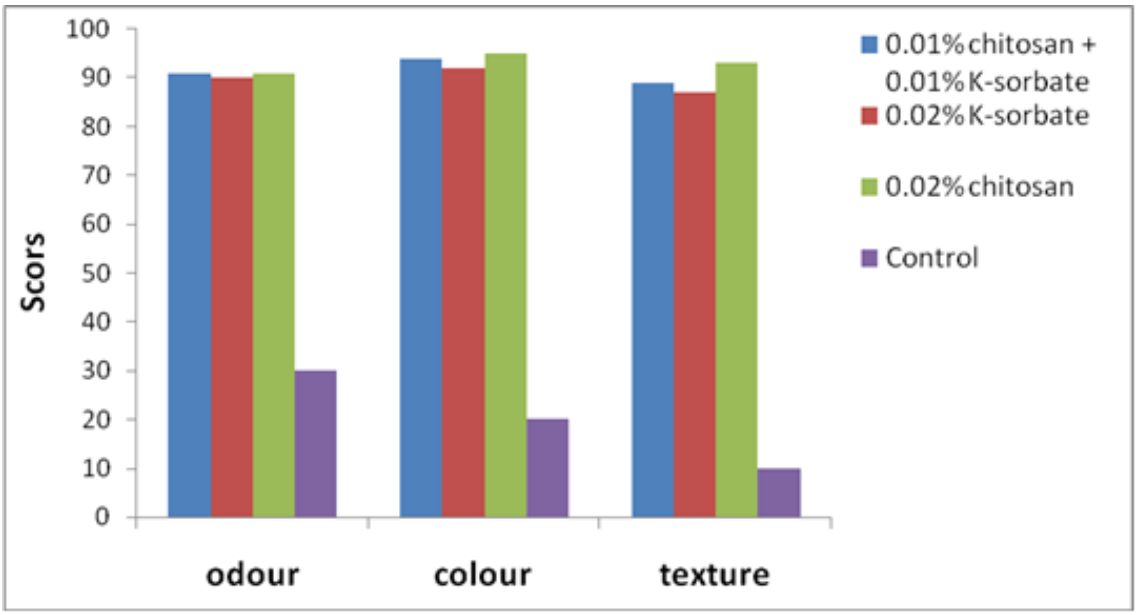

Figure 2: Effect of treating minced beef with chitosan and potassium sorbate on sensory characteristics

Sensory evaluation

There was a significant improvement in the values of sensual attributes of minced beef as a result for usage of chitosan as a preservative. After ending the storage duration, the sensory attributes (e.g. colour, odour and texture) values of untreated beef samples were highly poor, compared with other samples contained chitosan or sorbistat-K (Fig. 2). Chitosan get a higher efficiency than sorbistat-K in maintaining meat texture and odour quality, whilst, meat samples integrated with sorbistat-K had the highest values in colour. The average for texture, odour and colour were 93.1, 91.0 and 94.9% for 0.02% chitosan preserved meat; 87.1, 89.9 and 92.0% for 0.02% sorbistat-K and 9.9, 30.0 and 20.1 % for control, respectively. Lipid auto-oxidation and myoglobin-oxidation are the essential reason for meat discoloration and oxidative rancidity or another un acceptable odour or flavour components (36). With minimizing the fat auto-oxidation level, there is a prospective that the deterioration in red meat colour will be minimized as they are related reactions (37).

Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

ISOLATION AND IDENTIFICATION OF SOME POSSIBLE CAUSATIVE AGENTS OF SWOLLEN HEAD SYNDROME (SHS) IN BROILER CHICKENS IN EGYPT

Nasif S. Abdelmoez[1], M. M. Shawky[2], Heba A. Abdelhady[3], M. A. Lebdah[2], Selim S. Salama[1*]

[1]Central Laboratory for Evaluation of Veterinary Biologics, Abbasia, Cairo – Egypt, [2]Avian and Rabbit Medicine Dept. Faculty of Vet. Med., Zagazig University, Egypt, [3]Animal Health Research Institute (AHRI), Alexandria branch, Egypt

*Corresponding author, E-mail: selimsalama2000@yahoo.com

Abstract: The current study was designed to detect, isolate and identify the etiological agents of SHS in chicken flocks in Egypt. Clinical samples, including the trachea, lung and choanal cleft swabs, from different flocks, ages and localities were collected from non-vaccinated commercial broiler flocks with respiratory signs then transmitted directly to the laboratory for further examination. Avian metapneumovirus RNA *(aMPV RNA)* was extracted then amplified using one step RT-PCR using both subtype A and B (G gene) primers then differentiation occurred by real time PCR (qPCR). Swab samples from subcutaneous edema and exudates were directly cultured and the suspected bacterial colonies were biochemically identified then confirmed by PCR. Four samples were positive and one doubtful for *aMPV* using RT-PCR confirmed by qPCR. Bacteria isolation revealed that out of 40 samples, 16 samples were positive for *E.coli*, 4 samples were positive for *P. aeruginosa*, 6 samples were positive for *P. mirabilis*, 4 were mixed *E. coli* and *P. aeruginosa*, 8 were mixed *E. coli* with *P. mirabilis* and 2 samples were mixed *P. mirabilis* and *P. aeruginosa*. Depending on these findings, we could prescribe a preliminary guide to decrease SHS outbreaks by primary control of bacterial complication.

Key words: Swollen Head Syndrome; PCR; bacterial complication

Introduction

The respiratory diseases considered one of the most serious problems affecting chicken flocks resulting in severe economic losses. Several etiological agents like viruses and bacteria have been incriminated. Among viruses infecting broiler flocks, avian metapneumovirus (*aMPV*), which cause a disease entitled as Turkey Rhinotracheitis (TRT) in turkey and swollen head syndrome (SHS) in chickens (1). In Egypt, aMPV infection was diagnosed through detection of the infected broiler chickens by RT-PCR that revealed circulation of both aMPV subtypes A and B in turkey flocks (2 and 3). Unfortunately till now, no identification to aMPV subtype circulating in broiler chickens in Egypt.

Swollen head syndrome is a disease of upper respiratory tract affecting broilers and broiler breeders and characterized by swelling of head

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and facial edema which resulted from accumulation of inflammatory exudate beneath the skin of the head in response to secondary bacterial infection usually *E. coli* following the initial upper respiratory viral infection and it has become a problem in many countries in the last few years. SHS has been described as a multi-factorial disease. The initial lesion mainly caused by virus, while the clinical signs were a consequence of bacterial complications. The severity of the disease depends on presence of some environmental factors such as accumulation of ammonia, dust, overcrowding and bad ventilation. So, *aMPV* cause the initial damage of the respiratory tract, allowing invasion of bacteria, especially E.coli, which were responsible for the clinical disease (4). SHS is a disease of chickens of all ages mainly 4 - 6 weeks old with morbidity may reach to 10% and mortality about 2% (5). The affected birds showed depression, decreased feed intake, nasal exudate, sneezing, coughing and conjunctivitis followed by facial edema which start around eye extending over the head and descending to submandibular tissues (6).

Therefore, the current study was designed to detect, isolate and identify the etiological agents of SHS in chicken flocks in Egypt.

Materials and methods

Virus isolation and identification

Clinical Samples

Samples were collected from non-vaccinated commercial broiler flocks with respiratory signs such as, sneezing, coughing, tracheal rales, nasal and ocular discharges, swollen infraorbital sinuses and foamy conjunctivitis then transmitted directly to the laboratory for further examination. Samples included the trachea, lung and choanal cleft swabs. Samples were collected from different flocks, ages and localities.

Extraction of viral RNA

Procedure adopted using QIAamp Viral RNA Mini Kit (Cat# 52906) according to manufacture instructions using reagents provided in the kit.

Amplification of aMPV RNA

The reaction was prepared according to QIAGEN One Step RT-PCR (Cat# 204443) manufactures instructions. The reaction was performed for both subtype A and B (*G gene*) as following: reverse transcription (42°C /30 min), initial denaturation step (95°C /10 min), followed by 40 cycles of denaturation (95°C /1 min), annealing (54°C /1 min), extension (72°C /1 min) and final extension (72°C /10 min). The PCR product was then analyzed by electrophoresis in 1.5% agarose gel with 100 bp DNA ladder using TAE running buffer and 100 volts for 40-50 min. The ethidium bromide stained PCR bands were visualized in the agarose gel using UV transilluminator.

Primers used for *aMPV* RT-PCR were designed as described by (7) Ga(gf)- forward CCGGGACAAGTATCTCTATGG for all subtypes, G2(gaf)-reverse CCACACTTG AAAGATCTACCC for A subtype and G12(gb)- reverse CAGTCGCCTGTAATC TTCTAGGG for B subtype. Also probes used for *aMPV* real time PCR were designed following (8) as:

SHf: TAGTTTTGATCTTCCTTGTTGC

SHr: GTAGTTGTGCTCAGCTCTGATA

MB-SH-A:HEXCGCGATCGTGGAC-CTCCT

GCACTGTGGATCGCG-Iowa Black FQ

MB-SH-B:FAMCGCGATCATT-GTGACAGC

CAGCTTCACGATCGCG-Iowa Black FQ.

qPCR was done using SuperScript® III Platinium ® One-Step qPCR system-Invitrogen (Cat# 11732-088) according to manufacture instructions.

Sampling and bacterial isolation

Swabs were collected aseptically from subcutaneous edema and sinuses exudate. Collected samples were inoculated in nutrient broth then 24 hr incubation at 37ºC aerobically. Loopfull transferred onto nutrient agar, MacConkey agar and blood agar media and incubated for 24 hr aerobically at 37ºC. Suspected colonies were identified by cultural, morphologically (9), biochemically (API 20E) (10), and molecularly by PCR.

Polymerase Chain Reaction (PCR)

Extraction of bacterial DNA from E.coli were propagated by inoculation on nutrient broth then injected subcutaneously in mice followed by re-isolation from heart and liver on MacConkey agar. A single bacterial colony was transferred into 2 ml of nutrient broth and incubated overnight at 37°C. Culture was centrifuged and the bacterial pellet was re-suspended in 400µl and DNA was extracted using DNA Extraction Kit (Presto^{TM} Mini gDNA Bacteria Kit Cat# GBB101).

E. coli isolates were screened for presence of 16s*rRNA* gene using specific primers (Eco-1F, GACCTCGGTTTAGTTCACAGA and Eco-2R, CACACGCTGACGCTGACCA giving rise a specific PCR product at a size of 585 bp (11). The cyclic conditions were performed as previously described (12).

Vitek2 compact system method for bacterial identification

Bacterial identification by Vitek-2 compact system was done according to the manufacture's instruction (Biomeriux VITEK-2 Compact ref Manual – Ref-414532)

Results

Clinical signs

The suspected samples were collected from birds showing coughing, snicking, wet or frothy eyes, conjunctivitis, sneezing, facial edema, unilateral or bilateral swelling of infraorbital sinuses and some chickens showed swelling of their entire face including wattles. At the same concern the postmortem lesions of suspected dead cases revealed often yellowish extensive gelatinous to purulent edema of subcutaneous tissues of head region, sinusitis, tracheitis, perihepatitis, pericarditis and few cases showed pneumonia and air saculitis.

Detection of aMPV by RT-PCR

The genomic RNA of *aMPV* was tested using Ga and G2 primers for subtype A that revealed all samples were negative while with using Ga and G12 primers for subtype B only 4 samples were positive giving rise to a product of 312 bp (Fig. 1).

Detection, confirmation and subtyping with qPCR

The positive, doubtful and negative samples were re-examined by qPCR for accurate confirmation, 5 samples out of 40 were positive and confirmed *aMPV* subtype B isolates (Table 1, Fig.2).

Colonial characteristics of isolated bacterial associated with SHS infection

Several bacterial causative agents were isolated from suspected samples on different media as shown in Table (2). The suspected colonies of *E. coli* showed slight turbidity on nutrient broth, circular, smooth surface, mucoidy colonies, and greyish in color on nutrient agar while on MacConkey agar showed Pink colonies due to lactose fermentation. Meanwhile the suspected colonies of Pseudomonas spp. showed abundant growth and turbidity with bluish green color on nutrient broth and convex, glistening, translucent with bluish green color on nutrient agar while on MacConkey agar it showed pale colonies of non-lactose fermenter. Proteus spp. showed uniform turbidity with a slight powdery deposit and an ammonical odour on nutrient broth while on the nutrient agar made swarms intermittently in the characteristic step-like pattern across the agar surface but it looked like pale colonies with non-lactose fermentation on MacConkey agar.

Bacterial isolation and biochemical identification

Frequency of isolation as confirmed by Vitek2 assay and PCR was illustrated in Table (2). The identification results revealed that 28 samples out of 40 were *E. coli* (16 as single infection, 8 mixed infection with *P. mirabilis* and 4 mixed infection with *P. aeruginosa*). Also 16 samples out of 40 were *P. mirabilis* (6 as single infection, 8 mixed infection with *E. coli* and 2 mixed infection with *P. aeruginosa*). Finally 10 isolates out of 40 were *P. aeruginosa* (4 as single infection, 4 mixed infections with *E. coli* and 2 mixed infection with *P. mirabilis*). The amplified PCR product of suspected *E. coli* samples were 585 bp using the specific primers (Fig. 3).

Table 1: Frequency of positive samples as detected by qPCR

AMPV type	No. of tested samples	No. of +ive samples	No. of -ve samples	Detection percentage
aMPV Subtype A	40	0	40	0%
aMPV Subtype B	40	5	35	12.5%

% calculated according to the number of positive to tested samples.

Table 2: Frequency and isolation rate of some bacterial isolates either single or mixed complication associated with *AMPV* infection

Bacterial type		Tested flocks No.	Frequency of isolation	Percent of isolation
E. coli		40	16	40%
P. aeruginosa		40	4	10%
P. mirabilis		40	6	15%
Mixed infections	*E.coli* + *P. aeruginosa*	40	4	10%
	E.coli + *P. mirabilis*	40	8	20%
	P. mirabilis + *P. aeruginosa*	40	2	5%

% calculated according to the number of positive to tested samples.

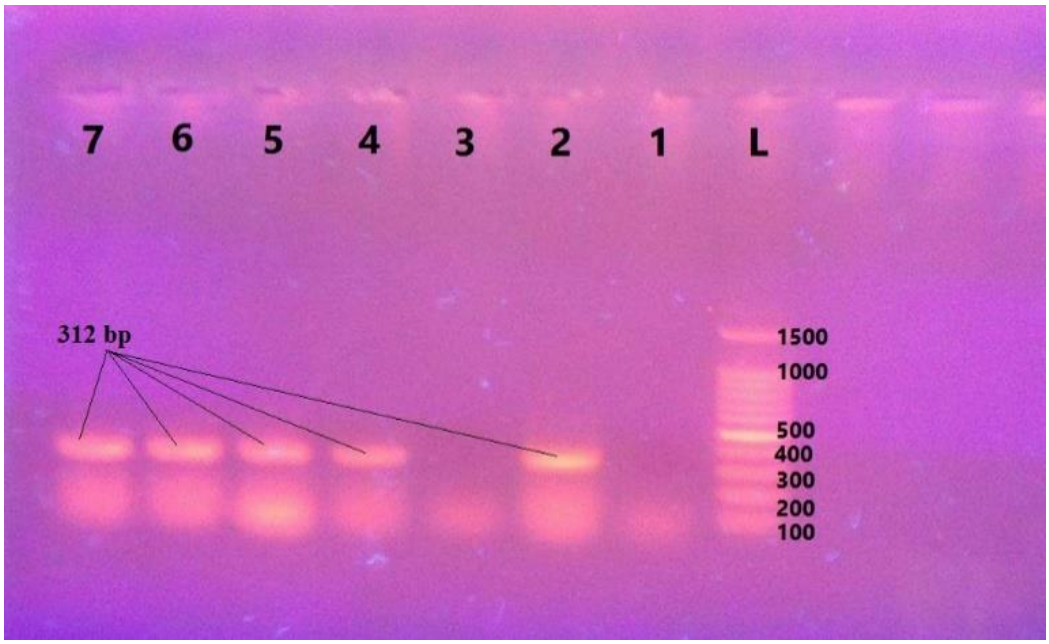

Figure 1: Ethidium bromide-stained 1.5% agarose gel showing RT-PCR amplification products of *aMPV* subtype B (312 bp). (L: 100 bp DNA Ladder; Lane 1: Negative control; Lane 2: Positive control; Lane 3: doubtful sample and Lanes 4, 5, 6 and 7 were positive sample

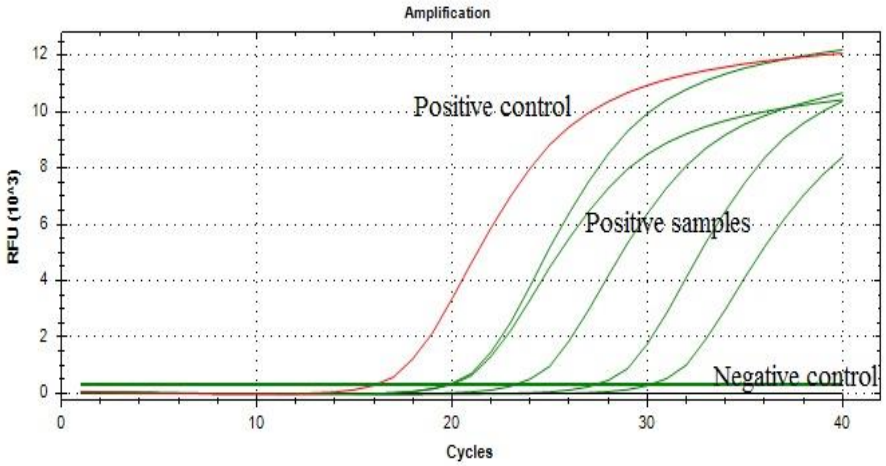

Figure 2: Amplification curves of qPCR showing cycle threshold (Ct) values of *aMPV* isolates where red line: positive control; green lines: positive samples and black line: negative control

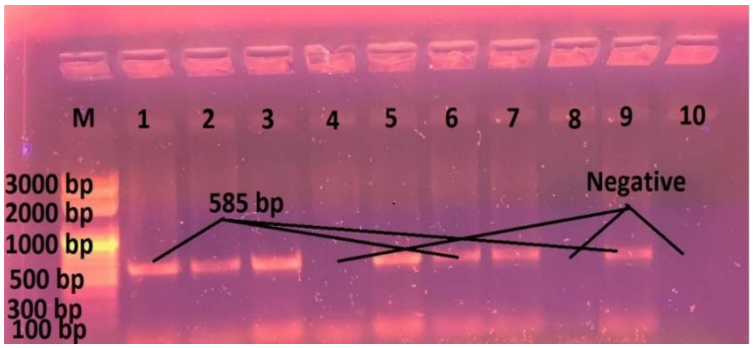

Figure 3: Ethidium bromide-stained 1.5% agarose gel showing PCR amplification products of 16s*rRNA* gene of *E.coli* (585 bp). M, marker; positive samples are 1, 2, 3, 5, 6, 7 and 9; while negative samples are 4, 8 and 10

Discussion

Infection associated with respiratory tract has significant economic impact on poultry production worldwide. SHS is a disease of upper respiratory tract and considered as one of these problems in last few years. The disease affects broilers and broiler breeders which resulted in inflammatory exudate beneath the skin (13). SHS has been described as a multi-factorial disease where the initial lesion mainly caused by *aMPV*, while the clinical signs are a consequence of bacterial complications and the severity of the disease depends on environmental factors (4).

This study was planned to detect and try to isolate some of the possible etiological agents of swollen head syndrome from different 40 broiler flocks showing swollen heads and respiratory manifestations with frothy conjunctivitis, snicking, wet or frothy eyes, sneezing, facial edema, unilateral or bilateral swelling of infra-orbital sinuses. Samples included lung tissues, trachea and choanal cleft swabs and scrabs from sinuses and turbinates. These observations were parallel to the findings obtained by (5) who reported that, the first symptoms of swollen head syndrome in broiler chickens were sneezing, redness of conjunctiva with swelling of lacrimal glands followed by subcutaneous edema of head starting around eye. Similar findings had been reported by (14). At the same concern (4) reported that the severity of the SHS depends on environmental factors.

The postmortem lesions of suspected freshly dead cases revealed often yellowish extensive gelatinous to purulent edema of subcutaneous

tissues of head region and extended in some cases to include the submandibular area, sinusitis and tracheitis. On the other hand, few cases showed perihepatitis and pericarditis, pneumonia and airsaculitis. These findings were the same that recorded by (15). Also (16) observed other lesion in the form of various amounts of casious exudate in lacrimal gland. In addition to (17) found that, under field conditions gross pathology depends largely on the presence of secondary infections in form of airsaculitis, pericarditis, perihepatitis and pneumonia.

Regarding detection of *aMPV* using conventional RT-PCR, only 4 samples out of 40 in a percentage of 10% were detected meanwhile, when the same 40 samples were examined using qPCR, 5 samples out of the collected 40 flock samples were detected, in a percentage of 12.5% and the detected virus isolate belonged to subtype B of *aMPV*. The difference between the two results could be attributed to the higher sensitivity of qPCR than conventional PCR. Parallel results were recorded in Egypt by (18) who detected TRT antibodies in broiler chickens from ten farms showing swollen heads and in other five healthy broiler farms in different Egyptian provinces using two different ELISA kits indicating seroprevalences of virus in Egypt. Also (3) detected the TRT virus (*aMPV*) subtype B in turkey flocks in Egypt. On the other hand, our results were in agreement with that of (19) who detected *aMPV* subtype B in 17 broiler flocks out of 133 examined flocks constituting about 12.5% of tested flocks in Jordan.

Regarding bacterial complications in the examined 40 flocks regardless to presence or absence of *aMPV* infection, our results revealed that twenty eight samples out of 40 were positive for *E. coli* isolation (16 as single infection, 8 mixed infections with *P. mirabilis* and 4 mixed infection with *P. aeruginosa*), sixteen samples out of 40 were carried *P .mirabilis* (6 as single infection, 8 mixed infection with *E. coli* and 2 mixed infection with *P. aeruginosa*) and ten isolates out of 40 were infected with *P. aeruginosa* (4 as single infection, 4 mixed infection with *E. coli* and 2 mixed infection with P. mirabilis).

Concerning the secondary bacterial infection accompanying the *aMPV* infection among the 5 infected flocks, the *E. coli* was the predominant secondary bacterial spp. in the 5 flocks either alone in 2 flocks or complicated with either *P .mirabilis* (2 flocks) or *P. aeruginosa* as in one flock. Similar results were recorded by (20) and (21) who isolated pure *E. coli* culture from cases of *aMPV* infection. In the same context many authors reported mixed bacterial infection complicating *aMPV* infection in broilers including (22) who had isolated *E. coli*, *Pseudomonas* spp. besides *Moraxella* spp. from SHS cases. Also, (23) who had isolated *P. aeruginosa*, *E. coli*, *P.* mirabilis and Staphylococcus spp. complicating viral infection from young chicken during 1992 with SHS. Furthermore, (24) could isolate Pneumovirus for the first time together with *E. coli* and *P .mirabilis* from a broiler flock with swollen head syndrome in Japan.

Regarding the other 35 flocks that were negative for *aMPV* and showing SHS signs, our results revealed incrimination of *E. coli* alone or mixed with *P. mirabilis* and /or *P. aeruginosa* to be the causative agents of SHS in that chicken flocks besides the recorded bad environmental and/or managemental factors. These findings were parallel to those obtained by (25) who reported that TRT virus did not play a causal role in SHS in commercial broilers in Greece but other bacterial agents together with bad environmental factors seemed to be essential in the occurrence and severity of the disease.

The lower detection rate of *aMPV* from the affected broiler flocks may be due to short period of presence of that virus in the tissues of affected birds, nearly not more than 4 days, in addition to presence of secondary bacterial infection in most of affected cases. From the above mentioned findings, we could prescribe a preliminary guide to subside and decrease SHS outbreaks by primary control of bacterial complication either by application of biosafety and biosecurity measures, optimization of environmental factors, application pf available vaccination programs or curative and prophylactic treatment will help and support the reduction of

prevalence of SHS infections among broiler and broiler breeder flocks in Egypt.

Conclusion

It could be concluded that these results confirming the prevalence of *aMPV* subtype B among poultry flocks and its detection for the first time among broiler chickens in Egypt, through isolation, identification of the *aMPV* virus using RT-PCR and qPCR. In addition to, isolation and identification of some bacterial co-infection as *E. coli*, *P. mirabilis* and *P. aeruginosa* using traditional methods, PCR and Vitek2 compact system.

Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

FIELD APPLICATION FOR EXPERIMENTAL INACTIVATED MULTIVALENT *P. multocida* AND AVIAN INFLUENZA (H9N2) VACCINE IN POULTRY

Selim S. Salama[1]*, Heba A. Abdelhady[2], Lamiaa Atia[3]

[1]Central Laboratory for Evaluation of veterinary Biologics (CLEVB), Cairo, Egypt, [2]Animal Health Research Institute (AHRI), Alexandria branch, Alexandria, Egypt, [3]Animal Health Research Institute (AHRI), Banha branch, Qalyubia, Egypt

*Corresponding author, E-mail: selimsalama2000@yahoo.com

Abstract: Fowl cholera (FC) and Avian Influenza (AI) are two of the major economically important respiratory and septicaemic disease of poultry in Egypt and allover the world. A field trails was conducted to evaluate the immunogenicity of an experimentally multivalent inactivated Fowl cholera (P. multocida) and Avian Influenza (H9N2) vaccine. The In this study a combined inactivated Montanide ISA fowl cholera and AI vaccine was prepared then the immunization potency and protective efficacy were evaluated through experimental application on different poultry breeds including broiler breeder, broiler and turkey flocks.The peak of humeral immune response against *P. multocida* as measured by ELISA was at the 5th week post vaccination and at 3rd week post boostering all over the used poultry flocks. At the same time, AI immune response as measured by HI reached the peak at the 6th week post vaccination and at 2nd week post boostering. Regarding the challenge test, the protection levels were 76.6, 70 and 80% and 90, 86.6 and 93.3% against *P. multocida* while protection levels were 83.3, 80 and 86.6% and 93.3, 90 and 96.6% against AI virus after challenge with the used virulent strains post single and booster dose respectively. In conclusion the combined prepared fowl cholera and AI vaccine succeeded in eliciting protective antibody titres and full protection against both fowl cholera and AI diseases.

Key words*: Pasteurella multocida*; AI; vaccine; ELISA; PHA; HI; challenge test

Introduction

Fowl cholera (1) and AI (2) are two of the major economically important respiratory and septicaemic diseases of poultry in Egypt and allover the world. They are highly contagious diseases causing devasting economic losses to the poultry industry through death, weight loss and condemnation of carcasses worldwide. Fowl Cholera caused by *P. multocida* which belongs to the family *Pasteurellaceae and* classified into five groups based on capsular antigens and into 16 serotypes based on LPS antigens (3). It occurs sporadically or enzootically as peracute, acute or chronic form all over the world (4). Mortality may range from only few percent to nearly 100% and recovered birds may remain as carriers even after 9 weeks after infection (1).

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Avian influenza (AI) is an infectious respiratory disease of birds caused by avian influenza type A viruses that are members of the family Orthomyxoviridae (5). The H9N2 avian influenza virus (AIV) was reported to be of low pathogenicity in chickens (6, 7) causing minimal clinical signs other than a slight drop in egg production but greatly has immunosuppressive effect (8) confirmed that the control of H9N2 viruses in poultry is important.

Combination of bacterial and viral vaccines which contain multiple antigens has many benefits for the manufacturer as it reduce production costs, for the administrator as it save time, effort and simplify the immunization schedule and for the animals as it minimize stress of multiple vaccinations (9).

The aim of this study is to evaluate the usage of a combined vaccine against FC and AI diseases prepared with Montanide ISA 206 as adjuvant under field condition.

Materials and methods

Strains used

a- Pasteurella multocida serotypes (1, 3, and 4): obtained from the Strain Bank, Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Cairo, Egypt.

b- AI H9N2: isolated in the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), yearling 2015, Cairo, Egypt.

Vaccine Preparation

a- Preparation of inactivated Pasteurella multocida antigenic phase

A virulent local strains of *P. multocida* Serotypes 1, 3, 4 and D2 were propagated in brain heart infusion broth (BHI) at 37 ^{O}C for 24 hours to obtain a dense culture containing approximately 2×10^9 CFU of each strain. After inactivation by addition of 0.1% formalin, each culture was tested for purity, safety and sterility as mentioned by (10). Finally, cultures were equally mixed together then preserved with 0.01% of thiomersal and stored at 4 C° until use.

b- Preparation of inactivated AI antigenic phase

Propagation and titration of AI H9N2 that locally isolated were done in SPF 9 -10 day old ECE according to (11) and (12). Its titer was 10^9 EID_{50}/ml. Inactivation of AI virus was carried out using binary ethelenamine 0.1M with final concentration 0.01M. Inactivation and testing of inactivation was done according to (13).

c- Preparation of combined inactivated FC and AI vaccine by using ISA 206

A combined vaccine of *P. multocida* and AI was prepared water in oil in water emulsion by mixing equal volumes of the inactivated *P. multocida* antigenic phase and inactivated AI antigenic phase to form an aqueous phase and immersed in equal volume of Montanide ISA 206 oil adjuvant (SEPPIC, cosmetics, pharmacy division, Paris, France).

Quality control of the prepared vaccine

The vaccine was tested for sterility and safety following the standard international protocol as described by OIE (14) before the usage in the field trial.

Experimental design

A separate broiler breeder, broiler and turkey flock houses were used through the experimental work of this study. Birds were vaccinated I/M at the age of six weeks, 2 weeks and 4 weeks respectively with 0.5ml / bird with the prepared combined vaccine. Random 60 birds from each group were transferred to a specific isolator to be challenged 3 weeks post first vaccination. A group of the rest birds of the different flocks were then received booster dose and 3 weeks apart challenge test were done. Also a group of the broiler were kept to continue in a special house to complete the period of experiment. A group of bird per each flock were kept as non-vaccinated control group. Random serum samples were collected from all flocks all over the experimentation period.

Enzyme Linked Immunosorbent Assay (ELISA) ELISA was performed according to the manufacturer instruction (ID-VET) for determining *P. multocida* antibody titer.

Haemagglutination inhibition (HI) test

It was carried out according to OIE (14) for the evaluation of immune response against AI.

Challenge test

*a- Challenge with virulent P. multocida strains***.**

This test was done according to (15). Vaccinated birds (30 per each flock) as well as non-vaccinated (15 per each flock) were challenged by inoculation of 0.1ml of $2x10^2$ CFU of *P. multocida* Serotypes 1, 3, and 4 (10 vaccinated and 5 non vaccinated birds for each serotype) via intramuscular route 3 weeks after both first and booster dose vaccination. All birds were observed for 14 days and mortalities were recorded.

b- Challenge with AI H9N2 strain

This was done according to (16). 30 vaccinated as well as 10 non-vaccinated birds per each flock were challenged by inoculation of 0.1ml of AI 10^6 EID_{50} strain via intranasal route 3 weeks after first and booster dose of vaccination then cloacal swaps were collected on 1, 3 and 5 days post challenge to check virus shedding and protection obtained.

Results and discussion

Control of fowl cholera and avian influenza are still subject of interest and usually attract the attention of researchers to know more about diseases, epidemiology and how to control in susceptible birds. Despite the prophylactic measures for protection against both diseases using various vaccines and different vaccination programmes, mortality and losses still occur due to respiratory diseases. Combination of bacterial and viral vaccines has many benefits for the manufacturer as it reduce production costs, save time, effort and simplify the immunization schedule. Also it minimize stress of multiple vaccinations on the animals. Vaccination is still considered one of the major tools for controlling both of the two diseases. Vaccine efficacy depend on many variables, such as the nature, the amount of antigen administrated and the presence of adjuvant to enhance immunogenicity (17).

FC and AI vaccine was prepared using Montanide ISA 206 and evaluated by measuring humeral immune response and vaccination challenge assays against both in this field study.

Assessment of Quality control measures for the prepared inactivated vaccine indicated that, the prepared vaccine was completely sterile, haven't any bacterial or fungal contaminants when tested on specific bacteriologic and fungal media. In addition there was no local, systemic reaction or mortalities were recorded in inoculated chicks, and these denote to the safety of the prepared vaccine.

Regarding the potency of the prepared vaccine, the serological tests carried out on serum samples obtained from vaccinated chickens with the combined FC and AI vaccine revealed that such birds exhibited detectable *P. multocida* antibodies from the first week post vaccination as shown in Table (1). The ELISA antibody titers increased gradually till reach its maximum level at the 5th week post first vaccination where it records 1247, 1197 and 1226 in case of broiler breeder, broiler and turkey flocks respectively. This is consistent with (18) who reported that the antibody titres significantly increased 3 weeks after primary vaccination. Booster doses caused a rapid shooting in the antibody titer and reached its highest level at the end of 3rd week post boostering. The highest antibody titers were 1832, 1578 and 1801, respectively.

It is clear that all birds showed a secondary response greater than that seen in birds vaccinated once, these findings was in accordance with that recorded by (15) who stated that a significant immunological stimulus had been elicited by the 2nd exposure and (19) who reported that the immune response of secondary immunization was significantly higher than the primary immunization. On the other hand (20) evaluated the immune response induced by several adjuvants included in *Pasteurella multocida* vaccines and stated that there is no significant decrease of antibody titres was observed between 4 and 8 weeks after vaccination.

The inoculation of booster dose of combined vaccine at the start of 4th week caused a rise of antibodies titer where reached the peak against

P. multocida at the 3rd week post boostering. From these investigations, it was clear that for longer term protection against FC, the vaccines should be given at two doses, at least three weeks apart.

As regards to the HI test that carried out on serum samples obtained from vaccinated chickens with the prepared combined FC and AI vaccine, it revealed that there was an increase in AI antibodies with titer of 6.4, 5.8 and 6.4 $\log_2$ when measured at the 3rd week post single dose of vaccination which raised gradually up to its maximum level at the 5th week recording 7.8, 7.2 and 7.6 in broiler breeder, broiler chickens and turkey respectively as shown in Table (2). Meanwhile the inoculation of booster dose after 3wks caused a rapid rise of antibodies titer where the peak of AI antibody titer was 8.6, 8.0 and 8.2 $\log_2$ by the 3rd week post boostering respectively. The same results obtained by (21) who determined the efficacy of two oil emulsion (ISA 206, ISA70) HA subunit vaccine derived from H5N1 virus.

The same results obtained by (22) who evaluated the quality of oil emulsion AI H9N2 vaccine in-vitro by HI test without boostering. They found that the antibody titer increased till reach its peak (6.24 $\log_2$) at 5th week post vaccination. On the other hand, (23) evaluated a reverse genetics H5N3 avian influenza virus cell cultured vaccine by single dose and the HI titer reached its peak (6.2 $\log_2$) at the 4th week. (21) Found that chickens which received one dose of rHA- H5 vaccine had a markedly poor response and priming by two dosages appeared to improve the antibody response. (24) evaluated inactivated H9N2 vaccine with gel-primed and mineral oil- boosted regimen in broiler breeders. They concluded that a single dose regimen of inactivated H9N2 vaccine provided not enough anti body level in the broiler breeder flock and recommended a two doses regimen than single dose. Also stated that a gel- primed and oil- boosted regimen might be an economical and effective vaccine strategy for poultry producers.

Regarding the challenge test, the protection percentages of birds against *P. multocida* strains were 76.6, 70.0 and 80.0 %, among broiler breeder, broiler and turkey groups respectively when challenged 3weeks after single dose, while they were 90.0, 86.6 and 93.3 %, among the same groups when challenged 3 weeks after booster dose as shown in Table (3).

The results obtained by (25) who reported that the protection % in ISA 206 combined FC and ND vaccine was 100%. Also (26) noticed that chickens vaccinated with FC vaccine containing ISA 206 showed 90% protection. From these data it was clear that the antibody titer was measured with ELISA test was highly correlated with protection against challenge with virulent organisms as reported by (15).

For the protection against AI H9N2 virus, it was 83.3, 80.0 and 86.6% among the broiler breeder, broiler and turkey bird groups when challenged 3weeks after single dose as shown in Table (4). These protection percentages were raised up to 93.3, 90.0 and 96.6 % for the same bird groups when challenged 3 weeks post booster of vaccination.

An inactivated ISA 70 H9N2 vaccine was evaluated by (27) and concluded to this vaccine induced protection 100% 3weeks post vaccination where the virus isolated from 0/7 cloacal samples collected on 1, 3 and 5 days post challenge. In contrast (23) concluded that ISA 206 adjuvant vaccine provides only 50% or even less protective efficiency to SPF chickens. Meanwhile (28) recorded that the protection levels were found to increase up to 90 % when the Montanide ISA 70 and Montanide ISA 206 formulations were used.

The findings of this study indicated that the vaccine is valid and has a satisfactory result according to specifications of OIE (14).

Conclusion

Conclusively, it is very beneficial to apply the inactivated polyvalent *Pasteurella multocida* and AI H9N2 vaccine for the protection against both FC and AI outbreaks as it reduce production costs, for the administrator as it save time, effort and simplify the immunization schedule. Also for the birds, it minimizes stress of multiple vaccinations.

Table 1: *P. multocida* ELISA mean titer of different bird flocks vaccinated with the prepared FC and AI (H9N2) vaccine post single and booster dose vaccination

Type of Birds	Weeks post vaccination								Control
		1	2	3	4	5	6	7	
Broiler breeder	Single dose	523	841	1011	1176	1247	1208	1185	36
	Booster dose				1381	1566	1832	1792	41
Broiler	Single dose	469	719	904	1066	1191	1174	1081	39
	Booster dose				1218	1466	1578	1511	38
Turkey	Single dose	497	819	973	1118	1226	1211	1172	44
	Booster dose				1341	1494	1801	1716	29

Table 2: AI H9N2 HI mean titer of different bird flocks vaccinated with the prepared FC and AI (H9N2) vaccine post single and booster dose vaccination

Type of Birds		Weeks post vaccination							Control
		1	2	3	4	5	6	7	
Broiler breeder	Single dose	3.6	4.8	6.4	6.6	7.8	7.6	7.2	0.0
	Booster dose				7.2	8.2	8.6	8.2	0.0
Broiler	Single dose	3.2	4.0	5.8	5.8	7.2	6.8	6.2	0.0
	Booster dose				6.8	7.4	8.0	7.6	0.0
Turkey	Single dose	3.6	4.6	6.4	6.2	7.4	7.2	6.8	0.0
	Booster dose				7.2	7.6	8.2	7.8	0.0

Table 3: Protection percentages obtained in birds vaccinated with single and booster doses of the prepared FC and AI vaccine against virulent *P. multocida* strain

Type of Bird	Challenge 3 weeks post						Control		
	Single dose vaccination			Booster dose vaccination					
	Bird No	M.	P %	Bird No	M.	P %	Bird No	M.	P %
Broiler breeder	30	7	76.6	30	3	90.0	15	12	20.0
Broiler	30	9	70.0	30	4	86.6	15	14	07.0
Turkey	30	6	80.0	30	2	93.3	15	13	13.4

M = Mortalities P% = protection percent

Table 4: Protection percentages obtained in birds vaccinated with single and booster doses of the prepared FC and AI vaccine against AI H9N2 virus

Vaccination	Type of bird	ECE No.	HA		Protection %
			Positive	Negative	
Single dose	Broiler breeder	30	5	25	83.3
	Broiler	30	6	24	80.0
	Turkey	30	4	26	86.6
Booster dose	Broiler breeder	30	2	28	93.3
	Broiler	30	3	27	90.0
	Turkey	30	1	29	96.6

Conflict of interest

The authors declare that they have no conflict of interest.

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