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SLOVENIAN VETERINARY RESEARCH

SLOVENSKI VETERINARSKI ZBORNIK

Supplement 25

7th International Conference on
Veterinary Medical Science

Veterinary Medicine and The One Health Concept

Faculty of Veterinary Medicine
Zagazig University, Egypt

Hurghada, Egypt
October 2022

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SLOVENSKI VETERINARSKI ZBORNIK

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Original Research Article
Veterinary Medicine and The One Health Concept

THE EFFECT OF *Neem* LEAVES EXTRACT AS IMMUNSTIMULANT BEFORE CHICKEN INFECTIOUS ANEMIA VACCINATION IN BROILERS

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Abstract: Chicken infectious anemia (CIA) has lately arisen as a major problem in poultry production due to poor growth, high mortalities. We successfully used PCR on seven flocks of chickens - (three breeder and four broiler) in the Egyptian governorates of Dakahlia and Damietta to detect CIAV in tissues of probable CIAV-infected birds. Numerous substances, including triterpenoids and glycosides, are found in *Neem* and are assumed to be the source of its antiviral effects. All birds were randomly allocated into eight groups (n=20 each) for different treatments, including vaccination, feeding *Neem* 8%, feeding infected birds 8%, vaccination and infected birds, feeding infected birds 8%, and finally feeding infected birds 8% and vaccination. One-hundred and sixty birds were randomly divided into eight groups (n=20 each). The groups were differentially-treated as the followings; healthy birds, birds infected with CIAV, birds fed *Neem* 8%, infected birds fed *Neem* 8%, vaccinated birds, vaccinated and infected birds, birds fed *Neem* 8% and vaccinated, and finally the infected ones fed *Neem* 8% and vaccinated. The study is aimed to detect the immunostimulant effect of *Neem* leaves extract 8% on immune response post vaccination against CIAV in broilers. The group taken immunstimulant (*Neem* leaves extract 8%) along with vaccination increased immune response of birds since titers of the enzyme-linked immunosorbent assay were 15200 ($P \leq 0.05$) than the group valued 14732 of vaccination only. However, in groups (*Neem* vaccinated infected, infected and vaccinated infected) were 14663, 12600 and 12091 ($P<0.05$, $P<0.05$ and $P<0.01$, respectively). Infected group exhibited indications of CIAV as despondency, while group vaccinated and treated with *Neem* leaves extract 8% appeared normal after challenging with CIAV. The hematocrit values of infected group and vaccinated treated infected group were 21 and 30, respectively. Histopathological changes in *Neem* vaccinated group after challenging with CIAV showed increasing in the thickness of both cortex and medulla of thymic lobules beside over population. In the latest group, bone marrow showed activation and proliferated hemopoitic elements with regenerative centers. We conclude that the combination of *Neem* leaves extracts 8% and CIAV vaccination is a potent antiviral and has immunstimulant properties during the production cycle of broilers.

Key words: CIAV; PCR; histopathology; enzyme-linked immunosorbent assay; broilers

Introduction

One of the most prevalent viral diseases in the poultry industry is the chicken infectious anemia (CIA), resulting in significant mortality, lower productivity, and expensive preventive medicine costs. Chicken infectious anemia virus (CIAV) infection can show up as clinical or subclinical symptoms of birds such as anemia, thymus degeneration, bone marrow depletion, and immunosuppression characterize the disease's outbreak (1,2). The immunosuppressive effect, whether direct or indirect, induces significant lymphocyte depletion in main and inferior lymphoid organs (3). Moreover, erythroblastoid cells of the bone

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marrow and immune cells in the thymus cortex of one-day old chick are destroyed (4). CIAV detection via molecular technology, like as polymerase chain reaction (PCR) is a rapid, sensitive and simple way for detecting viral nucleic acid (5). blood and tissue samples were used from diseased broiler breeder chicks and detected CIAV-specific 418-base-pair by PCR (6). However, there are limited investigations on the CIAVs that are prevalent in Egypt (7, 8). the efficacy and safety of live CIAV vaccines was assessed and it was found that live vaccines give the birds measurable titers to CIAV by the first week reaching the peak titers by the eighth week (9). Non-vaccinated hens took the virus shed by the vaccinated ones and had lower antibody titers. Medicinal plants (Azadirachta indica) are used by humans to fight ailments. It known as "*Neem*", has gotten a lot of attention from the medical community throughout the world because of its vast range of medicinal characteristics (10). *Neem* contains numerous components, including triterpenoids and glycosides, which are thought to be responsible for the herb's antiviral properties (11). The current study is aimed to determine the prevalence of CIAV infection in some flocks in two Egyptian governorates (Dakahlia and Damietta) by using PCR in broiler and breeder chicken flocks, and then to examine the hematological, pathological, and value of live CIAV vaccine in developing immunity against the illness with or without Neem leaves extract 8% treatment in experimental chicks against CIAV infection with estimation of immune response.

Material and methods

PCR for detection of CIAV

DNA was taken out by QIA amp DNA mini kit according to producer's directions. Amplification of fractional VP1 gene was done according to the previous protocol (13). The definite intensified PCR product was illustrious using agarose gel electrophoresis according to previ-ous methodology (14).

Titration (Dose)

The virus was intramuscularly injected at day 21 by 0.1ml contained 102CID50 (18). The 50 SPF chicks.

To determine the Chicken Infectious Dose (CID), two groups of specified pathogens free (SPF) chicks received injections at one day old. Tenfold sequential dilution (10-1-10-5) with five chicks each dilution was carried out using the Reed and Meunch technique (17).

CID 50: It means half of the inoculated an-imals shows the positive reaction. Reed and Muench method computing a 50% end point of a virus titration. Calculate the proportionate distance between dilutions which infect above and below 50% of the chicks.

Equation

$$PD: \frac{\text{No of mortality above 50\% - 50\%}}{\text{(No of mortality above 50\% - No of mortality belowe 50\%)}}$$

Table 1: The grouping of birds in the study.

Groups no.	Birds no./group	Treatment of *Neem* 8%	Period of *Neem* treated (days)	Vaccination against CIAV at day 5	CIAV challenge day
1	20	-	-	-	-
2	20	-	-	-	21
3	20	+	1-30	-	-
4	20	+	1-30	-	21
5	20	-	-	+	-
6	20	-	-	+	21
7	20	+	1-30	+	-
8	20	+	1-30	+	21

Vaccine used

CIAV vaccine Nobilis® CAVP4 is a live attenuated vaccine against CIAV strain with active components per dose 26P4 ≥ 3.0log10TCID50 with dose 0.2 mL per bird (Patch no. A043AJ01) at the 5th days of age.

Bird management

The birds were housed under standard environmental and hygienic conditions for 30 days and fed organic feed (2 weeks' starter feed 21% protein and 2950 k.cal. /kg and complete the experiment with grower feed 19% and 2800 k.cal. / kg feed), with continuous lighting throughout the experiment and water ad-libitum. The eight groups were differentially treated as showed in Table 1.

The birds reared for thirty days in rigorous biosafety procedures and standard conditions (level 3). Moreover, CIAV strains were obtained from tissue homogenate from flock no.1 at age of 6 weeks (Ross breeder species), which more pronounced postmortem lesions. The homogenized tissues with PBS have been treated with antibiotics. After repeating the freezing and thawing process three times, the sample was centrifuged at 3000 rpm for 20 minutes. The supernatant was PCR-tested for Avian Influenza (H5 or H9), Infectious Bursal Disease, and Newcastle Disease to confirm that the sample were devoid of these viruses. The breeder hens and the flock we choose the chicks from were free from Adeno virus. The virus was taken from the samples of the infected flocks that we performed a PCR (the first flock in the field infected flocks). The dose of the virus measured according to Reed and Meunch technique.

Tissue specimens

The samples were taken from seven chicken flocks (4 commercial broilers, aged 2 ~ 4 weeks old) and 3 breeder chicken, aged 6 ~ 8 weeks old) located in two Egyptian governorates (Dakahlia and Damietta) during 2021 (shown in Table 2) - All the birds were not got vaccine against CIAV. Moreover, the tissue samples taken from freshly dead birds (postmortem) suffered from suspected CIAV infection with anemia, stunting, depression, pale comb & wattles, poor growth, weakness, and droopy appearance in chicken (Figure 1). Various tissues were used for sampling such as (thymus, bone marrow, bursa of Fabricious, and spleen), following the previous protocol (12).



Figure 1: Clinical examination of naturally infected bird with CIAV

Table 2: Data of the birds suspected to be infected with CIAV within Dakahlia and Damietta governorates in 2021

Flock no.	Locality	Breed	Age (week)	Total no.	Mortality % (daily)
1	Dakahlia	Ross Breeder	6	5500	0.3
2	Dakahlia	Ross Breeder	8	4500	0.3
3	Damietta	Avian 48 Breeder	7	7500	0.4
4	Dakahlia	IR Broiler	4	10000	0.35
5	Damietta	Cobb Broiler	3	2500	0.4
6	Dakahlia	Cobb Broiler	4	4000	0.3
7	Damietta	Cobb Broiler	3	4500	0.3

Herbal antiviral and immunstimulant (Neem leaves extract 8%)

Aqueous *Neem* leaves extract was prepared according to the protocol of (15). Eighty mL from this aqueous solution was then collected and mixed with one liter of drinking water, also 8% *Neem* leaves extract supplemented as ad-libidum (16).

Hematocrit values

Blood samples were collected weekly from day-1 old till day-28 from jugular vein of chicks on 3.5% sodium citrate solution. PCVs were determined (19).

ELISA test

Serum samples from non-heparinized blood samples were obtained individually at 7, 14, 21, and 28 days. Commercial CIAV antibody ELISA kit of IDEXX (ref. no: 99-08702 – LOT: FU728) was used and performed based on the manufacturer instructions.

Histopathological examination

For microscopic inspection, small sections of the thymus, bursa of Fabricious, bone marrow, and spleen were fixed in buffered neutral formalin solution at a 10% concentration (20).

Statistical analysis

Data from ELIZA titers were utilized to analyze the variance using SPSS program through One way-ANOVA.

Results

Clinical symptoms and postmortem lesions of infected flocks

The examined infected flocks were suffered from anemia, stunting, depression, pale comb & wattles, poor growth, weakness, and droopy appearance with high daily mortality rates ranging from 0.3-0.4%. Although necropsy results revealed paleness in the bone marrow, hemorrhage under the skin and bursa of Fabricious and thymus atrophy can range from mild to severe cases. Additionally, many cases of subcutaneous and muscle bleeding were reported in Table 1.

Using PCR for detection of CIAV-DNA in tissues

Liver, spleen, thymus and bone marrow of seven commercial broiler and breeder chicken flocks were examined. The primer set was designed to amplify a 418 bp fragment of the vp1 gene (2,4,8) - At the proper size band of 418 bp, five flocks tested positive with an incidence rate of 71.42%, as shown in Table 3. The findings revealed that all flocks of various ages except for flocks 5 and 6 were positive for CIAV.

Clinical and postmortem findings of the experimental birds

The CIAV-infected group displayed anorexia, despondency, general weakness, a droopy appearance, pale comb and wattles, stunting, and growth retardation (Group. 2). Thymus glands that have atrophied, subcutaneous bleeding, fatty yellow bone marrow, regressed bursa of Fabricious, liver and spleen showing and enlargement and paleness, and slowing of keel bone ossification were among the pathological findings. The groups given *Neem* leaves extract (8%) had normal immune organs, while the control group did not. The parameters used in the experiment were exclusive for histopathological changes, hematocrite values and ELIZA because the aim of the experiment is to assess the immunstimulant effect and the mortality rates. The occurrence of other factors rather than infection (like blood sampling or faulty injection for example) may give a faulty figure to the *Neem* effect. Also, the clinical finding was not a main parameter in assessing the immunstimulant effect. The chicks in all groups appeared normal may be due to strictly hygienic conditions unlike the field situation in low biosecurity farms.

Hematocrit values

At 28 days of age, the CIAV-infected group (Group. 2) had symptoms of normocytic normochromic anaemia which represented a significant decrease in PCV to less than 22% (21.1%). When compared to the normal group (27.5%), the PCV

Table 3: Frequency of CIAV detection by PCR among examined flocks in Dakahlia and Damietta governorates

Flock No.	Locality	Breed	Age (week)	No of examined birds	Vaccination of CAV	PCR results
1	Dakahlia	Ross Breeder	6	5	-	+
2	Dakahlia	Ross Breeder	8	5	-	+
3	Damietta	Avian 48 Breeder	7	5	-	+
4	Dakahlia	IR Broiler	4	5	-	+
5	Damietta	Cobb Broiler	3	5	-	-
6	Dakahlia	Cobb Broiler	4	5	-	-
7	Damietta	Cobb Broiler	3	5	-	+
Total	**35** samples				**25** (+ve) samples, **(71.4%)**	

values of the CIAV-infected *Neem*-treated group (Group 3) and the infected vaccine group (Group 4) decreased by 50% and 75%, respectively. In infected treated and vaccinated group Interestingly, group 5 showed increase in PCV values (30%) compared to vaccinated infected and/or treated infected groups as shown in (Figure 2).

ELISA results

The immune response increased in *Neem* vaccinated and vaccinated groups as the ELISA titers were 15200 and 14732, respectively while ELISA titers in groups (*Neem* vaccinated infected, infected and vaccinated infected) were 14663,12600 and 12091. The negative control group had maternal antibodies as it is broiler chicks not SPF as shown in Table 4.

On day 21, the results showed that supplementation of *Neem* 8% improved significantly the immune status of birds infected with CIAV, vaccinated, and vaccinated then infected with the CIAV infection (P=0.02, P=0.011, and P=0.034, respectively) compared with those only vaccinated birds followed by the viral infection. Further, on day 28, Moreover, *Neem* 8% plus vaccination significantly (P=0.048, P=0.036, and P=0.025) the birds' immunity compared to infected, infected and supplemented with *Neem* 8%, and vaccinated ones, respectively. Supplementation of *Neem* 8% plus vaccination optimized the immunity compared with vaccinated birds only. It was clear that the birds' immunity was shaped on day 28, and therefore the birds adapted to the viral load through the compensatory mechanism and targeted the homeostatic immune balance - It comes from the fact that our results showed that supplementation of Neem 8% plus infection improved the negative feedback mechanism of birds to slightly represented as the control birds Immune response was significantly increased in groups treated with 8% neem.

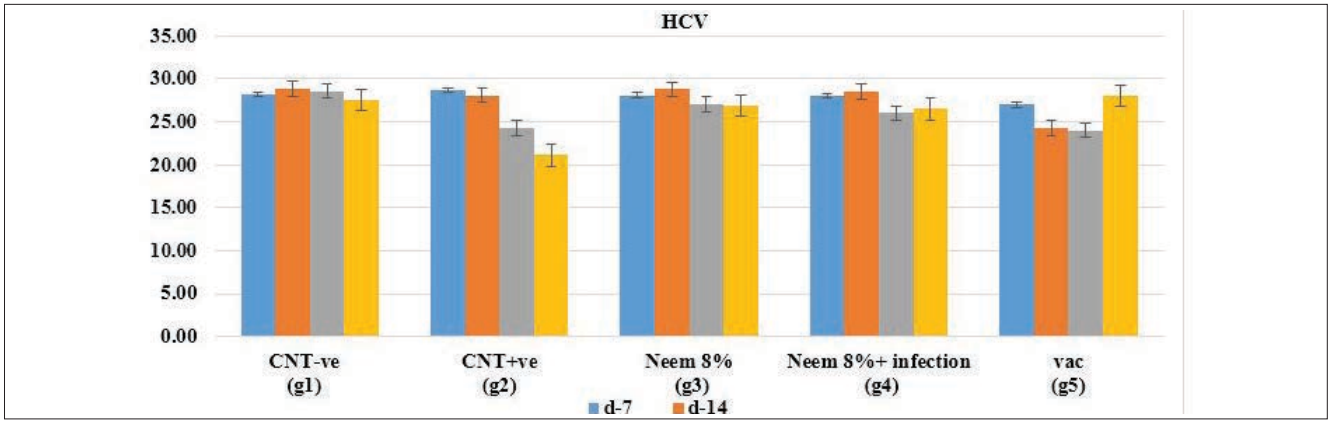


Figure 2: Hematocrit value at 7, 14, 21, and 28 days of age showing increased PCV values in "infected treated and vaccinated group" compared to vaccinated infected and treated infected groups.

Table 4: The ELISA results

Group no.	Group name	Total no.	Serum samples no.	Time of samples collected							
				7 days		14 day		21 day		28 days	
				GMT	CV (%)	GMT	CV (%)	GMT	CV (%)	GMT	CV (%)
1	CNT-ve	20	5	15250	2.2	14952	3.3	14752	2.5	14644	7.3
2	CNT+ve	20	5	15300	3.5	12525	7.7	12600	5.7	15489 c	2.5
3	*Neem* 8%	20	5	14995	3	13884	3.5	15200	3.6	15500	1.5
4	*Neem* 8%+infec.	20	5	12694	23	13584	7.2	14472a	2.1	15601 c	1
5	vac.	20	5	14824	1.4	13554	12.5	14732b	13	15695 c	1.7
6	vac+infec.	20	5	13226	8.9	14356	3.3	12091	11	15.35	7
7	*Neem* 8%+vac.	20	5	13781	8.4	13830	2.4	14197a	2.3	15183	6.8
8	*Neem* 8%+vac+ infec.	20	5	13028	15	13418	12	14663a	7.3	13606	17

Vac; vaccinated, inf; infection, GMT: Geometric mean titer, CV%: Coefficient variance.
Statistical analysis represented by SPSS-One ANOVA.
[a]$P \leq 0.05$ compared with vac+infec group.
[b]$P \leq 0.01$ compared with vac+infec group.
[c]$P \leq 0.05$ compared with *Neem* 8% + vac group.

Histopathological changes

Histopathological pictures of lymphoid organs of negative control (Group. 1) and treated group (Group. 3) showed a typical normality of histological pictures, thymic lobules' cortex & medulla and lymphoid follicles of the bursa. Splenic elements, capsule & hemopoitic centers and matrix of the bone marrow are within the histo-morphological picture (Figure 3: A-D). Infected group (Group. 2) (Figure 3: E-H) showed hemorrhages and edema in medulla of thymic lobules (arrow) beside intense lymphoid depletion from cortex. The bursal lymphoid follicles showed atrophy besides intra and inter follicular heterophilic infiltration. Moreover, the spleen picture showed severe lymphoid depletion while treated challenged group (Group. 4) showed activated hemopoitic centers with reduction of stromal cells in thymus with bursal regenerative attempts in some bursal lymphoid follicles. Besides, the spleen showed hyperplastic white pulps, and the bone marrow showed activated hemopoitic centers with regenerative attempts and numerous stem cells (Fig. 3I, Figure 3L). In (Group. 8); treated vaccinated group and challenged revealed an increase in the thickness of both cortex and medulla of thymic lobules and hyperplasia with proliferated hemopoitic elements. In addition, bone marrow showed numerous regenerative hemopoitic centers within fatty marrow (Figure 3Q, T) compared with vaccinated and challenged group (Group. 6) which showed moderate atrophy with moderate necrotic changes in all lymphoid organs (Figure 3M, P). On the other hand, the treated vaccinated group (Group. 7) displayed nearly normal thymic, Fabricious bursa, and splenic architecture. Additionally, the bone marrow region had few fat globules and nearly normal architecture, including all the hematological series (Figure 3A).

Discussion

Because of the increased susceptibility to subsequent infections, viral infections that impair the immune system have a huge financial impact on the poultry industry. One of the most common immunosuppressive diseases in poultry is chicken infectious anemia virus (CIAV). CIAV has emerged as a major poultry virus, causing serious financial harm to the global poultry industry (8,21-24). Furthermore, earlier research has revealed that CIAV is widely distributed in Egypt's commercial poultry sectors (8). The pathophysiological profile is characterized by atrophy of the thymus, generalized hemorrhages in the skin muscles, bone marrow aplasia, immunological suppression, and anemia in chickens; which are the only known hosts afflicted by this virus (22,25). In our study, three breeder farms and four broiler

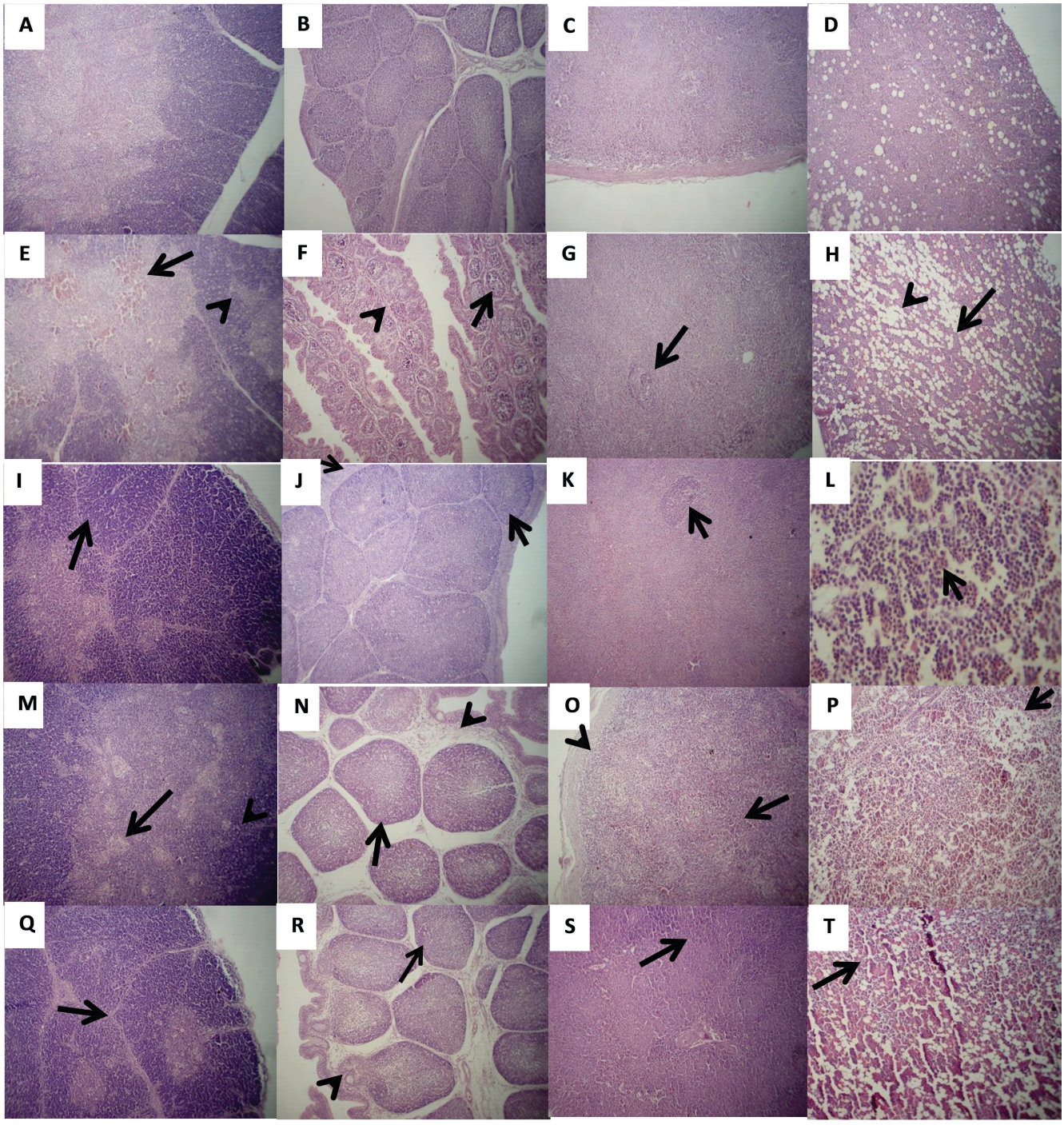


Figure 3: Histopathological findings of experimentally infected chicks with CIA virus thymus, bursa of Fabricious, spleen and bone marrow: (A-D) negative control group; (E-H): positive control group; (I-L): The group treated with *Neem* 8% and challenged; (M-P): vaccinated and challenged group; (Q-T): group treated with *Neem* 8%, vaccinated and challenged.

farms are used as sources for the thymic loops, bone marrow, bursa of Fabricious, and spleen in Dakahlia & Damietta governorates. The suspected flocks giving suspected signs and lesions of CIAV such as drooping aspect, pale combs and wattles, overall weakness, depression, high mortalities, stunting, and growth retardation. Necropsy findings include watery blood, yellow, fatty bone marrow, particularly atrophied thymus glands, atrophied bursa of Fabricious, and enlarged livers and spleens. Clinical symptoms and postmortem lesions were consistent with the findings of (3,26-

30). In flocks of broiler and breeder chickens, polymerase chain reaction (PCR) was employed to identify the presence of the chicken infectious anemia virus (CIAV). In Damietta and Dakahlia provinces of Egypt, out of 7 tested commercial flocks, 5 flocks were positive as reported in the study. For the recognition of CIAV-DNA in birds' tissues, the PCR test was the method of choice. Compared to virus isolation through cell culture, it was more precise and sensitive; especially because the same tissues used to isolate viruses can also be utilized to extract DNA (8,31,32). CIA has no specific therapy because it is a viral disease, and its significant immunosuppressive effect raises its economic danger to poultry producers. Herbal immunomodulation is a new field in research for establishing effective and healthy chicken production due to the birds' increased sensitivity to several diseases as a result of constant genetic selection and intensive production practices (33-35). Therefore, in our study, the immunostimulant effects of *Neem* were evaluated with concentration 8% compared with vaccination against CIAV. Infected group displayed CIAV symptoms such anaemia, stunting, sadness, and a pale comb and wattles, poor growth, weakness and droopy appearance with high daily mortality rates ranging from 0.3-0.4% hence the mentioned mortality rate is for the infected flocks in the survey not in the experiment. However, chicken's group vaccinated and treated with *Neem* leaves extract 8% appeared normal after challenging with CIAV. The hematocrit values were 21.10 (in infected group) and 30.00 (in vaccinated and treated with *Neem* 8%). Hematocrit value of less than 27%, along with yellowish changes in the bone marrow and thymic atrophy, may be symptoms of CIA (3,29,30). The groups treated with *Neem* 8% showed faster recovery to the hematocrit values than positive control, and therefore these findings supported by (36). The latest author referred that the ameliorative effects of Azadirachta indica on CIAV-induced hematological alterations in chicks treated with Azadirachta indica (*Neem*). It indicates a significant improvement in hematological profiles and recovered from anemia within two weeks after infection. These findings emphasize the influence of *Neem* on the hematological parameters in birds by its hepatostimulatory and hepatoprotective effects – It indicates the release of erythropoietic factors by liver cells, which causes the production of more hemoglobin in the bone marrow. the effectiveness and safety of live CAV vaccines was assessed and we found measurable antibody titers by the first week after receiving a live CAV vaccination and the author evidenced that specific CAV antibodies were detectable in the birds, reaching maximal titers by the eighth week (9). When non-vaccinated birds encountered vaccinated chickens, the chickens shed virus and the non-vaccinated birds' antibody titers were lower. Concerning to the ELISA readings, the immune status of the birds was compared after different treatments. It was found that the immune response increased in *Neem*-treated and CAIV vaccinated, and vaccinated group as the ELISA titers were 15200 and 14732 respectively. However, birds treated with *Neem* 8% and infected gave higher ($P<0.05$) antibody response than which vaccinated and infected with virus. It means that using of *Neem* is better than vaccination while birds treated with *Neem* 8% and vaccinated after 5 days then infected at day 21 give higher antibody response ($P<0.05$) than vaccinated after 5 days and infected at day 21 without *Neem* which indicates immunstimulant effect of *Neem*. Treated group with *Neem* 8% and vaccinated at day 5 gives higher antibody response ($P=0.01$) than vaccinated group at day 5 and infected at day 14, indicating neutralization of antibodies by field strain and immunstimulant effect of *Neem*. It was clear that our results showed that supplementation of *Neem* 8% plus vaccination improved the negative feed-back mechanism of birds to slightly represented as the control birds. Histopathological changes in *Neem* vaccinated group post-challenge showed a wideness with increase in the thickness of both cortex and medulla of thymic lobules beside over population of cells (hyperplasia). Also, bone marrow showed activation and proliferated hemopoitic elements with regenerative centers. The bursal lymphoid follicles appeared broad and larger in diameter beside their overpopulation by lymphocytes. Herein, spleen showed intense hyperplastic changes in lymphocytes of white and red pulps than other groups. However, in vaccinated group challenged with virus showed atrophy and necrotic changes in all lymphoid organs. In group treated with *Neem* 8%, vaccinated and challenged revealed an increase in the thickness of both cortex and medulla of thymic lobules and hyperplasia with proliferated hemopoitic elements. In the same group, the bone marrow showed numerous regenerative

hemopoitic centers within fatty marrow which are compatible with the findings of former studies (37,38). The previous authors showed that the immunosuppressive outcome is due to the virus destructive effect of hematological and lymphopoietic tissues, which results in a weakened immune response. CIAV infection in splenic T cells is characterized by decreased phytohemagglutinin reactivity, concanavalin A production, and a reduction in interleukin output (39). In addition, it was discovered that 8% *Neem* resulted in 100% recovery of leukocytes distributed in the hepatic parenchyma following bacterial infection (16). Macrophage concentration, interleukin-1 (IL-1) creation, Fc-receptor expression, phagocytosis, and bactericidal action are significantly reduced. Immunological response was altered due to the harmful effects of virus on lymphocyte and macrophage activities. It leads to increasing the risk of concomitant infection with other antigens and makes immunization ineffective (2). In this work, mild lymphoid follicle exhaustion was visible in the bursa. The finding is consistent with other authors who discovered substantial hypoplasia in hematopoietic cells in the bone marrow as well as significant lymphocyte depletion in the thymus and bursa of Fabricious (40). In addition, bursal alterations in lymphoid follicles with varying degrees of atrophy and dispersed necrotic foci was identified which attributed to secondary infections (6). The thymus and bone marrow samples were also positive because CIAV targets erythroid and lymphoid progenitor cells in these tissues (2).

Conclusions

It is concluded that CIAV damages the spleen, thymus and bone marrow, leading to high level of aplastic anemia, immunosuppression and a significant drop in PCV. The obtained results revealed that the use of *Neem* leaves extracts 8% in addition to vaccination rather than vaccination only with CIAV vaccine is a potential combination during the rearing period of chicken due to its high antiviral and immunstimulant effect. The finding could also help in preventing secondary infections of chicken and large financial losses for the chicken producers.

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Institutional Review Board Statement: Animal Experimental Guidelines were followed, and the Animal Care and Use Committee of the Animal Health Research Institute, Zagazig University, Egypt.

The data that supports their conclusions is available from the authors of this manuscript upon request.

There are no conflicts of interest in the current work, according to the authors.

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Original Research Article
Veterinary Medicine and The One Health Concept

EVALUATION OF THE EFFICACY OF DIFFERENT DIETARY RUMEN BUFFERS ON PREVENTION OF RUMINAL ACIDOSIS IN GOATS

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Abstract: This study was realized to evaluate the efficacy of dietary supplementation of sodium bicarbonate plus magnesium oxide "MgO", calcareous marine algae (AcidBuf) and essential oils (Actifor®Boost) on the animal performance and prevention of ruminal acidosis in goats moreover, evaluation the diagnostic accuracy of some biochemical parameters including blood pH, total antioxidant capacity (TAC), bicarbonate (HCO_3^-), partial pressure of carbon dioxide (pCO_2), beta-hydroxybutyric acid (βHBA) and plasma $_L$-lactate versus ruminal pH in diagnosis of ruminal acidosis. A total of 30 goats were divided into five equal groups (G1-5) according to the type of dietary treatment. G1 served as a control group and fed the concentrate diet only, G2 fed the concentrate diet supplemented with 10 g Na bicarbonate plus 4g MgO, G3 supplemented with 4g AcidBuf, G4 supplemented with 10 g Na bicarbonate plus 4g MgO plus 4g AcidBuf and G5 supplemented with 1g Actifor® boost/head/day for five days. Tachycardia, tachypnea and decreased ruminal motility were recorded in G1 only. The ruminal pH tended to return to its toward normal for G3 and G4 with a mean of (6.3±0.03 and 6.3±0.01, respectively) which significantly higher than those of G1 and G2 (6.19±0.01 and 6.17±0.02 respectively). A significant increase in plasma $_L$-lactate and a significant decrease in blood pH, HCO_3^-, pCO_2, TAC, and βHBA were recorded in G1 when compared with other groups. Calcareous marine algae with or without adding other compounds found to be a promising rumen buffer agent, moreover TAC and plasma $_L$-lactate showed better diagnostic performance versus rumen pH in the diagnosis of ruminal acidosis.

Key words: rumen buffers; ruminal acidosis; goats; diagnostic accuracy

Introduction

Today, both small and large producers look to goat farming as a source of income, thus their feed management has been modified to promote efficient growth and achieve quick weight gain through the use of highly fermentable carbohydrates, which puts the animals at risk for developing ruminal acidosis (RA) (1).

The microbial fermentation of these carbohydrates by the amylolytic bacteria present in the rumen produces volatile fatty acids and lactic acid leading to dropping of ruminal pH below the physiological levels, death of many gram -ve bacteria and favours the growth of other gram +ve bacteria, particularly lactate-producing bacteria like *Streptococcus bovis* and *Lactobacilli spp*. This has detrimental effects on animal health (2).

When the microbial production of lactic acid exceeds its utilization, lactic acid absorbed into the blood circulation and causing systemic changes that can be assessed by measuring some hematobiochemical parameters that give useful information in the diagnosis, prognosis, and treatment of RA (3, 4).

Dietary buffers play an important role in preventing ruminal pH from falling below optimal limits. There are a variety of them which could be used to control the rumen fermentation and

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improve the animal productivity and health including sodium bicarbonate (SB), sodium bentonite, magnesium oxide, calcium carbonate and calcified seaweed (5)

Sodium bicarbonate is the most widely used mineral buffer in the animal industry for its rumen buffering capacity and its inclusion in animal diet has become a common practice in most parts of the world (6), several reviews highlight the efficiency of SB in raising the ruminal pH, and there are practical recommendations for adding SB at 7–10 g/kg (DM) (7). MgO is a slow-release neutralizing agent that has been used to raise the ruminal pH; however, due to its low water solubility, its effect developed slowly and occurred only after 24 h of treatment (8). Two to three parts of SB to one part of MgO is the proper ratio to use it in the diet (9). Recently, calcareous marine algae (CMA), natural product made from calcified seaweed "Lithothamnion calcareum" and have been used recently to stabilize the rumen pH and prevent development of ruminal acidosis (10). Essential oils could be considered a natural alternative to modify the rumen microbial fermentation (11).

So, this study was planned to evaluate the efficacy of dietary supplementation of SB plus MgO, AcidBuf and Actifor®Boost on prevention of ruminal acidosis in goats fed on acidogenic diet moreover, evaluation the diagnostic accuracy of some biochemical parameters versus the ruminal pH in the diagnosis of RA.

Materials and Methods

All the procedures used in this experiment were reviewed and approved by the Zagazig University Animal Research Ethics Committee (ZU- IACUC, approval number: ZU- IACUC/2/F/30/2022).

Animals, diet and experimental design

A total of 30 clinically healthy goats of both sexes weighed (20-25 kg) and aged (2–3 years) were admitted to the isolation section at Faculty of Veterinary Medicine, Zagazig University, Egypt.

Experimental design

Adaptation period

Upon arrival, the goats underwent to a thorough clinical examination and dewormed through subcutaneous injection of Ivomec® plus (Ivermectin/ Clorsulon, Merial) at a dose of 1ml/50kg body weight. This period lasted for 20 days during them the goats were fed on a forage diet and water ad libitum and kept on wheat straw beds in natural light and room temperature conditions.

Feeding period

This period began after the 20 days of the adaptation period and lasted for five days during them all goats received basal concentrate diet, crushed corn at 50 g/kg body weight per day. According to the type of dietary treatment, the goats were randomly divided into five equal groups, each with six goats. The dietary treatments were; G1 fed the basal concentrate diet only and served as an unbuffered control group, G2 supplemented with 10 g SB plus 4g MgO, G3 supplemented with 4g AcidBuf, G4 supplemented with 10 g SB plus 4g of MgO plus 4g of AcidBuf and G5 supplemented with 1g of Actifor ® boost / head / day for five days.

Clinical examination

All experimental goats underwent to thorough clinical examination according to Smith and Sherman (12), focusing on the evaluation of vital signs (heart rate, respiration rate, body temperature and rumen motility).

Sampling

Blood and ruminal fluid samples were collected before morning feeding at zero day (last day of adaptation period) then at interval of 24, 48, 72 and 96 h post feeding the concentrate diet.

Blood samples

Three blood samples were collected from each goat through jugular vein puncture: one on vacuum-heparinized tubes containing freeze-dried lithium heparin for hemogasometry analysis; measurements of blood pH, pCO_2 and HCO_3^-, were made within half an hour after collection, using blood gas analyzer (RAPIDlab 348EX SIEMENS blood gas system), the second sample was collected in clean and dry tubes without anticoagulant to obtain serum for the determination of βHBA, using commercial spectrophotometric kits (Pointe Scientific, Inc. USA) according to Koch and Fledbruegge (13) and TAC, using a commercial test kit (Sigma-aldrich, USA) according to Miller and

Evans (14); and the third sample in tubes with sodium fluoride for plasma $_{L}$-lactate determination using kits produced by spin react according to Burtis (15).

Ruminal fluid samples

Ten mL ruminal fluid was obtained via stomach tube to determine the pH. A digital pH meter (Adwa, AD11, ROMANIA) was used, calibrated by standard pH buffer according to Constable et al. (16).

Statistical analysis

Data were analyzed using R 4.1.0 (R Foundation for Statistical Computing, Vienna, Austria). Descriptive statistics for the different clinical and biochemical parameters in ruminal acidotic +ve and -ve cases were presented. The two-way analysis of variance (ANOVA) was used to test the effect of groups and different sampling times on the ruminal pH, while the one-way ANOVA was used to investigate the ruminal pH within each group in the different sampling times.

Ruminal acidosis case definition was based on the ruminal pH value of ≤ 5.5 as described previously (17), the receiver operating characteristic curve (ROC) was executed for some biochemical parameters to test their diagnostic accuracy versus the ruminal pH, in-between such parameters the blood pH, TAC, HCO3-, pCO2, βHBA and L-lactate. The optimal cut-off values plus sensitivities (Se) and specificities (Sp) were presented for each of the tested parameters, such Se and Sp were calculated from the following formulas:

$$Se = TP / (TP + FN)$$
$$Sp = TN / (FP + TN), \text{ where}$$

TP: true positive: Rumen pH ≤ 5.5 and positive biochemical test (value > cut off point)
FP: false positive: Ruminal pH >5.5 and positive biochemical test (value > cut off point)
FN: false negative: Rumen pH ≤ 5.5 and negative biochemical test (value < cut off point)
TN: true negative: Ruminal pH >5.5 and negative biochemical test (value < cut off point)

Results

Clinical examination

The results of clinical findings of all groups were summarized in Table 1. The vital parameters (temperature, heart rate, respiratory rate, and rumen contraction) showed non-significant changes between the different buffered groups (G2-5), while the unbuffered control group G1 recorded significant increase in the mean of respiratory and heart rate per minute. Non-significant changes in the mean of body temperature between all groups, while the ruminal contraction per 2 minutes was significantly lower in G1 only (P-values between G1 and other groups were < 0.001).

Hemogasometry and biochemical parameters

Mean blood pH was significantly lower in G1 compared to other groups (7.37±0.07) and was significantly higher in G2 group compared to other groups (7.48±0.01) and mean TAC and βHBA were significantly lower in G1 in comparison to other groups (6.39±5.38, 0.49±0.18) respectively. The mean plasma L-lactate level was significantly higher in G1 compared to other groups (26.4±15.5), all such comparisons and others are summarized in Table 1.

Efect of dietary treatments on the ruminal pH

Results of the two-way ANOVA for the effect of groups, times of sampling, and their interaction were presented in Table 2. There was a significant effect of the groups, times, and their interaction on the level of the ruminal pH (P-value for all was <0.001). When the post-hoc test was implemented, the mean ruminal pH for G3 and G4 groups at the five sampling times was (6.3±0.03 and 6.3±0.01, respectively) that was significantly higher than those of G1, G2 and G5 (5.34±0.07, 6.19±0.01 and 6.17±0.02, respectively) as shown in Table 3. Figure 1 is showing the different ruminal pH values across groups and times.

Table 4 is showing the rumen pH in the different sampling times was compared within each of the treated groups. Interestingly, in groups G2-G5, there was a significant difference between rumen pH at zero h and 96h, but for all group the rumen

pH values were not less than 5.5, this indicating the efficiency of all buffers in preventing the occurrence of ruminal acidosis.

Diagnostic accuracy for some biochemical parameters versus ruminal pH

Based on the selected cut-off point to define the +ve RA, the ruminal pH for animals in group C was 22 times equal to or below this selected cut-off point (≤ 5.5), indicating the presence of RA. Blood pH, TAC, HCO3-, pCO2, βHBA, and plasma L-lactate accuracy were tested versus the accuracy of the ruminal pH in RA diagnosis (Figure 2). The more the curve moved toward the upper left corner of the ROC space is an indicator of higher test accuracy. TAC has the best diagnostic accuracy among other parameters (Se 97.7%, Sp 81.8%, and AUC 0.92) while HCO3- showed the lowest accuracy (Se 77.3%, Sp86.4%, and AUC 0.88).

Table 1: The effect of dietary treatments on the physical and biochemical parameters in the different groups through the five sampling times

	G1	G2	G3	G4	G5
Respiratory rate/ min	29.7±4.95^{a}	22.6±1.19^{c}	24.2±1.12^{bc}	24.1±1.25^{bc}	24.5±1.38^{b}
Heart rate/min	87.2±8.3^{a}	77.1±1.4^{b}	77.6±2.2^{b}	77±1.8^{b}	76.4±1.14^{b}
Temperature °C	39.5±0.08 ^{a}	39.5±0.06 ^{a}	39.6±0.05^{a}	39.5±0.07 ^{a}	39.5±0.08 ^{a}
Blood pH	7.37±0.07^{d}	7.48±0.01^{a}	7.44±0.03^{b}	7.44±0.01^{b}	7.4±0.02^{c}
TAC (ng/ml)	6.39±5.38^{b}	9.33±3.85^{a}	11.5±3.01^{a}	9.77±2.21^{a}	9.07±3.23^{a}
Lactate (mg/dl)	26.4±15.5^{a}	16.1±9.05^{b}	12±4.81^{b}	11.2±4.5^{b}	15.1±6.11^{b}
βHBA (mmol/L)	0.49±0.18 ^{b}	0.64±0.09^{a}	0.66±0.08^{a}	0.61±0.04 ^{a}	0.61±0.11 ^{a}
HCO_3 (mmol/L)	20.4±4.9^{d}	29.5±3.02^{a}	26.4±1.03^{b}	23.6±1.5^{c}	24.2±2.6^{c}
pCO_2 (mmHg)	29.06±3.8^{d}	37.7±4.4^{b}	38.9±3.7^{b}	33.4±4.5^{c}	42.3±2.3^{a}

Results are expressed as means ± SD
Different superscripts within the row indicate statistically significant differences ($p<0.05$).
TAC: total antioxidant capacity; βHBA: beta hydroxybutyric acid; HCO_3^-: bicarbonate concentration; pCO_2: carbon dioxide partial pressure. Treatments: G1 = control; G2 = sodium bicarbonate + magnesium oxide; G3= calcareous marine algae; G4= calcareous marine algae+ sodium bicarbonate + magnesium oxide; G5 = essential oil.

Table 2: Two-way analysis of variance (ANOVA) table for groups and times effect on the rumen pH

	Sum of squares	Df	Mean square	F-value	P-value
Groups	19.8	4	4.9	384.9	<0.001
Time	25.8	4	6.4	501.04	<0.001
Group*Time	8.2	16	0.5	39.85	<0.001

Df: degree of freedom

Table 3: The effect of dietary treatments on the rumen pH in the different groups

	G1	G2	G3	G4	G5
Rumen pH	5.34±0.07^{d}	6.19±0.01^{b}	6.3±0.03^{a}	6.3±0.01^{a}	6.17±0.02^{bc}

Results are expressed as mean ± SD (i.e., mean pH through the five sampling times).
Different superscripts within the row indicate statistically significant differences ($p<0.05$).
Treatments: G1 = control; G2 = sodium bicarbonate + magnesium oxide; G3= calcareous marine algae; G4= calcareous marine algae + sodium bicarbonate + magnesium oxide; G5 = essential oil.

Table 4: The effect of rumen buffers on the rumen pH along the time in the different groups

Time / Groups	Zero h	24h	48h	72h	96h
G 2	6.87±0.08^{a}	6.08±0.07^{b}	5.98±0.19^{b}	6.07±0.1^{b}	5.97±0.05^{b}
G 3	6.87±0.05^{a}	6.42±0.16^{b}	6.07±0.13^{c}	6±0.09^{c}	6.15±0.16^{c}
G 4	6.85±0.05^{a}	6.28±0.11^{b}	6.1±0.09^{c}	6.13±0.15^{bc}	6.17±0.05^{bc}
G 5	6.83±0.05^{a}	5.95±0.12^{b}	5.97±0.15^{b}	5.97±0.13^{b}	5.97±0.1^{b}

Results are expressed as mean ± SD.
Different superscripts within the row indicate statistically significant differences ($p<0.05$).
Treatments: G1 = control; G2 = sodium bicarbonate + magnesium oxide; G3= calcareous marine algae; G4= calcareous marine algae+ sodium bicarbonate + magnesium oxide; G5 = essential oil

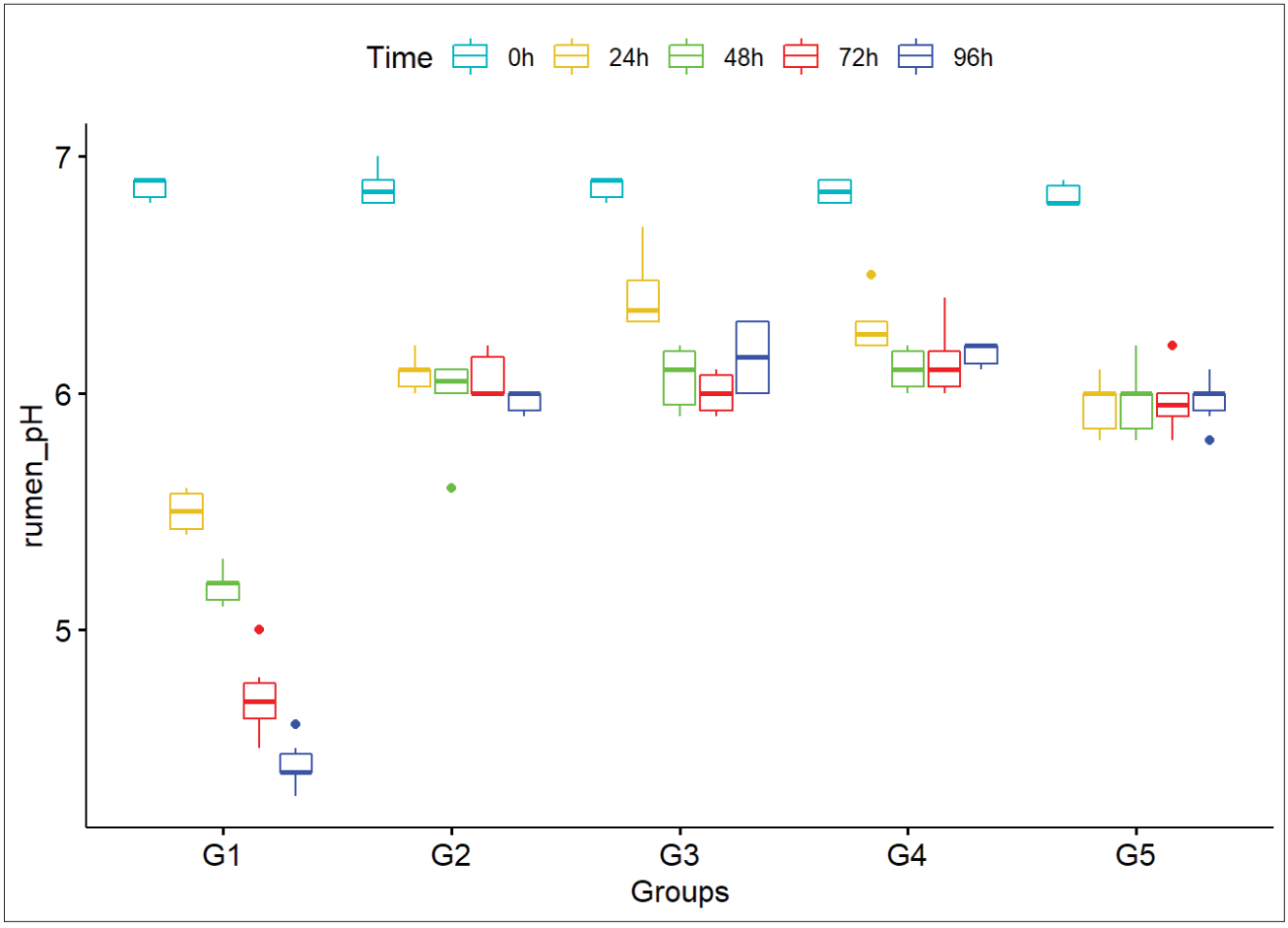


Figure 1: Box plot for the rumen pH values in different groups/times.

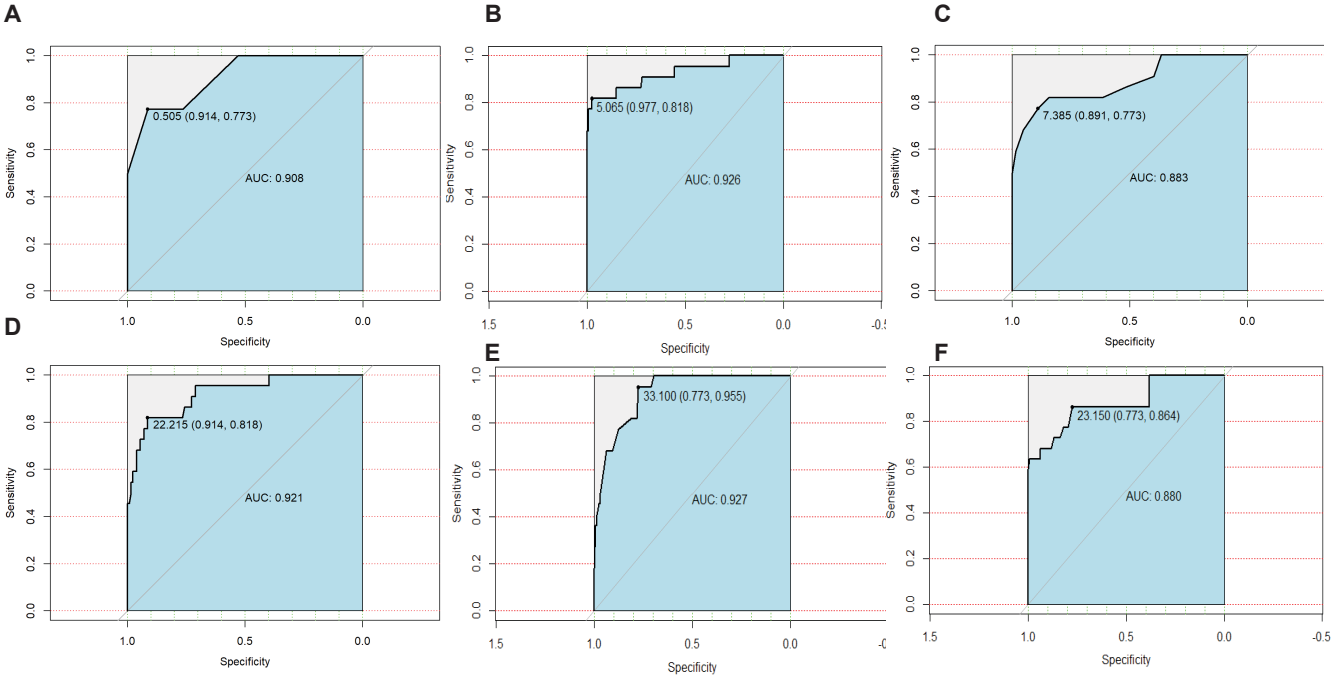


Figure 2: The receiver operating characteristic curves (ROC) for blood pH (A), TAC (B), bicarbonate (C), pCO2 (D), βHBA (E), and plasma lactate (F). The optimal cut-off points were shown for each parameter and the maximum sensitivity and specificity, also the area under curve (AUC)

Discussion

In order to improve animal performance and increase the weight gain, the highly and rapidly fermentable carbohydrates have been used widely in animal nutrition but this leads to some microbial disturbances and predispose the animal to the risk of RA (1) which considered an important and common clinical emergency of small ruminants and if not manipulated properly may lead to high economic losses due to the high morbidity and mortality, making detection of the disease in the field is very important (16).

On clinical examination, there were significant increase in heart and respiratory rate per minute found to be characteristic for the acidotic goats in the unbuffered control group (G1) compared to others. The recorded tachycardia in G1 coincided with the dropping of the ruminal pH to values < 5.5, increased ruminal osmolarity and consequently, mobilization of water from the circulation to the rumen causing hemoconcentration and increased heart rate. The increased respiratory rate may be attributed to stimulation of the respiratory center by the acidic blood pH that helps in removing the excess of CO_2 as a compensatory mechanism for correction of the metabolic acidosis occurred (18). Those results agree with previous studies (18-21).

The ruminal contraction/ 2minutes show significant decrease in G1 only which was similar to that was reported in previous studies (22-24) in which the ruminal contraction was significantly decreased with the ruminal acidosis that may be attributed to the low ruminal fluid pH and increased ruminal osmolarity (25).

Concerning ruminal pH and the buffer effect on it, the control diet used in this study caused ruminal pH to fall below 5.5 in the unbuffered control group only which had the lowest mean ruminal pH compared to other groups. This might be due to excess accumulation of lactate resulting from fermentation of the highly fermentable carbohydrates in the rumen (3).

These findings agree with previous studies (21, 26). The results show that the four dietary buffer treatments have a positive effect on the mean ruminal pH and all the buffers used in this study have the ability to prevent lowering of ruminal pH below 5.5 compared with the control group. The buffer effect on rumen pH at all sampling times in the different groups was greater for G3 and G4 that had the highest mean ruminal pH. Also, G3 and G4 groups were most effective in maintaining high rumen pH with a greater daily mean rumen pH as shown in table 4. It has been claimed previously that CMA is more effective than SB, has twice the buffering capacity of it probably due its high content of calcium, magnesium, and essential trace elements (10) and give better results when mixed with the SB (27). These findings agreed with previous studies (28, 29) where the authors reported that CMA decreased the time spent under pH 5.5 compared to SB and, agreed with (30) who mentioned previously that the addition of 90 g/day CMA to high concentrate diets was more efficient than addition of 180 g/day SB in prevention of ruminal pH reductions.

Blood gas analysis offers a proper assessment of acidosis while being less invasive than ruminal pH so, it can be considered a valuable tool to diagnose RA (31). Blood pH relies on the relative amounts of bases, acids, and buffers (bicarbonate) (32) and there is a tight relationship between the ruminal pH and blood pH (31). There were a significant decrease in the mean values of blood pH, HCO_3^- and pCO_2 in G1 group in comparable to other groups. Lactate produced from the digestion of concentrates absorbed into the blood circulation and metabolized by the liver until the amount of lactate overwhelmed the liver's capacity, lactate accumulates, causing a reduction of blood pH and development of metabolic acidosis (1, 3). Bicarbonate reserves consumed through buffering of the accumulated acid and cause a reduction of blood pH (1). Low blood pH acts as stimuli for the respiratory center and increasing alveolar ventilation for elimination of extra CO_2 from the lungs decreasing pCO_2 concentration (3). Those findings have been agreed with previous studies (1, 3) in which the authors mentioned significant decrease in blood pH, $HCO_3^-$and pCO_2 in sheep and goats during induction of ruminal acidosis.

Concerning plasma $_{L-}$lactate concentration, there was a significant increase in the mean values of plasma $_{L-}$lactate in G1 group compared to other groups. Lactate is produced in large amount during feeding of concentrates and absorbed into the blood circulation increasing its level in the blood. Similar results were reported previously (1, 33).

TAC and βHBA serum concentration recorded a significant decrease in their mean values in G1 group in comparable to others. Ruminal acidosis was associated with excessive reactive oxygen

species (ROS) and oxidative stress leading to exhaustion of enzymatic and non-enzymatic antioxidants in their neutralization as it was obvious by decreased TAC (22). Those results agree with previous studies (23). The high concentration of blood glucose reduced the βHBA concentration (34) as mentioned in a previous work (35).

To the best of our knowledge, our study is the first to report the diagnostic accuracy of some biochemical parameters versus the ruminal pH in RA diagnosis. The precision and accuracy of a diagnostic test serve as two independent descriptors of the test performance, Se and Sp are usually measured to represent the accuracy of such diagnostic test (36). The receiver operating characteristic curves are graphical plots that illustrate the diagnostic ability of a test as its discrimination threshold is varied (37). Recently, such diagnostic accuracy measures are used extensively in the veterinary practice.

In this study, testing TAC and plasma $_{L-}$lactate versus the ruminal pH in diagnosis of ruminal acidosis showed the best Se (97.7%, 91.4%, respectively) and Sp (81.8% for both) rather than other biomarkers, with outstanding area under ROC curves for both parameters (nearly 0.92 for both). Ruminal pH was found to be decreased along with decreasing TAC and increase the plasma $_{L-}$lactate as reported in previous studies (23, 38, 39). Reasons for such correlation were described earlier in the discussion section.

The interpretation of our diagnostic accuracy results could be limited to the small sample size , only 22 times RA was diagnosed, further studies with larger sample size are recommended. The same recommendation is advised to verify the buffer results on a large scale and longer study duration. In conclusion, calcareous marine algae with or without adding other compounds found to be a promising rumen buffer agent. TAC and plasma lactate found to be good diagnostic biomarkers for RA when tested versus the ruminal pH as a gold standard.

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Original Research Article
Veterinary Medicine and The One Health Concept

EVALUATION OF DIFFERENT NEWCASTLE DISEASE VIRUS VACCINATION REGIMES AGAINST CHALLENGE WITH RECENTLY ISOLATED GENOTYPE VII VIRUS FROM EGYPT

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Abstract: Newcastle disease virus (NDV) genotype VII is incriminated in the currently circulating NDV outbreaks in the Middle East region. In this study, evaluation of different vaccination regimes including genetically matched or mismatched vaccines to the currently circulating field virulent NDV (vNDV) genotype VII was performed. One-day-old Arbor Acres broiler chicks were divided into nine groups; groups 1 to 3 were vaccinated with live or inactivated genetic mismatched vaccines (genotype II) or both of them. Groups 4 to 6 were vaccinated with either live or inactivated genetic-matched vaccine to vNDV genotype VII or combination of them. Group (Gp) 7 was vaccinated with a combination of inactivated genetic-matched and live genetic-mismatched vaccines to vNDV genotype VII while groups 8 and 9 were kept as control non-vaccinated. The groups that received a combination of live and inactivated vaccines from either genetically-matched or mismatched origins had the highest serological responses and protection against mortality which was 100%. The two groups received a combination of inactivated genetic matched vaccine and live vaccines of either genetic-matched or mismatched origins had the lowest clinical index and were nearly completely protected against vNDV clinical signs. The virus tracheal and cloacal shedding titers and number of shedders were significantly reduced or nearly neglicable in the instance of application of inactivated genetic-matched vaccine to the challenge virus either alone or boosted with live genetic-matched or mismatched vaccine. In consistent inactivated genetic-matched vaccine inhibited the transmissibility of the challenged virus to contacted birds. We concluded from our results that application of NDV vaccination regimes included a combination of inactivated NDV genotype VII vaccine and live vaccine regardless of its genotype provides better clinical protection and minimize virus shedding and subsequently decrease transmissibility and virus load to the surrounding environment.

Key words: Newcastle disease virus; genotype VII; virus shedding; vaccine programs

Introduction

Poultry sector is the fastest growing industry, in Egypt. It offers the nation various forms of animal protein in the form of eggs and meat, as well as employment prospects for almost 3 million workers with investments totaling over 90 million Egyptian pounds.

Several economic losses occur in this industry as a result of various pathogenic agents, particularly those associated with respiratory issues such as Avian Influenza (AI), Newcastle disease, and Infectious Bronchitis (1-4). Newcastle disease (ND) is a highly contagious infectious disease that has a negative impact on poultry production across six continents, resulting in significant economic losses, prompting the Office International des Epizooties to classify it as list A disease (5). Newcastle disease was initially described in Egypt for the first time in 1948 by (6) as a real threat to the Egyptian poultry industry. Even in vaccinated flocks, the disease causes significant economic losses due to high mortality, decreased egg production, and decreased body weight of broiler chickens (7). The causative agent

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Newcastle disease virus (NDV) is an enveloped, single stranded, non-segmented RNA of negative sense virus. The NDV is also known as the avian Orthoavulavirus 1 (AOAV-1) and has been classified in the order *Mononegavirales*, family *Paramyxoviridae*, subfamily *Avulavirinae*, and genus *Metaavulavirus* (8, 9). Within a single serotype, NDV strains have been categorized into class I and II based on the size of the genome and the nucleotide sequences of the F and L genes (10). Class II NDVs have greater genetic diversity, with 20 newly identified genotypes named I to XXI (excluding XV because it contains recombinant viruses), which include virulent and avirulent strains and are widely distributed across the African continent (9, 11). However, discrepancy in clinical signs in infected birds are primarily determined by the pathotype of the infected strain, whether velogenic, mesogenic, lentogenic, or asymptomatic. The velogenic (extremely virulent) strains can produce severe clinical symptoms such diarrhea, neurological and respiratory signs, egg difficulties, hemorrhagic lesions in the intestinal tract and proventriculus, and significant mortality rates (7, 12). Although there is no cure for ND infection, culling infected birds, combined with strict biosecurity and aggressive vaccination protocols, are the most effective ways to control NDV outbreaks. There are three types of NDV vaccines: live, inactivated and recombinant vaccines. Many such studies have demonstrated the ability of different types of NDV vaccines to reduce or prevent clinical disease and mortality, reduce virus shedding into the environment, and increase the amount of virus that necessary to infect vaccinated birds (10, 13). Even in well-vaccinated farms, ND outbreaks continue to occur frequently despite the application of intensive vaccination programs and intense usage of ND vaccines. The former has raised questions about the protective efficacy of the commercially applied vaccines and the role antigenic variations of NDV in the NDV vaccination defect phenomena. LaSota and Hitchner B1 NDV strains used to produce the most commercially available NDV vaccines, both have the same genotype of viruses isolated in the 1940s and are classified within class II as genotype I or II (14). Nowadays, both strains are genotypically different or mismatched from strains causing the recent outbreaks of ND. These conventional vaccines could partially or completely protect chicken from mortalities and clinical signs but not able to prevent viral dissemination from infected birds to the environment through its dropping and respiratory secretions which represent source of infection to non-vaccinated birds (15). Genotype VII of NDV was firstly isolated by (16) then it was reported many times in concurrent outbreaks among Egyptian poultry flocks with high mortality percentage (17-20). Recent investigations on the usefulness of genotype VII-based vaccinations in the prevention of NDV epidemics indicated that they offered improved protection and decreased viral shedding from infected birds against challenge with vNDV genotype VII (21-23). In Egypt, even with strict preventive measures, NDV genotype VII outbreaks have been still occurred which makes it mandatory to improve the applied vaccination strategy. Therefore, the goal of this study was to evaluate the efficacy of different NDV vaccination programs composed of genetic-matched or mismatched vaccine or even combination of them in experimentally infected broiler against the velogenic genotype VII strain.

Materials and methods

Experimental birds

One hundred seventeen Arbor Acres broiler chickens, one day of age, were obtained from EL-Abrar Company for poultry production at Ismailia Governorate. The birds were raised in a floor-based system in the animal house experimental facility at Faculty of Veterinary Medicine, Zagazig University. Throughout the experiment, all birds had free access to food and water. The birds were observed for any changes that could indicate the presence of infection. All the procedures were carried out under strict biosafety conditions that comply with local animal welfare regulation on experiments with live birds. The study was approved by the head of Institutional Animal Care and Use Committee (ZU-IACUC) under acceptance number ZU-IACUC/2/F/ 187/2019, Zagazig University

Vaccines

1. IZOVAC B1 Hitchner (IZO, Italy with Batch n.0463M), a freeze-dried live attenuated strain, contain at least 10^6 EID_{50}/dose of live NDV B1 strain. The vaccine was administrated via intraocular route.

2. Nobilis Clone30 (MSD, Kenilworth, NJ, USA with Batch n. A320AJ0) a freeze dried live attenuatedstrain; contain at least 10^6 EID_{50} of live NDV Clone 30 virus particles /dose. The vaccine was administrated via intraocular route.

3. Himmvac Dalguban N (Plus) Live Vaccine (KBNPC4152R2L strain), KBNP Inc., Gyeonggi, Korea with Lot number CMRL1218, a live freeze-dried attenuated strain, contain at least 10^6 EID_{50}/dose of live NDV KBNP-C4152R2L strain (genotype VII). The vaccine was administrated via intraocular route.

4. Volvac® ND KV (Boehringer Ingelheim, Germany), oil emulsion inactivated vaccine, contains LaSota NDV with lot number 19030104. The vaccine was injected at a dose of 0.5 ml per bird, subcutaneously in the middle third part of the back of the neck.

5. Himmvac Dalguban N (Plus) Oil Vaccine (KBNP, Inc., Gyeonggi, Korea oil emulsion inactivated recombinant vaccine, contains KPNB-C4152R2L NDV strain, with Lot number CMR0319. The vaccine was injected at a dose of 0.5 ml per bird, subcutaneously in the middle third part of the back of the neck.

Virus

A reference genotype VII vNDV strain termed as (NDV/chicken/Egypt/1/2015), with accession number KX231852 was used as the challenge virus. In 9-day-old embryonated chicken eggs (ECEs), the virus was propagated and titrated. The infective dose was adjusted to contain $10^{6.5}$ embryo infective dose 50 (EID_{50})/ 0.1 ml and administrated via oculonasal route.

Experimental design

One hundred and seventeen Arbor Acres broiler chicks obtained from EL-Abrar Company for poultry production at Ismailia Governorate were divided into 9 groups, each of 13 birds (10 challenged and 3 in contact added post challenge). From group one to seven, several NDV vaccination regimens were administered and two groups were kept non-vaccinated as control groups. The vaccination programs applied for the different groups are described clearly in Table (1).

All the groups except (group 9) were challenged with $10^{6.5}$/EID_{50}/100 uL per bird of vNDV genotype VII strain (NDV/chicken/ Egypt/1/2015) at 31st day old via oculonasal route. Three sentinel birds to each group were inserted between the vaccinated birds forty-eight hours post challenge. Blood samples were collected at first day old for evaluation of maternal-dervied NDV immunity and weekly from the wing vein according to the approval of (ZU-IACUC/ 2/ F/ 187/2019). Tracheal and cloacal swabs were taken from five challenged birds and three contact sentenile birds from each group and were collected into sterile phosphate buffer saline containing antibiotics at 3 and 5 post challenge from challenged birds and at 3, 5 & 7 days post contact from sentenile birds. Challenged birds were examined daily for clinical signs and mortality. The clinical indicies were calculated as previously described by Grund et al (24)

Table 1: Experimental design of different NDV vaccination regimes in chicken Arbor Acres broiler broilers

Number of groups	Number of chicks		1 day old	10 days old	18 days old	31 days old
	Challenged	Contact				
I	10	3	HitchnerB1		Clone 30[1]	Challenged[5]
II	10	3	HitchnerB1	LaSota Inactivated[2]		Challenged[5]
III	10	3	HitchnerB1	LaSota Inactivated[2]	Clone 30[1]	Challenged[5]
IV	10	3	HitchnerB1		Dalguban Live[3]	Challenged[5]
V	10	3	HitchnerB1	Dalguban Inactivated[4]		Challenged[5]
VI	10	3	HitchnerB1	Dalguban Inactivated[4]	Dalguban Live[3]	Challenged[5]
VII	10	3	HitchnerB1	Dalguban Inactivated[4]	Clone 30[1]	Challenged[5]
VIII	10	3	-			Challenged[5]
IX	10	3	-			-

1& 3 were applied via intraocular route with at least 10^6 EID_{50}/dose. 2 &4 were injected at a dose of 0.5 ml per bird, subcutaneously. 5 was applied oculonasal with $10^{6.5}$/EID_{50}/100 uL per bird.

Table 2: Primers and Probe used to detect the amount of vNDV in tracheal/ cloacal swabs after challenge

Gene	Primer/ probe sequence 5'-3'
NDV Genotype VII (F)	F-Egy-289-FW CGSARGATMCAAGGGTCT
	F-Egy-378-Rev CTACACTGCCAATAACRGC
	F-Egy-361-Probe [FAM] AGGAGACRAAAACGYTTTATAGGTCG[TAMRA]

The primers and probes in this study were received as a gift from prof Dr Christian Grund, FLI (Friedrich Loeffler institute; The Federal institute for Animal Health of Germany 2016 (unpublished)

Evaluation of humoral NDV immunity

Blood samples were taken from chicks at various ages, including 1 day, 7, 14, 21, 28, 35, and 38 days. Serum samples were separated and haemagglutination inhibition (β procedure) assay was performed for measuring the NDV antibody titer. Serum was initially diluted into 1/2 and a two-fold dilution series of each tested serum sample was prepared according to OIE (5). Four HA units of the NDV LaSota antigen were added to the serially diluted serum samples, and 1% chicken red blood cells were utilised as an indicator.

Measurement of viral shedding using qRT-PCR:

Tracheal and cloacal swabs were randomly taken from 8 birds in each group (5 infected + 3 sentinel birds). Viral RNA samples were extracted using an RNeasyPlus Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Specific probes and primers were designed based on the F protein gene to detect viral RNA using qRT-PCR (Table 2).

The primers, probes, and PCR conditions for the assay was applied according to the manufacturer's instructions. The reaction was subjected to the cycling conditions of primers and probes using Real time PCR machine (Stepone applied biosystem). The amount of viral shedding was be expressed as the number of viral RNA copies. Absolute quantification of the number of viral RNA copies in the swabs was be based quantification cycle (Cq) values for each sample and the standard curve method for qPCR.

Statistical analysis:

Using a One-Way Analysis of Variance (ANOVA) and SPSS (ver. 21.0; IBM, USA), statistically significant variations in the serological analysis data between various groups was assessed.

Results

Serological responses following vaccination with different NDV vaccination regimes

Prior to challenge, antibody responses from zero day and weekly after different NDV vaccination regimes were evaluated (Figure 1 and Table 3). The birds had high maternal antibody arithmatic mean titers against NDV of (7.5±1.50) $\log_2$, which gradually decreased to < 4 log2 at 14 days old. The results showed no significant differences between the different vaccinated groups at 14 days old, regardless of the genotype of the used inactivated or live vaccine. At 21 days old, the antibody titers in all vaccinated groups began to increase gradually. One week later, groups (3, 6, 7) that received a combination of live and inactivated vaccines either of genotype II or VII origin provided the highest pre challenge HI- NDV Ab titers (6±0.00, 5.6±0.24 & 5.8±0.20)$\log_2$, respectively. The NDV-HI-Ab titers in the aforementioned groups were statistically significant at ($P< 0.05$) when compared with the other vaccinated groups. Vaccination with inactivated ND vaccine only of either genotype II (Gp2) (5.2±0.20^{bc}) or VII (Gp5) (5±0.00^{c}) led to NDV- higher and statistically significant HI-Ab titers when compared with vaccination with live vaccine only (Gp1 & Gp4) (4.2±0.24^{d} & 4±0.00^{d}), respectively.

Table 3: Statistical analysis of HI result following vaccination with different NDV regimes

Age of birds	Arithmatic mean titers of differentially vaccinated NDV vaccinated groups								
	Gp1	**Gp2**	**Gp3**	**Gp4**	**Gp5**	**Gp6**	**Gp7**	**Gp8**	**Gp9**
1st day	7.5±1.5	7.5±1.5	7.5±1.5	7.5±1.5	7.5±1.5	7.5±1.5	7.5±1.5	7.5±1.5	7.5±1.5
7th day	4.5±0.50	4.5±0.50	4.5±0.50	4.5±0.50	4.5±0.50	4.5±0.50	4.5±0.50	4.5±0.50	4±0.00
14th day	2.3±0.29	2.4±0.37	2.7±0.42	2.6±0.37	2.4±0.37	2.6±0.43	2.6±0.20	2±0.44	2±0.44
21st day	2.8 ± 0.20^{b}	3.4 ± 0.24^{ab}	4 ± 0.00^{a}	2.8 ± 0.20^{b}	3.2 ± 0.20^{ab}	3.2 ± 0.20^{ab}	3.6 ± 0.24^{ab}	1 ± 0.32^{c}	1 ± 0.32^{c}
28th day	4.2 ± 0.20^{d}	5.2 ± 0.20^{bc}	6 ± 0.00^{a}	4 ± 0.00^{d}	5 ± 0.00^{c}	5.6 ± 0.24^{ab}	5.8 ± 0.20^{a}	0.4 ± 0.24^{e}	0.4 ± 0.24^{e}
35th day	2.4 ± 0.24^{c}	3.4 ± 0.24^{ab}	4 ± 0.00^{a}	2.4 ± 0.24^{c}	3.2 ± 0.24^{b}	3.4 ± 0.24^{ab}	3.6 ± 0.24^{ab}	3.6 ± 0.24^{ab}	0.0 ± 0.00^{e}
38th day	9 ± 0.00^{a}	9 ± 0.00^{a}	8.8 ± 0.20^{a}	8.6 ± 0.24^{ab}	8.8 ± 0.20^{a}	8 ± 0.00^{ab}	8 ± 0.00^{ab}	2.8 ± 1.71^{c}	0 ± 0.00^{d}

Data are represented as arithmatic Mean titers ±SE; Gp: Group
Means within the same raw with different superscript letters are significantly different (P< 0.05) .

Protective efficacy following vNDV challenge

As shown in Figure 2, the mortality of sham-vaccinated birds challenged with virulent NDV genotype VII (Gp8) began on day 4 and ended on day 6 post challenge, with 20% protection%. The two groups that received either live genotype II (Gp1) or VII (Gp4) vaccine demonstrated 80% protection%, with mortality occurring on days 5 and 6 following challenge. Both groups of birds that received either inactivated genotype II (Gp2) or VII (Gp5) vaccine demonstrated 90% protection, with mortality occurring only 6 days post challenge in both cases. The three groups that received a combination of live and inactivated vaccines from genotype II or VII origin (Gp3, Gp6, and Gp7) demonstrated 100% mortality protection. The negative control group (Gp9) exhibited neither mortality nor clinical signs throughout the experimental period.

Clinical indices following virulent NDV challenge

As shown in Figure3, following challenge with $10^{6.5}/EID_{50}/100$ uL per bird of vNDV genotype VII strain (NDV/chicken/ Egypt/1/2015) at 31 days old via oculonasal route, the clinical index was lowest (00.08) in birds received a combination of live and inactivated vaccines originated from NDV genotype VII (Gp6), indicating longer time for clinical disease to manifest. Furthermore, birds received a combination of inactivated NDV genotype VII vaccine and Clone 30 (Gp7) had clinical index of 00.10. However, birds given

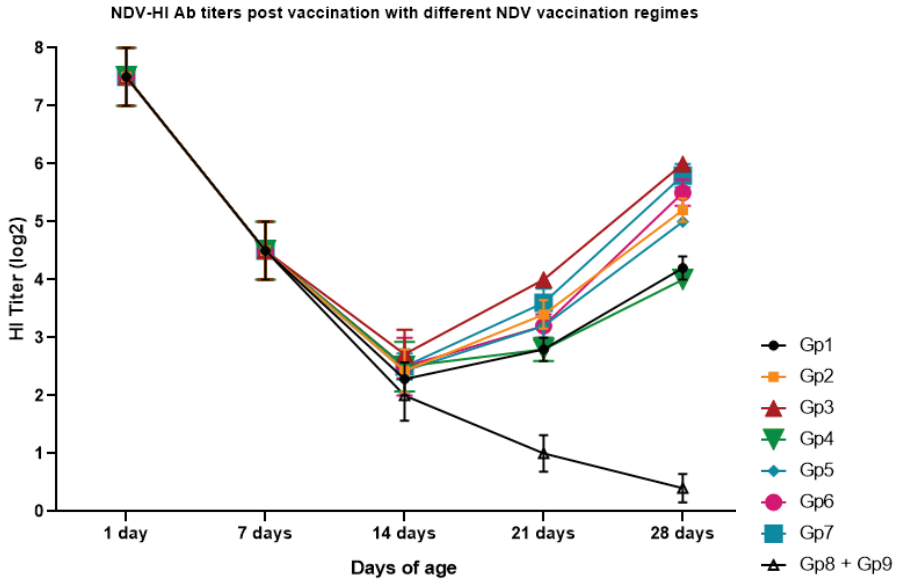


Figure 1: Arithmetic mean HI titers results: pre-challenge serological response of broiler chickens after vaccination with different types of vaccines, serum samples were collected weekly and tested against LaSota to measure the level of specific antibodies (mean HI titer ±SE) in vaccinated and non-vaccinated birds

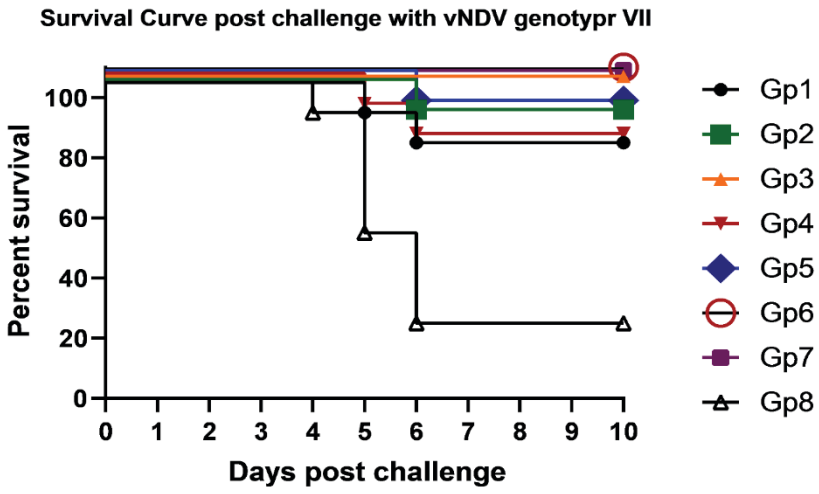


Figure 2: Survival curve: duration of protection following challenge of the vaccinated groups with $10^{6.5}$/EID_{50}/100 ul per bird of vNDV genotype VII strain (NDV/chicken/ Egypt/1/2015) at 31 days old via oculonasal route. The percent survival versus day post challenge was monitored for 10 days. Gp9 survival was 100% as it is non-infected group

Table 4: Clinical observations of the differentially NDV- vaccinated groups 10 days post challenge

Groups	Conjunctivitis	Swollen Eye lid	Greenish Diarrhea	Nasal discharge	Respiratory sign	Nervous signs	Depression
GP1	4/10	2/10	4/10	3/10	4/10	1/10	4/10
GP2	3/10	1/10	2/10	2/10	3/10	0/10	2/10
GP3	2/10	1/10	1/10	1/10	3/10	0/10	2/10
GP4	3/10	2/10	3/10	2/10	4/10	0/10	4/10
GP5	2/10	1/10	2/10	1/10	1/10	0/10	2/10
GP6	1/10	0/10	1/10	0/10	1/10	0/10	2/10
GP7	1/10	0/10	2/10	0/10	0/10	0/10	2/10
GP8	10/10	8/10	10/10	4/10	6/10	3/10	10/10
GP9	0/10	0/10	0/10	0/10	0/10	0/10	0/10

a combination of inactivated LaSota and live Clone30 (Gp3) had a clinical index of 00.12, while birds given only inactivated LaSota or genotype VII had a clinical index of 00.23 or 00.20, respectively. Higher clinical indices were observed in birds received live vaccines only either of genotype II (Gp1) or genotype VII origin (Gp4) with clinical index of ≥ 00.40. It is worth noting that the clinical index of the non-vaccinated group (Gp8) was 1.14. Regarding to the control negative group (Gp9) the clinical index was 00.00. The recorded clinical signs and the number of sick birds for the differentially vaccinated groups are represented in Table (4).

Virus shedding after challenge with vNDV genotype VII

Three days post virus challenge, virus replicated and was shed efficiently in the upper respiratory (Figure 4A) and intestinal tract (Figure 4C) of control positive chickens (GP8) with arithmetic mean titers of approximately $10^{5.23 \text{ and } 3.7}$ EID_{50}/ml, respectively. Virus shedding in upper respiratory and intestinal continued at high levels at 5th day post challenge with mean titers of approximately $10^{5.67 \text{ and } 4.61}$ EID_{50}/ml, respectively (Figure 4B & D). Surprisingly, three days post challenge, birds that vaccinated with genotype II vaccines (genetic mismatched vaccine) either live, inactivated or a combination of both failed to significantly reduce viral tracheal shedding when compared to control birds. As a result, the tracheal mean virus titers in any of genotype II vaccinated groups (1, 2 & 3) were ($10^{4.96, 4.66 \text{ \& } 3.47}$) EID_{50}/ml, respectively and all the tested birds were 100% shedders (5\5) in (Gps1 & 2). Whereas 80% of birds that received both inactivated LaSota and Clone 30 vaccines (Gp3) were shedders (4\5). On the fifth day post challenge, the tracheal mean virus titers significantly reduced in Gp1, Gp2, and Gp3 and were ($10^{1.64, 1.37 \text{ and } 1.36}$) and 40% of birds were positive shedders (2\5) in each group. Regarding the intestinal shedding, 3 days post challenge, only

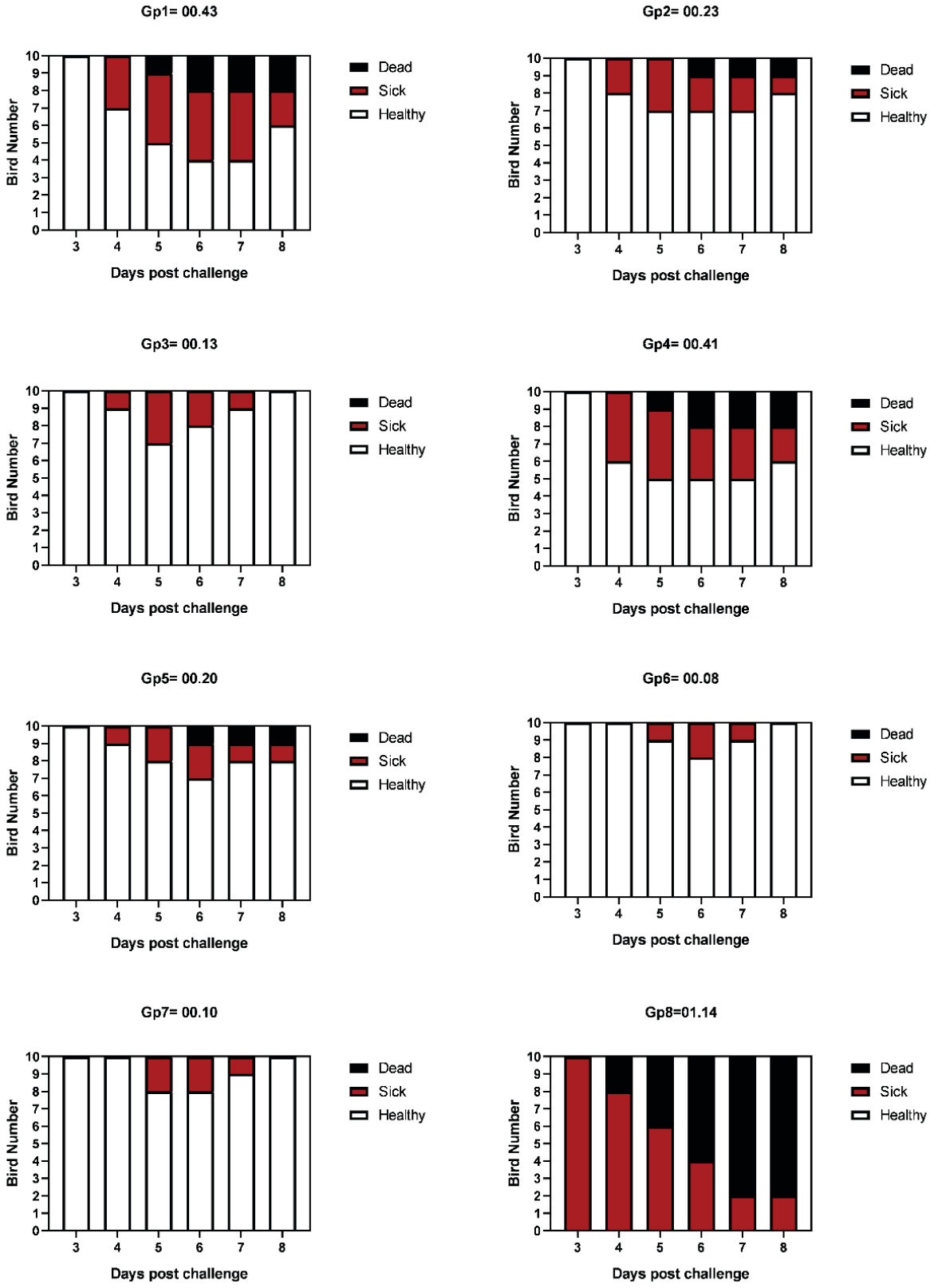


Figure 3: Course of NDV: broiler chickens vaccinated with different vaccine programs were challenged with $10^{6.5}$/ EID_{50}/100 uL per bird of vNDV genotype VII strain (NDV/chicken/ Egypt/1/2015) at 31 days old via oculonasal route. The birds were monitored for clinical signs and the numbers indicate the clinical indices that were calculated as previously described by Grund et al (24). Group 9 was non-infected group and all the birds remain in normal conditions with no clinical signs all over the experimental period

Gp1 that received live genetic mismatched vaccine had mean cloacal titers >10^2 EID_{50}/ml and the shedding was 100% (5\5). However, in Gp2 & 3 the mean titer of viral cloacal shedding were < 10^2 EID_{50}/ml and 40% (2\5) of the birds were shedders in those groups (Figure 4C). Those groups (Gp1, 2 & 3) demonstrated reduced levels or no shedding by day 5 pc with mean cloacal titers of < 10^2 EID_{50}/ml and the shedding were 40% (2\5), 40% (2\5) and 0% (0\5), respectively. In contrast, virus shedding was significantly reduced following using homologous vaccines (genotype VII) to the challenge virus when compared to control group and the genotype II vaccinated groups. The tracheal mean virus titers, 3 days post challenge, in Gp4, Gp5, Gp6 and Gp7 were ($10^{4.40, 2.97, 1.43 \& 1.36}$) EID_{50}/ml, respectively and the shedding were 100% (5\5) in Gp4, 80% (4\5) in Gp5, 20% (1\5) in both group (Gp6 and Gp7). On fifth day post challenge, the tracheal mean virus titers were decreased in Gp4 and Gp5 ($10^{1.39 \& 1.23}$) and 40% of birds were shedders (2\5) in both groups and birds of Gp6 and Gp7 had no detectable tracheal shedding (Figure 4A & B). Regarding the intestinal shedding, group of birds that received live genetic-matched vaccine only (Gp4) had mean cloacal titers of < 10^2 EID_{50}/ml on day 3 & 5 post challenge and 40% of the birds (2\5). Birds immunized with inactivated genetic-matched vaccine to the challenge virus (Gp5, Gp6 and Gp7) had no detectable cloacal viral shedding on day 3 & 5 post challenge (Figure 4C & D).

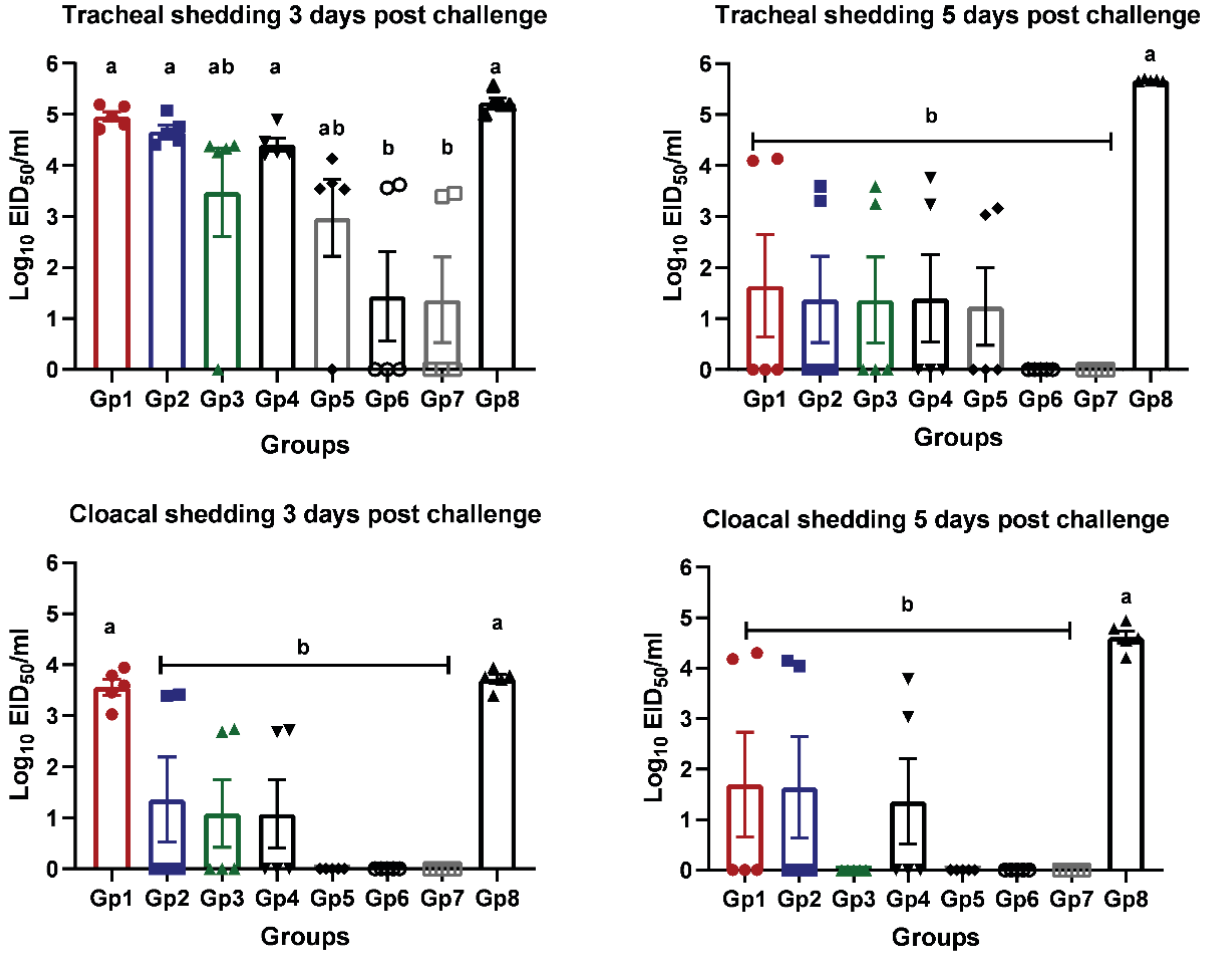


Figure 4: NDV viral shedding of experimentally infected birds with $10^{6.5}$/EID_{50}/100 uLper bird of vNDV genotype VII strain (NDV/chicken/ Egypt/1/2015) at the 31st day of age via oculonasal route. (A) Scatter blot represents mean viral titers of tracheal shedding 3 days post challenge and number of shedders. (B) Scatter blot represents mean viral titers of tracheal shedding 5 days post challenge and number of shedders. (C) Scatter blot represents mean viral titers of cloacal shedding 3 days post challenge and number of shedders. (D) Scatter blot represents mean viral titers of cloacal shedding 5 days post challenge and number of shedders. Titers were calculated as $\log_{10}$ EID_{50}/1 mL (±SE). Group 9 was non-infected control group and showed negative shedding from both tracheal and cloacal routes during all the measuring time points

Virus shedding and mortality of sentinel birds

Three sentinel birds were placed in each group 48 hours after the vNDV genotype VII virus challenge. Virus tracheal shedding was not observed in any of the sentinel birds three days after contact and five days after post challenge. The highest NDV tracheal shedding was observed in contacted sentinel birds with Gp1. The mean NDV virus titers were $10^{4.80\ \&\ 5.99}$ EID_{50}/ml on day 5 & 7 post contact, respectively and 100% of the birds (3/3) were shedders at the two time points. Groups 6 and 7 contacted birds, which received inactivated genotype VII vaccine in addition to either live genotype VII or genotype II vaccine, respectively, did not experience illness or viral shedding. Vaccination with a single dose of live or inactivated genotype II or VII vaccines did not prevent virus transmission, and tracheal virus shedding was (2/3) birds (Figure 5A & B).

Mortalities in sentinel birds were highest (2/3) in birds placed in contact to birds of Gp1, 2 and 3. Only one sentinel bird died in contact to birds of Gp4. No mortality in sentinel birds was observed in sentinel birds in contact to birds of Gp5, 6 and 7.

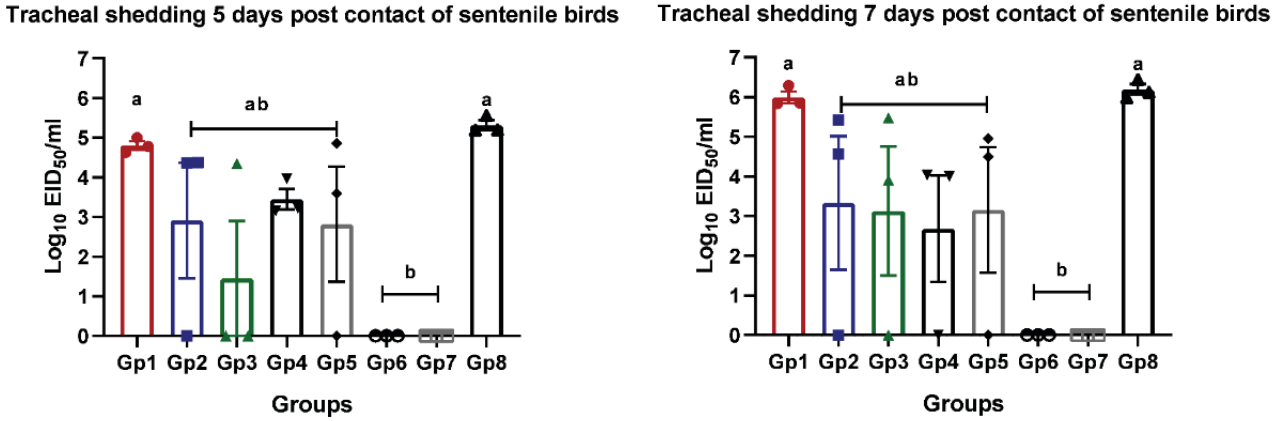


Figure 5: NDV viral shedding of sentinel birds in-contact with experimentally infected birds with 106.5/ EID50/100 uL per bird of virulent NDV genotype VII strain (NDV/chicken/ Egypt/1/2015) at 31 days old via oculonasal route. (A) Scatter blot represents mean tracheal viral titers at 5 days post contact to infected birds and numbers of shedders. (B) Scatter blot represents mean tracheal viral titers at 7 days post contact to infected birds and numbers of shedders. Titers were calculated as log_{10} EID_{50}/1 mL (±SE).

Discussion

Newcastle disease (ND) is one of the most devastating diseases that considerably cripple the global poultry industry, because of its enormous socioeconomic importance and potential to rapidly spread to nattive birds in the vicinity. Newcastle disease is included among the list A of avian diseases that must be notified to the OIE immediately upon recognition (25).

Prevention of the disease includes, in addition to biosecurity, the immunization of birds using different type of commercial NDV vaccines either lentogenic or mesogenic in either live or inactivated form. The right parameters for evaluation of the NDV vaccines are the ability to decrease or eliminate clinical disease as well as bird's mortality, decrease the amount of virulent virus shed and increase the infectious dose of the challenge virus (26). In the past, the inactivated or live NDV genotype II-based vaccines had provided only adequate protection against clinical disease and mortality (26). Although, they can reduce vNDV shedding but the virus shedding still cannot be completely inhibited in the vaccinated birds (13). In the last ten years, an increased number of NDV outbreaks were reported in the vaccinated birds which caused by genotype VII (27, 28). The later suggested that the well-established inactivated or live genotype II-based vaccines, LaSota strain as example, may not produce enough clinical protection against the newly emerged genotype (10, 26, 27, 29). Therefore, in the past few years antigenically matched engineered vaccines to vNDV genotype VII strains were constructed using reverse genetics in either live or inactivated form (26). One such vaccine based on the use of the

LaSota vaccine backbone with the replacement of the fusion and hemagglutinin neuraminidase genes, which replaced with their correspondence in genotype VII virus. Furthermore, the cleavage site of the F gene was modified to be identical to the cleavage site of the LaSota vaccine strain (30). There has not yet been a report that is clear and highlights an appropriate vaccination schedule for using these promising vaccines in the most beneficial way to reduce NDV. Therefore, in this study we designed several vaccination programs using either the genetic-matched or mismatched NDV vaccines as well as combination of them to combat the recently isolated vNDV genotype VII challenge (17), in comprehensive experimental trials. The different vaccination regimes were evaluated from different prospective. Accordingly, the birds were bled weekly to monitor the vaccine serological responses. Serological monitoring of vaccinated birds is one of the important parameters for evaluation of the protective level afforded by applied vaccine (31). The experimental birds had high maternal derived immunity of (7.5±1.5) $\log_2$, which decreased gradually to < 4 $\log_2$ at 14 days old in non-vaccinated birds (32). At the fourth week of age, significant immune responses were peaked in all vaccinated groups with protective antibodies titers ranged from 4-6 $\log_2$ (33). In fact, only when at least 75-80% of the flocks have antibody titers that are equivalent to or higher than 4 $\log_2$ does the herd immunity against NDV exist (33). In the present work, regardless of the vaccine's genetic background, the type of vaccination used had a significant impact on the level of NDV vaccine-induced Ab. Hence, the highest antibodies titers were recorded in the three groups that were vaccinated with combination of live and inactivated NDV vaccines, followed by the two groups vaccinated with inactivated vaccine only. The application of live vaccine only had the lowest seroconversion results regardless of its genetic type. However, the seroconversion results in the present study did not distinguish between the responses of genetic-matched and mismatched vaccines. The previous mainly due to the presence of the both genotypes II and VII in one serotype and the using of LaSota strain as HI antigen.

Following challenge the birds were monitored and scored daily according to their clinical conditions as well as the clinical indices were calculated according to Grund et al (24). The non-vaccinated birds had the highest clinical index (1.14) and clinical signs reflecting the characteristic of velogenic NDV infection and its pneumotropic and viscerotropic properties (7). Hence, the non-vaccinated birds suffered from depression, loss of appetite, feverish, conjunctivitis, swollen eyelids, greenish diarrhea and respiratory signs in form of nasal discharge and rales. On the other hand, the lowest clinical indices were found in the two groups that received combination of genetic-matched inactivated NDV vaccine together with live vaccine of either genetic-matched or mismatched origin with 100% survival. The consistent presence of genetic-matched inactivated vaccine in the two groups induced the superior clinical protection indicate the protective importance of the inactivated vaccine in the designing of NDV vaccination programs. Although vaccination with combination of live and inactivated genetic-mismatched vaccine (Gp3) inhibited vNDV induced-mortality, this certain group had higher clinical index and the clinical signs started one day earlier, compared to that birds vaccinated with genetic-matched inactivated NDV vaccine and live vaccine of either genetic-matched or mismatched origin. Further, in order to maximize protection, it has been recommended that the adjuvant-based ND inactivated vaccine be made from local strains of velogenic NDV (29). Regarding to birds that received live vaccine only (Gp1 & Gp4) had high clinical indices and mortality was approximately 20% in the two groups. These results confirm that whatever the genetic relatedness of the applied live vaccine, it cannot be effective alone for inhibition of NDV infection. Even vaccination with inactivated vaccine without boostering with live vaccine could not provide the birds with complete protection from mortality and clinical signs. It has been found earlier inactivated vaccines based on genotype VII strain could protect birds against clinical disease, especially when used together with live attenuated vaccines (34).

The level of viral shedding in poultry house is also considered an important parameter for evaluation of vaccine and for determination of NDV transmissibility across surrounding poultry flocks (35). The genotype II commercial applied NDV vaccines can protect against clinical disease but not able to prevent virus replication and shedding from infected vaccinated birds to the surrounding environment. The previous could be attributed to the genetic distance between the

vaccine strains and the circulating virus strains (as example; genotype VII) (13, 36, 37). Previous studies showed that using of antigenically matched vaccines homologus with the field virus increases the capacity to prevent or reduce challenge virus shedding in the terms of amount and number of shedders (10, 38). In the present work, the application of live vaccine only of either genotypes II or VII failed to significantly reduce viral tracheal and cloacal shedding 3 days post challenge when compared to control group. However, the duration of virulent virus tracheal shedding was lesser in these groups compared with control groups. In consistent, application of heterologous inactivated vaccine, either alone or boost with heterologous live vaccine, could not prevent viral tracheal shedding of vNDV genotype VII at the third day post challenge. However, it minimize the duration of vNDV shedding from the same route (10). The previous reports stated that vaccination with a combination of live and inactivated LaSota vaccines can protect against clinical disease but not prevent infection and virus shedding (13, 31, 33, 38, 39). On contrary, the two groups vaccinated with inactivated homologues vaccine that boosted with live homologues or heterologous vaccines had minimal or completely diminished viral tracheal shedding at the two evaluated time points (40). From these results we can conclude that when inactivated genotype VII matched vaccines was used, viral shedding was significantly reduced or nearly inhibited (23, 36, 41). Beside the importance of the vaccine genetic homology (27), this result could be attributed to the high humoral immunity specific for NDV genotype VII induced by inactivated vaccine which could prevent virus replication in experimentally infected birds (42). Virus replication in challenged birds was sufficient to transmit to sentinel chickens 3 days post exposure to infected birds

For Newcastle disease control, it is important to take into account the environmental contamination that exists in an outbreak scenario and/or with any population, that has received vaccinations. This is particularly valid for nations where vNDV is endemic. In the present work and inconsistent with the shedding study and the number of shedders results, the virus transmissibility was lowest in the instance of the two groups vaccinated with inactivated homologues vaccine that boosted with live homologues or heterologous vaccines with no recorded mortality. It is thought that for a bird to infected with NDV, between 10^3 and 10^4 EID_{50} of virus must be administered (43) and those two particular groups had lesser amount of virus shedding from both routes. Additionally it is believed that vaccination programs used genotype VII inactivated and live vaccines initiated a high level of monospecific genotype VII antibody titers that neutralize the virus and limit the transmission and virus shedding to the contact birds (42).

In conclusion, the use of NDV vaccination regimens based on heterologous vaccine to the field strain could reduce losses from death, but it did not reduce losses from NDV caused clinical symptoms and transmission among poultry flocks. In order to prevent the transmission and circulation of the NDV genotype VII virulent strain among chicken flocks, a stringent control strategy based on the use of homologous inactivated NDV vaccines with a prime-boost strategy would be helpful.

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Original Research Article
Veterinary Medicine and The One Health Concept

POTENTIAL PERFECTION EFFECTS OF SILVER NANOPARTICLES AGAINST CISPLATIN SIDE EFFECTS IN HEPATOCELLULAR CARCINOMA INDUCED IN SPRAGUE DAWLEY ALBINO RATS: HEMATOLOGICAL, BIOCHEMICAL, HISTOPATHOLOGICAL, AND IMMUNOHISTOCHEMICAL ALTERATIONS

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Abstract: Silver nanoparticles (AgNPs) have various applications such as their use in the medical field. As they have been reported to show antimicrobial and anti-tumor effects. The main purpose of the current study was to explore the anti-cancer effects of AgNPs administration alone or in a combination with cisplatin (CP) against hepatocellular carcinoma (HCC) in rats. Seventy-five rats (Sprague Dawley albino rats) were used in the present study. Rats were assigned to 5 groups. Group 1 served as normal control. Group 2 was injected intraperitoneally (IP) with a single dose (200 ml/kg) of diethylnitrosamine (DEN), then one week later carbon tetrachloride (CCl4) was injected IP (0.2 ml/kg) two times weekly for 14 successive weeks for the induction of HCC. Group 3 was treated with AgNPs (4 mg/kg) daily. Group 4 was treated with CP (6 mg/kg) once a week. Group 5 was treated with a combination of AgNPs (4 mg/kg/daily) and CP (2.5 mg/kg/weekly). Groups 3, 4, and 5 were treated for 3 successive weeks after induction of HCC. Hematological, biochemical, antioxidant activities, proinflammatory cytokines, and apoptotic genes were evaluated in the current study. Hematological results denoted normocytic normochromic anemia in all examined groups except group 2 which showed macrocytic hypochromic anemia, with thrombocytopenia and leukocytosis in all groups. Significant hypoproteinemia, hypoalbuminemia, and hypoglobulinemia were detected in all groups, with no significant changes ($P < 0.05$) in globulin in group 5. Significantly decreases in GSH and SOD were recorded in all groups. While the serum AST, ALT activities, and levels of total bilirubin, urea, creatinine, IL-6, TNF-α, AFP, VEGF, BAX, and caspase-3 were markedly elevated. The results revealed a remarkable improvement in group 5 than groups 3, and 4. The obtained results were supported by immunohistochemical and histopathological investigations of the liver tissue..

Key words: AgNPs; cisplatin; hepatocellular carcinoma; hematology; biochemical; TNFα; IL6

Introduction

Nanoparticles are an important branch of nanotechnology (1). Nanotechnology is concerned with particle structures ranging from 1-100 nm (2). Nowadays, there is a significant increase in nanoparticle usage, especially in the biomedical field. One of the most important nanoparticles is AgNPs, which are used in medicine, pharmaceutics, and dentistry. AgNPs have anti-cancer, anti-inflammatory, anti-bacterial, anti-fungal, and anti-viral activities. AgNPs are expected to present a new perspective on the detection, protection, and treatment of tumors (3). AgNPs are considered potentially ideal choices for cancer therapy due

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to their novel and unique antiproliferative and apoptosis-inducing properties (4). Hepatocellular carcinoma (HCC) is primary liver cancer. HCC is the sixth most common cancer in the world (5) and is considered a great challenge for clinicians owing to its increasing morbidity and mortality. HCC progresses from fibrosis, and cirrhosis to cancer (6, 7). Chemotherapy is considered one of the strategies of cancer treatment that may be used alone or in a combination with the other types (8, 9). Cisplatin is one of the most well-known chemotherapeutic drugs used for various types of cancer. Cisplatin interferes with DNA repair mechanisms causing DNA damage and then inducing apoptosis in cancer cells. Cisplatin causes several harmful side effects despite its anticancer activity such as nephrotoxicity (10, 11), decrease immunity, allergic reactions, gastrointestinal problems, and hemorrhage (12). Moreover combined treatment of cisplatin with other sensitizing agents is an effective method to overcome the resistance of cisplatin (13).

This study aimed at investigating the potential ameliorative effects of silver nanoparticles against cisplatin side effects in hepatocellular carcinoma induced in Sprague Dawley albino rats.

Material and methods

The experimental design was approved by the Committee of the Animal Welfare and Research Ethics, Faculty of Veterinary Medicine, Zagazig University, Egypt. (ZU-IACUC/2/F/124/2022).

Induction of Hepatocellular carcinoma:

The induction of Hepatocellular carcinoma (HCC) occurs in two steps (initiation and promotion) (14). For initiation, DEN was dissolved in corn oil and then injected intraperitoneally by a single dose (200 mg/kg bwt) (15). For promotion, CCl4 (CCl4/ olive oil; 1:1; 1 ml/kg) was given intraperitoneally one week later after DEN administration by a dose of 0.2 ml/kg bwt two times weekly for 14 successive weeks (16).

Synthesis of AgNPs

Preparation of aqueous silver nitrate:

The preparation of colloidal AgNPs was according to a previous report (17).

Reduction by tri-sodium citrate

$AgNO_3$ of high purity of more than 99% (Alpha Chem. Company, India) was dissolved in 100 ml of distilled water. Heat the Silver nitrate solution near boiling (95–98°C), then Add 2% tri-sodium citrate solution (reducing and stabilizing agent) dropwise, one drop per second, and continue heating at 95–98°C (near boiling) for 15 min. The solution turned into a light yellow color which indicates the formation of AgNPs. Wait until reaching room temperature, and then store in the dark at 2–8°C (17).

Characterization of AgNPs.

Nanoparticles' size and shape were measured by transmission electron microscope (TEM) descriptions (Figure 1A, and 1B), Dynamic light scattering (DLS) determined the particle size and particle size distribution as described in (Figure 1C) and Ultraviolet (UV) visible spectrophotometry of AgNPs shows a typical absorption peak (Figure 1D).

Experimental animals and management

A total number of 75 clinically apparent healthy adult male albino rats of 6 to 8 weeks with average body weight (200-250 g) were used in the experimental trials. These rats were obtained from the central animal house of the Faculty of Veterinary Medicine, Zagazig University. The experimental animals were assigned to five groups, each consisting of fifteen rats, and were kept under standard hygienic conditions in metal cages, fed on a balanced ration, and a good source of water and light (12 hours dark/ light cycle). Daily cleaning and changing of water and food were done for experimental animals. One week before the experiment, all animals were kept under observation to ensure that they were free from bacterial infections and parasitic infestation.

Experimental design

The 75 male adult albino rats were assigned into 5 groups, 15 rats in each group. Group 1 was kept as normal control. Group 2 was injected intraperitoneally by DEN (200 mg/kg bwt) at a single dose, then one week later, CCL4 was

injected intraperitoneally by (0.2 ml/kg) 2 times weekly for 14 successive weeks for the induction of HCC (16) and served as an HCC induced group. Group 3 was injected intraperitoneally by AgNPs (4 mg/kg b.wt) daily for three successive weeks (18) continuously after the induction of HCC. Group 4 was injected intraperitoneally by cisplatin (6 mg/kg b.wt) once a week for three successive weeks after induction of HCC (19). Group 5 was treated with a combination of AgNPs (4 mg/kg/IP/daily) and CP (2.5 mg/kg/IP/weekly) for three successive weeks continuously after induction of HCC.

Blood sampling

The blood samples from all individuals in all experimental groups were collected from the tail vein at the end of the experiment (20). Blood samples collected for hematological examination were collected on EDTA in clean Wassermann tubes, while that used for biochemical and antioxidant parameters were collected in a plain centrifuge test tube without anticoagulant to separate serum.

Tissue sampling

Specimens from the liver were collected within 21 days of treatments from all groups for immunohistochemical and histopathological examination.

Hematological studies

Red blood cells (RBCs), hemoglobin (Hb) concentration, packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), and total leukocytic counts were determined using an automatic cell counter (ABX micros 60 hematology analyzer manufactured by HORIBA ABX SAS).

Biochemical and antioxidant assays

The biochemical tests were performed by using test kits of Diamond–Egypt, Spectrum, Spinreact, Cloud–Clone Corp (USA), (Reagents for ELIZA kit), H CUSABIO, DRG ELISA, BIO VISION, Egyptian Company for Biotechnology (S.A.E), and Bio-Med Diagnostic). Serum biochemical profiles including aspartate transaminase (AST), and alanine transaminase (ALT) were estimated (20). Total protein was measured by ELISA Kit (Cat. No MBS1600724), albumin was measured by ELISA Kit 09/17 (Catalog NO E4364-100), and Serum globulins were calculated by subtracting the obtained albumin level from the obtained total proteins level (20), while urea was measured by using a colorimetric assay kit (Catalog NO K375-100), moreover, creatinine was estimated by ELISA Kit rev 03/18 (Catalog NO E4370-100), total bilirubin was estimated by using ELISA Kit 12/19 (Catalog NO E4794-100), serum glutathione (GSH) was measured according to (21) and superoxide dismutase (SOD) was assessed (22).

Pro-inflammatory cytokines and HCC markers

Tumor necrosis factor alpha (TNFα) was estimated by ELISA Kit (Catalog Number SEA133Ra), while ELISA Kit SEA079Ra was used in the estimation of interleukin 6 (IL6), for VEGF, ELISA Kit (Catalog Number CSB-E04757r) was used, and for alpha-fetoprotein (AFP) ELISA Assay kit (SEA153Ra) was used.

Gene expression analysis by quantitative real-time PCR

Liver samples were used for evaluating the expression of apoptosis-related genes, BAX and caspase-3. Total RNA was extracted from tissue lysate by Direct-zol RNA Miniprep Plus protocol (Cat NO R2072, ZYMO RESEARCH CORP. USA), then the quantity and quality were calculated by Beckman dual spectrophotometer (USA). For reverse transcription of extracted RNA (RT-PCR), Super Script IV One-Step RT-PCR kit (Cat NO12594100, Thermo Fisher Scientific, and Waltham, MA USA) was used. After RT-PCR, the data were expressed in Cycle threshold (Ct) to calculate the Relative Quantification (RQ) (relative expression) using the 2-ΔΔCt method (23).

Histopathological examination

Liver tissue Specimens of the sacrificed rats were examined macroscopically, then fixed in neutral buffered formalin (10%). From all liver

samples, the Paraffin sections (5-micron thickness) were prepared, stained by H&E (hematoxylin and eosin), and then examined microscopically (24).

Immunohistochemical analysis of Bcl2 in the hepatic tissue

This technique was performed on 3–5 μm thickness sections of liver tissue (25). then anti-Bcl-2 of the rat was diluted at 1:200 and then incubated with a liver section for 60 min (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Then the primary antibodies were diluted in TBS (Tris-buffered saline) 1% and BSA (bovine serum albumin). Then add the secondary antibody against rat immunoglobulin which was incubated for 15 min (Biotinylated Link Universal–DakoCytomation kit). Then a reddish-to-brown color appeared after adding 3-amino-9-ethyl carbazole (AEC) for 15 min at the sites of immunolocalization of the primary antibodies. Moreover, the specimens were counterstained with hematoxylin (1 min) and mounted with Aquatex fluid (Merck KGaA, Germany). All sections were incubated under the same conditions with the same concentration of antibodies and at the same time; for the immunostaining to be comparable among the different experimental groups.

Statistical analyses

The obtained data were analyzed using the One-way ANOVA procedure, by using PASW statistics 28 (SPSS Inc., USA). $P \leq 0.05$ was considered statistically significant.

Results

Hematological studies:

The hematological parameters were described in table (1). Regarding the erythrogram, all groups (2, 3, 4 & 5) showed significant reduction ($P < 0.001$) in erythrocyte count, Hb level, and PCV concentration (normocytic normochromic anemia) except for group 2 which showed macrocytic hypochromic anemia in comparison with the normal control group. Moreover, all groups (2-5) showed thrombocytopenia in comparison with the normal control group. Concerning the leukogram, the total leukocyte count (TLC) revealed leukocytosis in groups 2, 3, 4 & 5 when compared with the normal control. But when compared with group 2 (HCC inducted group) it showed a significant decrease ($P < 0.001$) as declared in groups 3, 4 & 5.

Biochemical parameters and serum antioxidant activities

The changes in the biochemical parameters and serum antioxidant activities were clarified in the table (2).

Regarding liver functions, the serum total protein and albumin levels in the present study showed a significant reduction ($P < 0.001$) in all groups compared with group 1. While serum globulin level revealed a significant decrease ($P < 0.001$) in all groups except group 5.

Moreover, the serum total bilirubin, ALT, and AST activities illustrated a significant increase ($P < 0.001$) in all groups in comparison with the control. But when compared with group 2 (HCC inducted group) it showed a significant decrease as shown in groups 3, 4 & 5.

Concerning kidney function, the serum levels of creatinine and urea in groups (2-5) showed a significant increase when compared with the normal control.

Regarding antioxidant activity, the serum GSH and SOD activities showed a significant decline ($P < 0.001$) in groups 2, 3 & 4) in comparison with the normal control group, except group 5 which showed a non-significant decrease ($P > 0.05$) when compared with the normal group.

Pro-inflammatory cytokines and HCC markers

There was a significant increase ($P < 0.001$) in serum TNF-α, IL-6, AFP, and VEGF levels in all groups when compared with group 1. But it showed a significant reduction in groups 3, 4 & 5 when compared with group 2 (HCC inducted group).

Gene expression analysis by quantitative real-time PCR

The pro-apoptotic genes (BAX and caspase-3) expressed up-regulation ($P < 0.001$) in the liver of groups 3, 4 & 5, while group 2 (HCC inducted group) showed insignificant difference ($P > 0.05$) when compared with group 1 (Fig. 2 and 3).

Table 1: Hematological changes in the different experimental groups

Treatment / Parameter	Gp.1	Gp.2	Gp.3	Gp.4	Gp.5	P value
RBCs x 10^6/µl	8.60^{a} ±0.20	3.40^{d} ±0.17	7.38^{b} ±0.15	6.30^{c} ±0.19	7.44^{b} ±0.18	<0.001
Hb g/dL	16.24^{a} ±0.59	6.70^{c} ±0.25	12.92^{b} ±0.38	12.42^{b} ±0.60	13.76^{b} ±0.49	<0.001
PCV %	41.54^{a} ±0.62	22.32^{d} ±0.99	37.04^{b} ±0.62	30.66^{c} ±1.75	35.48^{b} ±1.04	<0.001
MCV fl	50.07^{b} ±0.80	65.82^{a} ±2.39	50.11^{b} ±0.93	49.37^{b} ±1.05	47.57^{b} ±0.39	<0.001
MCH pg	18.94^{a} ±0.64	19.91^{a} ±1.44	17.54^{a} ±0.66	20.03^{a} ±0.48	18.44^{a} ±0.28	0.20
MCHC %	$39.06a^{b}$ ±1.00	30.33^{c} ±2.07	35.01^{b} ±1.13	40.68^{a} ±1.50	38.76^{ab} ±0.36	<0.001
Platelets x 10^3/µl	631.00^{a} ±7.48	194.80^{e} ±4.21	394.80^{c} ±4.21	317.60^{d} ±28.42	553.00^{b} ±35.22	<0.001
T.L.C. x 10^3/µl	10.61^{d} ±0.43	23.92^{a} ±0.61	17.14^{b} ±0.42	13.32^{c} ±0.76	12.60^{c} ±0.29	<0.001

Values within the same row carrying different superscript letters are significantly different at P < 0.01. Values represent mean ± SE.

Table 2: Some biochemical parameters, antioxidant activities, pro-inflammatory cytokines, and HCC markers

Treatment / Parameter	Gp.1	Gp.2	Gp.3	Gp.4	Gp.5	P value
ALT U/L	35.70^{e} ±0.46	159.21^{a} ±2.77	118.81^{b} ±2.92	100.86^{c} ±2.72	77.40^{d} ±1.80	<0.001
AST U/L	48.32^{e} ±0.22	188.78^{a} ±2.48	148.62^{b} ±2.93	118.14^{c} ±0.51	89.21^{d} ±2.59	<0.001
Total Bilirubin mg/dL	0.16^{e} ±0.005	1.69^{a} ±0.07	1.16^{b} ±0.01	0.90^{c} ±0.04	0.64^{d} ±0.01	<0.001
Total protein g/dL	7.43^{a} ±0.32	3.57^{d} ±0.10	4.55^{b} ±0.11	3.64^{cd} ±0.26	4.34^{bc} ±0.30	<0.001
Albumin g/dL	4.00^{a} ±0.27	1.98^{b} ±0.20	2.22^{b} ±0.20	1.76^{bc} ±0.07	1.34^{c} ±0.20	<0.001
Globulins g/dL	3.42^{a} ±0.12	1.59^{c} ±0.18	2.34^{b} ±0.20	1.88^{bc} ±0.26	3.00^{a} ±0.28	<0.001
Creatinine mg/dL	0.54^{e} ±0.006	8.62^{a} ±0.32	5.47^{b} ±0.39	4.18^{c} ±0.07	3.28^{d} ±0.07	<0.001
Urea mg/dL	6.35^{e} ±0.16	68.93^{a} ±1.00	31.03^{c} ±0.90	51.69^{b} ±3.32	25.37^{d} ±0.57	<0.001
GSH m. mol/mg	3.54^{a} ±0.16	1.26^{d} ±0.13	2.60^{b} ±0.09	2.13^{c} ±0.10	3.55^{a} ±0.13	<0.001
SOD U/ml	5.49^{a} ±0.09	0.80^{e} ±0.05	2.74^{c} ±0.06	1.97^{d} ±0.02	3.10^{b} ±0.08	<0.001
TNF (pg/ml	83.08^{e} ±0.25	420.83^{a} ±13.33	224.38^{c} ±8.65	298.09^{b} ±6.01	169.12^{d} ±6.39	<0.001
IL6 (pg/ml	62.45^{e} ±0.04	276.00^{a} ±8.78	182.56^{c} ±13.68	219.87^{b} ±2.73	120.83^{d} ±6.41	<0.001
VEGF pg/mg	8.65^{e} ±0.01	90.45^{a} ±6.21	42.60^{b} ±0.49	33.13^{c} ±2.04	20.91^{d} ±1.10	<0.001
AFP ng/mg	1.39^{e} ±0.09	17.79^{a} ±1.02	11.97^{b} ±0.26	9.30^{c} ±0.43	5.68^{d} ±0.22	<0.001

Values within the same row carrying different superscript letters are significantly different at P < 0.01. Values represent mean ± SE

Table 3: Effect of treatment of AgNPs and/or cisplatin and their combination on lesions scoring and quantitative assessment for the immunoexpression of the Bcl2 of rat liver

Lesions	Group 1	Group 2	Group 3	Group 4	Group 5
Thickening of the hepatic capsule	0	3	2	2	0
Macrosteatosis	0	3	2	2	0
Mononuclear cells infiltration	0	3	2	3	1
Portal congestion and haemorrhage	0	3	3	1	0
Karyomegaly of hepatocytic nucleui	0	3	3	2	0
Cytoplasmic vacuolation of hepatocyte	0	3	2	1	1
Cellular atypia	0	0	3	2	0
Cellular vaculation	0	3	2	1	1
Fibroplasia	0	1	1	3	0
Immunoreactivity of Bcl–2	***	*	**	**	**

Absent (0) mild (1) moderate (2) sever (3)
Mild expression (*) moderate expression (**) strong expression (***)

Histopathological examination

The normal control revealed normal histological structure, in which the hepatocytes orderly were arranged in normal lobular architecture with central veins and radiating hepatic cords, moreover, the portal triads showed normal histological structure (normal branches of the hepatic artery, portal vein, bile duct, and portal area) (Fig. 4A). Group 2 (HCC induced group) showing atypical mitosis with abundant accumulation of fibroplasia with inflammatory cells infiltration around hepatic lobules (Fig. 4B), with numerous mitotic figures with deep esinoohilic cytoplasm and focal aggregation of mononuclear cells infiltration (Fig. 4C) Liver from group 3 showing mononuclear cells infiltration in the portal area, cellular atypia and cellular vacuolation (Fig. 4D). While in group 4 (cisplatin group) showing abundant accumulation of fibroplasia with mononuclear cells infiltration around hepatic lobules (Fig. 4F). The Liver tissue from group 5 showing mild vacuolation of the hepatic parenchyma with few mononuclear infiltrations (Fig. 4F).

Immunohistochemical analysis of Bcl2 in hepatic tissue

Immunolabelling of Bcl-2 in the liver tissue of the negative control group had a strong expression of Bcl-2 in the hepatic parenchyma (Fig. 5A). While In group 2 (HCC induced group) showing + of Bcl-2 expression (Fig. 5B, C). The rats of group 3 had ++ of Bcl-2 expression (Fig. 5D). Group 4 possessed ++ of Bcl-2 expression (Fig. 5E). In liver sections from group 5, the reaction of Bcl-2 was ++ expression (Fig. 4F).

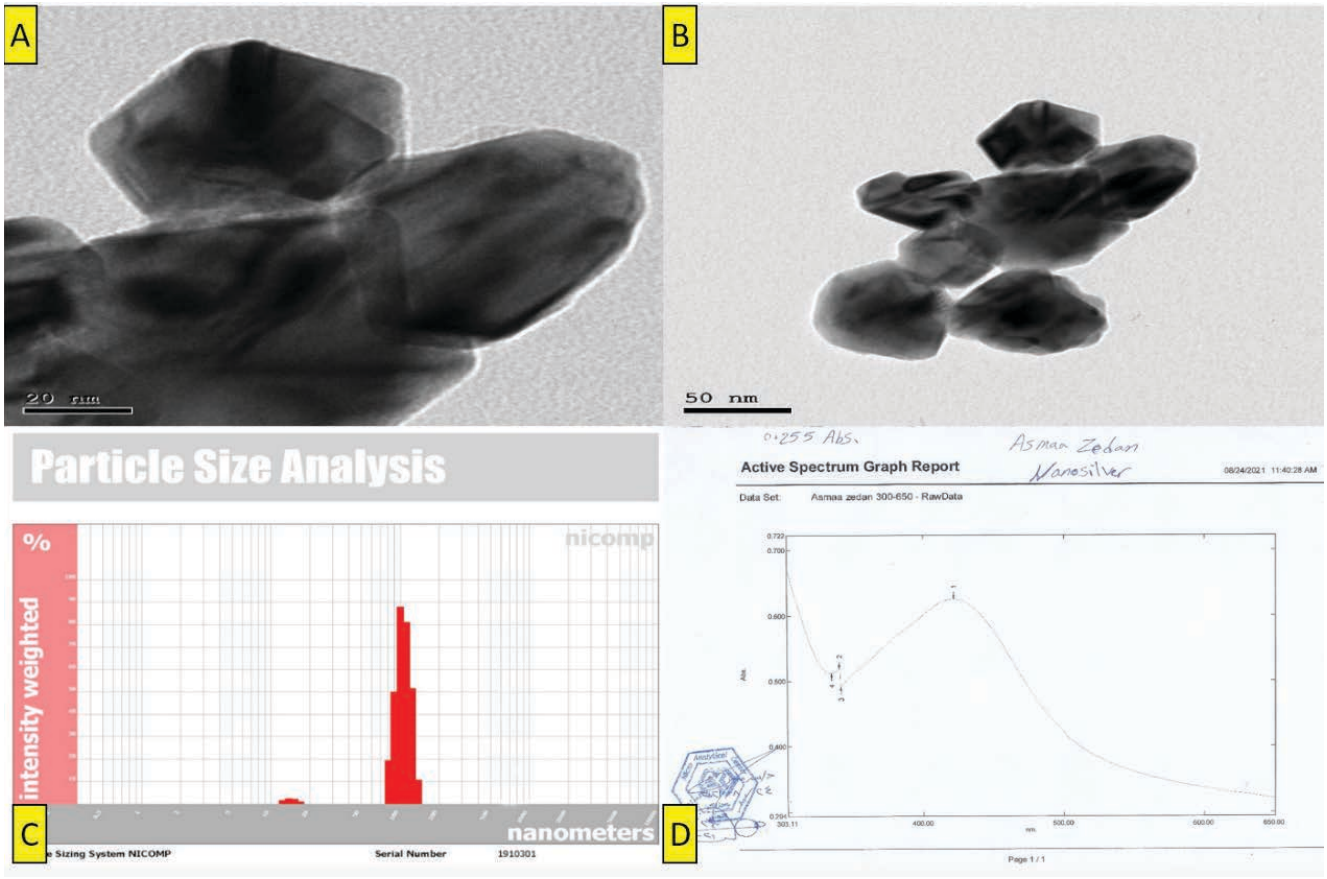


Figure 1: Characterization of the prepared silver nanoparticles; TEM image (A, B), DLS image (C), and UV (D)

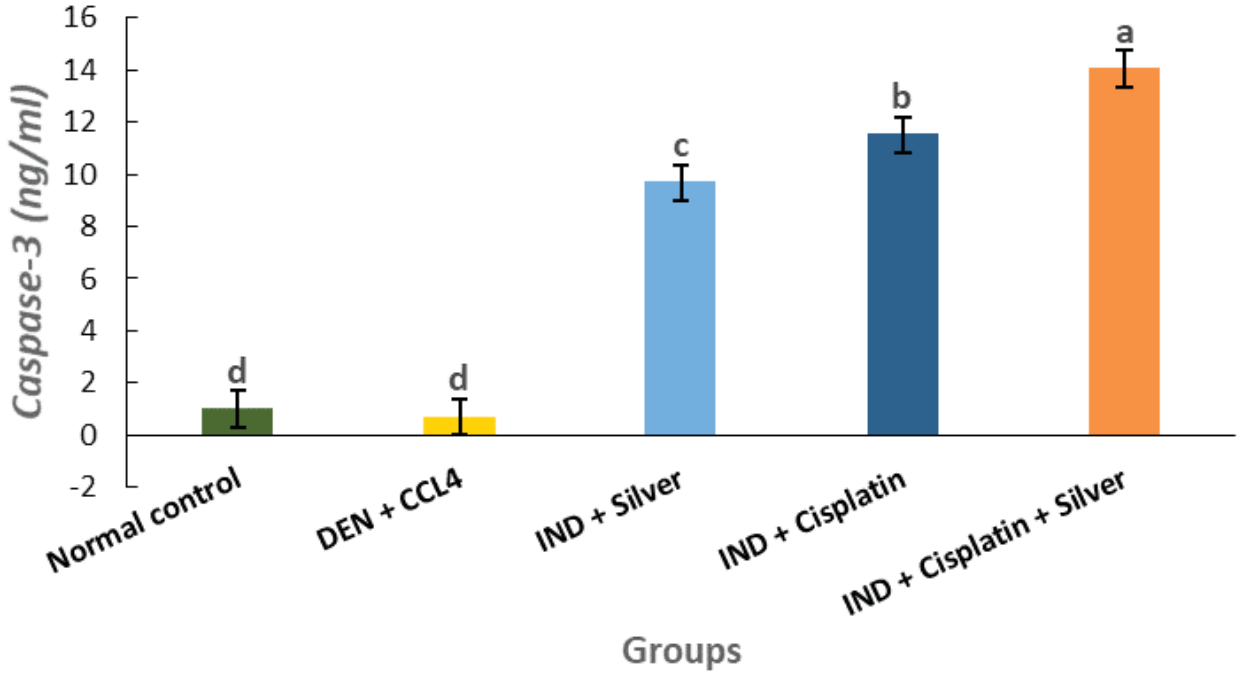


Figure 2: The changes in serum Caspase-3 level in different experimental groups. Columns carrying different letters are significantly different

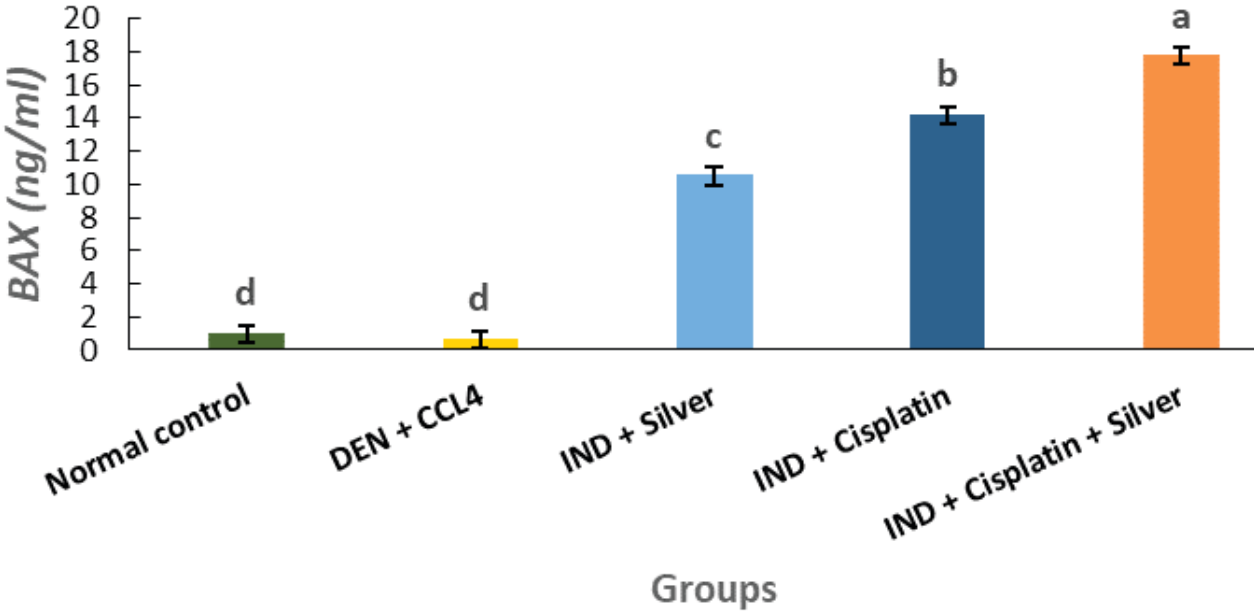


Figure 3: The changes in serum BAX level in different experimental groups. Columns carrying different letters are significantly different

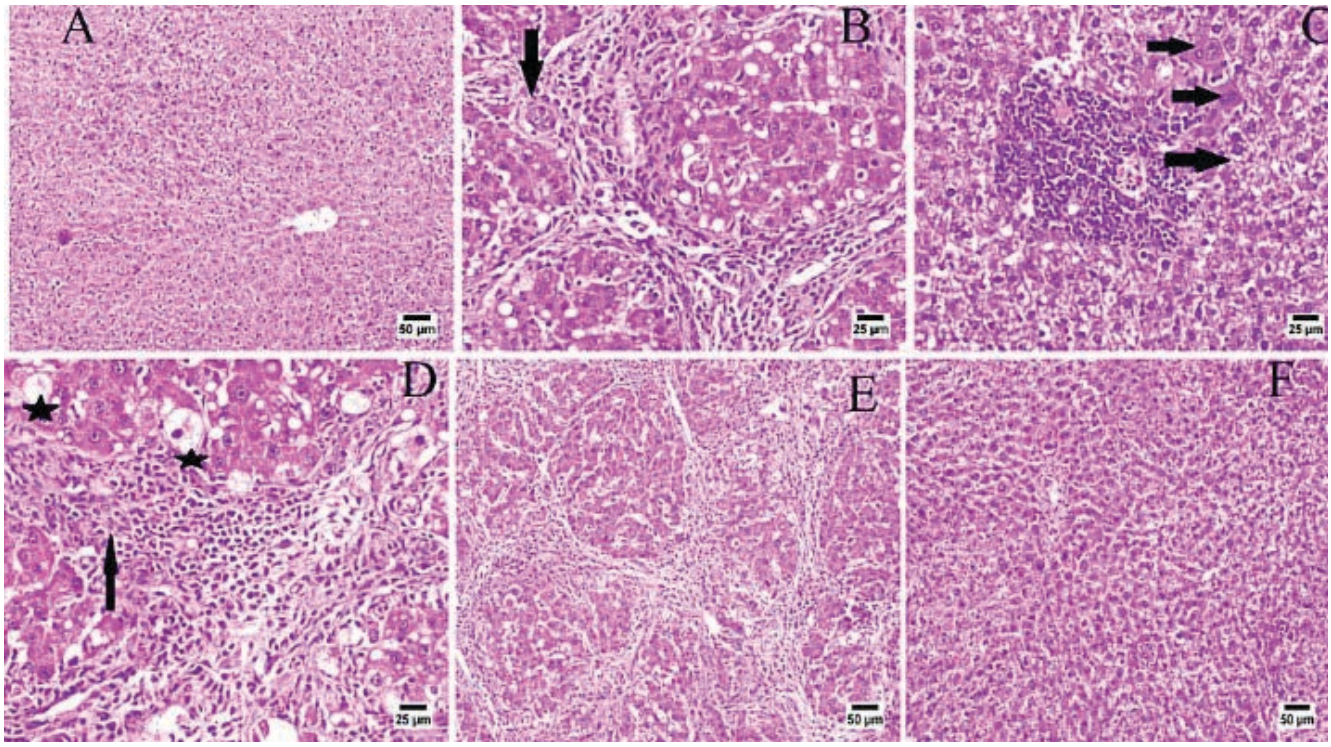


Figure 4: Photomicrographs of liver tissue. (A) normal histological structure, in which the hepatocytes are orderly arranged in normal lobular architecture with central veins and radiating hepatic cords, moreover, the portal triads showed normal histological structure (normal branches of the hepatic artery, portal vein, bile duct, and portal area) in the normal negative control. (B) atypical mitosis (arrow) with abundant accumulation of fibroplasia with inflammatory cells infiltration around hepatic lobules, (C) with numerous mitotic figures with deep esinoohilic cytoplasm (arrow) and focal aggregation of mononuclear cells infiltration of hepatocyte (C) Portal congestion and hemorrhage (arrow) and karyomegaly of hepatocytic nuclei (star) in addition to cytoplasmic vacuolation of hepatocyte (zigzag arrow) in group 2. (D) Mononuclear cells infiltration in the portal area (arrow), cellular atypia (zigzag arrow), and cellular vacuolation in group 3. (E) Abundant accumulation of fibroplasia with mononuclear cells infiltration around hepatic lobules in group 4. (F) Mild vacuolation of the hepatic parenchyma with few mononuclear infiltrations in group 5

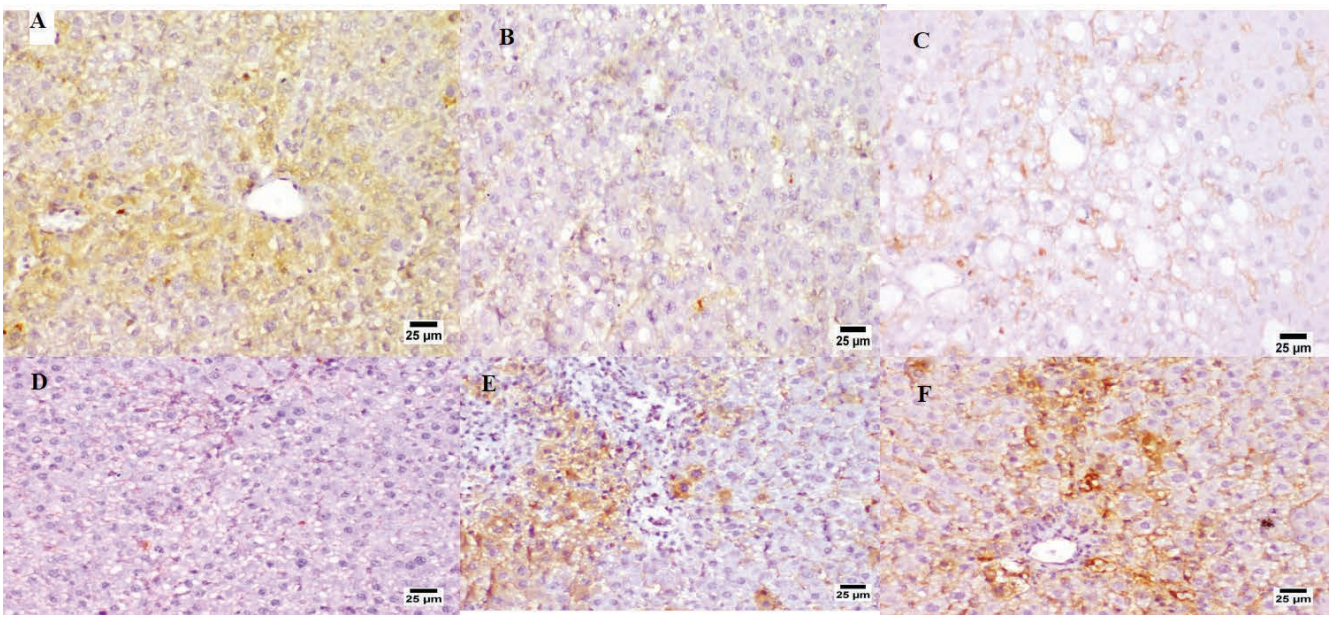


Figure 5: Photomicrograph of liver showing the immunoreactivity of Bcl–2, (A) Immunolabelling of Bcl-2 in liver tissue of negative control group showing a strong expression of Bcl-2 in the hepatic parenchyma. (B &C) group 2 (HCC induced group) showing + of Bcl-2 expression. (D)The rats of group 3 revealed + + of Bcl-2 expression. (E) The liver of rats treated with cisplatin (group 4) demonstrated a moderate positive cytoplasmic labeling of Bcl-2. (F) In liver sections from group 5, the reaction of Bcl–2 was ++ expression

Discussion

Regarding the hematological parameters changes in group 2 which exhibited a significant decline in erythrocyte count, Hb level, PCV concentration (macrocytic hypochromic anemia), and the thrombocyte count (thrombocytopenia) with leukocytosis and these results might be attributed to hemolysis and hemoglobinopathy (26). In the AgNPs-treated group, the hematological studies showed a significant decrease in red blood cell count, Hb level, PCV concentration (normocytic normochromic anemia), and thrombocyte count (thrombocytopenia). Moreover, leukocytosis had been observed, these alterations in the blood picture may be attributed to the effect of NPS on hemoglobin syntheses (during red blood cells maturation in bone marrow) (27) or suppression of circulating hormones such as erythropoietin hormone (28) resulting in normochromic normocytic anemia (29). Leukocytosis might be due to a toxic or allergic reaction against drugs or chemicals, as white blood cells represent the first line of defense, as well as it has an important role in the immunity of the body (30). The erythrogram and leukogram in rats of group 4 (cisplatin-treated group) revealed a significant decrease in the RBCs count, Hb level, and PCV concentration with the development of normocytic normochromic anemia when compared with normal control this may be attributed to the nephrotoxicity occurred by cisplatin resulting in a negative effect on erythropoietin hormone, which leads to reduction of erythropoiesis (31). The reduction in the platelet count is possibly owing to inhibiting bone marrow activity by cisplatin or due to decreased production or increased consumption of platelets (32). Group 5 (AgNps + cisplatin-treated group) revealed a significant decrease in erythrocyte count, Hb level, PCV concentration (normocytic normochromic anemia), and thrombocyte count. This might be attributed to the effect of nanoparticles on hemoglobin syntheses (27) and the suppression effect of cisplatin on hematopoietic tissues leads to impaired erythropoiesis by inhibition of the production of renal erythropoietin, which may be attributed to the same causes previously mentioned in both in groups 3 and 4.

Regarding the biochemical results, the liver function revealed a significant decline in total protein, albumin, and globulin levels with a significant increase in total bilirubin level, ALT, and AST activities in the HCC-induced group. Such results were attributed to the effect of DEN/CCl4 which induces liver dysfunction (19), moreover, DEN produced hepatic injury which is related to the disturbance in hepatocytes membrane instability and metabolism resulting in alterations in liver function (33). Such results were supported by the histopathological finding which revealed atypical mitosis, abundant accumulation of fibroplasia, inflammatory cells infiltration around hepatic lobules, numerous mitotic figures, deep esinoohilic cytoplasm and focal aggregation of mononuclear cells infiltration. n group 3, there was a significant decrease in total protein, albumin, and globulin levels. By contrast, the total bilirubin level, ALT, and AST activities are elevated, these alterations may denote stress on the liver imposed by the nanoparticles (34). But these results showed a significant improvement when compared with the HCC-induced group. Such results were supported by the histopathological findings which revealed mononuclear cell infiltration in the portal area, cellular atypia, and cellular vacuolation. In the cisplatin-treated group, the biochemical results revealed a significant decrease in total protein, albumin, and globulin levels with a significant increase in total bilirubin level, ALT, and AST activities. This result may reflect the metabolism alteration that resulted in liver malfunction (19). However the previous liver function showed significant improvement when compared with the HCC group and this is attributed to the significant reduction of HCC by cisplatin (35), this is evidenced by the enhancement of the histopathological finding which showed abundant accumulation of fibroplasia with mononuclear cells infiltration around hepatic lobules. The rats in group 5 showed significant improvement in the biochemical parameters (total protein, albumin, globulin levels, total bilirubin level, ALT, and AST activities) when compared with the HCC group (group 2), but did not reach the normal levels. Such enhancement is attributed to the effect of AgNPs and cisplatin in the treatment of HCC and this is supported by the histopathological findings which showed mild vacuolation of the hepatic parenchyma with few mononuclear infiltrations.

Regarding the changes in kidney function tests (serum urea and creatinine levels) in group 2. It showed a significant increase in the normal group, this may be attributed to alterations in the renal tissue induced by DEN (36).

In rats of group 2, AgNPs significantly reduced the levels of urea and creatinine than the HCC group but are still higher than normal and this improvement may be due to the protective action of AgNPs against alterations in the renal tissue induced by DEN (36).

The cisplatin-treated group showed a significant increase in urea and creatinine levels, these elevations might be due to the nephrotoxicity induced by cisplatin through induction of oxidative stress resulting in elevation of ROS, lipid peroxidation, and reduction of the antioxidant defense system (37, 38). Rats in group 5 revealed a significant improvement in kidney function (serum urea and creatinine level) when compared with group 2, this improvement owing to the protective effect of synthesized AgNPs against the side effect of cisplatin on renal tissue during HCC treatment, as cisplatin has a nephrotoxic effect (39). But it was still elevated than normal.

Regarding antioxidant activities, group 2 showed a significant decrease in GSH and SOD levels, this may be attributed to the oxidative toxic effect of DEN/CCl4 which increases MDA level (40). GSH and SOD are critically needed for the scavenging of MDA (19). Also, hepatic dysfunction induced disturbances in the antioxidant defense systems and increases the reactive oxygen species (ROS) (41) which leads to increased oxidation of thiol groups of GSH to overcome the increased level of ROS (42). In group 3 there was a significant increase in both GSH and SOD levels in the HCC group (group 2), but still less than the normal control level, this improvement in GSH and SOD levels was explained by (43) who reported an enhancement in GSH and SOD levels, which probably protect the cells from functional damage (44). Moreover, in group 4, the improvement in anti-oxidant activity might be attributed to the anti-cancer and potential inhibitory effect of cisplatin on DEN and CCL4 which induced intracellular oxidative stress. AgNPs and cisplatin in group 5 had a repair effect in remodeling the oxidative stress induced by DEN and CCL4 (45, 46). In addition to the raised levels of GSH, and SOD, AgNPs could induce apoptosis in different types of cancers (47).

TNF-α and IL-6 (proinflammatory cytokines) revealed a significant increase ($P<0.001$) in group 2 owing to the effect of DEN and CCL4, which induced the expression of pro-inflammatory markers under the direct transcriptional directive of NF-κB. (48). Group 3 revealed a significant improvement compared to group 2 in the levels of TNF-α and IL-6 but did not reach the normal level, AgNPs reduced the activity of the TNFR1/NF-KB transcriptional pathway, resulting in a reduction in the TNFα- and suppress IL-6 (49). In group 4 (cisplatin-treated) there was a significant elevation in TNF-α, and IL-6 levels, cisplatin causes cellular damage and forms a platinum-based DNA adduct (Pt-DNA), which activates the p38 mitogen-activated protein kinase (MAPK) pathway and the inflammatory pathway (50) however it had lesser effects when compared with HCC group, in our opinion, this is attributed to the anticancer effect of cisplatin. Group 5 (AgNPs and cisplatin-treated) revealed a significant improvement compared to groups 2, 3 & 4 in the levels of TNF-α and IL-6, but did not reach the normal level, such improvement is resulting from the anti-inflammatory effects of Ag-NPs which had a strong inhibitory effect on Th1cells production which secrete TNF- α, IL-1β and INF-γ that involved in chronic inflammatory disorders and cellular immunity (51).

Regarding HCC markers, both AFP and VEGF showed significant elevations in the HCC group such elevation is owing to the hepatic damage and development of HCC (19, 52) and the carcinogenic effect of DEN and CCL4 (53). The previous parameter levels were reduced in groups (3, 4 & 5) than HCC inducted group but still raised than the normal control, this improvement in results may be owing to the apoptotic effect of AgNPs (54) and cisplatin (55). The apoptotic effect of AgNPs was clarified through the activation of caspase-3 and the ability of AgNPs to inhibit VEGF (56) as shown in group 3. Moreover, the downregulation of VEGF level in group 4 may be attributed to the suppressing effect of cisplatin on cell proliferation and inhibition of the angiogenesis-associated proteins (57), this is supported by our result (increasing Casp-3 and BAX levels), so cisplatin induces apoptosis and down-regulated anti-apoptotic BCL-2 gene (40), in the same trend combined group AgNPs and cisplatin-treated group revealed much improvement in the AFP and VEGF levels and also increased both Casp-3 and BAX. Such results were supported by the immunohistochemical analysis of Bcl2 in hepatic tissue, which showed strong expression of Bcl-2 in the hepatic parenchyma. While HCC induced group showed a slight expression of Bcl-2. The rats of groups 3 and 4, and 5 illustrated moderate Bcl-2 expressions.

Conclusion

AgNPs have anti-inflammatory, anti-angiogenic, and apoptotic properties so they are more recommended as a cytotoxic therapy for HCC. Despite cisplatin being the most commonly used chemotherapy now, it has side effects on hematology and biochemical assay, as well as, apoptotic and antioxidant status in HCC animals. To overcome the side effect of cisplatin, a combination of AgNPs and cisplatin (at a lower dose) enhanced the therapeutic effect on HCC with improved hematology and biochemistry, as well as, apoptotic and antioxidant status in HCC animals.

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Original Research Article
Veterinary Medicine and The One Health Concept

CHANGES IN THE VAGINAL CYTOLOGY AND PROGESTERONE AND ESTROGEN SE-RUM CONCENTRATIONS AFTER TREATMENT OF ANESTRTUM BITCHES WITH A COMBINATION OF FSH AND LH

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Abstract: The current study was designed to evaluate the changes in vaginal cytology and serum progesterone and estrogen concentration in anestrum bitches treated with a combination of FSH and LH. Seven healthy Mongrel anestrum bitches were treated with daily administration of 75 I.U. FSH plus 75 I.U LH for consecutive nine days and another three bitches were kept as a control without any treatment. Treated and control bitches were examined for the percentages of different vaginal-cytology cells, serum concentration of estrogen and progesterone, and signs of proestrus and estrus in a scheduled timeline. Five of the seven treated bitches (71.4%) showed a gradual decrease in the percentage of parabasal cells, a gradual increase in the percentage of cornified and RBCs, a rapid decrease in the estrogen concentration, and an increase in the progesterone concentration. These five bitches showed signs of proestrus and four of them showed male acceptance and were conceived. These five bitches had high estrogen concentrations before initiation of the treatment. The other two bitches had a low estrogen concentration before the initiation of the treatment and after treatment, they showed a gradual increase in the estrogen concentration, but they did not show characteristic changes in vaginal cytology or progesterone concentration. Control bitches had high estrogen concentrations before treatment, but they did not show characteristic changes in vaginal cytology, estrogen and progesterone concentrations, or signs of proestrus up to one month after the end of the treatment. In conclusion, treating anestrum bitches with a mixture of FSH and LH induced characteristic changes in vaginal cytology and serum estrogen and progesterone concentrations in the majority of bitches. These changes were associated with the induction of fertile estrus in more than half of the treated bitches, and this percentage increased to about 80% in bitches having initial high estrogen concentration.

Key words: bitch; estrus-induction; FSH-LH, vaginal cytology, progesterone; estrogen

Introduction

Domestic bitches spontaneously ovulate once or twice per year (1). The inter-estrus intervals range from 5 to 12 months in fertile, non-bred, and non-fertile cycles (2), and some bitches may enter into either primary or secondary pathologically anestrum (3). The inter-estrus interval consists of about 2 months of the luteal phase (diestrus) followed by a prolonged anestrus phase which extends to 3 to 10 months (4). During anestrum phase, progesterone and basal concentration LH is low, but the basal FSH concentration is somewhat high, ranging from 50 – 400 ng/ml (5). Moreover, there is follicular growth in the ovary, but small follicles usually regressed without progressing to ovulatory size (6). The main factors that maintain the bitch in the anestrum phase are the low concentration of the LH and the high concentration of prolactin (7). Therefore, the induction of estrus depends mainly on inducing a drop in prolactin concentration or inducing an increase in the gonadotrophins mainly LH concentration.

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A practical method to induce estrus may be applied to reduce the inter-estrus interval in the cases of missed breeding opportunities or conception failure, and for treatment of primary or secondary anestrus (8). Several treatment protocols using different medications such as anti-prolactin, gonadotropin-releasing hormones, and gonadotrophin active preparations have been developed to induce estrus in the bitches (8). Administration of anti-prolactin results in a reduction in prolactin concentration that indirectly leads to an increase in LH pulse frequency which stimulate follicular development up to ovulation in bitches (9, 10). Administration of anti-prolactin to anestrum bitches has successfully induced estrus within 14 to 40 days with a success rate ranging from 80 to 83% and a conception rate ranging from 60 to 80% (11-14). The administration of different GnRH agonists has successfully induced estrus in anestrus bitches within 7 to 10 days, with an estrus induction rate ranging from 70 to 80% and a conception rate ranging from 58 to 100% (15-17). Thus, administration of GnRH-agonist elicits the release of a sufficient concentration of endogenous gonadotropins.

Gonadotropin-active preparations such as purified LH, purified FSH, hCG, and/or eCG provide a stimulus similar to that provided by the endogenous LH pulses during the natural estrus cycle and may stimulate further development and maturation of the follicles up to ovulation (18 – 21). Administration of gonadotropin active preparations to anestrus bitches safely induces estrus within 5 to 7 days with the estrus induction rate ranging from 70 to 80% and a conception rate ranging from 50 to 80% (19-22). All the studies that employed gonadotropin-active preparations have focused on the estrus induction rate without studying the detailed changes in the vaginal cytology and the serum estradiol and progesterone concentrations in response to the treatment with gonadotropin-active preparations. Examination of detailed changes in the vaginal cytology and the serum estrogen and progesterone concentration will be helpful to understand the detailed response of bitches to the treatment protocol (23). Therefore, the current study was designed to investigate the detailed changes in the vaginal cytology and the estradiol and progesterone concentrations in anestrum bitches treated with a combination of FSH and LH.

Material and methods

Management of Bitches

The experimental design was approved by the Institutional Animal Care Use Committee Zagazig University (ZU-IACUS, approval number ZU-IACUC/2/F/161/2021). Ten healthy mongrel bitches with average age of 15 to 36 months and weights from 25 to 30 kg were used. All bitches were housed in the experimental animal unit faculty of veterinary medicine, Zagazig University from two weeks before the initiation of the treatment protocol up to 30 days after the end of the treatment protocol. Before initiation of the treatment, bitches were dewormed by using Drontal tablets with a dose of 1 tab/10kg (Praziquantel 50 / Pyrantel embonate144 / Febantel 150 mg tablet for dogs, Bayer, Germany). To overcome tick infestation, the bitches were dipped in Mitaban Solution for dogs (Amitraz 10.6 ml, Zoetis, USA). During the whole experiment, bitches were supplied with a continuous source of clean water, fed on cooked meat with vegetables, and regularly exposed to natural light.

Defining the stage of the estrous cycle

There was no data about the previous fertility or the number of days since the previous estrous. To define the stage of the estrous cycle, bitches were subjected to progesterone hormonal assay and vaginal cytology examination 2 weeks before (day -14) and just before initiation of the treatment (day -2). An anestrum phase was defined by the dominance of parabasal cells with the presence of a few proportions of RBCs, WBCs, and intermediate cells in the vaginal cytology, and progesterone concentration <1 ng/ml, (2, 24).

Treatment Protocol

Bitches were randomly allocated to either the treated (n=7) or control group (n=3). Bitches in the treated group were treated with daily intramuscular administration of 75 I.U. FSH plus 75 I.U FSH (Epigonal 75, Eipico, Cairo, Egypt) for nine days according to the method described by (18). Control bitches did not receive any treatments. Control bitches or treated bitches that did not respond to the treatment were observed

for the signs of proestrus until one month after the end of the treatment protocol.

Assessment of the serum estrogen and progesterone concentrations

Blood samples were collected from both treated and control bitches on day -14 and then day after day beginning from day -2 till day 12 (with day 0 being the day of treatment initiation). The serum was separated and stored at – 20 °C until the assessment of the serum progesterone and estrogen concentrations. The serum concentrations of progesterone and estrogen were measured using the electrochemiluminescence immunoassay "ECLIA" (Cobas e 801 immunoassay analyzers). The procedures were applied according to the guidelines of the manufacturer (Roche Diagnostics GmbH, Mannheim, Germany). The intra-assay coefficients of variation were below 6.81% and 9.39% for the estradiol and progesterone, and the inter-assay coefficients of variation were 2.3% and 3.66% for estradiol and progesterone, respectively. The sensitivities were 5 pg/ ml and 0.3 ng/ml for estradiol and progesterone, respectively.

Assessment of vaginal cytology

Vaginal smears were collected on day -14 and every 2 days beginning from day -2 until the beginning of the proestrus or day 12 according to the method described by (24).

After the preparation of a stained film, 100 cells were examined and categorized into different types. Parabasal cells which are small, round, or ovoid with a marginal big nucleus, occasionally, contain cytoplasmic vacuoles or neutrophil granulocytes in the cytoplasm. Intermediate cells are characterized by a polygonal border and a well-shaped nucleus. Cornified cells are large squamous none nucleated cells that take dark stains. Leucocytes which are polymorph nuclear-granulocyted cells, and finally the RBCs. The proportion of each type of cell was calculated as a percentage of the total number of cells.

Monitoring the behavioral and external signs of proestrus and estrus

Bitches in treatment and control groups were examined daily for the external signs of proestrus. The beginning of the proestrus was characterized by the appearance of pronounced vulvar swelling and the presence of bloody tinged discharge from the vulva. The beginning of the estrus was defined based on changing the vaginal discharge into straw-colored and receptivity to the male, and the end of estrus was defined by the disappearance of male acceptance.

Mating and pregnancy diagnosis

Bitches that showed the signs of the protesters were teased daily by a male beginning from the first day of proestrus to test their male acceptance. Bitches that showed male receptivity were matted with a proven fertile three males on the first day of male acceptance and every 2 days until the refusal of the male. Bitches were examined for pregnancy diagnosis using ultrasound on day 25 of post-coitus. Pregnant bitches were maintained up to the day of birth. The pregnancy period was estimated from the day of natural mating up to the day of parturition, conception rate and the number of puppies were recorded.

Statistical Analysis

The statistical differences for the effect of examination time points within the same variable and the same group were analyzed using a linear intercept mixed-effects model. The models included each measured variable as a fixed-effect. These variables include the percentages of different vaginal cytology cells and the concentrations of estrogen and progesterone. Bitch was included as a random effect in the models to account for the dependent nature of the data where variables were measured at multiple time points in each bitch. The statistical differences between treated and control groups or respond and non-respond bitches were evaluated using a t-test. The descriptive statistics were calculated for all variables over the different time points and the results are presented as mean ± SE. All analyses were performed in R software version 3.5.3, and the critical probability was set at $P < 0.05$ for all analyses (25).

Results

The changes in the vaginal cytology

On days -14 and -2, the vaginal cytology of treated bitches showed a dominance of parabasal

cells (81.3 ± 2.7%) and few percentages of RBCs (8.5 ± 1.3%), WBCs (2.8 ± 1.2%), and intermediate cells (7.4 ± 2.4%). After the initiation of the treatment, the vaginal cytology revealed a significant decrease in the mean percentage of the parabasal cells beginning from day 6, and the lowest percentage was recorded on day 12 (22.7 ± 38.7%). Meanwhile, there were marked increases in the mean percentages of the RBCs and cornified cells beginning from day 10 (26.7 ± 12.8 and 30 ± 20.8 %, respectively). The mean percentage of the intermediate cells showed a significant increase on days 6 and 8 followed by a reduction on days 10 and 12 (Figure 1 a).

The untreated control bitches showed a significant decrease in the mean percentage of parabasal cells during the examination time points, however, the parabasal cell was the dominant cell up to day 12 (79.6 ± 0.5%) (Figure 1 b). Significant differences between the control and treated groups in the percentage of parabasal, cornified, and RBC cells were recorded beginning from days 4, 8, and 10, respectively. Untreated bitches did not show any cornification in the vaginal cytology. Before the appearance of a significant increase in the percentage of cornified cells in the treated group, there was a significant increase in the percentage of intermediate cells on days 4,6, and 8 days.

Examination of the vaginal cytology of individually treated bitches revealed the presence of two groups of bitches. Five of the treated bitches showed characteristic changes in the vaginal cytology that ended with the appearance of the signs of proestrus, but the other two bitches did not show pronounced changes in the vaginal cytology during the examination period. The changes in the vaginal cytology in the five respond and the two non-responded bitches are present in Figure 2.

Estrus induction and conception

Five out of the 7 treated bitches (71.4%) showed the external signs of proestrus. These bitches were the bitches that had high estrogen concentration before initiation of the treatment. The other two bitches (28.6%) did not show any signs of proestrus. These two bitches were the bitches that had low estrogen concentration before initiation of the treatment. One bitch showed the signs of the proestrus before the end of the treatment (day 8),

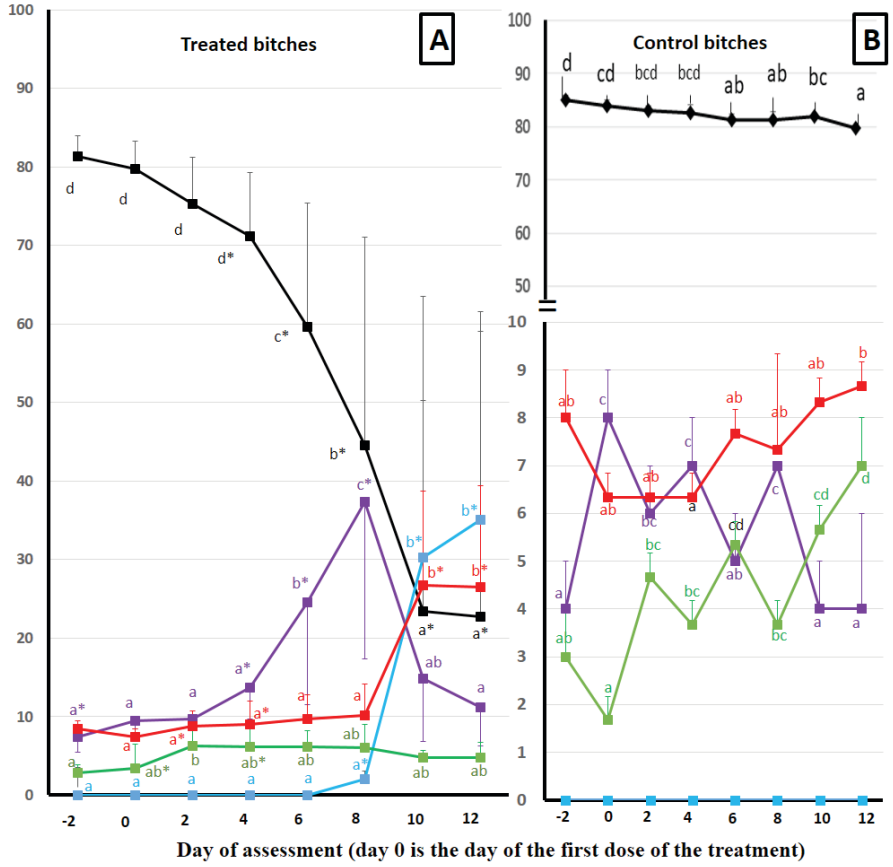


Figure 1: The mean percentages of different superficial vaginal cells in the vaginal smear of treated (A) and untreated bitches (B) at examination time points. Parabasal cells (black line), intermediate cells (violet line), cornified cells (blue line), WBCs (green line), and RBCs (red line). Values that have different superscripts are significantly different between different time points in the same type of cells at a p-value <0.05. * Indicate significant differences between treated and control groups at the same examination time point in the same type of cells. Note that the y-axis in untreated bitches was split

and the interval from the initiation of treatment until the appearance of the signs of the proestrus ranged from 8 to 13 days with a mean of 11 days. The duration of the proestrus ranged from 7 to 10 days with a mean of 8.4 days. Four treated bitches (4/7 57.1%) showed male receptivity and were matted. The duration of the estrus ranged from 8 to 18 days with a mean of 13.5 days. All mated bitches get conceived (conception rate of 100% (4/4) and pregnancy rate of 57.1% (4/7)). All pregnant bitches maintained the pregnancy till parturition, and the duration of pregnancy ranged from 59 to 65 days with a mean of 62 days. The litter size ranged from 2 to 6 puppies with an average of 4 puppies. There were no side effects recorded from the drug during or after the end of the treatment protocol.

None of the untreated control bitches showed signs of proestrus up to one month after the end of the treatment

The changes in estrogen and progesterone concentrations

On day -2, the mean concentration of progesterone and estradiol in the serum of treated bitches were (0.5 ± 0.14 ng/ml and 76.5 ± 51.9 pg/ml). Examination of the serum estradiol and progesterone concentration of individual bitches before the initiation of the treatment revealed that five bitches showed a high estrogen concentration with a mean of 101 pg/ml and the other two bitches showed low estrogen concentration with a mean of 5.5 pg/ml. After the initiation of treatment, treated bitches showed a rapid decrease in the mean serum concentration of estradiol beginning from day 2, and the mean serum estradiol concentration continue low up to day 12 (16.5 ± 4.3 pg/ml). Meanwhile, there was a gradual increase in the mean serum concentration of progesterone till reached a significantly high concentration on days 10 and 12 (2.7 ± 0.8 and 5.9 ± 1.9 ng/ml) (Figure 3). The untreated control bitches did not show a pronounced change in the mean concentration of estradiol (ranging from 111.7 to 116.7 pg/ml) and progesterone (ranging from 0.34 to 0.38 ng/ml) during the examination period although there were some significant differences. The concentrations of estrogen and progesterone in treated bitches were significantly different from that of untreated bitches beginning from day 2 and 6, respectively (Figure 3).

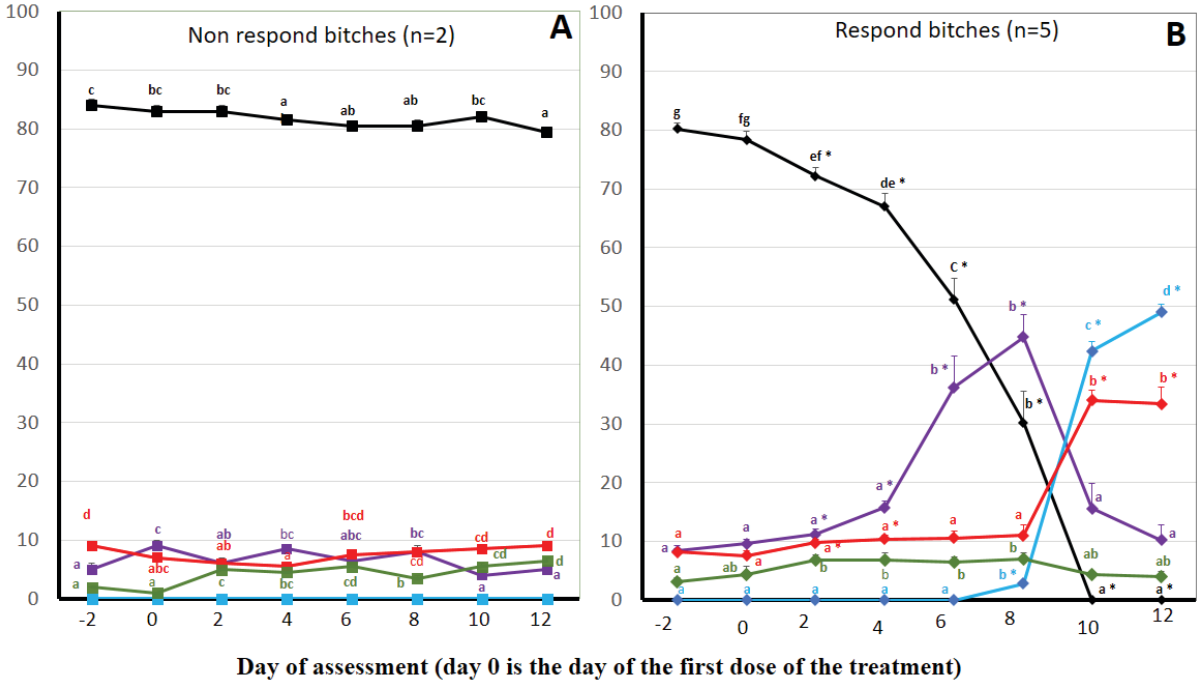


Figure 2: The average percentages of different superficial vaginal cells in the vaginal smear of the two treated non-respond bitches (panel A) and the five treaded respond bitches (panel B) at examination time points. The responded bitches showed the signs of proestrus but the non-responded bitched did show the signs of proes-trus. Parabasal cells (blue line), intermediate cells (red line), cornified cells (grey line), WBCs (violet line), and RBCs (yellow line). Values that have different superscripts are significantly different between different time points in the same type of cells at a p-value <0.05. * Indicate significant differences between responded and non-responded bitches at the same examination time point in the same type of cells.

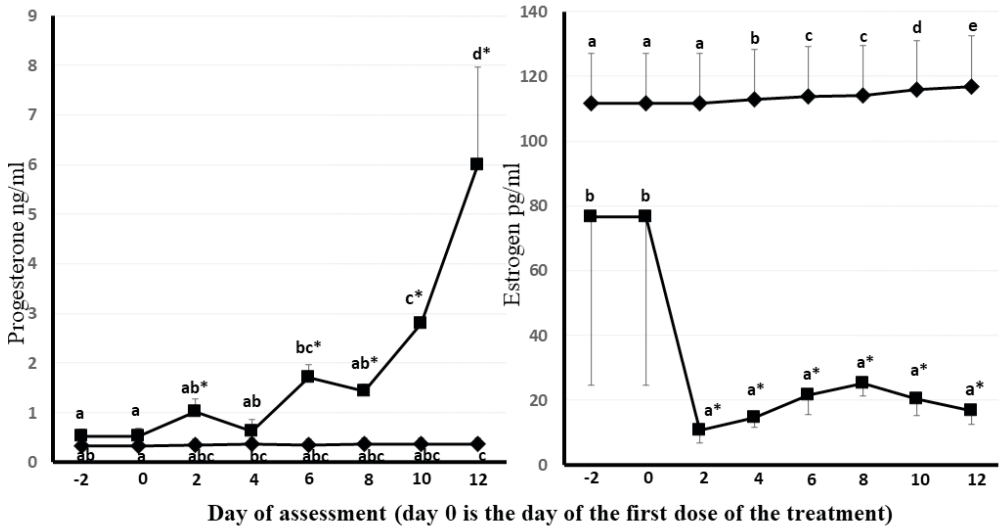


Figure 3: The mean concentrations of progesterone and estradiol in serum of treated (■) and untreated control bitch-es (◆) at examination time points. Values have different superscripts that are significantly different between different time points at a p-value <0.05. * Indicate significant differences between treated and control bitches at the same examination time point in the same hormone

Examination of the hormonal changes in individually treated bitches revealed that five bitches showed the previously described changes, these five bitches were the bitches that had a high estrogen concentration before the initiation of the treatment. But the other two bitches that had low estrogen concentration before the initiation of the treatment showed a different pattern of hormonal changes. These two bitches showed an increase in the estrogen concentration beginning about six days after the initiation of the treatment followed by a decrease in the estrogen concentration at about day 10 after the initiation of the treatment. These two bitches showed a moderate increase in the progesterone concentration beginning from day 6 (Figure 4). The changes in the estrogen and progesterone concentrations in the five respond and the two non-respond bitches are present in Figure 4.

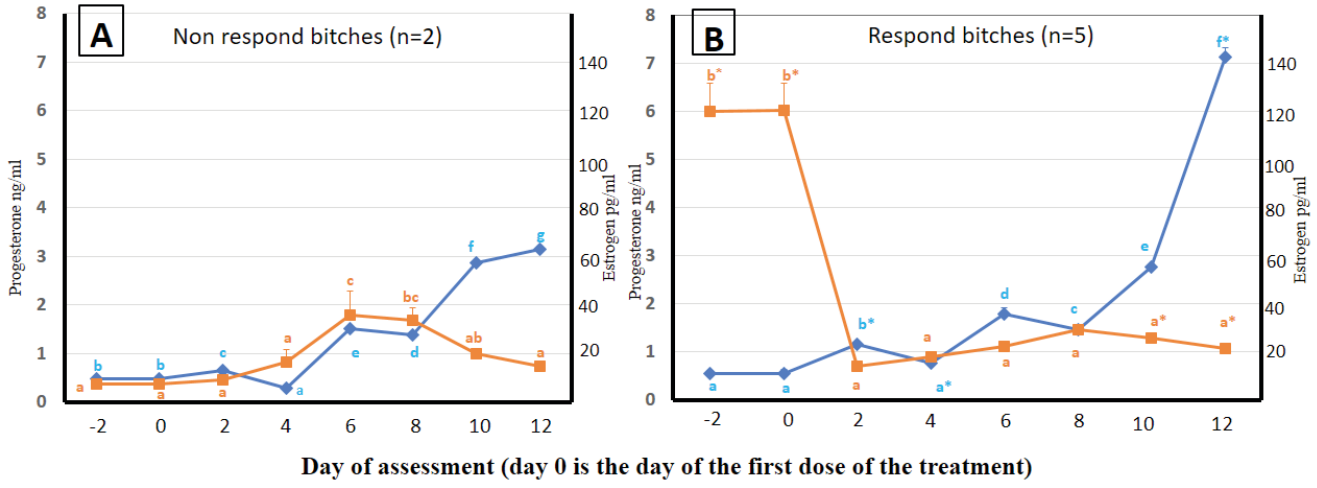


Figure 4: The average serum concentration of estrogen (orange line) and progesterone (blue line) in the two treated non-respond bitches (panel A) and the five treaded respond bitches (panel B) at examination time points. The responded bitches showed the signs of proestrus but the non-responded bitched did show the signs of proes-trus. Values that have different superscripts are significantly different between different time points in the same type of cells at a p-value <0.05. * Indicate significant differences between responded and non-responded bitches at the same examination time point in the same hormone.

Discussion

In the current study, we investigated the detailed changes in vaginal cytology, estradiol, and progesterone concentration in anestrum bitches treated with a combination of FSH and LH.

In the current study, 5 out of 7 treated bitches showed a considerably high estradiol concentration before the initiation of the treatment but the other two bitches showed low estrogen concentration. This may indicate differences in the steroidogenic activity of the follicles that are present in the ovaries before initiation of the treatment (6, 26). The five treated bitches that had initial high estradiol concentration showed a rapid decrease in the serum estradiol concentration from the second day of the treatment. However, the other two treated bitches that had low initial estrogen concentration showed an increase in the estrogen concentration for 4 to 6 days followed by a decrease in the estrogen concentration. Regardless of the concentration of the estradiol before the initiation of the treatment, treating anestrum bitches either with pure porcine LH or eCG and hCG combination was accompanied by an initial increase in the serum estrogen concentration followed by a decrease in the estrogen concentration (27, 28). The variations in the estradiol concentration before the initiation of the treatment may reflect differences in the nature of the gonadotropin receptors in the ovarian follicles at this time (29, 30). These differences may be responsible for the different responses to the treatment as reviewed by (8).

The two treated bitches that showed an increase in the estrogen concentration after treatment may respond to the administration of FSH and LH combination by the development of new follicular growth or higher estrogen production from the current follicles (31). However, the five treated bitches that show a rapid decrease in the estrogen concentration may respond to the treatment by early luteinization of the current follicles (31, 32). This theory was supported by the fact that the five treated bitches that showed a rapid decrease in estradiol concentration showed a gradual increase in progesterone concentration. The increase in the progesterone concentration was reported after treatment of anestrum bitches with porcine LH or a combination of eCG and hCG (18, 27, 28, 32). The interval from the initiation of the treatment until detection of a high progesterone concentration varied among studies. In the current study, treated bitches showed a somewhat early increase in the progesterone concentration which reach above 5 ng/ml on day 12 after the initiation of the treatment with the interval from initiation of the treatment till the proestrus was 11 days, which agrees with a previous result after using eCG (32).

The five treated bitches that showed a remarkable increase in the progesterone concentration showed an increase in the percentage of RBCs and cornified cells in the vaginal cytology in addition to the appearance of a serosanguinous vulvar discharge that indicate the proceeds of these bitches to the proestrus at about day 9 of the treatment. The proestrus induction rate in treated bitches reported in the current study was 71.4% which is comparable to the 70 to 80 % reported after using different combinations of the eCG and hCG (31), but it is lower than the 100% reported after using purified pig LH (28). The interval to the proestrus reported in the current study was 9 days which is longer than the 4-6 days previously reported after using different combinations of eCG and hCG (32), but it was shorter than the 14-15 days reported in another study used eCG and hCG combination (22). Four out of five bitches that showed signs of proestrus showed male acceptance, but the fifth bitch did not show signs of male acceptance. This indicates that this bitch may respond to the treatment by follicular growth, but the follicles did not reach the required stage to induce male acceptance. Twenty-five percent of the anestrum bitches that were treated with porcine LH and showed the signs of proestrus did not proceed to estrus (28). The estrus induction rate reported in the current study was 57% which is comparable to the 64% reported after using a combination of eCG and hCG (32), but its lower than the 80-100% reported after using eCG and hCG combinations (33, 34)

The current study did not have a clear indication of ovulation, but four treated bitches conceived which indicates ovulation of good-quality ova. The pregnancy rate reported in the current study was 57% which is comparable to the 40 to 57 % recorded after treating anestrum bitches with cabergoline (0.6µg/kg/day), deslorelin, Lutrelin, and hCG injections (13, 16, 18, 35, 36), but it is lower than the 67 to 100 % reported after treatment of the bitches with deslorelin vulvar implant, metergoline and cabergoline (5µg/kg/day) (14, 34, 37, 38), respectively. In the

current study, all treated bitches that conceived maintained the pregnancy until the full term which excludes the luteal insufficient suggested after treatment of anestrum bitches with eCG and hCG combination (39). In the current study, all the three untreated control bitches had a high initial estrogen concentration, but none of them showed any change in the hormonal profile or vaginal cytology during the sampling period and none of them showed any signs of proestrus or male acceptant up to one month after the end of the treatment. This indicates that the treatment applied in this current study is efficient to induce estrus in anestrus bitch that had initial high estrogen concentration and that this treatment shortened the inter-estrus interval by at least 40 days.

The efficiency of the current treatment on the two bitches that had an initial low estradiol concentration was completely different from the five bitches that had an initial high concentration of estrogen. The two bitches that had an initial low estradiol concentration showed a marked increase in the estrogen concentration but fail to show any pronounced changes in the progesterone profile or the vaginal cytology. These findings agreed with a previous result that treatment of anestrum bitches with a combination of eCG and hCG increases the estrogen concentration in all five bitches but only two bitches showed an increase in the progesterone concentration (31). This may indicate that the treatment protocol applied in the current study may be efficient to induce follicular growth but unable to achieve follicular maturation and proceeding to ovulation, and growing follicles are mostly regressed (31). It seems that the initial concentration of estrogen may be related to the success of the hormonal treatment. In the current study, the number of bitches is few a further study to define the threshold concentration of estrogen that may predict the successful response after treatment will be helpful. Moreover, administration of a higher dose of LH may be required to induce follicular maturation and ovulation in bitches that have initial low estrogen concentration, further investigation is required to prove this hypothesis.

In conclusion, treating anestrum bitches with a mixture of FSH and LH induced characteristic changes in the vaginal cytology and the serum estrogen and progesterone concentrations in the majority of bitches. These changes were associated with induction of fertile estrus in more than half of the treated bitches, and the estrus induction rate increased to about 80% in bitches having initial high estrogen concentration.

Acknowledgment

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Original Research Article
Veterinary Medicine and The One Health Concept

CLINICAL, HEMATOBIOCHEMICAL AND RADIOGRAPHICAL STUDIES OF CAPRINE PNEUMONIA

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Abstract: Fever, dullness, tachypnea, bilateral nasal discharge, cough, dyspnea, and abnormal lung auscultation were all symptoms of pneumonic goats. To evaluate the hematological, biochemical and pulmonary function changes in 45 pneumonic goats compared with 10 apparently healthy ones, whole blood and serum samples were collected. The results revealed that pneumonic goats had significantly lower (P<0.05) RBCs, Hb, and PCV levels than healthy goats. A decrease in lymphocyte count and an increase in WBCs and neutrophils were found to be significant (P<0.05) in pneumonic goats compared to healthy ones. Blood pH and pO_2 were significantly reduced (P<0.05) while pCO_2, tCO_2, and HCO_3 were significantly elevated (P<0.05) in pneumonic goats compared to healthy ones. Biochemically, K, MDA, Hp and SAA showed significant (P<0.05) increase, whereas Cl and TAC showed a significant (P<0.05) decrease in the pneumonic goats compared to control group. The echotexture of the pneumonic consolidation on ultrasound resembles that of the liver. The chest x-ray showed increased opacity with a cotton-wool-like look that may be more grey or white. According to the findings, the diagnostic techniques mentioned above are helpful in diagnosing goat pneumonia. So, this study aimed to evaluate some selected biochemical parameters and different diagnostic methods including ultrasonography and radiography in pneumonic goats.

Key words: goats; pneumonia; radiography; antioxidants; SAA

Introduction

Goats are the most versatile domestic animals, they may be found in a variety of climates, including those that are dry, humid, tropical, chilly, desert-like, and mountainous (1). Goats are important primarily because they consistently provide more meat, milk, fiber, and skin (2).

Small ruminant respiratory disorders are complicated, and the respiratory disease complex has a number of different etiological causes. In particular, when bacterial and viral illnesses are present together, unfavorable weather conditions that cause stress frequently contribute to the onset and spread of such disorders (3).

The risk of respiratory disorders is greatest for small ruminants in hot, humid climates like those found in the Tropics (4). Transportation, weather changes, unfamiliar environments or feed, poor management and nutrition, and excessive stocking density are other stressors that contribute to the development of respiratory disease (5). The primary risk factor for transmission is overcrowding along with involuntary inhalation of polluted air with a variety of potentially harmful compounds (6).

The presence of fever (pyrexia), loss of appetite, nasal discharge (serous or mucoid), coughing, sneezing, signs of difficulty breathing (dyspnea), and abnormal lung sounds upon auscultation are

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the typical clinical signs and responses of animals infected with respiratory diseases (7).

Acute phase proteins (APPs) are a group of blood proteins that vary in concentration in animals exposed to external or internal stresses, such as infection, inflammation or surgical trauma (8). Major APPs in goats include haptoglobin (Hp) and serum amyloid A (SAA) (9).

If at all possible, chest radiography should be utilized to determine the location, size, and extent of the lesion, followed by lung ultrasonography to confirm the diagnosis of pneumonia. After that, use ultrasonography to obtain more details about the identified lesions (10). So, this study aimed to evaluate some selected biochemical parameters and different diagnostic methods in pneumonic goats.

Material and methods

The study protocol was approved by the animal care committee of the Faculty of Veterinary Medicine, Zagazig University, Egypt No. ZU-IACUC/2/F/99/2022**.**

Animals

A total of 45 goats of both sexes (31 females and 14 males Baladi goats) aged between 3 months to 1 year belonged to different private farms in Sharkia governorate and the hospital of Faculty of Veterinary Medicine – Zagazig University, Egypt. This study started from April 2021 to March 2022. Goats were suffering from respiratory manifestations in addition to 10 goats from private farm were apparently healthy and used as control group.

A thorough clinical history, clinical examinations including general clinical examinations for temperature, pulse, respiration, mucous membranes, superficial palpable lymph nodes, rumen motility, heart rate, and physical examination of the respiratory system by percussion and auscultation were carried out on each goat (11).

Diseased goats showed symptoms of respiratory distress including nasal “serous or mucoid” and ocular discharge, rhinitis and congested mucous membranes, dry or moist cough, respiratory distress, cough, depression and inappetence.

Sampling

Using a jugular vein puncture, three blood samples were obtained from each goat. Two milliliters of blood were drawn freely from the jugular vein into EDTA blood collection tubes, and immediately analyzed. The hematological indices total red blood cells (RBCs), hemoglobin concentration (Hb), packed cell volume (PCV%), total leucocytic count (TLC), and differential leucocytic count were measured in these samples (12).

Additionally, 10 ml of blood from the jugular vein is allowed to flow freely into a clean, dry, and labelled centrifuge tube without the use of an anticoagulant for serum preparation (12). After that, it was preserved for the analysis of potassium, chloride, sodium, acute phase proteins (Hp & SAA), and MDA &TAC in a deep freezer set to -20°C.

Heparinized syringes were used to draw venous blood samples from the jugular vein. The blood was immediately examined using a blood gas analyzer for blood pH, partial oxygen pressure (pO_2), carbon dioxide pressure(pCO_2), and bicarbonate (HCO_3).

Ultrasonographic examination

Using the ultrasound machine (Esoate My lab, Neitherland with a 7 MHz linear probe), the lung area of each goat was examined for ultrasonographic changes of lung abnormalities (edema, congestion, or consolidation) (13).

Radiography

The radiography was taken to confirm the pneumonia in order to evaluate the various pulmonary function scores in pneumonic goats (14). Radiographs were performed using an X-ray machine (Pox-300 BT, Toshiba, Rotanode TM, Japan) from both dorsal and lateral views without tranquilizer (15). The exposure factors ranged from 45-50 kV and 50 mA-S with 70-75 cm as focal film distance (FFD). The films were manually processed in a darkroom.

Postmortem and histopathological examination

were applied if mortality occurred (six goats) or after emergency slaughter (three goats). For

histopathological analysis, small lung specimens with a thickness of 0.5 cm were taken from recently dead or emergency slaughtered goats with respiratory disorders. In order to protect the pulmonary alveoli's architectural integrity, lung tissues were inflated with formalin as previously done by Ghanem et al. (16).

Statistical analysis

A Student's T-test was used to statistically examine the data, and the findings were recorded as mean ± SE. The values were considered significant at $P<0.05$.

Results

Clinical findings and physical examination:

Anorexia, dullness, depression, dyspnea, nasal discharge, moist painful cough and dry cough were the most prevalent clinical symptoms on pneumonic goats (Table 1). Physical examination revealed that the pneumonic goats had fever (40.22 ± 0.13 °C), shallow rapid breathing (35.00 ± 1.14 breaths/min), an accelerated heart rate (94.80 ± 1.65 beats/min), redness of the ocular mucous membrane and conjunctiva, and crusts around the nasal opening. In contrast to apparently healthy goats, lung auscultation revealed pronounced wheezing, crackling sound, moist rales, and exaggerated vesicular sound (Table 2).

Hematobiochemical changes

Upon hematology of pneumonic goats, RBCs count, Hb and PCV revealed significant ($P<0.05$) decrease while WBCs and neutrophils revealed significant ($P<0.05$) increase with a significant ($P<0.05$) decrease in lymphocytes count. Monocytes and eosinophils revealed no significant changes. Serum mineral analysis revealed a significant ($P<0.05$) decrease in Cl, a significant ($P<0.05$) increase in K, and no significant changes in Na. TAC showed significant ($P<0.05$) decrease while MDA, Hp and SAA levels showed significant ($P<0.05$) increase in pneumonic goats when compared with control goats (Table 3).

Pulmonary function tests

In comparison to the control group, the pulmonary function tests of pneumonic goats revealed a significantly ($P<0.05$) lower pH and pO_2 and a significantly ($P<0.05$) higher pCO_2, HCO_3, and tCO_2 (Table 4).

Table 1: Incidence of clinical signs in diseased pneumonic goats.

Clinical sign	No. of affected goats	%
Fever	45	100
Cough	39	86
Nasal discharge	23	51
Decreased appetite	41	91
Congested mucous membrane	34	75.5
Cyanosed mucous membrane	11	24.5
Dyspnea	7	15

Table 2: Physical examination of diseased pneumonic goats compared with control

Physical examination	Control group (N=10)	Pneumonic group (N= 45)
Temperature (°C)	39.26 ± 0.08	**40.22 ± 0.13***
Pulse (/minute)	82.60 ± 1.02	**94.80 ± 1.65***
Respiration (/minute)	26.20 ± 0.58	**35.00 ± 1.14***
Mucous membrane	Light rosy red and somewhat bluish, no secretion and no swelling	Congested m.m. in some cases and cyanosed in other cases
Appetite	Normal appetite	Anorexia
Depression	Animals were alert	Depression and recumbency were observed in late stages of pneumonia

Cough	Absence of cough	Some cases showed dry and nonproductive cough while others showed moist cough
Nasal discharge	Absence of nasal discharge	Scanty serous to mucoid in some cases, mucopurulent to purulent nasal discharge in other cases.
Dyspnea	No dyspnea	Some cases showed signs of dyspnea
Lung auscultation	Normal vesicular sound	In some cases, dry rales, moist rales, crepitation and wheezes were heard on auscultation. While, in others there were absence of vesicular sounds and audible heart sounds.
Lung percussion	Normal resonant sound	incomplete to complete dull sound

Data are presented as means ± S.E. (S.E. = Standard error). * Means significantly different from control at P< 0.05

Table 3: Hematobiochemical changes of diseased pneumonic goats compared with control

Parameters (Units)	Control group (N= 10)	Pneumonic group (N=45)
RBCs (10^6/ mm^3)	6.09 ± 0.03	4.82 ± 0.17*
Hb (gm/dl)	12.21 ± 0.05	11.19 ± 0.16*
PCV (%)	33.09 ± 0.35	27.07 ± 0.52*
WBCs (10^3/ mm^3)	12.82 ± 0.20	16.12 ± 0.24*
Lymphocytes (%)	69.05 ± 0.29	63.34 ± 0.22*
Neutrophils (%)	21.14 ± 0.18	26.26 ± 0.30*
Monocytes (%)	4.65 ± 0.19	5.00 ± 0.08
Eosinophils (%)	4.72 ± 0.07	5.49 ± 0.12
Na (mmol/L)	144.40 ± 0.18	143.92 ± 0.32
K (mmol/L)	4.32 ± 0.02	5.16 ± 0.15*
Cl (mmol/L)	97.69 ± 0.23	83.04 ± 0.88*
SAA (mg/L)	88.60 ± 1.01	122.61 ± 0.81*
Hp (g/L)	2.98 ± 0.10	7.03 ± 0.09*
TAC (ng/ml)	15.04 ± 0.08	12.95 ± 0.33*
MDA (nmol/ml)	6.83 ± 0.22	9.54 ± 0.47*

Data are presented as means ± S.E. (S.E. = Standard error). * Means significantly different from control at *P*< 0.05

Table 4: Pulmonary function test changes of diseased pneumonic goats compared with control

Parameters (Units)	Control group (N= 10)	Pneumonic group (N=45)
pH	7.43 ± 0.01	7.30 ± 0.01*
***p*CO_2 (mmHg)**	38.42 ± 0.14	48.11 ± 0.33*
***p*O_2 (mmHg)**	39.45 ± 0.17	36.82 ± 0.19*
***t*CO_2 (mmol/L)**	19.92 ± 0.31	25.23 ± 0.35*
HCO_3 (mmol/L)	19.26 ± 0.14	23.81 ± 0.32*

Data are presented as means ± S.E. (S.E. = Standard error). * Means significantly different from control at *P*< 0.05

Ultrasonographic Findings in normal and pneumonic goats:

The normal ultrasonographic picture of the lungs were typically aerated. This was evidenced by the presence of numerous parallel and regular reverberation artefacts underneath the uppermost hyperechoic linear image. Both pleural leaves were visible as a broad, smooth, hyperechoic line. Pneumonic lung consolidation was visible as a homogenous or heterogeneous hypoechoic to echoic structure without reverberation artefacts. Due to acoustic enhancement from pleural exudates and the presence of comet-tail artefacts, the visceral pleura appeared thicker and more hyperechoic than normal (Figure 1).

Radiographic findings in normal and pneumonic goats:

Lung radiodensity changed in accordance with lung inflation during radiography, with expiration processes having a higher radiodensity than inspiration processes. Goats with pneumonia had considerable opacity on the radiograph. Opacity appeared as high gray- or white-density areas with the look of cotton wool. The cranioventral region was mostly affected (Figure 2).

Postmortem findings in pneumonic goats:

Upon gross examination of six recently dead and three emergency slaughtered pneumonic goats, trachea revealed frothy serous blood-tinged exudate (Figure 3 A). The lungs were found to have hard, congested, consolidated, or hepatized parts (Figure 3 B, C & D).

Histopathological findings in pneumonic goat:

Microscopically, fibrin threads and leukocytes infiltration with eosinophilic substances (serous exudates) in the alveoli of some cases (fibrinous bronchopneumonia). Other cases showing thickening of interlobular septa by fibrin threads with alveoli filled with alveolar macrophage (interstitial pneumonia). Some cases showed thickening in the pleura by fibrin thread and leukocytes infiltrations with presences of thrombotic mass inside pulmonary blood vessels (Figure 4).

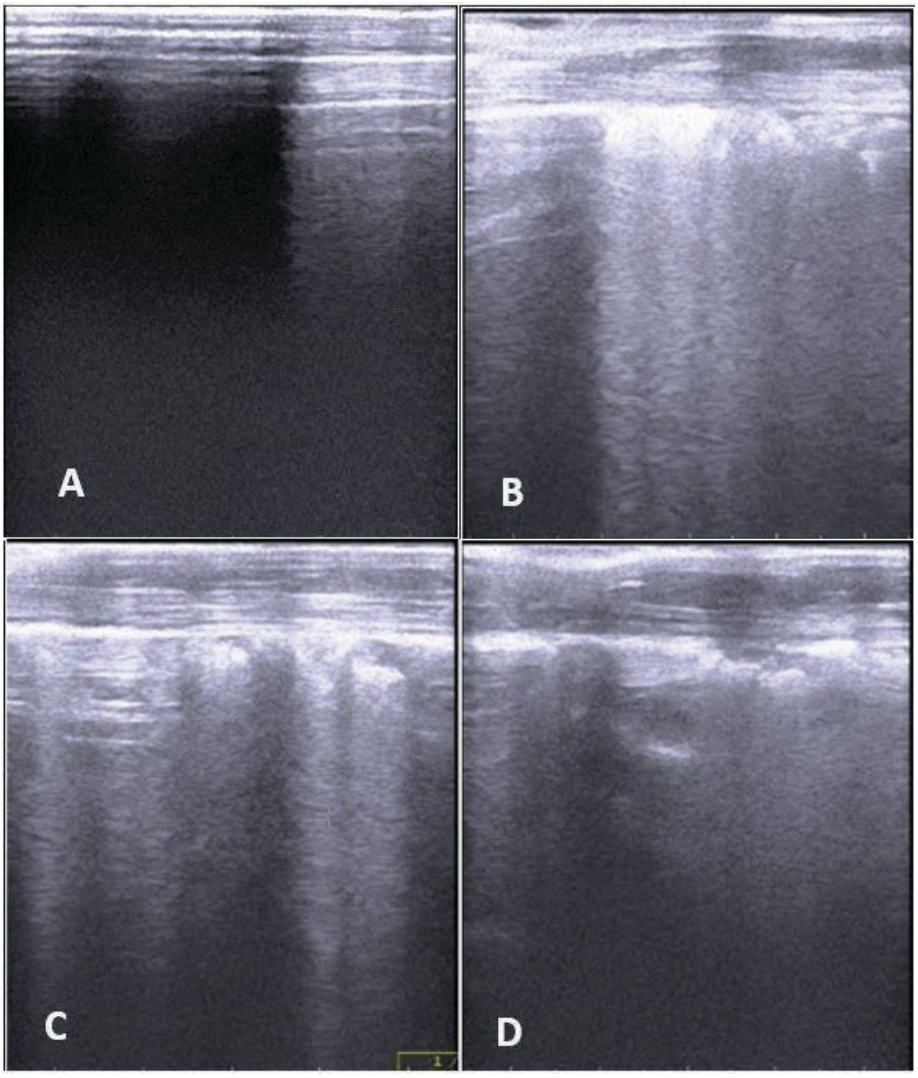


Figure 1: Ultrasonographic imaging of lung. A: normal lung with reverberation artifacts, B, C& D: Absence of reverberation artifacts, consolidation and presence of comet-tail artifacts

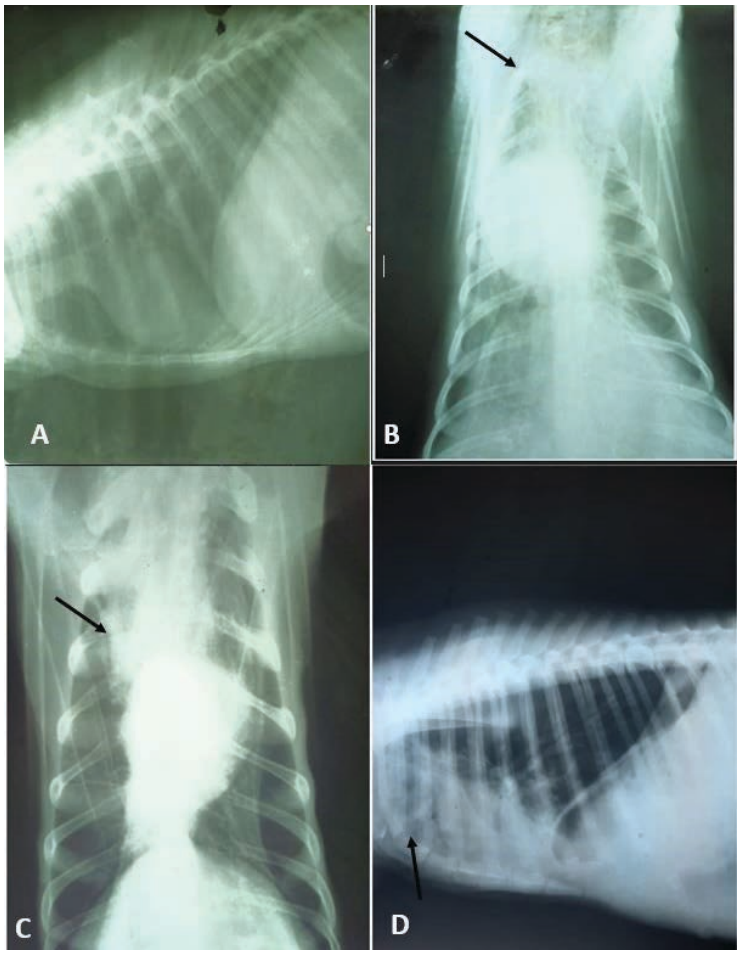


Figure 2: Radiographic imaging of lung. A: Normal radio-density of lung (lateral view) B, C& D: Increased radio-opacity of the lung (B &C: Dorsal views and D: Lateral view)

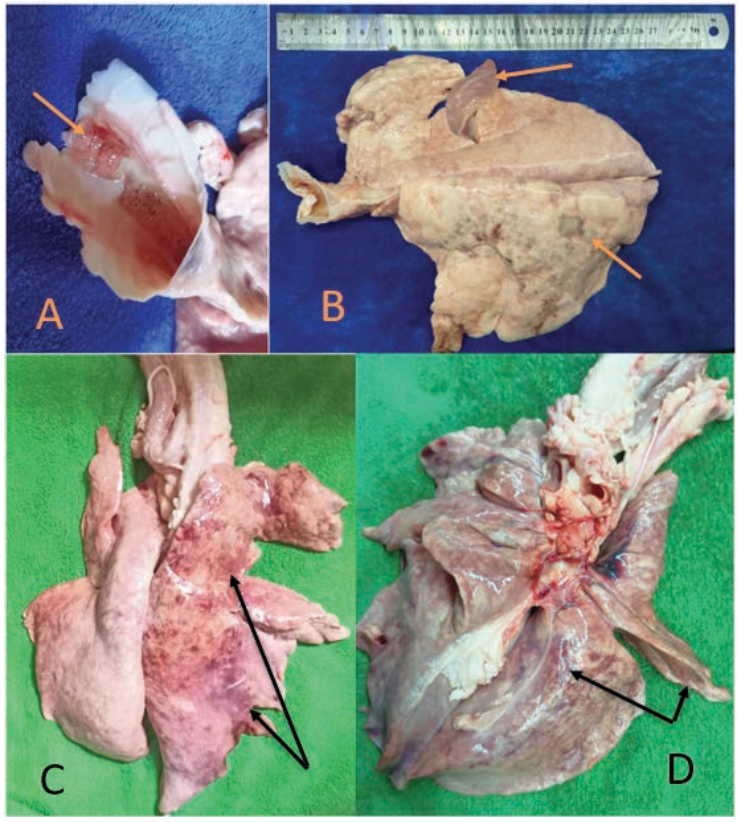


Figure 3: Trachea and lungs of goat. A: frothy serous exudate in trachea. B, C, D: congestion and consolidation of the pneumonic lung (B: lesions in right middle and left caudal lobes, C: lesions in right lung and D: lesions in left lung)

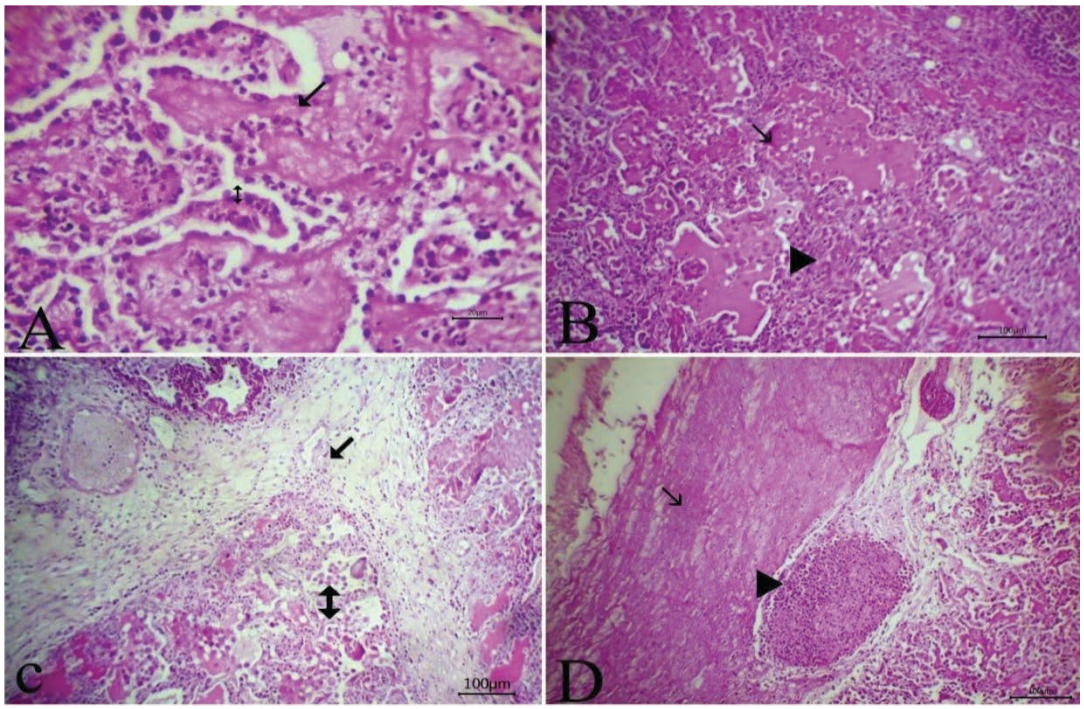


Figure 3: photomicrograph of the lung A: showing alveoli filled with fibrin thread (arrow) and leukocyte infiltration (arrow with 2head) HE, bare20. B: the alveoli filled with eosinophilic substances (serous exudates) (arrow) and leukocytes infiltration (arrowhead) HE, bare100. C: thickening of interlobular septa by fibrin thread (arrow) some alveoli filled with alveolar macrophage (arrow with 2head) HE, bare 100. D: thickening in the pleura by fibrin thread and leuko-cytes infiltrations (arrow) and presences of thrombotic mass inside pulmonary blood vessels (arrowhead), HE, bare 100.

Discussion

Goatindustry in Egypt is considered one of the main important sources for meat and milk production and consequently important for human life maintenance (17). Respiratory diseases in goat continues to be a major problem and generally resulting from exposure of animals to environmental and managerial stressors (18).

Particularly in poorer nations, pneumonia poses a serious danger to the production of small ruminants (19). Physical examination, laboratory results, and other imaging methods should all be used to make an accurate clinical diagnosis of pneumonia (20).

The clinical symptoms of pneumonia in goats included pyrexia, sneezing, and coughing, followed by mucous membrane congestion, bilateral nasal discharge, tachypnoea, dyspnea, tachycardia, ocular discharge, anorexia, and mucopurulent nasal discharge and crackles sound on lung auscultation. The results concurred with those of Chung et al. (5).

The hematological examination of pneumonic goats revealed anemia, which may be caused by an iron deficiency that might be attributed to a decrease in protein and energy intake as a result of anorexia associated with pneumonia, or the sequestration of iron in bone marrow macrophages and hepatocytes during infection, making it unavailable for utilization in hemoglobin synthesis and inhibiting erythropoiesis. It could also be resulted from destruction of red blood cells by micro-organisms secretions (21). The significant increase in both WBCs and neutrophils may be related to inflammatory lesions and presence of bacterial infection in pneumonic goats. However, the significant reduction in lymphocytes could be explained by the adrenal gland's activation under stress in response to the tissue being infiltrated by toxins (22).

Hyperpyrexia during the acute stage of the disease and metastatic infection of the liver and kidneys leading to hepatic and renal dysfunction may be responsible for the hypochloremia with hyperkalemia (23). Hyperkalemia might be occurred in respiratory disorders, especially if acidosis is present. This might be attributed to the exchange of potassium from intracellular to extracellular fluid with H+ ions (24).

Higher SAA may be attributed to its function in moving, binding, and scavenging lipoproteins from hepatic cells during the inflammatory phase (25). SAA is also necessary for the APR through the activation of phagocytic cells (macrophage and neutrophil) or the eradication of coliform germs (8, 26).

Following inflammation and/or infection, tissue injury (pleuropneumonia in this study) may induce higher serum Hp levels (27). According to a recent publication, Hp has strong bacteriostatic potentials through the binding of free hemoglobin (Hb), which leads to the creation of Hp-Hb complexes that are then eliminated by the reticuloendothelial system (28).

Pneumonic goats had significantly higher MDA values than control values, whereas significantly lower TAC values were seen in the diseased group compared to the control group (29). As is well known, inflammatory diseases are associated to stimulated oxidative responses and reduced antioxidant defenses. This might be the result of excessive lipid peroxidation in plasma and cells due to many factors or diseases that cause excessive NADPH production, which in turn enhance lipid peroxidation in the presence of the cytochrome P450 system (30).

The changes in pulmonary function tests, which included considerably lower pH and pO_2 and significantly higher pCO_2, tCO_2, and HCO_3, were similar to those observed by Nagy et al. (31) in pneumonia-affected calves. These results could be explained by respiratory acidosis, which causes pneumonia-related hypoxic conditions and pulmonary hypoventilation. The findings agreed with those of Ghanem et al. (16).

Pulmonary air content makes it more difficult to evaluate the lung parenchyma using ultrasound in a healthy animal. Where the lungs are filled with air, total reflection causes the intercostal transmission of ultrasound to only reach the visceral pleura and cease at the air-filled alveoli (32). When the pulmonary air content is decreased and the lung resembles a liver, an accurate ultrasonographic examination of the lung tissue can be achieved. Visceral pleural surface irregularity may be a precursor to consolidation (33).

The consolidations in the current investigation were consistently hypoechoic and uniform, which may have been caused by the accumulation of exudate, blood, and mucus. Similar results have already been published (34). In this investigation, the right lung's cranioventral region exhibited the majority of the consolidation. The findings were in contrast to those of Tharwat and Al-Sobayil (32), who noted that consolidation in the right lung was predominantly caudo-dorsally.

For confirming pneumonia in goats, radiography is regarded as one of the most reliable tests (35). According to Masseau et al. (36), radiography research had a 94% sensitivity for identifying cattle lower respiratory tract infections. According to the results of the radiographic examination, the severity of pneumonia in goats was determined and scored as 1, 2, and 3 by looking at the parts of the lung that were affected and the infiltration density in relation to healthy goats. The findings were supported by Falcon et al. (37). Radiographic examination of the lungs that reveals severe lung alterations may be used to determine the condition's severity and prognosis (14).

The present study's findings are supported by the observation that pneumonic lungs with dark red patches and consolidation of several lobes with firm consistency are the most prevalent gross lesions detected in the lungs of most cases (38).

Different investigated goats showed variable degrees of bronchopneumonia, according to the histopathological examination. These results might be explained by the variable susceptibilities of the infected goats. The toxic proteins leukotoxin, lipopolysaccharide, and polysaccharide (39), as well as the inflammatory factors produced by neutrophils and other inflammatory cells (40), may be the cause of these histopathological alterations.

Conclusion

Diagnosis of caprine pneumonia could be confirmed by hematological, biochemical mainly SAA, Hp, TAC and MDA, blood gas analysis, ultrasonographic, and radiographic examination, supported with postmortem and histopathological examination.

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All authors contributed in the study design and in the writing of the manuscript. The samples were collected by SMA, who also monitored the animals. The data was analyzed by SMA, and the results were reviewed by NEA. The final manuscript was read and approved by all authors.

The authors declare that they have no competing interests.

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Original Research Article
Veterinary Medicine and The One Health Concept

CARNOSINE-LOADED ORAL NIOSOMES AMELIORATE HIGH-FRUCTOSE-INDUCED METABOLIC SYNDROME IN RATS VIA MODULATION OF SIRT1, A METABOLIC MASTER SWITCH

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Abstract: Metabolic syndrome is a crucial health challenge, and the available therapeutic agents are still not effective. Carnosine, a cytoplasmic dipeptide, is a potent anti-glycation, anti-oxidant, anti-inflammatory and chelating agent. However, whether carnosine would be assumed as a potential hypoglycemic agent or not, no decisive report with detailed mechanisms is found yet. As such, we suggest the carnosine-loaded in niosomes as a prospective solution to bypass its unwanted fast degradation by carnosinase which is considered as a major obstacle with the clinical application of carnosine as an oral drug therapy. Toward this, the purpose of our study is to assess the profits of oral administration of carnosine, and carnosine-loaded niosome in HFD-induced metabolic syndrome rats and to inspect some of the involved mechanisms. Initially, carnosine-loaded niosomes were prepared and characterized. Then, metabolic syndrome was provoked by 60% fructose diet in male Sprague Dawley rats where carnosine and carnosine-loaded niosomes were orally administered at doses 50 mg/kg and 25 mg/kg, respectively. In addition, biochemical and molecular studies were performed to clarify the possible mechanisms of action. Data showed that the consumption of 60% fructose diet displayed a tremendous increment in body weight, body mass index as well as a significant elevation in levels of serum glucose, insulin, TAG, TC, LDL-c, VLDL-c and FFA. Also, it showed a significant reduction in levels of serum HDL-c. Furthermore, HFD provoked up-regulation of SREBP-1c and FAS mRNA levels in adipose tissue. Also, it induced down-regulation of SIRT1, GLUT-4 mRNA levels in adipose tissue. We found that oral administration of either carnosine or carnosine-loaded niosome effectively reversed HFD-mediated alterations via SIRT1 activation. Overall, oral delivery of carnosine-loaded niosome had a better efficacy than oral carnosine, attenuating HFD-mediated alterations. Carnosine nano-formulation is a new excellent candidates for metabolic syndrome management and needs further exploration of its mechanisms.

Key words: carnosine; niosome; metabolic syndrome; fructose; SIRT1; SREBP-1c; GLUT-4

Introduction

Metabolic syndrome (MetS) is identified as a group of at least three of the five subsequent conditions: central obesity, hyperglycemia, hypertension, hypertriglyceridemia, and low serum high-density lipoprotein (1). MetS is mainly recognized by central adiposity, that is closely related to insulin resistance and facilitate further metabolic risk factors development (2). In the last years the MetS has acquired specific attention, since diabetes, obesity, and hypertension increase the risk for additional forms of COVID-19 and associated mortality (3). The co-morbidities control via efficient medications which are available for hyperglycemia, arterial hypertension, and dyslipidemia treatment is the most likely approach to hinder MetS-related outcomes (4, 5).

Carnosine (β-alanyl-L-histidine) has anti-glycation, anti-inflammatory, anti-oxidant, and chelating properties (6). carnosine has achieved an enormous attention as a possible therapy in metabolic disorders but the mechanism of the effect of carnosine on metabolic syndrome

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manifestations needs to be elucidated (7). The action of carnosine is limited in the body due to low lipophilicity and its fast hydrolysis via carnosinase. Thus, carnosine require modification to increase stability and improve bioavailability after its systemic administration (8) through incorporating it into nanostructured constructions. This is a promising strategy that may help to resist carnosinase activity and improve its target delivery (9, 10).Also, nanomaterials can reduce the amount of drug utilized and its related side effects in metabolic disorders treatment (11).

Niosome is closed vesicles which composed of non-ionic surfactants organized in concentric bilayers with cholesterol Incorporation. Niosomes are analogous to liposomes in their structure, but they provide tremendous advantages including being untoxic, having more chemical stability, and having simple and unexpensive production techniques (12). There are various studies has been displayed to improve the delivery system of some hypoglycemic agents with niosomal delivery system (13, 14). Recent studies showed that niosomes display protection from the proteolytic enzymes to the peptide and protein drugs (15). For oral delivery, niosomes of trimethyl chitosan-coated insulin are developed in order to increase insulin permeation (16). Thus this research in the field of niosomes will increase further and may lead to good market formulation in the pharma industry (15).

However, carnosine in a nano-formulation has not yet been examined for metabolic syndrome treatment. We proposed the carnosine-loaded niosomes as a potential solution to bypass its unwanted fast degradation by carnosinase which is considered as a major obstacle with the clinical application of carnosine as an oral drug therapy and to achieve high therapeutic outcome at lower dose than that previously reported with free carnosine. Toward this, the aim of our study is to evaluate the efficacy of oral free carnosine comparing to oral carnosine-loaded niosomes administration on the development of MS in a rat model provoked by high fructose diet. Also, the underlying mechanism of carnosine in the metabolic syndrome management remains mysterious. So, at the same time, there is a clear need to investigate the possible mechanism of carnosine action.

Materials and methods

Chemicals

Carnosine, Cholesterol, and Span 60 were purchased from Sigma Aldrich, USA. Fructose was purchased from uni-pharma, Egypt. All other chemicals utilized in our study were obtained from Sigma-Aldrich unless stated otherwise.

Laboratory animals

Sixty adult male Sprague Dawley rats weighing 168–170 g were procured from the animal house of the Faculty of Veterinary Medicine, Zagazig University, Egypt. They were housed for one week under standard laboratory conditions. They were kept at a temperature of 20-25 °C, relative humidity of about 60%, 12-hour light-dark cycle and free access to water and food. The study protocol got the approval of the Institutional Animal Care and Use Committee, Zagazig University (ZU-IACUC/2/F/45/2022).

Preparation and characterization of Carnosine-loaded niosomes

Carnosine-loaded niosome was prepared at Nanomaterials Research and Synthesis Unit, Animal Health Research Institute, ARC, Giza, Egypt. Niosome was prepared as described by Moulahoum *et al.,* 2019 and by using the thin film method. Span 60 (100 mg) and cholesterol (20 mg) were dissolved in 10 ml chloroform. Then, by using a rotary evaporator (Buchi R-3, Switzerland), the solvent was eliminated to form a thin lipid film (120 rpm, 60 °C, 1 h). The obtained thin film was hydrated with a carnosine solution to obtain carnosine-loaded niosome. In a 10 ml solution of phosphate buffer saline (PBS), Carnosine (50 mg/ml) was dissolved at pH 7.4, 60°C to obtain the final concentration. Then, with the aqueous solution, the lipid layer was dispersed and sonicated for 5 minutes in an ultrasonic bath (Sonics & Materials Inc. USA) to obtain the niosomal formulation (9). Using the Zetasizer device, the particle size and zeta potential of carnosine-loaded niosome were measured. Using transmission electron microscopy, Its surface morphology was visualized.

Induction of metabolic syndrome

The rats were given the high fructose diet for 10 weeks to induce metabolic syndrome. The high fructose diet provides 60% of the calories from fructose and prepared by mixing standard rodent diet with 60% wt/wt fructose (17). After the high-fat diet administration for 10 weeks**,** the success of fructose-induced metabolic syndrome was verified by monitoring body weight changes and significant elevation of fasting serum glucose, insulin and triglycerides. Rats were further used for the post-treatment period (4Weeks).

Animal groups and treatments

After one week from adaptation, rats were assigned in six groups (n = 10 each) for 14 weeks:

Group I: served as a control group and fed standard chow diet throughout the experimental period. Instead of supplements, 0.9% saline was orally administered for equivalent handling to other experimental groups.

Group II: served as the MetS group and fed on high-fructose diet and received saline solution similar to control throughout the experimental period for 14 weeks.

Group III: rats were fed high-fructose diet and received carnosine (50 mg/kg/day; p.o) dissolved in saline throughout the experimental period as a co-treatment.

Group IV: rats were fed high-fructose diet and received carnosine-loaded niosome (25 mg/kg/day; p.o) dissolved in saline throughout the experimental period as a co-treatment.

Group V: rats were fed high-fructose diet and received carnosine (50 mg/kg/day; p.o) dissolved in saline for 4 weeks starting from week 11 of the experimental period (after confirmation of metabolic syndrome) as a post-treatment.

Group VI: rats were fed high-fructose diet and received carnosine-loaded niosome (25 mg/kg/day; p.o) dissolved in saline for 4 weeks starting from week 11 of the experimental period (after confirmation of metabolic syndrome) as a post-treatment.

The oral dose of carnosine used in the current report was selected based on the effective dose of a previous study which revealed that carnosine had a hypoglycemic effect in diabetic rats (18). Body weight and body length were recorded at baseline and at the end of the study protocol to calculate the BMI:

Body mass index (BMI) = body weight (g)/ length^2 (cm^2)

Blood and tissue samples

Following an intervention period of 14 weeks, rats were euthanized by an intraperitoneal injection of sodium thiopental (60 mg/kg), and fasting (12 hr). For biochemical estimation, the blood samples were immediately collected through the heart puncture. At room temperature for 15 min, they were allowed to clot. Then, for serum separation, they were centrifuged for 15 min at 3,000 rpm. For gene expression analyses, adipose tissues were collected, immediately frozen in liquid nitrogen and stored at - 80^o C.

Biochemical assay

Biochemical estimation of serum glucose, insulin levels and HOMA-IR

Using the glucose oxidase method (Spinreact, Girona, Spain), serum glucose levels were measured. Using rat insulin ELISA kit (BioVendor Laboratory Medicine, Brno, Czech Republic), serum insulin levels were measured according to the manufacturer's protocol. The homeostasis model assessment for insulin resistance (HOMA-IR) index was calculated as follows: HOMAIR= fasting blood glucose (mg/dl) × fasting insulin (ng/ml)/405 (19).

Biochemical estimation of lipid profile markers and free fatty acids

Serum lipid profile including serum total cholesterol (TC), high density lipoprotein-cholesterol (HDL-C), low density lipoproteins-cholesterol (LDL-c) and triacylglycerol (TAG) were determined using commercially available kits (Spinreact, Spain). Serum levels of very low density lipoproteins-cholesterol (VLDL-C) were calculated by Friedewald equation (20) Serum concentrations of free fatty acids (FFA) were assessed by a rat ELISA kit (Cusabio Biotech Co., China) according to the manufacturer's protocol.

Quantitative real-time PCR

Using TRIzols Reagent (15596026, Life Technologies, USA), total RNA was extracted from freshly isolated adipose tissues following the manufacturer's information. Using nanodrop, RNA concentrations were measured, then using QuantiTects Reverse Transcription Kit (Qiagen,

Table 1: the primer sequences

Gene	Sequence	Accession number	References
SIRT1	Forward 5′TGG ACG AGC TGA CCC TTG A3′ Reverse 5′TCC TGC GGA TGT GGA GAT T3′	XM_017601788	(21)
SREBP-1c	Forward 5‘GCTCACAAAAGCAAATCACT3‘ Reverse 5‘GCGTTTCTACCACTTCAGG3‘	NM_001276707.1	(22)
FAS	Forward 5‘CTATTGTGGACGGAGGTATC3‘ Reverse 5‘TGCTGTAGCCCAGAAGAG3‘	NM_017332.1	(22)
GLUT-4	Forward 5‘TCATTCCTGTGAAAGTGATGACGA3‘ Reverse 5‘CTGCCACAGTGTCATATCATCCAA3‘	NM_012751.1	(23)
β-actin	Forward 5‘CCGTAA AGACCTCTATGCCAACA3‘ Reverse 5‘GCTAGGAGCCAGGGCAGTAATC3‘	NM_031144.3	(23)

USA), cDNA was synthetized based on the manufacturer's protocol. Rotor-Gene Q (Qiagen, USA) was used to determine sirtuin-1 (SIRT1), sterol regulatory element binding protein-1c (SREBP-1c), glucose transporter isoform 4 (GLUT-4) and fatty acid synthase (FAS) mRNA levels. Quantitative PCR was performed in duplicate on Maximas SYBR Green/Fluorescein qPCR Master Mix. The relative levels of SIRT1, SREBP1c, FAS and GLUT4 mRNA were calculated by the $2^{-\Delta\Delta ct}$ method, which was normalized to the β-actin housekeeping gene mRNA level. The primer sequences for SIRT1, SREBP-1c, FAS and GLUT-4 are listed in table 1.

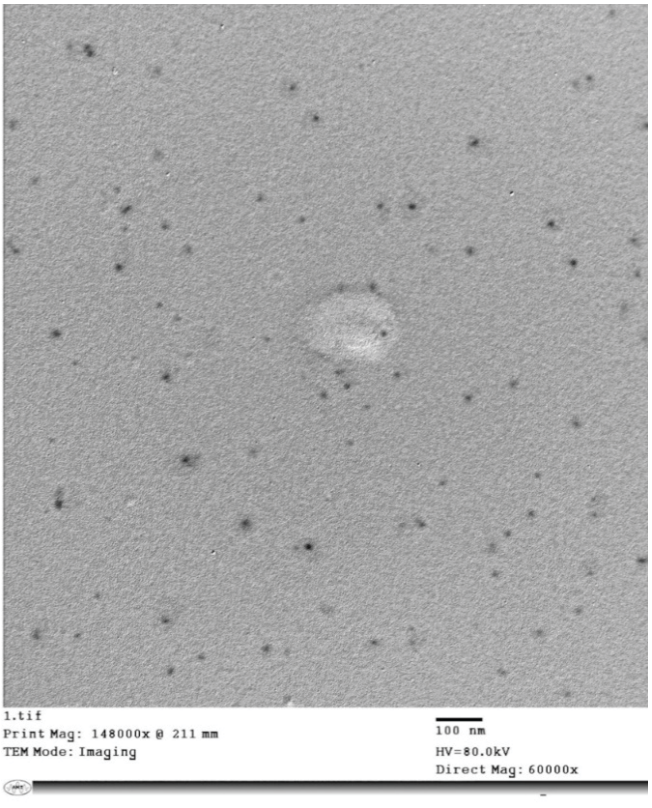


Figure 1: TEM micrograph of carnosine-loaded niosome

Statistical analysis

Data were presented as mean ± SEM and analyzed by one-way ANOVA using IBM SPSS software.

Results

Preparation and characterization of carnosine-loaded niosome

The prepared carnosine-loaded niosome showed 168 nm particle size. The zeta potential of the niosome was -2.8 mV, which indicates that the niosomes are stable against fusion due to increasing of repulsive forces between the particles (24). The morphology of carnosine-loaded niosome is shown in Figure 1.

Effect of oral carnosine, and carnosine-loaded niosome on body weight and body mass index (Anthropometric indices) in experimentally induced metabolic syndrome in rats

As shown in table 2, The baseline weight and body mass index of rats in different groups was not significantly different. It was observed that the final body weight and body mass index of all groups increased as compared to the initial measurements. The results showed that final body weight and body mass index in HFD-fed rats increased significantly when compared with the control group. The body weight and body mass index of HFD-fed rats treated with either oral carnosine or oral carnosine-loaded niosome in both co-treatment (14 weeks) and post-treatment

conditions (4 weeks) showed statistically significant decrease compared to HFD-fed rats. This decrease was significantly ameliorated especially in the co-treatment conditions. Moreover, HFD-fed rats treated with oral carnosine-loaded niosome showed the greatest effect in both co-treatment and post-treatment conditions.

Effect of oral carnosine, and carnosine-loaded niosome on serum glucose and insulin levels and the HOMA-IR index in experimentally induced metabolic syndrome in rats.

On rats fed with HFD, there was a significant increase in serum glucose and insulin levels in addition to the HOMA-IR values as compared to the normal control group, as shown in table 3. However, a significant decline in these parameters was detected in HFD-fed rats treated with either oral carnosine or oral carnosine-loaded niosome in both co-treatment (14 weeks) and post-treatment conditions (4 weeks) in comparing to HFD group. This reduction was more pronounced with the co-treatment conditions than the post-treatment conditions. Moreover, HFD-fed rats treated with oral carnosine-loaded niosome showed the greatest effect in both co-treatment and post-treatment conditions.

Effect of oral carnosine, and carnosine-loaded niosome on Lipid profile markers in experimentally induced metabolic syndrome in rats.

Serum total cholesterol (TC), triacylglycerol (TAG), low-density lipoproteins (LDL), very-low density lipoproteins (VLDL) and free fatty acids (FFA) displayed markedly raised levels, unlike HDL-cholesterol showing a dropped level, in rats fed with HFD relative to the control group, as shown in table 3. These effects were reversed by administration of either oral carnosine or oral carnosine-loaded niosome in HFD-fed rats in both co-treatment (14 weeks) and post-treatment conditions (4 weeks) and were substantially improved especially in the co-treatment conditions. Moreover, HFD-fed rats treated with oral carnosine-loaded niosome showed the greatest effect in both co-treatment and post-treatment conditions.

Effect of oral carnosine, and carnosine-loaded niosome on mRNA levels of SIRT1, SREBP-1c, FAS and GLUT-4 in adipose tissues of experimentally induced metabolic syndrome in rats.

As shown in figure 2, RT-PCR showed that HFD-fed rats exhibited a significant up-regulating

Table 2: Effect of Oral Carnosine, and Oral Carnosine-Loaded Niosome on Body Weight and Body Mass Index in HFD-fed rats

		I	II	III	IV	V	VI
B.W(g)	**Day 0**	168.85±2.63^{a}	169.43± 2.65^{a}	168.28±2.60^{a}	168.71±2.91^{a}	170.57±3.41^{a}	168.57±2.68^{a}
	14 weeks	206.43±5.98^{d}	311.00±6.23^{a}	281.86±5.07^{b}	258.42±5.17^{c}	309.43±4.95^{a}	302.71±4.74^{a}
BMI	**Day 0**	0.35±0.005^{a}	0.36±0.005^{a}	0.35±0.006^{a}	0.35±0.006^{a}	0.36±0.007^{a}	0.35±0.005^{a}
	14 weeks	0.44±0.01^{d}	0.66±0.01^{a}	0.59±0.01^{b}	0.54±0.01^{c}	0.65±0.01^{a}	0.64±0.01^{a}

Means within the same row having different superscript letters are significantly different. Significant at 0.05 probability.

Table 3: Effect of Oral Carnosine, and Oral Carnosine-Loaded Niosome on Biochemical Parameters in HFD-fed rats

	I	II	III	IV	V	VI
GLU (mg/dl)	99.42±0.75^{f}	183.71±0.84^{a}	134.14±0.59^{d}	122.71±0.78^{e}	164.85±0.74^{b}	153.28±0.92^{c}
INS (ng/ml)	1.85±0.11^{f}	4.35±0.08^{a}	2.85±0.04^{d}	2.57±0.03^{e}	3.72±0.04^{b}	3.35±0.04^{c}
HOMA-IR index	0.45±0.03^{f}	1.97±0.05^{a}	0.94±0.02^{d}	0.78±0.01^{e}	1.52±0.02^{b}	1.27±0.02^{c}
TC (mg/dl)	85.67±1.35^{f}	107.68±0.68^{a}	94.45±0.36^{d}	91.49±0.34^{e}	100.77±0.50^{b}	96.55±0.30^{c}
TAG (mg/dl)	114.01±3.85^{f}	210.70±2.80^{a}	156.94±0.82^{d}	145.58±1.62^{e}	186.26±1.40^{b}	173.63±0.69^{c}
HDL-C (mg/dl)	57.92±0.72^{a}	44.43±0.24^{f}	51.19±0.35^{c}	54.68±0.40^{b}	46.95±0.26^{e}	48.97±0.20^{d}
LDL-C (mg/dl)	4.95±0.30^{f}	21.10±0.25^{a}	11.87±0.19^{d}	7.69±0.52^{e}	16.56±0.14^{b}	12.85±0.19^{c}
VLDL-C (mg/dl)	22.80±0.77^{f}	42.14±0.56^{a}	31.39±0.16^{d}	29.11±0.32^{e}	37.25±0.28^{b}	34.72±0.14^{c}
FFA (ng/ml)	72.85±0.80^{f}	102.71±0.60^{a}	87.28±0.42^{d}	82.71±0.78^{e}	96.14±0.46^{b}	91.28±0.42^{c}

Means within the same row having different superscript letters are significantly different. Significant at 0.05 probability.

mRNA levels of SREBP-1c and FAS and a marked decrease in mRNA levels of SIRT1 and GLUT-4 compared to normal rats. In contrast, mRNA levels of all the aftermentioned genes were reversed in HFD-fed rats which received either oral carnosine or carnosine-loaded niosome in both co-treatment (14 weeks) and post-treatment conditions (4 weeks). These effects were significantly improved especially in the co-treatment conditions. Moreover, HFD-fed rats treated with oral carnosine-loaded niosome showed the greatest effect in both co-treatment and post-treatment conditions.

Discussion

Obesity is one of the prime components of metabolic syndrome (25). Body weight and body mass index are important anthropometric indices for assessing obesity (26). The oral administration of carnosine-loaded niosome was more effective than oral administration of carnosine in reducing obesity in HFD-fed rats as observed with decreased body weight and body mass index. The current results are in agreement with previous studies which revealed that carnosine administration reduced body weight (7, 27).

Dyslipidaemia was observed with reduced HDL-C, as well as increased VLDL-C, TAG, and LDL-C levels in HFD-fed rats. The changes in lipid metabolism are associated with free fatty acids flux secondary to insulin resistance (28). The oral administration of carnosine-loaded niosome was more effective than oral administration of carnosine in regulation of lipid metabolism. Various studies with carnosine proved altered lipid metabolism (27, 29). There are conflicting

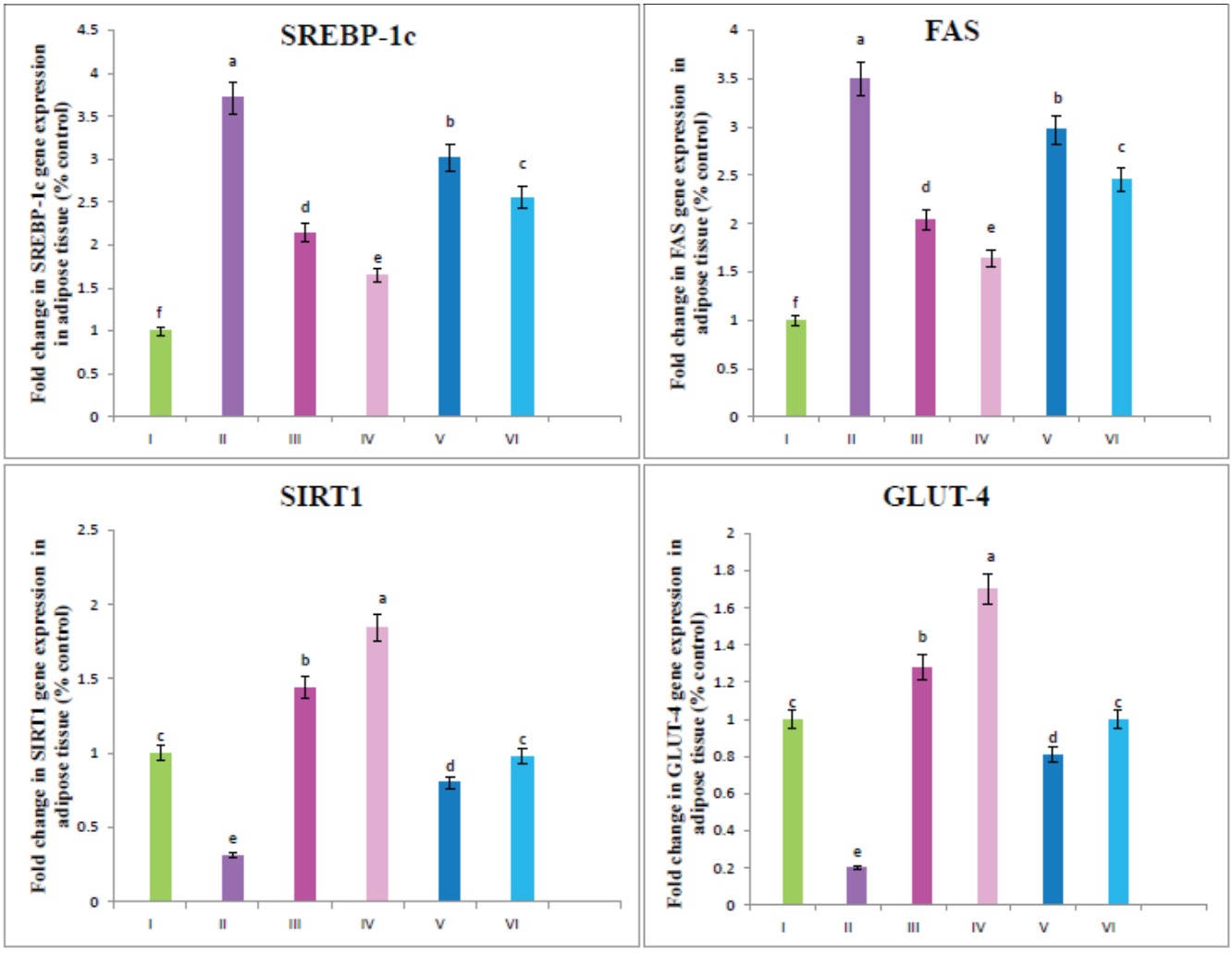


Figure 2: The effect of oral carnosine, and oral carnosine-loaded niosome on the mRNA levels of SIRT1, SREBP-1c, FAS and GLUT-4 in the adipose tissue of HFD-Induced Metabolic Syndrome in Rats (data are presented as mean ± SEM)

studies regarding the effect of carnosine on HDL (29, 30).

To further elucidate the mechanism of the anti-obesity and hypolipidemic effects of either oral carnosine or oral carnosine-loaded niosome in HFD-fed rats, we conducted a real-time PCR analysis in which we compared gene expression profiles of SREBP-1c and FAS of adipose tissue in different treatment groups. SREBP-1c, a lipogenic transcription factor, regulates lipid metabolism in cells (31, 32, 33). In the fatty acid biosynthesis pathway, FAS is the rate-limiting enzyme and its mRNA expression is under control of SREBP-1c (32). In normal tissues and cells, SREBP-1c and its regulated enzymes always have low expression and activity. But in tissues with abnormal lipid metabolism, they are stimulated (34). Our study showed that either oral carnosine or oral carnosine-loaded niosome administration, in particular carnosine-loaded niosome, downregulated the expression of SREBP-1c and FAS, indicating that they could significantly suppress lipogenesis, resulting in their anti-obesity and hypolipidemic effects in HFD-induced metabolic syndrome in rats. A previous study using carnosine also reported Similar observations (35).

Insulin resistance is a key component of MetS. The body fails to response to insulin, resulting in raising the blood glucose levels (36, 37). The oral administration of carnosine-loaded niosome was more effective than oral administration of carnosine in reducing hyperglycemia, hyperinsulinemia and insulin resistance.The hypoglycemic effect of carnosine is in agreement with previous studies (7, 30, 38, 39). In contrast, carnosine administration didn't affect the plasma glucose level (27). The reduction of insulin level and HOMA-IR by carnosine was consistent with previous studies (27, 35). There are conflicting studies regarding the effect of carnosine on insulin. A previous report did not display any significant effect of carnosine on insulin levels (7). Other studies are in contrast with our finding, it confirmed that carnosine did not alter the insulin resistance (40) and also increased plasma insulin levels (39, 41).

To determine the molecular mechanism of hypoglycemic and hypoinsulinemic effect of either oral carnosine or oral carnosine-loaded niosome in HFD-fed rats, we conducted a real-time PCR analysis for gene expression profiles of GLUT-4 of adipose tissue in different treatment groups. GLUT4, an insulin-regulated glucose transporter, is found mainly in adipose, skeletal and cardiac tissues. Insulin stimulates the GLUT4 translocation to the plasma membrane leading to a rapid elevation in the glucose uptake and therefore the decline of blood glucose level (42,43). Our results showed that either oral carnosine or oral carnosine-loaded niosome treatment, in particular carnosine-loaded niosome, significantly increased the expression of GLUT4 indicating that they are able to induce a plasma glucose-lowering effect. Therefore, they enhanced insulin sensitivity in HFD-induced metabolic syndrome in rats. A previous study is in line with our finding, it confirmed that 10 mM carnosine supplementation to glucolipotoxic-media resulted in a significant GLUT4 translocation improvement (44).

SIRT1, a NAD^+- dependent deacetylase, modulates gene expression via deacetylation of histones (45, 46). Various studies of Sirt1 agonists have showed contrasted results in the metabolic syndrome management (47, 48, 49). In high glucose-treated adipocytes, SIRT-1 enhances glucose homeostasis via regulating GLUT-4 proteins (50). SIRT1 depletion in 3T3-L1 adipocytes suppressed GLUT4 translocation and glucose uptake. But, SIRT1 activators administration to the cells led to stimulation of glucose uptake (51). In contrast, the Sirt1 overexpression does not improve IR in skeletal muscle in vivo (52). SREBP-1c is believed to be a direct target of SIRT1 (53, 54, 55). SIRT1 with AMP-activated protein kinase (AMPK) regulated SREBP-1c and FAS expression levels to alleviate NAFLD (56, 57). These data highlight the Sirt1 inhibition as a main component of the molecular pathways that produce the metabolic syndrome outcomes. Remarkably, these favorable effects of either oral carnosine or carnosine-loaded niosome are leading to relieve the metabolic disorders. Resveratrol, a direct SIRT1 activator, and carnosine appear to share many characteristics (anti-oxidants, anti-carcinogenic, anti-inflammatory and platelet anti-aggregation activity) (58). Therefore one has to consider, accepted very speculatively, the potentiality that carnosine may also stimulate SIRT1 in a method analogous to that suggested for resveratrol (59); at the minimum the two molecules are almost the same size (58). There are few studies in the literature examining whether carnosine may alter SIRT1 expression or not. The

pretreatment with carnosine in both ultraviolet -A- and 4-hydroxynonenal-treated fibroblasts restored the SIRT1 expression and prevented the acetylated proteins accumulation (60). In high glucose-induced podocytes, Carnosine upregulated the SIRT1 expression to reduce the glycative and the lipoperoxidative stress (61).

Another prime finding of our report was that encapsulation of carnosine in niosome form is predicted to enhance carnosine's bioavailability, therefore positively impact its therapeutic action. Our study gives for the first time new evidence that the developed carnosine-loaded niosome displayed superior *in-vivo* effectiveness and facilitated the way for a predicted positive effect of niosomes on enhancing carnosine therapeutic efficacy, which could benefit pharmacological studies in the future.

Finally, Carnosine has been characterized as an enigmatic and a forgotten dipeptide (58, 62) and perhaps the mystery surrounding carnosine's "true" action is just a reflection of the truth that there hasn't been a lot of experimental work in some of the areas that have been discussed; much more research is needed to discover whether any of these hypotheses are justified (58).

Conclusion

Our results emphasize the potent efficacy of oral carnosine- loaded niosome in management of metabolic syndrome. Also, this study demonstrates a novel mechanistic insights by which oral carnosine, and carnosine loaded niosome can ameliorate hyperglycaemia, hyperinsulinaemia and insulin resistance via upregulation of GLUT-4 gene expression and also can protect against obesity and dyslipidemia via downregulation of SREBP-1c and FAS gene expression in adipose tissue of HFD-induced metabolic syndrome rats. Oral carnosine and oral carnosine- loaded niosome displayed a multimodal activity through activation of SIRT1, a master metabolic switch. Conclusively, oral carnosine- loaded niosome achieved the maximum therapeutic outcome with no changing in activity. Therefore, Carnosine-loaded niosomes could be a valuable and optimistic treatment choice for metabolic syndrome. Nevertheless, this remains to be further confirmed by clinical studies.

The authors have declared no conflict of interests.

Abbreviations

HFD, high fructose diet; MetS, metabolic syndrome; TC, total cholesterol; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoproteins-cholesterol; TAG, triacylglycerol; VLDL-C, very low density lipoproteins-cholesterol; FFA , free fatty acids; SIRT1, sirtuin-1; SREBP-1c, sterol regulatory element binding protein-1c; GLUT-4, glucose transporter isoform 4; FAS, fatty acid synthase; BMI, body mass index; HOMA-IR, homeostasis model assessment for insulin resistance; AMPK, AMP-activated protein kinase; NAFLD, Non-alcoholic fatty liver disease.

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Original Research Article
Veterinary Medicine and The One Health Concept

RISK FACTORS FOR GASTROINTESTINAL FOREIGN BODIES IN CATS: A RETROSPECTIVE STUDY IN EGYPT

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Abstract: Gastrointestinal foreign body (FB) obstruction in felines is one of the emergencies and commonly required surgical interventions. The purpose of this study was therefore to investigate the risk factors that could prone cats to ingest FB. This study was retrospectively conducted on sixty-five cats that had been diagnosed with FB obstruction. On the other hand, sixty-five cats that had no clinical findings suggestive of gastrointestinal illnesses were used as controls. Among cats with gastrointestinal illnesses, we found that 9.97% had FB obstructions. The most common presenting breeds of both cat patients with FB obstruction and controls were Persian and Siamese. Similarly, most of the presented normal and diseased cats were males. Cats with FB were younger ($p<0.05$) than those of control cats. There was no significant difference in the body weight for cats with FB versus control cats. Among the investigated factors, we also observed that living style, anxiety/stress, and frequency of exercise were the only factors that were strongly associated with the occurrence of FB obstruction. In conclusion, observations of this study indicate that a living style, anxiety/stress, and frequency of exercise have a significant impact on cat behavior and should be seen by cat-owned clients as important to prevent pica. Thus, the likelihood of blockage of the gastrointestinal tract by foreign bodies can be reduced.

Key words: foreign bodies; gastrointestinal tract; risk factors; obstruction; pica; cats

Introduction

Feline gastrointestinal foreign bodies (FB) obstruction is one of the emergencies that always needs critical care and commonly surgical intervention (1). Pica is known in cats as the ingestion of non-edible substances such as fabric, plastic, rubber, soil, and threads. In general, the prevalence of feline and canine behavioral problems (e.g., pica) varies according to breed, age, sex, age, lifestyle, and geographic region. These behavioral problems may consequently produce other related disorders; thus, it is valuable for veterinarians in advising owners about counteracting behavioral problems (2-6).

The possible underlying factors in cats exhibiting pica include boredom, lack of social connectivity, genetic predisposition, early weaning, fasting, or anxiety disorders (7). In cats, it was proved in a case-control study that cats showing pica were less common in cats fed ad libitum. As has often been mentioned, pica and vomiting have been correlated, but the causative relationship is not well elucidated (8). In addition, this study also reported that most of the cats introduced with pica were shorthaired or longhaired domestic cats (8).

The recognition of these factors might help to reduce exposure and increase the protection against FB obstruction. Since in our animal hospital we

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receive a considerable number of felines suffering FB obstruction, we became concerned to address the predisposing factors that may be associated with the incidence of FB obstruction in feline patients admitted to our veterinary hospital.

Therefore, the main purpose of this study was to figure out the predisposing factors that are likely associated with gastrointestinal tract FB obstruction in cats.

Materials and Methods

Animals and data collection

The current work was performed following the rules of the ethical committee and animal welfare of the Faculty of Veterinary Medicine, Zagazig University, Egypt. The animal study protocol was approved by the animal Ethics Committee of Zagazig University (ZU-IACUC/2/F/29/2022).

From the medical case records, the feline cases admitted to the Animal Clinic at Surgery, Anesthesiology and Radiology Department, Zagazig University, Zagazig, Sharkia, Egypt, from February 2019 to November 2021 were reviewed. From these records, cat patients diagnosed with GIT illness with radiologically and ultrasonographically confirmed FB obstruction were selected. The inclusion information of each cat included sex, age, breed, and body weight. The diagnosis of cats with FB obstruction was initially based on the case history, clinical investigation, blood analysis data, radiographic and ultrasonographic examination as described previously (17). Feline cases (n = 65) admitted to the clinic during the study period for reasons other than gastrointestinal disorders were employed as controls. The data concerning the proposed predisposing factors that could prone cats to ingest FB were collected from feline owners, and statistically analyzed.

Statistical analysis

The association of categorical variables and the presence of foreign bodies was determined by the Pearson chi-square test or Fisher's exact test as needed. To estimate the contribution of each risk factor toward foreign body formation, Odds ratios (ORs) with their 95% CIs were first determined in a univariate logistic regression model. Then a multivariate logistic regression model was constructed after a backward stepwise elimination procedure against foreign body formation in the cat. Factors associated with foreign body formation with a *P-value* of 0.05 were included in the multivariate logistic regression analysis. This analysis was done using the Generalized linear model (GLM) Function in R software (version 4.1.1) (9). Student-t test (unpaired two-tailed) was used to test the differences in blood parameters in the presence and absence of a foreign body.

Results

Figure 1 shows representative images for FBs retrieved from gastrointestinal tract in cats. Of a total of 1921 cats admitted during the studied period, we found that 652 cats (33.94%) had gastrointestinal tract illness. Among these 625 cats, sixty-five (9.97%) with FBs obstruction were identified and analyzed against the normal cats (n=65).

As shown in Table 1, the most common presenting breeds of cat patients with FBs obstruction were Persian (30/65) and Siamese (21/65). Similarly, for controls, the most common presenting cats were Persian (25/65) and Siamese (28/65). Most of the presented cats were males (50/65 for cat patients and 39/65 for controls). The mean ± SD age for Cats with FB (2.7 ± 0.86 years) was younger ($p<0.05$) than those of control cats (4.35 ± 1.25 years). There was no significant difference in body weight for cats with FB (3.53 ± 1.03 kg) versus control cats (3.93 ± 0.89 kg) (Figure 2).

Table 1: Distribution of breeds and gender of cats diagnosed with gastrointestinal FB obstruction (n=65) and control cats (n=65)

Breeds	Cats with FB	Control Cats
	No. (%)	No. (%)
Persian	30 (46.15)	25 (38.46)
Siamese	21 (32.31)	28 (43.08)
Domestic shorthair	10 (15.38)	7 (10.77)
Egyptian Mau (native breed)	2 (3.08)	1 (1.54)
Crossbreed	2 (3.08)	4 (6.15)
Male	50 (76.92)	39 (60.00)
Female	15 (23.08)	26 (40.00)

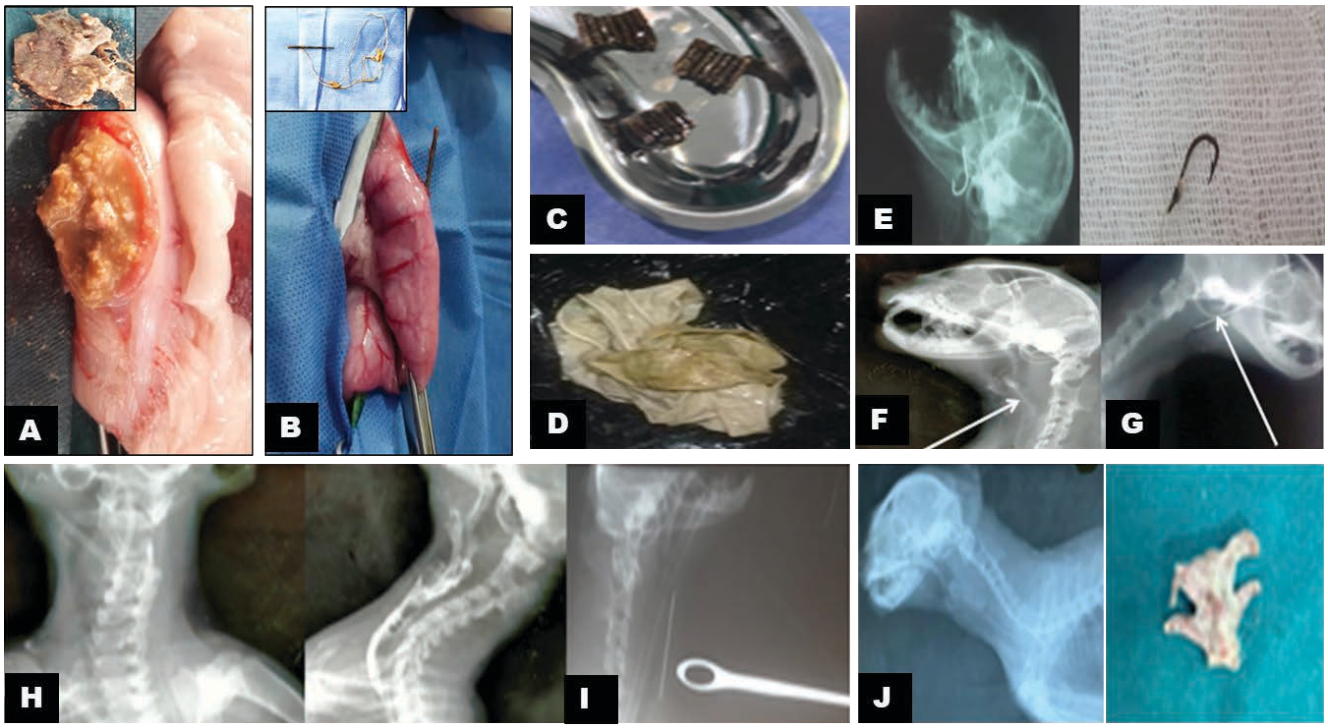


Figure 1: Representative images showing gastrointestinal foreign bodies in cats. and gastric hairball (A), an intestinal needle with string (B), leather parts retrieved from the stomach (C), rubber gloves removed from the intestine (D), a fishing hook in the mouth of a cat impeded in the tongue (E), chicken clavicle bone in the esophagus and application of opaque contrast material for testing perforation of the esophagus (H), needle migrating out of the esophagus into surrounding muscles (I), and chicken cervical bones lodged in the esophagus and one of the retrieved bones (J)

Table 2 shows the proposed risk factors that were investigated in this study for cats diagnosed with FB obstruction and control cats. The results extracted from univariate binary logistic regression analysis and Chi-2 analyses indicated that the Egyptian Mau breed was less likely to have FB obstruction compared to crossbred cats (P-value = 0.04). However, other breeds did not differ significantly from crossbreeds in this regard (Persian vs. crossbreed: P-value = 0.4; Shorthair vs. crossbreed: P-value = 0.3; Siamese vs. crossbreed: P-value = 1).

There was no significant difference between male and female cats for experiencing FB obstruction (P-value = 0.1). Cats that live only outdoors, or only indoors had a significantly higher risk (P-value < 0.0001, each) of FB obstruction than those with mixed living styles. While parent's/siblings' detachment did not significantly differ from weaning on time + relatives (P-value = 0.1), early weaning significantly differed from weaning on time + relatives in cases associated with FB obstruction (P-value < 0.0001). The data also indicated that exposing cats to once or twice exercise a week differed significantly from performing 5-times exercise per week (P-value = 0.006 and 0.009, respectively), but doing exercise 3- and 4-times did not show any significant difference from exercising 5-times a week (P-value > 0.05). Neither

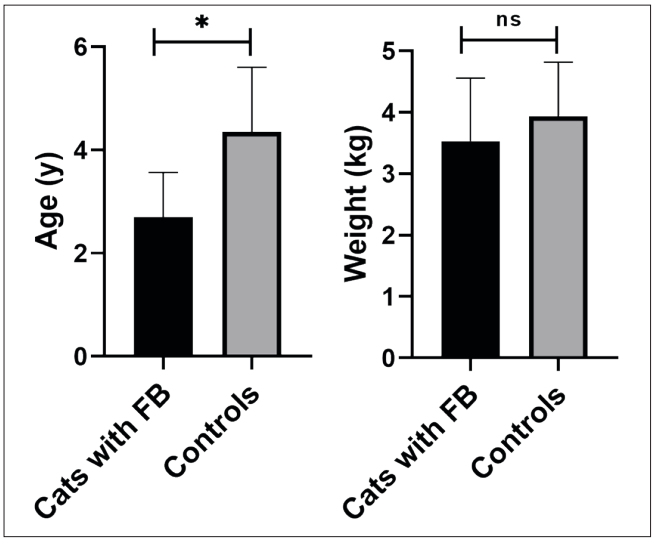


Figure 2: Distribution of age and body weight of the cats with gastrointestinal FB obstruction (n=65) and the control cats (n=65). *p<0.05; ns: non-significant

the studied cat diets nor household felines number had a significant impact on the occurrence of FB obstruction (P-value > 0.05). The univariate binary logistic regression models suggested that some of the factors (namely living, anxiety/stress, and frequency of exercise per week) were strongly associated with a diagnosis of FB obstruction in studied felines, whereas other factors (Breed, sex, kind of food and number of felines in the house) were not associated with the occurrence of FB obstruction in cats.

The multivariate model applied herein was better than the null model (*P*-value = 2.2) with no predictors and confirmed the results from the univariate model. It showed that cats that appeared within parents/siblings' detachment or early weaning category were more likely to have FB obstruction diagnosis than cats that appeared within weaning on time + relatives' category (odds ratio = 1.3 and 0.02, *P*-value = 0.00 and 0.6, respectively). Compared to 5-times exercise a week, fewer time exercises had a higher probability for cats to ingest FB and concurrently led to gastrointestinal obstruction (odds ratio ranges from 0.01-0.1). The living patterns showed that cats dwelling fully indoors or fully outdoors were more likely for having FB obstruction than cats with a mixed system of living (odds ratio = 0.00 and 0.16, *P*-values = 0.00 and 0.01, respectively).

Discussion

It is well known that FB obstruction in felines is a progressive life-threatening condition. For this reason, the awareness of feline owners of the factors that lead to the ingestion of foreign objects is significantly associated with early diagnosis and a shorter duration of the signs, accordingly, a higher survival rate of the animal can be obtained. It has been demonstrated that the treatment success rate declined with a longer duration of experiencing clinical signs in pets diagnosed with FB obstruction (10).

Persian and Siamese cats were the most likely breeds to be affected with FB obstruction, whereas it has been reported that Domestic shorthair cats were the most affected than other

Table 2: Distribution of the proposed risk factors investigated in this study to cats with gastrointestinal FB obstruction (n=65) and control cats (n=65)

Factors	Criteria	Number (%)	
		Cats with FB (n = 65)	Controls (n = 65)
Anxiety/stress	Weaning on time + relatives	25 (38.46)	50 (76.92)
	Parents'/siblings' detachment	12 (18.46)	12 (18.46)
	Early weaning	28 (43.08)	3 (4.62)
Frequency of exercise (by times) per week	5	7 (10.77)	16 (24.62)
	4	9 (13.85)	21 (32.31)
	3	7 (10.77)	9 (13.85)
	2	12 (18.46)	12 (18.46)
	1	13 (20)	4 (6.15)
	0	17 (26.15)	3 (4.62)
Living style	Mixed	8 (12.3)	48 (73.8)
	Full outdoor	26 (40)	14 (21.5)
	Full indoor	1 (47.69)	3 (4.6)
Kind of food	Mixed food	9 (13.85)	12 (18.46)
	Canned food	10 (15.38)	13 (20)
	Dry food	42 (64.62)	31 (47.69)
	Homemade food	4 (6.15)	9 (13.85)
Number of household felines	1 feline	23 (35.38)	22 (33.85)
	2 felines	10 (15.38)	14 (21.54)
	3 felines	9 (13.85)	8 (12.31)
	4 felines	23 (35.38)	21 (32.31)

inspected breeds (Siamese) (10), and this could be attributed to country-to-country variation. As well, some longhair cat breeds such as Persian and Siamese are often predisposed to grooming themselves daily and may reach to the point of over-grooming, chewing and swallowing it forming trichobezoars (hairballs) (11), and endure a pica "ingestion of non-edible items". Also, we found that the Egyptian Mau breed (native breed) was less likely for having been diagnosed with FB obstruction. As reported, the Egyptian Mau as our native breed is known as an intelligent and observant breed, thus are known to have less tendency to chew or eat a foreign object.

It has been demonstrated that the young ages (3.5 -3.7 years in dogs and 1.6-4 years in cats) have a higher chance of swallowing a large FB (10, 12-14). Inconsistent with our study, the ages of the cat patients with FB (2.7 ± 0.86 years) were younger than those of control cats (4.35 ± 1.25 years). Regarding the sex of the cats, in this report, we found that the occurrence of FB was commonly found in male cats, and this observation coincided with other investigations that reported male dogs swallowed FB more than females (12, 13).

The univariate binary logistic regression models suggested that some of the factors (namely living, anxiety/stress, and frequency of exercise per week) were strongly associated with a diagnosis of FB obstruction in studied felines, whereas other factors (Breed, sex, kind of food and number of felines in the house) were not associated with the occurrence of FB obstruction in cats.

This all could be associated with feline stresses-triggering odd behaviors involving eating foreign objects and consequently, may lead to gastrointestinal obstruction (3, 4, 6, 15). Additionally, environmental enrichment such as offering a bed with vinyl covering led to gastric FB obstruction in a 7-years-old Beagle crossbred dog (16).

Conclusion

The univariate binary logistic regression models suggested that some of the factors (namely living, anxiety/stress, and frequency of exercise per week) were strongly associated with a diagnosis of FB obstruction in the studied felines, whereas other factors (breed, sex, kind of food and number of felines in the house) were not associated with the occurrence of FB obstruction in cats. The addressed predisposing factors should be carefully considered by feline-owned clients to reduce the possibility of foreign bodies-mediated bowel obstruction.

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Original Research Article
Veterinary Medicine and The One Health Concept

PROPOLIS LOADED POLYVINYL ALCOHOL ATTENUATES CCL4 INDUCED HEPATIC FIBROSIS VIA MODULATION OF LET-7B/TGF-B/SMAD SIGNALING PATHWAY

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Abstract: Chronic liver disorders are a serious global health issue due to their widespread incidence. Nephropathy described the deterioration of kidney function. Safe drug delivery by nanoparticles is a rapidly developing field with promising applications in the treatment of a wide variety of diseases. The current study aimed to evaluate the use of propolis nanoparticles for managing carbon tetrachloride (CCl4)-induced hepato-nephropathy on rats. Seventy adult males Spargue Dawley rats were allocated into 7 equal groups 10 rats of each. Control, CCl4, CCl4 +Silymarin, CCl4 + propolis, CCl4+Nano-propolis, CCl4 +Silymarin +Propolis, CCl4 +Silymarin +Nanopropolis. Hepato- nephropathy was induced with oral administration of CCl4 dissolved in olive oil at dose of (1gm/kg) for 4 weeks. Silymarin, propolis and nanopropolis were orally administrated at a dose of (200mg /kg), (100 mg/kg) and (30 mg /kg) respectively for 4 weeks post hepato-nephropathy onset. Biochemical, molecular analysis, histological assessment of liver and kidney and serum oxidative stress were done. CCl4 caused a marked deterioration in biochemical, oxidative stress markers (MDA, TAC, CAT), serum TNF-α, IgM, molecular markers (SMAD-2, SMAD-3, SMAD-7, MMP-9, Desmin, TGF-β1, and let-7b), and the histopathological pictures of both liver and kidney. The above-mentioned parameters were restored with administration of silymarim + Nano-propolis, silymarin + propolis, silymarin, Nano-propolis, and propolis in order. Based on the previous findings we could speculated that combined therapy of nano-propolis and silymarin could be implicated in managing hepato-nepheropathy since it improves both liver and kideny function by targeting let-7b/TGF-β/Smad Pathway.

Key words: gene expression; hepatic fibrosis; nanoparticles; nephropathy; propolis; CCl4

Introduction

Hepatic fibrosis is the body's wound-healing response to liver injury from many causes. Cirrhosis is the most progressive form of fibrosis, meaning not just fibrosis but also liver parenchyma distortion, formation of septae and nodules, altered blood flow, and the likelihood of liver failure (1). Many medicines with strong antifibrotic properties *in vitro* have only moderate effects *in vivo* because insufficient quantities accumulate surrounding the target cell, causing detrimental effects in non-target cells. Hepatic stellate cells are the target cells of antifibrotic therapy because they play a key role in hepatic fibrogenesis (2).

The decline in renal function was termed nephropathy. End-stage renal disease, often known as ESRD, is the terminal stage of nephropathy. Reduced glomerular filtration rate, elevated albuminuria with time, high arterial blood pressure, and fluid restriction are the results of three types of nephropathies: nodular glomerulosclerosis, thickened glomerular basement membranes, and mesangial expansion. The two forms of nephropathies are diabetic nephropathy (DN) and hypertensive nephropathy. Hypertensive nephropathy is a kidney disease caused by a history of high blood pressure. It is

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a chronic disorder that poses a significant risk of developing ESRD (3).

Nanotechnology is the study and application of the unique chemical and physical properties of objects smaller than 100 nm in size, including enhanced chemical and physical reactivity and solubility. Because of its rapid and accurate motions, as well as its superior bioavailability and biodegradability, it offers a scientific advantage. The output of livestock animals, economic losses, and the creation of nutritious food and feed are all greatly impacted by all of these positives (4).

The chemical molecule CCl4 is a colorless and volatile liquid. Hepatotoxicity and nephrotoxicity are side effects. Hepatic fibrosis and nephropathy are two of the world's leading causes of death (5).

Honeybees create propolis, a natural resinous mixture made up of components collected from plant parts, buds, and exudates. Propolis is currently used as an antibacterial, anti-inflammatory, antiviral, anti-oxidant, anti-protozoal, anesthetic, anti-tumoral, anti-hepatotoxic, etc (6).

Propolis samples from all over the world were analysed, and researchers found that the major component varied between tropical and temperate regions. Despite the widespread availability of propolis in the Americas, the vast majority of research has focused on Brazilian green propolis. Liquidritigenin, formononetin, and biochanin A are the main components of red propolis, and they are extracted mostly from Dalbergia ecastophyllum. Flavonoids, phenolic acids, and their derivatives like caffeic acid phenethyl ester (CAPE), pinocembrin, and naringenin make up the bulk of Mexican poplar propolis. Acids like abietic acid, isopimaric acid, cycloartenol, and isocupressic acid can be found in the Mediterranean propolis that is harvested in Turkey, Egypt, and Greece (7).

The phenolic acids and polyphenols like flavonoids, are responsible for propolis biological actions. These substances have a low water solubility and bioavailability. To circumvent the physiological limits of propolis nanocarriers were mostly utilized as drug delivery methods. Furthermore, propolis in nanoform has been found to be more effective than propolis in terms of antibacterial and antifungal (8).

Milk thistle seeds, or silymarin, have been used to treat liver problems for undreds of years. The protective effects of silymarin on healthy liver cells or cells that have not yet been irrevocably damaged have been suggested by preclinical research (9).

This work aimed to evaluate the anti-inflammatory and apoptotic effects of propolis nanoparticles to that of the medication silymarin in a rat model of carbon tetrachloride (CCl4)-induced hepatic fibrosis and nephropathy. Biochemical and molecular analyses of blood and tissue samples were performed to evaluate hepatotoxicity, nephrotoxicity, and the results of treatment.

Material and methods

Materials and reagents

The propolis was purchased from Sigma Aldrich. Propolis nanoparticles, were prepared at NanoTech Egypt for photo-electronics (Dreamland,El-Wahaat Road, 6th October, Giza, Egypt, Email; sales@nanotecheg.com). Silymarin was purchased as powder from SEDICO-Egypt. All other chemicals used in this experiment were purchased from sigma Aldrich (St. Louis, MO).

Preparation of propolis nanoparticles

The emulsion diffusion technique was used to create propolis nanoparticles. Both the polyvinyl alcohol (PVA) (Fisher Scientific, UK) and the propolis solutions were dissolved in 10 ml of distilled water and then swirled for 30 minutes on a magnetic stirrer. The propolis solution was then dropped per drop onto aqueous PVA, stirred for 30 minutes, then centrifuged for 30 minutes at 2000 rpm. A transmission electron microscope was used to examine the clear supernatant containing propolis nanoparticles ([10]). TEM was carried out on a JEOL JEM-2100 high resolution transmission electron microscope with a 200 kV accelerating voltage.

Animal model and study protocols

Male albino rats (n = 70) weighing 200 to 250 g, obtained from the Animal House of the Faculty of Veterinary Medicine, Zagazig University were housed in a specific pathogen free animal laboratory and permitted free access to food and water. After a week of adaptation, rats were randomly divided into 7 groups each group containing 10 rats. Negative control group 1 in which rats have not received any treatment. Positive control group 2

in which rats were received orally CCl4 1 gm /kg (50% in olive oil) twice a week for 4 weeks. Group 3 CCl4-silymarin group in which rats were treated orally with silymarin (200 mg/kg) per day (11). Group 4 CCl4-propolis group in which rats were treated orally with propolis (100 mg/kg) per day (8). Group 5 CCl4-nanopropolis group in which rats were treated orally with nanopropolis (30 mg/kg) per day (8). Group 6 CCl4-silymarin and propolis group in which rats were treated orally with silymarin (200 mg/kg) and with propolis (100 mg/kg) per day which called combination group. Group 7 CCl4- silymarin and nanopropolis group in which rats were treated orally with silymarin (200 mg/kg) and with nanopropolis (30 mg/kg) per day which called combination group. The experimental work was approved by the Institutional Animal Care and Use Committees Zagazig University (ZU-IACUC) with approval No. ZU-IACUC/2/F/69/2022.

Sample collection and processing

Overnight, the rats were kept without food or water before being given ketamine/xylazine to put them to sleep. The rats were sacrificed, and their tissues were removed, after blood samples were taken from the orbital venous plexus, allowed to clot, and centrifuged at 3,000 rpm for 15 minutes. The serum samples were stored at -20 °C. Rats were sacrificed so that their organs could be harvested for study. When performing real-time PCR analysis, we snap-froze a sample of tissue by enclosing it in aluminium foil and placing it in a container of liquid nitrogen to stop the activity of endogenous RNase. Histological analysis was performed on another sample of tumour tissue after it was fixed in a 10% buffered formalin solution for 24 hours at room temperature.

Molecular determinations of liver and kidney tissues

The PureLink® RNA Mini Kit (Catalog number: 12183018A) from Ambion by life technologies by Thermo Scientific was used to extract total RNA. The purity of RNA samples was determined using a NanoDrop® ND-1000 Spectrophotometer from NanoDrop Technologies in Wilmington, Delaware, USA.To create cDNA, we used a Thermo Scientific High-Capacity cDNA Reverse Transcription Kit (catalogue number 4374966). To measure the gene expression of small mothers against decapentaplegic protein SMAD-2, SMAD-3, SMAD-7, Matrix Metallopeptidase 9 (MMP-9), let-7b miRNA, Desmin and TGF-β1, we used a Maxima SYBR Green qPCR Master Mix (2X) kit obtained from Thermo Scientific (cataloug #K0251). GADPH was used to standardize gene expression. The $2^{-\Delta\Delta ct}$ formula was used to calculate the amount of target gene expression levels. The following primers were used described in (Table 1).

Table 1: Primer sequence for genes used in this experiment

Gene	Sequence	Gene bank accession No.
SMAD-2	F 5′- CAAACGTGCACAGGTGACAG-3′ R 5‘- GACTGGCGTTGGAAGAAGGA-3‘	NM_001277450.1
SMAD-3	F 5′- CTGGGCAAGTTCTCCAGAGTT-3′ R 5‘- AAGGGCAGGATGGACGACAT-3‘	NM_013095.3
SMAD-7	F 5′- GAGTCTCGGAGGAAGAGGCT-3′ R 5‘- CTGCTCGCATAAGCTGCTGG-3‘	NM_030858.2
MMP-9	F 5′- GATCCCCAGAGCGTTACTCG-3‘ R 5‘- GTTGTGGAAACTCACACGCC-3‘	NM_031055.2
Desmin	F 5′- ATCTGCGGGAGTACCAGGAT -3′ R 5‘- GCAGAGAAGGTCTGGATCGG -3‘	NM_022531.2
TGF-β1	F 5′- AGGGCTACCATGCCAACTTC -3′ R 5‘- CCACGTAGTAGACGATGGGC -3‘	NM_021578.2
let-7b	F 5′- AACACGTGTGAGGTAGTAGGTT -3′ R 5‘- GTCGTATCCAGTGCAGGGT -3‘	MI_0000063
GADPH	F 5′- GCATCTTCTTGTGCAGTGCC -3′ R 5‘- GGTAACCAGGCGTCCGATAC -3‘	NM_017008.4

Determination of liver functions as markers of hepatic injury

Enzymatic colorimetric kits from Spinreact, Girona, Spain were used for quantitative detection of Alkaline phosphatase (ALP), alanine transaminase (ALT), and aspartate transaminase (AST), and Serum lactate dehydrogenase (LDH) as inflammatory marker was assessed by using enzymatic colorimetric kits from Spinreact, Girona, Spain according to manufacture instruction as described by Reitman and Frankel (12).

Determination of kidney function as marker of kidney injury in Serum

Serum creatinine was quantitatively assessed colorimetrically, using enzymatic colorimetric kits from Spinreact, Girona, Spain.

Determination of IgM, TAC, MDA, CAT, TNF-a

The serum Immunoglobulin M (IgM) levels were evaluated by using the IgM ELISA Kit (rat) (CusaBio; Catalog No: CSB-E07978r-1), the serum total antioxidant capacity (TAC) levels were determined using OxiSelect™ TAC Assay Kit (Cell Biolabs, Inc, Catalog No: STA-360), the serum Malondialdehyde (MDA) levels were determined using Rat Malondialdehyde ELISA Kit (MyBioSource, Catalog No: MBS738685), and the serum catalase (CAT) levels were evaluated by using Rat catalase ELISA Kit (MyBioSource,, catalogue number: MBS701908). The serum tumor necrosis factor alpha (TNF-α) levels were evaluated by using Rat TNF-α ELISA kit (CusaBio; Catalog No: CSB-E11987r), according to the manufacturer's directions.

Histopathology

The tissues of the kidney and liver of rats were obtained and preserved with formaldehyde solution (10 % w/v) and processed in an automated tissue processor. Following that, follow-up techniques were used to do histological study of these tissues. The tissues were then fixed in paraffin blocks at the end of the operation. They were then cut into 5–6 m thick paraffin sections and blemished with haematoxylin–eosin.

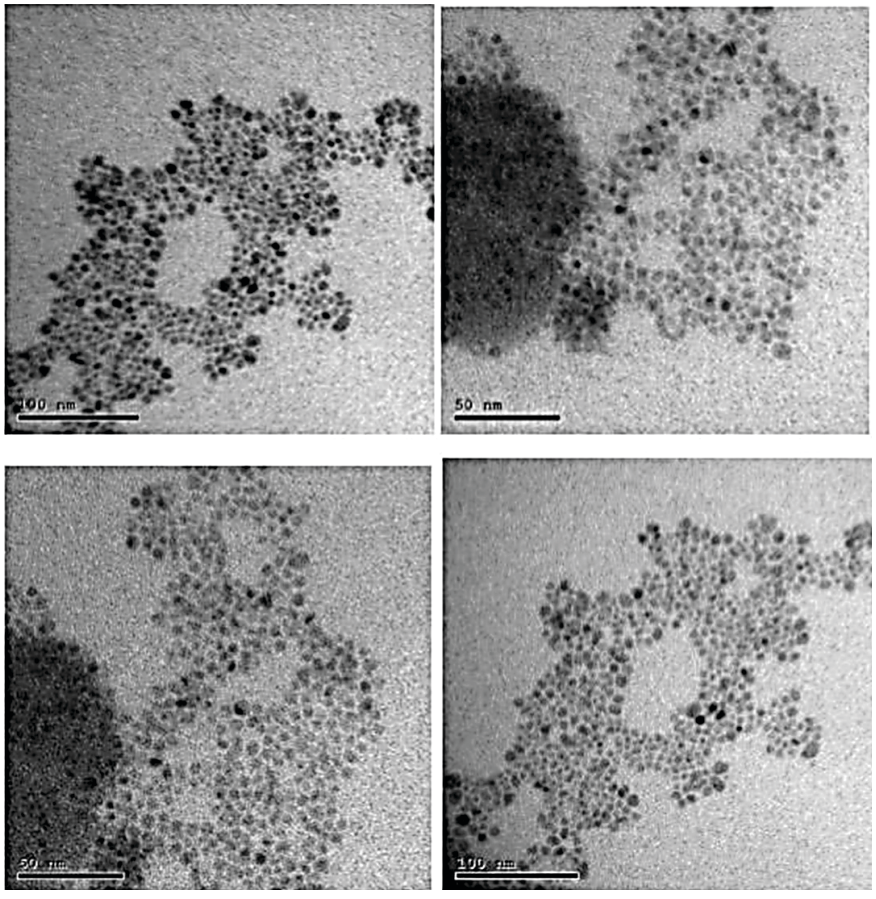


Figure 1: Size of Nano Propolis revealed by TEM

Statistical analysis

Standard deviations and means were used to summarise the data. The Shapiro-Wilk test establishes whether or not a distribution is normally distributed, the boxplot identifies outliers, and the Levene test evaluates for homogeneity. To compare group means, we used one-way analysis of variance (ANOVA), and to determine whether there were significant differences between groups, we used the Duncan's multiple range test. Having a P-value of less than 0.05 indicates statistical significance. Statistical analysis was performed using SPSS version 25. (Armonk, NY: IBM Corp).

Results

Transmission electron microscopy (TEM) validated the nanoparticle size and surface shape of the propolis (Figure 1). Nanoparticles were measured and determined to be spherical, with an average particle size of 10 nm and a standard deviation of 3 nm. Using a JEOL JEM-2100 high resolution transmission electron microscope and 200 kV accelerating voltage, we performed the real physical measurement.

Effect of silymarin (200 mg/kg/b.wt), propolis (100 mg/kg/b.wt) and nanopropolis (30 mg/kg/b.wt) on hepatic gene expression mRNA

The mRNA levels of a MMP-9 in male albino rats which exposed to silymarin, propolis and nanopropolis for 1 month were analyzed using real-time PCR. The normalization of target gene expression data was done using housekeeping gene. The GADPH gene is found in all cells and is one of the most widely used in gene expression data comparisons The results demonstrated that there was highly statistically different among groups in MMP-9 values (Figure 2 A). Negative control group revealed the lowest MMP-9 reading and the highest significant elevation of MMP-9 in CCl4 positive control group. The rats treated with CCl4+sylimarin, the group treated with CCl4+ propolis, the CCl4+ nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin showed significant downregulation of MMP-9 values in comparison with CCl4 positive control group. The lowest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group.

In our study, the expression SMAD-3 gene in the negative control group had revealed the lowest reading while it was significantly increasing in positive control group. The level of SMAD-3 gene expression was significantly downregulated in the group treated with CCl4+sylimarin, the group treated with CCl4+ propolis, the CCl4+ nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin in comparison with CCl4 positive control group. The lowest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group (Figure 2 B).

In reverse SMAD-7 gene expression was significantly the highest reading in the negative control group while in the CCl4 positive group showed the lowest reading. The level of SMAD-7 gene expression was significantly upregulated in the group treated with CCl4+sylimarin, the group treated with CCl4+ propolis, the CCl4+ nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin in comparison with CCl4 positive control group. The highest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group (Figure 2 C).

Also let-7b gene expression was significantly the highest reading in the negative control group while in CCl4 positive group showed the lowest reading that was non-significant with CCl4+ propolis. The level of let-7b gene expression was significantly upregulated in the group treated with CCl4+sylimarin, the group treated with CCl4+ propolis, the CCl4+ nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin in comparison with CCl4 positive control group. The highest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group (Figure 2 D).

It was revealed that there was highly statistically different among groups in TGF-β1 values. Negative control group revealed the lowest TGF-β1 reading and the highest significant elevation of TGF-β1 in CCl4 positive control group. The rats treated with CCl4+sylimarin, the group treated with CCl4+ propolis, the CCl4+ nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin showed significant downregulation of TGF-β1 values in comparison

with CCl4 positive control group. The lowest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group. This means that the group treated with CCl4+nanopropolis+silymarin can downregulate the TGF-β1 gene expression than other treatment groups (Figure 2 E).

Effect of silymarin (200 mg/kg/b.wt), propolis (100 mg/kg/b.wt) and nanopropolis (30 mg/kg/b.wt) on renal gene expression mRNA

It was revealed that there was highly statistically different among groups in TGF-β1 values. Negative control group revealed the lowest TGF-β1 reading and the highest significant elevation of TGF-β1 in CCl4 positive control group. The rats treated with CCl4+sylimarin, the group treated with CCl4+propolis, the CCl4+ nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin showed significant downregulation of TGF-β1 values in comparison with CCl4 positive control group. The lowest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group (Figure 3 A).

In our study, the expression SMAD-2 gene in the negative control group had revealed the lowest reading while it was significantly increasing in CCl4 positive control group. The level of SMAD-2 gene expression was significantly downregulated in the group

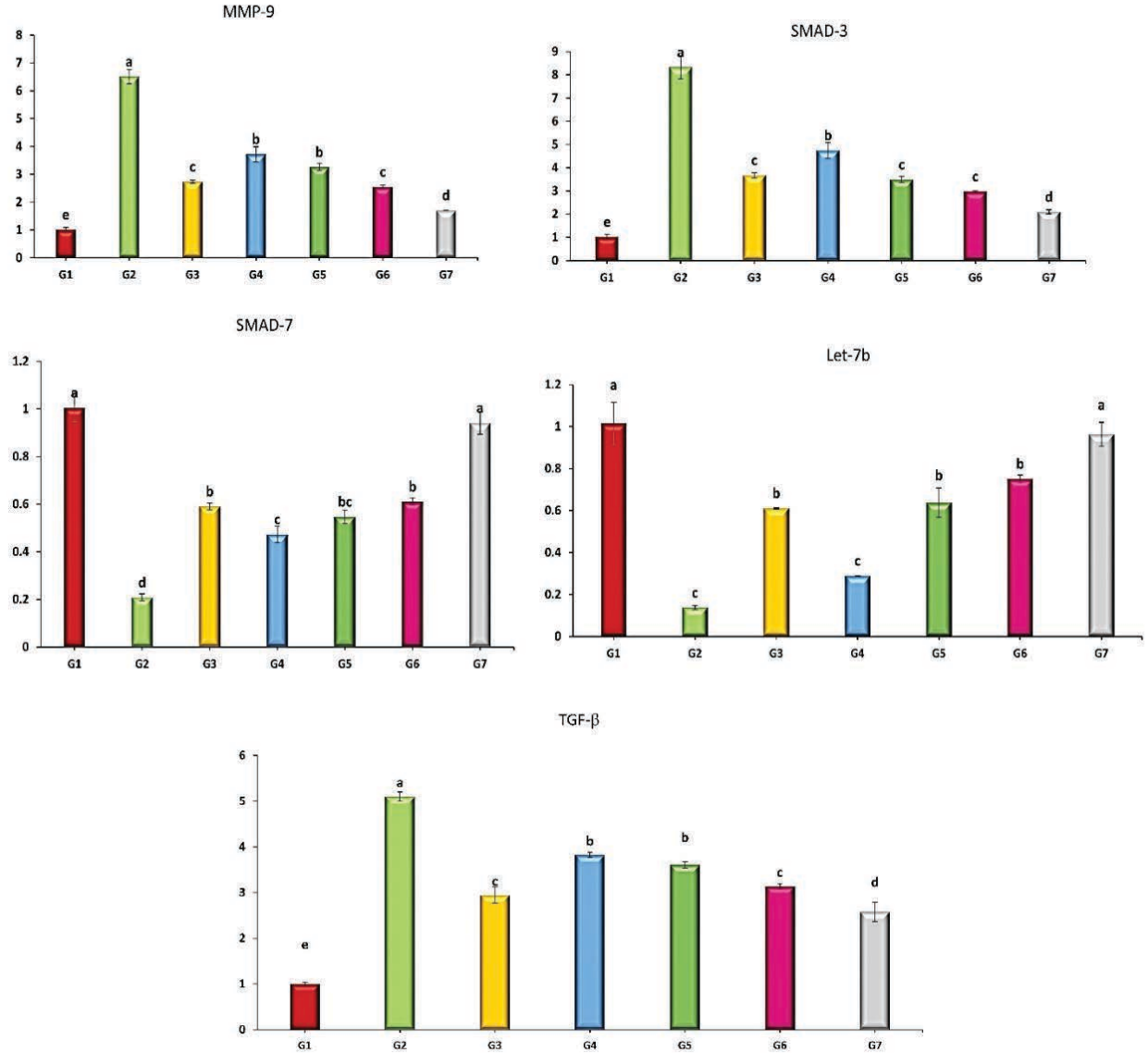


Figure 2: Effect of propolis (100 mg/kg/b.wt), silymarin (200 mg/kg/b.wt) and nanopropolis (30 mg/kg/b.wt) on hepat-ic gene expression mRNA. Small mothers against decapentaplegic protein (SMAD-3, SMAD-7), Matrix Metallopeptidase 9 (MMP-9), Transforming growth factor β (TGF-β)

treated with CCl4+sylimarin, the group treated with CCl4+ propolis, the CCl4+ nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin in comparison with CCl4 positive control group. The lowest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group (Figure 3 B).

The SMAD-7 gene expression was significantly the highest reading in the negative control group while the CCl4 positive control group showed the lowest reading. The level of SMAD-7 gene expression was significantly upregulated in the group treated with CCl4+sylimarin, the group treated with CCl4+ propolis, the CCl4+ nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin in comparison with CCl4 positive control group. The highest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group (Figure 3 C).

The let-7b gene expression was significantly the highest reading in the negative control group while the CCl4 positive control group showed the lowest reading. The level of let-7b gene expression was significantly upregulated in the group treated with CCl4+sylimarin, the group treated with CCl4+ propolis, the CCl4+ nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin in comparison with CCl4 positive control group. The highest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group (Figure 3 D).

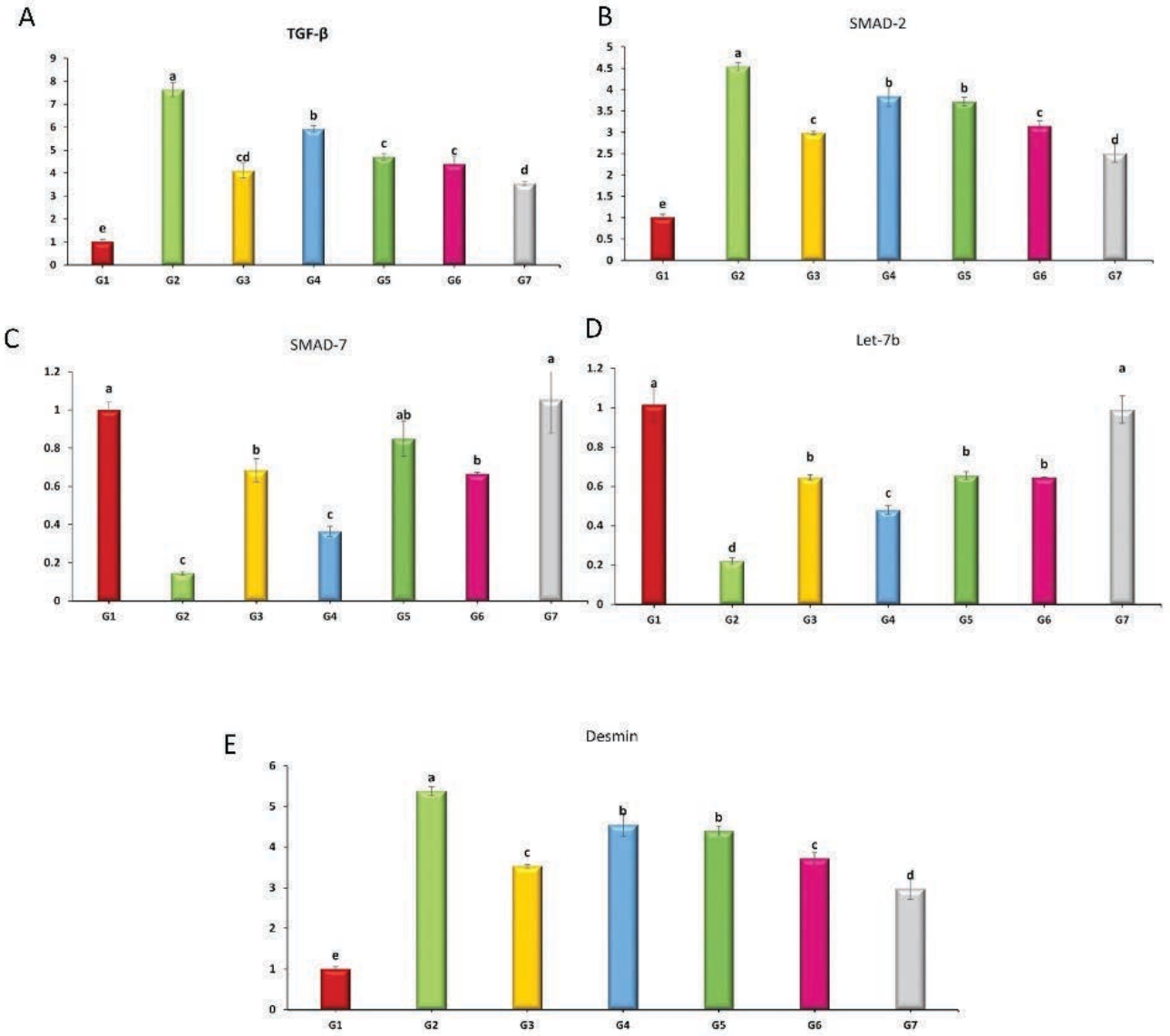


Figure 3: Effect of propolis (100 mg/kg/b.wt), silymarin (200 mg/kg/b.wt) and nanopropolis (30 mg/kg/b.wt) on renal gene expression mRNA. Small mothers against decapentaplegic protein (SMAD-2, SMAD-7), Transforming growth factor β (TGF-β)

The results demonstrated that there was highly statistically different among groups in Desmin values. Negative control group revealed the lowest Desmin reading and the highest significant elevation of Desmin in CCl4 positive control group. The rats treated with CCl4+sylimarin, the group treated with CCl4+ propolis, the CCl4+ nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin showed significant downregulation of Desmin values in comparison with CCl4 positive control group. The lowest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group (Figure 3 E).

Effect of silymarin (200 mg/kg/b.wt), propolis (100 mg/kg/b.wt) and nanopropolis (30 mg/kg/b.wt) on kidney function, LDH and liver functions

One-Way ANOVA results revealed that levels of the parameters of liver function test showed high significant differences among groups $p < 0.0001$***. The highest levels of ALT, AST, ALP, LDH, and creatinine were recorded in CCl4 positive control group. that showed significant difference with group treated with other groups. The lowest levels of ALT, AST, and LDH were in control negative group, CCl4+ nanopropolis +sylimarin

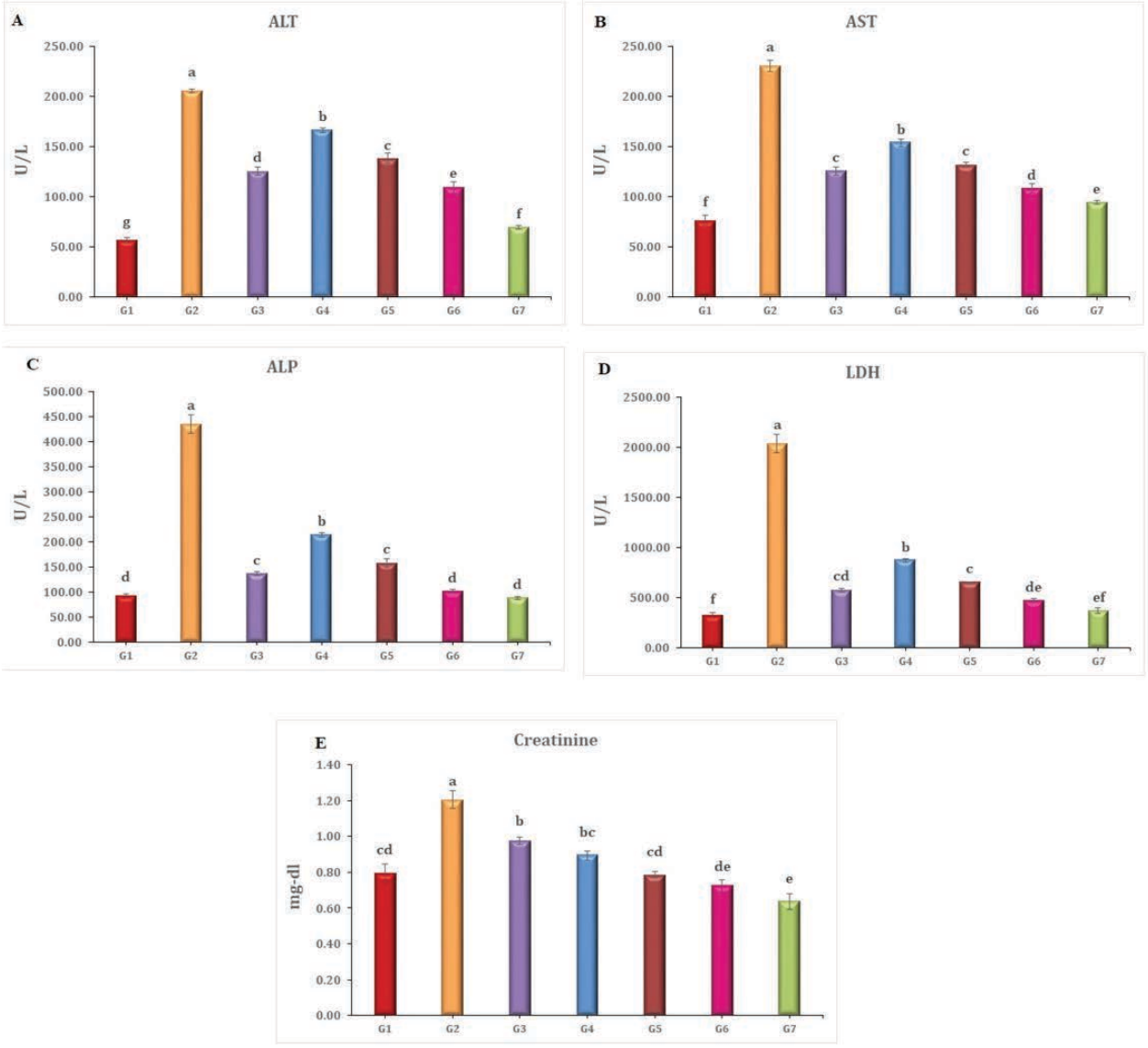


Figure 4: Effect of propolis (100 mg/kg/b.wt), silymarin (200 mg/kg/b.wt) and nanopropolis (30 mg/kg/b.wt) on the mean value of kidney function, LDH and liver functions of rats in different treated groups. Alkaline phospha-tase (ALP), alanine transaminase (ALT), aspartate transaminase (AST), serum lactate dehydrogenase (LDH)

treatment showed a significant up regulation in the levels of ALT, AST, ALP, LDH, and creatinine in comparison with CCl4 positive control group. These results revealed that the group of CCl4+ nanopropolis +sylimarin showed improvements in several markers than other groups (Figure 4).

Estimation of the mean value of oxidative stress markers

The result revealed that means with different superscript were statistically significant in CAT (Figure 5 A). The lowest significant value was revealed in CCl4 positive control group, and the highest significant value was found in control negative group. The rats treated with CCl4+sylimarin, the group treated with CCl4+ propolis, the CCl4+ nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin showed significant increase of CAT values in comparison with CCl4 positive control group. The highest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group.

Our data demonstrated that there was highly statistically different among groups in TAC levels (Figure 5 B). The lowest significant value was found in CCl4 positive control group, and the highest significant value was revealed in control negative group. The rats treated with CCl4+sylimarin, the group treated with CCl4+ propolis, the CCl4+

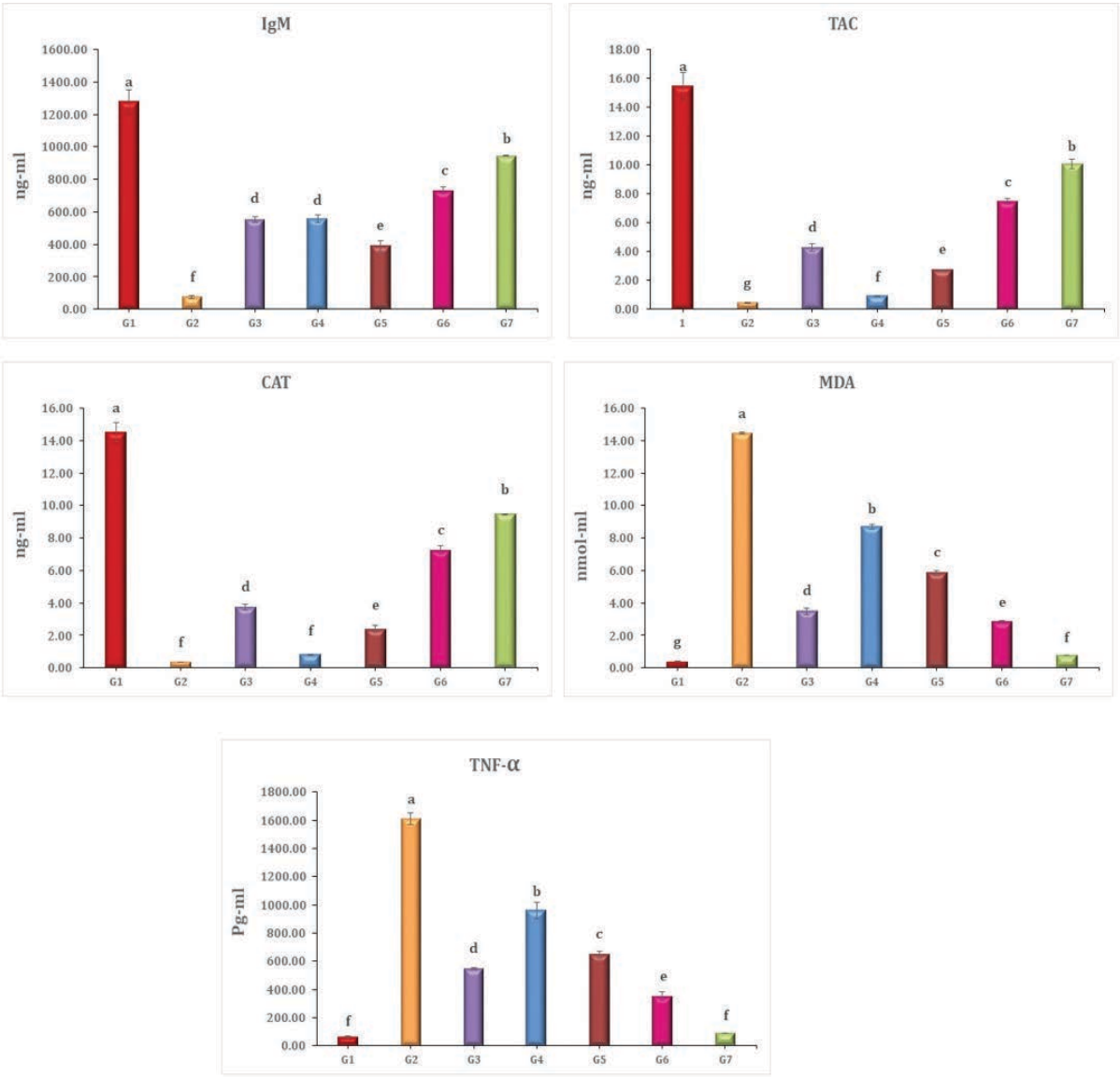


Figure 5: Effect of propolis (100 mg/kg/b.wt), silymarin (200 mg/kg/b.wt) and nanopropolis (30 mg/kg/b.wt) on the mean value of oxidative stress markers of rats in different treated groups. Immunoglobulin M (IgM), total antioxidant capacity (TAC) Malondialdehyde (MDA), catalase (CAT), tumor necrosis factor alpha (TNF-α).

nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin showed significant increase of TAC values in comparison with CCl4 positive control group. The highest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group.

There was highly statistically different among groups in IgM levels (Figure 5 C). The highest significant value was found in control negative group, and the lowest significant value was revealed in CCl4 positive control group. The treating rats with CCl4+sylimarin and the group treated with CCl4+ propolis showed non-significant difference to each other and showed a significant increase in IgM level in comparison with CCl4 positive control group. The rats treated with CCl4+ nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin were effective in increase of IgM value in comparison with CCl4 positive control group. The highest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group.

We also explored the influence of propolis and nanopropolis on MDA activity (Figure 5 D). The results revealed that the lowest significant value was found in control negative group, and the highest significant value was demonstrated in CCl4 positive control group. The rats treated with CCl4+sylimarin, the group treated with CCl4+ propolis, the CCl4+ nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin showed significant decrease of MDA values in comparison with CCl4 positive control group. The lowest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group.

One-Way ANOVA results revealed that levels of the parameters of TNF-α showed high significant differences among groups $p < 0..0001$*** (Figure 5 E). The results revealed that the lowest significant value was found in control negative group, and the highest significant value was demonstrated in CCl4 positive control group. The rats treated with CCl4+sylimarin, the group treated with CCl4+ propolis, the CCl4+ nanopropolis, CCl4+ propolis+ silymarin and CCl4+ nanopropolis+ silymarin showed significant decrease of TNF-α values in comparison with CCl4 positive control group. The lowest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group.

Histopathological Findings

Negative control group (G1): Liver and kidney sections from control group showed normal structures. Neither inflammatory, nor degenerative or apoptotic changes were recorded in any of the examined parts (Figure 6 A, B).

Positive control (CCl4 intoxicated group, G2): Identical feature of CCl4 toxicity were represented by moderate periportal hepatocytes, degenerative and necrotic changes accompanied by intense portal inflammatory reaction followed by fibroplastic hyperplasia with formation of fine fibrous strands enclosing hepatic lobules. Hepatocellular pathognomonic ballooning degeneration and necrosis with eccentrically situated pyknotic nuclei was pronounced. The portal blood vessels were dilated with occasional perivascular and interstitial hemorrhages (Figure 6 C). Renal sections demonstrated nephrotoxic changes represented by moderately dilated renal blood vessels, focal collecting tubular dilatation, moderate perivascular edema, hemorrhage and round cells infiltration beside tubular epithelial degenerative changes, glomerular lobulation and or atrophy (Figure 6 D).

CCl4-Silymarin treated group (G3): liver sections, demonstrated a comparatively moderate enhancing protective effect of the used compound as demonstrated by regenerative changes in the previously damaged hepatocytes with cytoplasmic basophilia and large hyperchromatic nuclei. Some hepatocytes were degenerated and or apoptotic (Figure 6 E). Renal pathology of this group gone parallel with the hepatic changes as a residual CCl4 toxic effects were seen and emphasized by perivascular edema, tubular epithelial degeneration, cystic dilatation and glomerular lobulation (Figure 6 F).

CCl4-Propolis treated group (G4): Sections from liver, showed a comparatively enhancing protective effect of the used compound as demonstrated by regenerative changes in the previously damaged hepatocytes with cytoplasmic basophilia and large hyperchromatic nuclei. Neither necrotic nor hemorrhagic or fibroblastic changes were recorded (Figure 7 A). Mild nephrotic changes represented by residual perivascular edema, a few dilated collecting tubules, tubular epithelial degeneration and lobulated glomerular tufts were recorded (Figure 7 B).

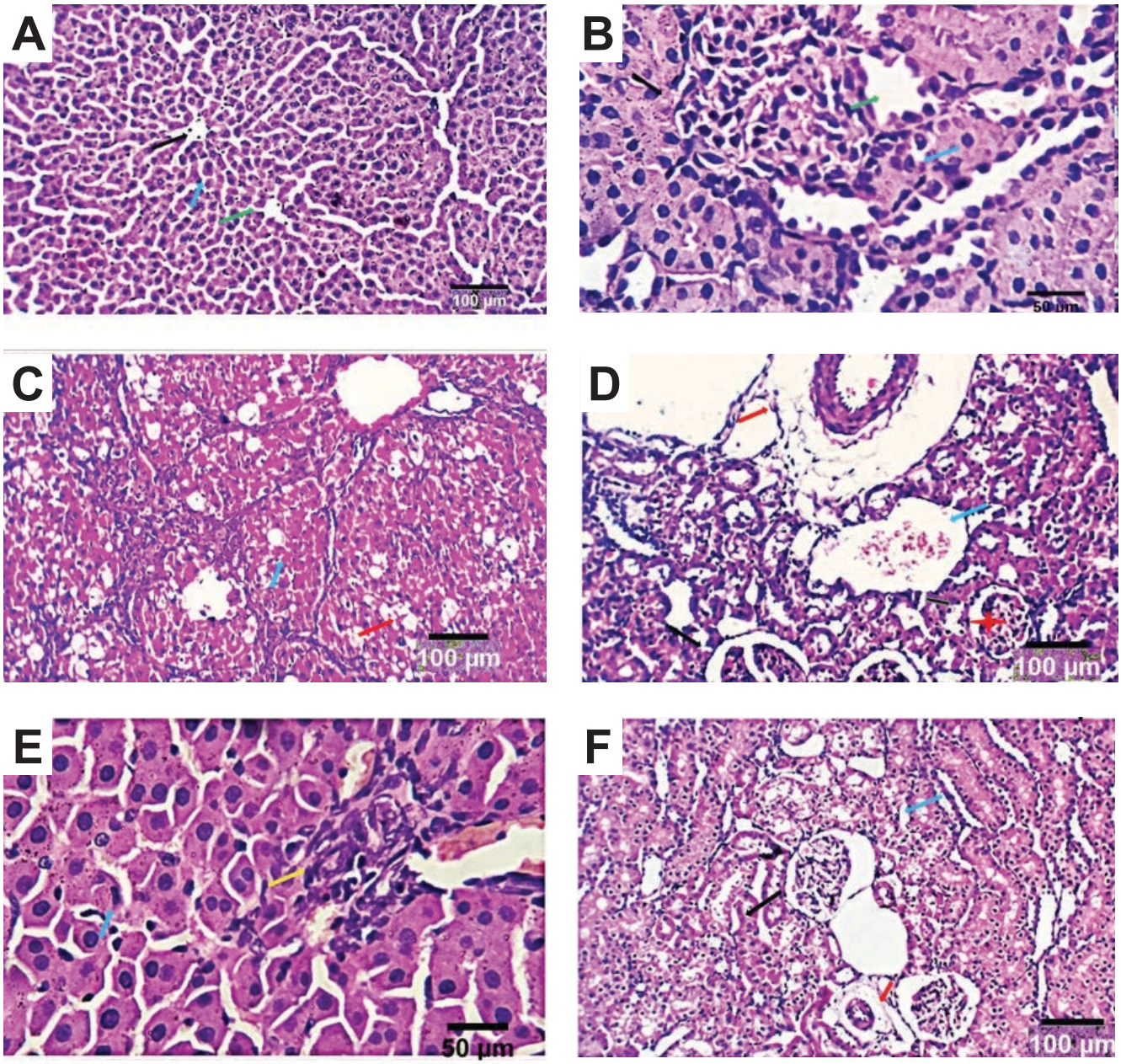


Figure 6: Photomicrograph from rat's liver (A), showing preserved hepatic cords, (blue arrows) portal triad's structures, vascular tributaries, and Von kupffer's cells (green arrows), and kidney of control negative group (B) showed apparently proximal and distal tubules besides loops of Henle (blue and green arrows). Photomicrograph from rat's liver (C) showed, moderate periportal hepatocytes, degenerative and necrotic changes accompanied by intense portal inflammatory reaction (blue arrow), hepatocellular pathognomonic ballooning degeneration and necrosis with eccentrically situated pyknotic nuclei is seen (red arrows), and Kidney (D) of control positive, CCl4-intoxicated group, nephrotoxic changes represents by moderately dilated renal blood vessels (red arrow), glomerular lobulation and or atrophy (red star). Photomicrograph from rat's liver (E), showed regenerative changes in the previously damaged hepatocytes with cytoplasmic basophilia and large hyperchromatic nuclei (blue arrow). Remnant of CCL4 toxic effects are seen and represents by periportal biliary proliferation and round cells infiltration (yellow arrow). and Kidney (F) of CCl4-Silymarin treated group (G3), renal pathology is seen as tubular epithelial degeneration (gray arrow) and glomerular lobulation (red star) (H&E X 200, 400)

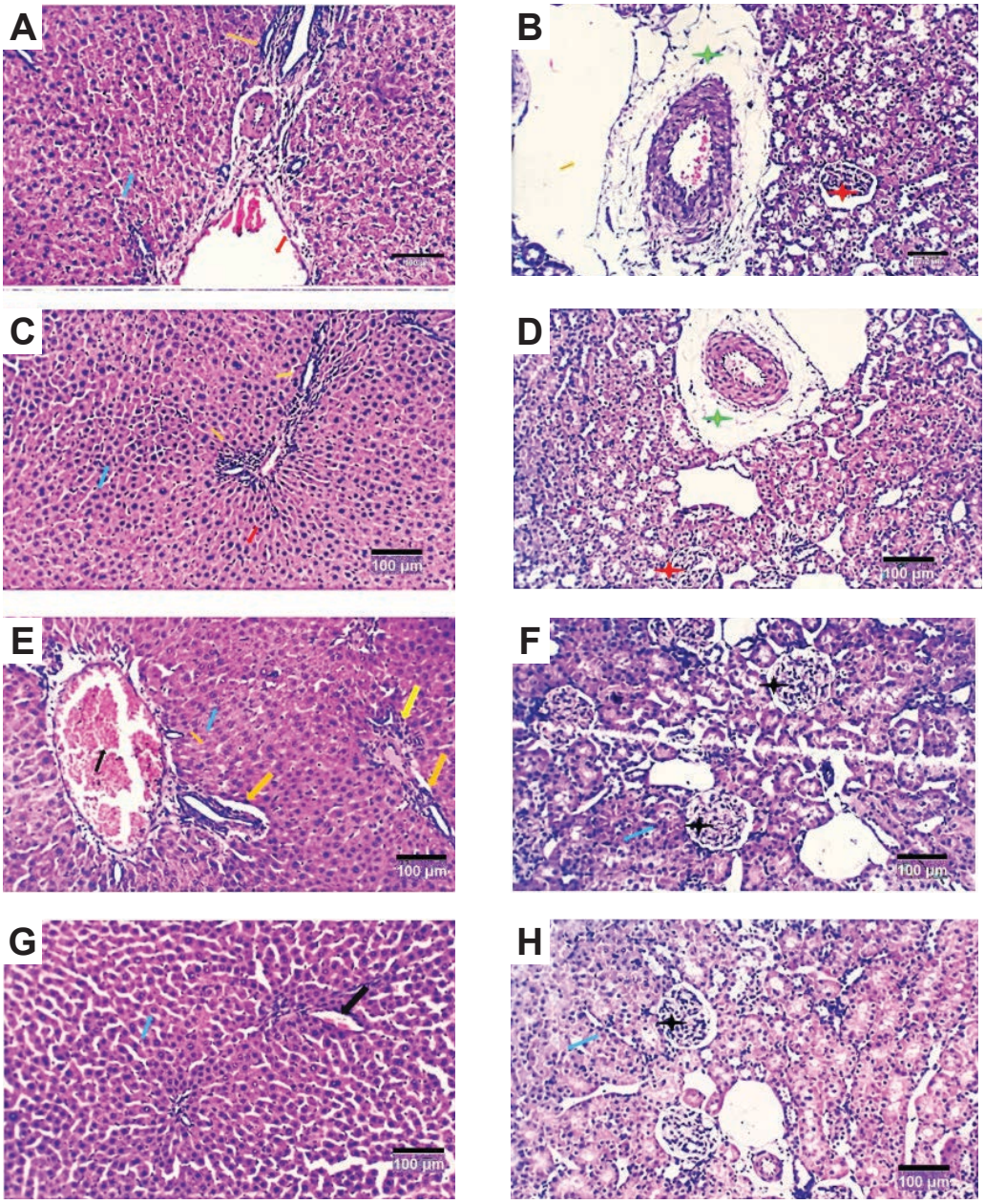


Figure 7: Photomicrograph from rat's liver (A) showed regenerative changes in the previously damaged hepatocytes with cytoplasmic basophilia and large hyperchromatic nuclei (blue arrow), portal vascular dilatation (red arrow), mild biliary proliferation and lymphocytic infiltration (orange arrows), and Kidney (B) of CCl4-Propolis treated group (G4), mild nephrotic changes represent by residual perivascular edema (green star), and lobulated glo-merular tufts (red star) were seen. Photomicrograph from rat's liver (C), showed apparently normal hepatic structures with preserved hepatic cords, some hepatocytes appeared with cytoplasmic basophilia and large hyperchromatic nuclei as a positively reacted anabolic ribosomal reactivities (blue arrow). A very few rats demonstrate residual portal round cells infiltration and biliary proliferation (orange arrows), and Kidney (D) of CCl4–Nano Propolis treated group (G5), showed an apparently normal nephron unit, excretory ducts, papillae and pelvis apart of residual focal perivascular edema and glomerular tufts lobulation (green and red arrows). Photomicrograph from rat's liver (E), showed normally arranged hepatic cords with active, occasionally binu-cleated cells (blue arrow). The portal triads structures, blood vessels and stroma were in a good histologic configuration. Mild portal blood vessels dilatation and blood engorgement (black arrow) biliary proliferation and round cells infiltration (orange and yellow arrows) are seen, and Kidney (F) of CCl4-Silymarin and propolis treated group (G6), showed normal histological structures of nephron units (black stars). Photomicrograph from rat's liver (G), showed hepatic tissue with normally arranged cords, active, occasionally binucleated cells (blue arrows), and Kidney (H) of CCl4-Silymarin and Nano propolis treated group (G7), showed absolutely normal histological structures of nephron units, collecting duct, papillae and pelvis beside normal stromal cells (blue arrows and black stars) (H&E X 100, 400)

CCl4-Nano-propolis treated group (G5): Sections from liver, showed apparently normal hepatic structures with preserved hepatic cords, portal triad's structures, vascular tributaries, biliary system, central veins, sinusoids. Some hepatocytes appeared with cytoplasmic basophilia and large hyperchromatic nuclei as positively reacted anabolic ribosomal reactivities. A few sections of the examined cases (10-15%) demonstrated residual portal round cells infiltration and biliary proliferation (Figure 7 C). Renal tissues of the same group declared a potent protective effect of the used compound as most of the examined sections showed apparently normal nephron units, excretory ducts, papillae and pelvis apart of residual focal perivascular edema and mild glomerular tufts lobulation (Figure 7 D).

CCl4-Silymarin and propolis treated group (G6): Hepatic tissue appeared with normally arranged cords with active, occasionally binucleated cells. The sinusoids comprised active phagocytic hypertrophic Von-Kupffer cells. The portal triads structures, blood vessels and stroma were in a good histologic configuration. A very few sections (8-10%) of the examined cases revealed mild portal blood vessels dilatation and blood engorgement, biliary proliferation and round cells infiltration (Figure 7 E). Renal sections demonstrated normal histological structures. A few renal blood vessels and collecting tubules were abnormally dilated. The latter showed atrophy of their lining epithelium. A few of the proximal and distal tubular epithelia were degenerated (cloudy swelling and hydropic degeneration) (Figure 7 F).

CCl4-Silymarin and Nano-propolis treated group (G7): The best protective effect was observed in this group. Hepatic tissue appeared with normally arranged cords and active, occasionally binucleated cells. The sinusoids comprise active phagocytic hypertrophic Von-Kupffer cells. The portal triads structures, blood vessels and stroma were in a good histologic configuration. A few residual round cells infiltration and fibroblasts were seen some portal areas (Figure 7 G). Renal sections demonstrated absolutely normal histological structures of nephron units, collecting duct, papillae and pelvis beside normal stromal cells (Figure 7 H).

Discussion

Nanocarriers are a new type of vehicle that is being developed to increase the solubility of medicinal compounds. Different nanonization procedures have increased the disintegration rates and bioavailability of certain medications by shrinking their size (13). Propolis nanoparticles had a substantial effect on the treatment of hepatic fibrosis; ALT, AST, LDH and creatinine levels gradually improved in the Propolis nanoparticles and silymarin group compared to CCl4 control positive group rats. The levels of ALT, AST, ALP, LDH and creatinine in the propolis nanoparticles and silymarin group had been restored to levels that were very near to control values. The recovery of hepatobiliary damage is indicated by lower level of ALP. In addition, no histological changes could be seen when compared to the control group. Our study indicated the beneficial effects of Propolis nanoparticles restoring on liver function and structure.

Our results show that propolis protects rats' livers from D-galactosamine and Lipopolysaccharide-induced toxicity, which is in line with previous studies that have linked propolis' hepatoprotective effect to its high concentration of phenolic components and their antioxidant, anti-inflammatory, and anti-apoptotic properties (14). Propolis protected rats' kidney from CCl4 damage, as shown by significantly lower serum creatinine activity in CCl4-injected rats. The antioxidant, anti-inflammatory, anti-hyperlipemic, and anti-hypertrophic properties of propolis may help to reduce cholestasis-related liver injury (15).

One more study examined at the haematological and biochemical effects of red propolis nanoparticles in dogs. Capsules containing 50 mg of polymeric nanoparticles loaded with 20% red propolis extract were orally administered once daily to eight otherwise healthy adult canines. Red propolis polymeric supplementation given orally once a day had no negative effects on hematologic, renal, or hepatic parameters in this study of healthy adult dogs. There was some evidence that nano propolis had a positive effect on the liver (16).

In a previous study utilizing propolis and nanopropolis, it was discovered that when oxidative stress was induced with alcohol, the levels of ALP and AST in male rats in the propolis-treated group and its and NP reduced dramatically (8). Another

study examined the effect of propolis and its nanoparticles on biochemical variables in the liver of tilapian fish. In comparison to the control groups, the meal containing *M. aeruginosa* cells induced significant increases in AST, ALT, ALP, creatinine, and urea. Co-administration of propolis and nanopropolis, results in significant reductions in these parameters. Propolis in nano form was more effective as a hepatoprotective agent and in competing with *M. aeruginosa* toxicity (17).

The antioxidant effect of propolis and its nano form was demonstrated in our study. The results approved with the subsequent researches described below. The highest values of IgM, TAC and CAT were revealed in CCl4+nanopropolis+silymarin treatment group after control negative group. While the lowest values of MDA and TNF-α were revealed in CCl4+nanopropolis+silymarin treatment group than other treatment groups and this confirms our theory that nanopropolis improved the effects of silymarin when administrated with it. Overall, these results indicated that nanopropolis has a significant inhibitory role in MDA and TNF-α levels.

Studies comparing propolis nanosuspension (PRO-NS) to free propolis have shown that the nano version is more effective at killing off Ehrlich ascites cancer (EAC) in female Swiss albino mice. PRO-NS inhibited the increase in blood AST and ALT activity, IgM, and creatinine and urea levels after EAC cells were implanted. In liver and EAC cells, PRO-NS boosted SOD activity and glutathione content. Their research revealed that PRO-NS significantly inhibits tumour growth through mechanisms that may include protection against oxidative damage, immune system activation, and apoptotic induction (18).

In Non-Alcoholic Fatty Liver Disease (NAFLD) mice, a similar pattern was observed; propolis alleviated symptoms by lowering liver lipids, enzymes, and advanced glycation end products. TNF-α and IL-6, pro-inflammatory cytokines are likewise reduced in NAFLD liver tissue by propolis (19). Propolis reduced liver damage in mice with diabetes and NAFLD in an experimental study as it lowered the levels of ALP, ALT, AST, LDH, and MDA and improved the protective antioxidant status of diabetic mice by upregulating antioxidant enzymes such as SOD, CAT, GPx, GST, and GR, as well as increasing glutathione levels and hepatic total antioxidant capacity. Propolis increases specific IgM and IgG titers while reducing serum IFN-γ, IL-1, and IL-6 cytokine levels in rats infected with *Toxoplasma gondii* (20).

In the current study we reported that nanopropolis improved the silymarin treatment through the inhibitory impact on the rat liver and kidney homogenate the gene expression of TGF-β1, MMP 9, SMAD-3, SMAD-2 and Desmin, while increased the expression of SMAD-7 and let-7b miRNA in comparison with other treatment groups. It was found that silymarin mediated reduction of CCl4-induced production of IL-17, TNF-α, and TGF-β was dosage dependent, and that levels were higher in CCl4-treated groups in on hepatic fibrosis and renal damage generated by CCl4 (21).

Hepatic stellate cells (HSCs) are active and display various markers, therefore the researchers used immunohistochemistry to look at the expression of α-SMA, vimentin, desmin, and MMP 9 in rat liver tissue to corroborate the fibrosis-related alteration of HSCs. CC14-administered group rats had greater expression of the above markers.Relative to the CC14 group, the expression of the aforementioned ECM proteins was reduced in the liver tissue of the rats given Silymarin (100 mg/kg) (22).

One of the most important epigenetic regulatory categories is microRNAs (miRNAs). They are non-coding, endogenous RNA molecules with a length of 20-23 nucleotides that play a function in either physiological or pathological situations. For example, one of the first miRNAs found was Lethal-7 (let-7). A member of the let-7 protein family, let-7b contributes to the onset of viral infection, alcoholic liver damage, and hepatocellular cancer. There is evidence that let-7b is down-regulated in a number of diseases. Let-7b-5p functions as a tumour suppressor in multiple myeloma by negatively regulating insulin-like growth factor receptor 1 at the post-transcriptional level (23). Let-7b's ability to increase p21 levels is an additional mechanism by which it suppresses HCC cell growth. Let-7b also goes after transforming growth factor-β1 (24).

In animal models of advanced chronic kidney disease, propolis inhibited pro-inflammatory signaling pathways in the TGF-β family signaling cascades, such as SMAD 2/3-dependent and SMAD-independent JNK/ERK activation, which have been associated to the development of tubulointerstitial fibrosis (25). Reduced oxidative stress and fibrosis, as well as co-inhibition of the

JAK2/STAT3/SOCS1 and TGF-β/Smad signalling pathways, are how silymarin nanoliposomes prevent STZ-induced kidney injury in diabetic rats (26).

Our histopathological findings appeared that groups treated with nano-propolis alone or with silymarin and nano-propolis restored the deterioration made by CCl4 in rat renal and hepatic tissues and the following results confirmed our data. Propolis protected the kidney from ischemic-reperfusion acute renal injury by reducing oxidative stress and increasing endothelial nitric oxide synthase and heme-oxygenase. After ischemic–reperfusion, propolis-treated renal tissue exhibited a considerably reduced tubular necrosis score, according to histological examination (27).

Bhadauria (28) discovered that propolis improved the kidney histoarchitecture, reduced glomerulus swelling, and created more consistent space between the glomerulus and capsule wall in CCl4-damaged mouse renal tissue. When administered to renal tissues of diabetic rats, propolis maintained glomerular basement membrane thickness. Diabetic rats that were not treated showed a considerable rise in the thickness of their glomerular basement membrane. Histopathological examinations showed that propolis protected the liver from further damage caused by diabetes. Propolis treatment reduced inflammation and immune cell infiltration in diabetic mice, and reduced the number of vacuolized cells (29).

Typical central vein, sinusoidal area, and portal triad sections were present in the liver tissue of negative rats. Neutrophil infiltration, sinusoidal congestion, and portal inflammation were observed in the liver tissue of the CCl4 group, while clear sinusoidal space and substantial regeneration were observed in the silymarin group (22).

CCl4's effect on kidney biochemical indicators is described by El-Haskoury et al. (30). Renal tubule dilatation and atrophy were observed on histology, showing that CCl4 interferes with glomerular function and causes acute nephropathy. Similarly, Yoshioka et al. (31) found an immediate histological alteration in the renal tissues of injected rats, which is consistent with our findings. The results we got line up with those from Wang et al (32). High fibrotic tissue, larger Kupffer cells, and a blubbing hepatocytes membrane were observed histologically in CCl4-injected rats, corroborating the biochemical result.

In another studies it was suggested that the histopathological findings, elevation of liver transaminases, elevation of lipid peroxidation (thiobarbituric acid reactive substances, TBARS), depletion of reduced glutathione (GSH), and reductions in the activities of antioxidant enzymes CAT, SOD, glutathione peroxidase (GPX), and glutathione-s-transferase (GST) were all indicative of CCL4 toxicity and this was in agreement with our study (33- 36).

Conclusion

Bees collect propolis, a resinous plant compound, which they combine with digestive enzymes and, on occasion, beeswax to create a sticky substance called propolis. Propolis possesses powerful anti-inflammatory, antiviral, anti-oxidant, anti-protozoal, anaesthetic, anti-tumoral, and anti-hepatotoxic qualities, and it also aids in the protection of the bee colony. Multiple in vitro, animal, and most importantly human clinical investigations have demonstrated the safety and usefulness of propolis. In conclusion, our research confirmed the importance of employing propolis nanoparticles for the management of hepatic fibrosis and nephropathy. Propolis nanoparticles boosted hepatocyte regeneration and alleviated hepatobiliary injury. The propolis nanoparticle acted as an anti-inflammatory by altering the relative expressions of SMAD-2, SMAD-3, SMAD-7, MMP-9, let-7b miRNA, Desmin, and TGF-β1 mRNA in liver and kidney tissues. Propolis nanoparticles were crucial in the treatment of nephropathy in rats given CCl4.Healing of renal tissue was aided by the nanoparticles of propolis in a definite and obvious fashion. It was shown that propolis nanoparticles regulate the apoptotic cascade by acting as antioxidants, protecting cells by altering the levels of IgM, TAC, MDA, CAT, and TNF-α. In addition, we proposed combining nanopropolis with other commercial medications for hepatic fibrosis and nephropathy.

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All authors contributed equally.

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The experimental work was approved by the Institutional Animal Care and Use Committees Zagazig University (ZU-IACUC) with approval No. ZU-IACUC/2/F/69/2022.

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Original Research Article
Veterinary Medicine and The One Health Concept

EFFICACY OF LYCOPENE ON AFLATOXIN B1- INDUCES OXIDATIVE STRESS, HEPA-TOTOXICITY, APOPTOSIS AND IMMUNODEFICIENCY IN JAPANESE QUAIL

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Abstract: Mycotoxins are harmful auxiliary metabolites delivered by species of filamentous organisms developing on grains some time recently collect and in capacity. The study was planned to evaluate the protective role of lycopene (LYC) against Aflatoxin B_1 (AFB_1) induces oxidative stress, hepatic toxicity, and apoptosis in Japanese quail. Sixty Japanese quail chicks (three-week-old) were randomly allocated into four groups; Negative control group; Aflatoxin -B_1 (AFB_1) group (1 mg/kg feed) as positive control; lycopene supplemented group (200 mg/kg feed) and AFB_1 (1 mg/kg feed) with lycopene supplemented group (200 mg/kg). The growth performance parameters, serum biochemical indices, and liver antioxidant activities as well as histopathological studies, and immunohistochemical pictures were performed. The results showed a significant increase on growth performance and a significant decrease on feed conversion ratio (FCR) in aflatoxin inclusion with lycopene supplemented group. Moreover, the liver enzymes (ALT, AST, and ALP) were significantly ($P < 0.05$) decreased. In addition, malondialdehyde (MDA) levels significantly decreased, while superoxide dismutase (SOD), Glutathione peroxidase (GSH-Px) and catalase (CAT) activities increased in liver tissues. Furthermore, when compared to the aflatoxin-inclusion with the lycopene supplemented group had improved liver tissue and lower levels of cytokine production (IL-6 and TNF-). In conclusion, these findings implied that dietary administration of lycopene has significantly alleviated AFB_1-triggered oxidative stress, inflammatory response and liver apoptosis in Japanese quail.

Key words: Lycopene; Aflatoxin B_1; hepatotoxicity; cytokine; liver apoptosis; Japanese quail

Introduction

Mycotoxins are poisonous compounds that are normally created by certain sorts of moulds (organisms). Moluds that can deliver mycotoxins develop on various foodstuffs, such as cereals, dried natural products, nuts, and flavors. Most mycotoxins are chemically stable and survive nutrient preparation. About a fourth of the cereals are globally estimated to be contaminated by fungal mycotoxins (1). Exposure to the danger of mycotoxins can occur either through contaminated cereals or through residues found in meat and eggs, such as aflatoxins, which are the end products of two fungal species named (*Aspergillus flavus* and *Aspergillus parasiticus*) that predominantly spoil feed ingredients during storage, especially in warm and humid climates (2). Aflatoxin B_1 (AFB_1) has been stated to induce various health problems, including hepatotoxicity, teratogenicity, mutagenicity, and carcinogenicity (3). AFB_1 has been categorized as a Group I carcinogen for humans via the "International Agency for Research on Cancer (IARC)" (4). The reactive metabolite (AFB1-exo-8,9-epoxide) produced from AFB_1 biotransformation by cytochrome P450. This metabolite robustly binds to DNA adducts, generating reactive oxygen species and leading to the AFB_1 carcinogenic effect

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(5). Indeed, aflatoxin poisoning has been linked to liver cancer in a number of countries, particularly in Asia and Africa, where grains are more likely to be contaminated with aflatoxin due to favourable humid conditions (6). Previous studies provided evidence that oxidative stress, inflammation, and cell apoptosis play a significant role in AFB_1-induced toxicity, suggesting that antioxidants could be used as a convenient approach to limit the progress of the toxic impacts associated with acute AFB_1 toxicity (7). Therefore, there is a dire need for finding antioxidant substances that might be effective in alleviating the deleterious effects of AFB_1 exposure. Lycopene (LYC) is a naturally occurring carotenoid that is mainly found in tomatoes, tomato by-products, grapefruit, guava, papayas, and red peppers (8). Lycopene proves to have anti-inflammatory, anticancer, and antioxidant activities as well as anti-cardiovascular disease capability and detoxification activity (9). A previous study reported that lycopene supplementation at a 5 mg/kg dose could relieve liver damage in mice (10). LYC has potent free radical scavenging efficacy, protecting the liver, kidney, mucous membrane, and immune system against AFB1-triggered oxidative stress (11). Based on the prior studies, we assume that Lycopene has a protective effect against AFB_1-triggered hepatotoxicity. Hence, the aim of this study is to investigate whether LYC supplementation exerts anti-inflammatory, antioxidant, and antiapoptotic effects on the damaged liver and kidneys in the Japanese quail chicks fed AFB1-contaminated diets.

Material and methods

Feed additives

Aflatoxin B1 was obtained from the Animal Health Research Institute, Dokki, Giza, Egypt. It was added to the diet at a dose of 1 mg/kg diet (12). Lycopene extract powder was purchased from Giftlover natural herbal company, China, and added with a dose of 200 mg/kg diet (13).

Birds

A total of sixty (21days-old) unsexed Japanese quail chicks (Coturnix japonica) with initial body weight of 128.56 ± 1.75g were used in this study. Birds were randomly divided into four treatments (n = 15), and each group included three replicates (n = 5) and were placed into galvanized wire cages (95×65×160 cm) and reared up to day 56 of age.

Experimental design

The experimental groups were as follow: 1) control group; fed basal diet, 2) AFB_1group; received AFB_1-supplemented diet (1 mg/kg diet), 3) Lycopene group; fed Lycopene supplemented diet (200 mg/kg diet), 4) AFB_1+ LYC group; fed 1mg AFB1 + 200 mg LYC /kg supplemented diet. Quail chicks were housed in battery cages rather than litter floor. Initial temperature was adjusted at 22°C. The nutritional requirements were prepared according to the recommendations of National Research Council (14). Free access for feed and water was provided on lightening schedule of 18 h of daylight and 6 h of darkness. During the experiment, body weight (BW) and feed intake (FI) were recorded weekly. The data were used to calculate average weekly gain (AWG), average weekly feed intake (AWFI) and feed conversion ratio (FCR) weekly and cumulatively. All experimental procedures of this study were conducted according to the guidelines of the "Local Experimental Animal Care Committee and the ethics of the institutional committee of Department of Pharmacology, Faculty of Veterinary Medicine, Zagazig University, Egypt".

Sampling

At 56 day of age, nine birds were chosen randomly (n = 3 birds per replicate). Sera were obtained by blood samples centrifugation at 2,500 rpm for 10 min at 4°C and stored at -20 °C until performing the biochemical analyses. Liver samples were rapidly dissected out and rinsed with NaCl 0.9% and divided into 2 parts; the 1st part was snap frozen then stored at -80 °C until used for hepatic antioxidant enzyme assay analysis and the 2nd part was immediately fixed in 10% buffered formalin for histopathological examination.

Liver and kidney functions

For assessment of liver function parameters, the concentrations of aspartic aminotransferase (AST), alkaline phosphatase (ALP), and alanine aminotransferase (ALT) were estimated. Creatinine

and uric acids were determined. These parameters were spectrophotometrically assayed by using semi-automated spectrophotometer (Erba-Chem7, Germany) and using commercial kits as mentioned by(15) following the manufacturer's instructions ("CUSABIO BIOTECH CO. Ltd., Houston, TX 77054, USA").

Serum inflammatory markers

The levels of cytokines including tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) were estimated in serum using commercial ELISA kits ("CUSABIO BIOTECH CO. Ltd., Houston, TX 77054, USA").

Hepatic antioxidant enzyme assay

The activities of hepatic antioxidant enzymes were assayed according to (16). Malondialdehyde content (MDA), catalase (CAT) Glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activities were assessed using specific kits from ("CUSABIO BIOTECH CO. Ltd., Houston, TX 77054, USA") according to the manufacturer's instructions. These parameters were spectrophotometrically assayed by using semi-automated spectrophotometer (Erba-Chem7, Germany).

Histopathological study

Excised liver specimens were fixed in 10% buffered formalin. Fixed specimens were dehydrated through graded concentrations of ethanol, cleared in xylene and then embedded in paraffin wax. Afterwards, paraffin blocks were cut into sections of 5 µm in thickness and stained with hematoxylin-eosin (H&E).The method was carried out as previously explained by (17).

Histochemical study for caspase-3 expression in liver:

The procedure was performed according to (18). Briefly, for immunohistochemical (IHC) staining, sections from the selected paraffin blocks were cut into 4 µm thick sections. The endogenous peroxidase was blocked by immersing slides in 3% H_2O_2 for 10 min at room temperature. After washing, slides were incubated with primary anti-caspase 3 antibodies. Afterwards, incubation with the appropriate secondary antibody was performed. Prior to dehydration and mounting, all slides were lightly counterstained with hematoxylin for 30 sec. Apoptotic cells were seen by microscope as cells with brown nuclei.

Immunohistochemical scoring

Cytoplasm of hepatocytes reacting to Caspase 3 was considered positive. Stained tissue sections undergo semi-quantitative analysis which accomplished through modified Allred scoring system guidelines (19). The average number of positive cells was estimated after counting them in three different high-power fields (HPF) (400 xs). Individual percentages of positive cells (0–5) and cytoplasm staining intensity (0–3) were summed to get the final grades. The percentage of positive cells was determined as follows: 1) less than 10 positive cells; 2) between 10-20 positive cells; 3) between 20-50 positive cells; 4) between 50-70 positive cells; and 5) for more than 70 positive cells. The staining intensity was scored as: 1- weak; 2- medium; and 3-strong.

Statistical analysis

Data were analyzed using The Graph Pad Prism software (version 9, San Diego, USA). Kruskal Wallis was used to determine the statistical significance of the various groups. Dunn's multiple comparisons test, post hoc test was performed for in-between groups' comparison. ($P < 0.05$) was selected as the significance level. The data is displayed as mean ± standard error.

Results

Effect of Lycopene and aflatoxin on growth performance

(Table 1), Non-significant ($P > 0.05$) increase was detected in FI throughout the experimental trial. Nonetheless, birds fed dietary AFB_1 showed a reduction in AWG ($P < 0.05$) and increase in FCR as compared to the group fed the basal diet. Japanese quail group fed a combination of AFB_1 and LYC exhibited an improvement in AWG compared to the group fed AFB_1 only. Besides, a decrease ($P < 0.05$) in FCR was observed in AFB_1 + LYC group compared to AFB_1 group. Moreover, feeding AFB_1 supplemented with LYC improved BW (+14.72%) and BWG (+25.66%) compared to the AFB_1 treated group.

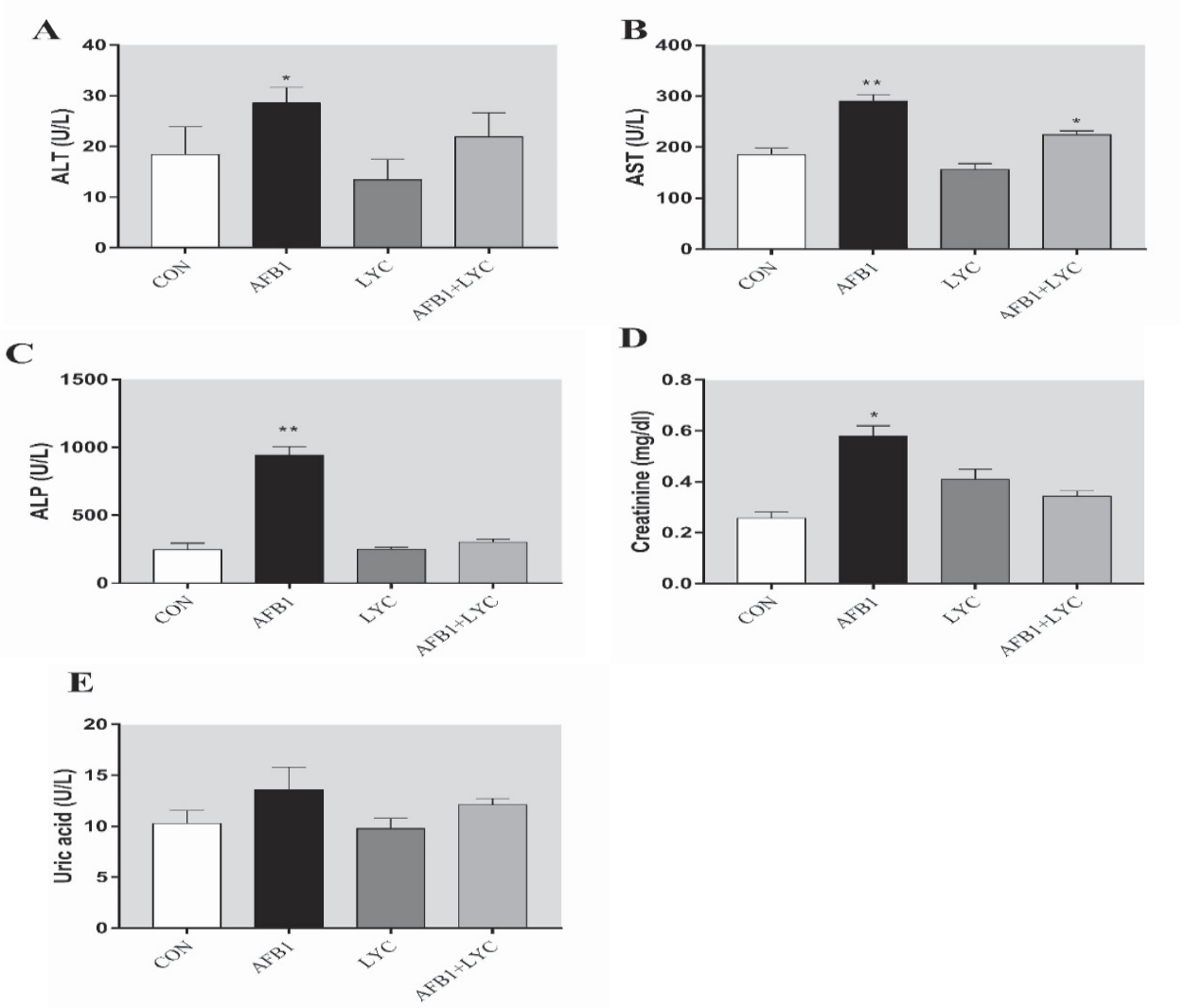


Figure 1: Effect of LYC on quails' liver and kidney functions of fed diets contaminated with AFB1. Values are represented as the mean ± SE (n = 9). Means within the column with different stars are significantly different, $p< 0.05$

Table 1: Effect of dietary supplementation of LYC and AFB1 on growth performance

Diets	Final weight(g)	Weight gain (g)	Feed intake(g)	FCR (%)
CON	297.43±8.17	177.14±11.07	270.35±5.91	1.53±0.086
AFB1	279.43±7.19 *	151.43± 5.38*	266.31±8.12	1.77±0.064*
LYC	330.57±4.81	191.71±3.508	271.51±4.93	1.41±0.024
AFB1+LYC	320.57±3.26	190.29±4.58	282.03±6.33	1.49±0.036

Values are represented as the mean ± SE (n = 15 per group). Mean values within a column with (*) were significantly different (P < 0.05). CON: control; AFB_1: Aflatoxin B1; LYC: Lycopene; FCR: feed conversion ratio

Ability of Lycopene to restore abnormal liver and kidney function enzymes caused by AFB1

As summarized in Fig. 1, AFB_1 exposure for 35 days elicited a noticeable increase in the levels of serum ALT, AST and ALP compared to the control group. Conversely, LYC co-administration decreased (P <0.05) the levels of these biomarkers in the group received dietary AFB_1. No remarkable (P > 0.05) alterations were detected in serum ALT, AST and ALP levels between the control and Lycopene groups. Whereas, AFB_1 inclusion significantly ($P< 0.05$) elevated creatinine level compared to that of the control group. Thus, LYC addition improved($P<0.05$) kidney function, as evidenced by the decreased creatinine level when compared to the AFB_1 group.

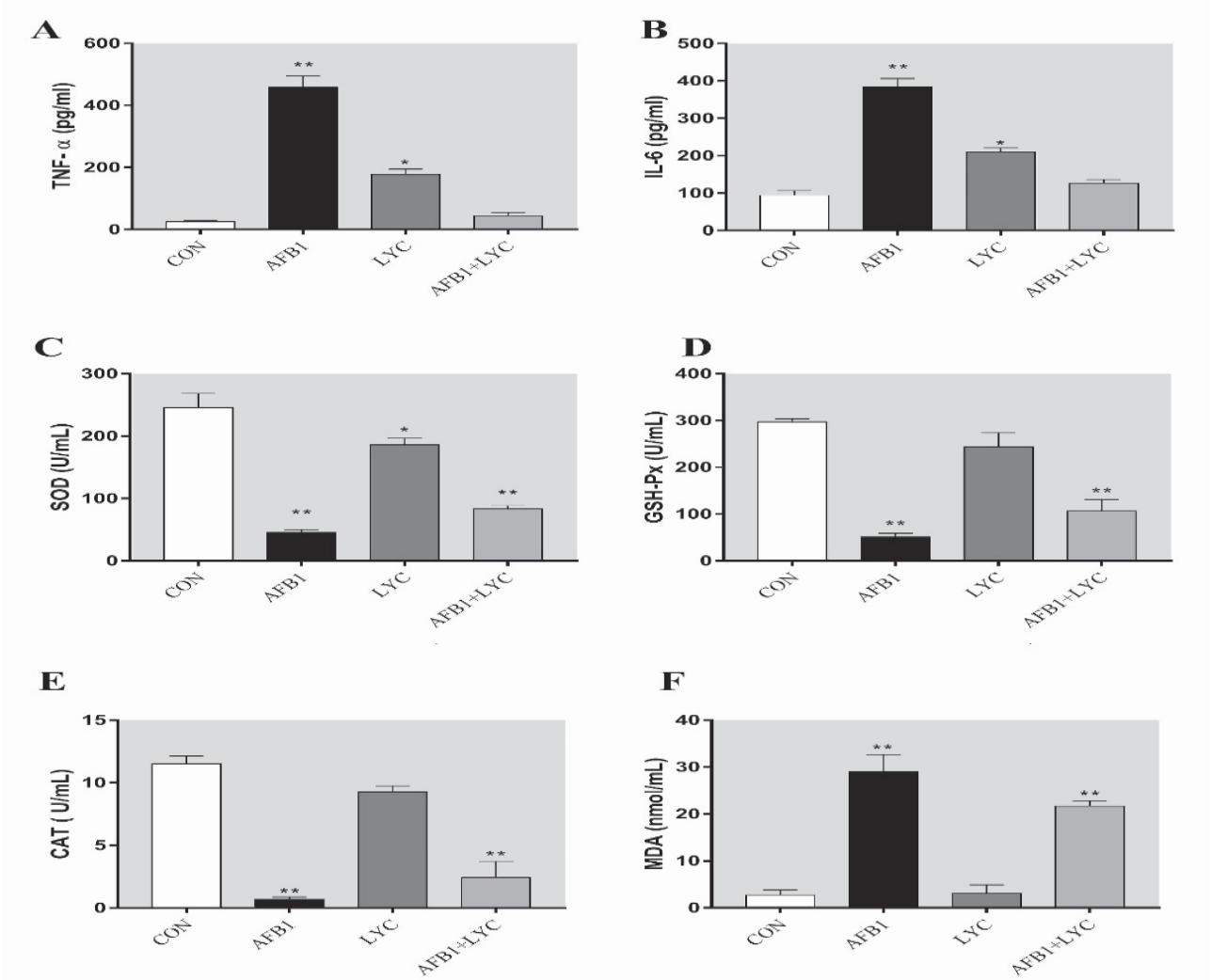


Figure 2: Effects on AFB1-induced liver oxidative stress markers and serum inflammatory cytokines. Values are repre-sented as the mean ± SE (n = 9). *column with different superscript letters were significantly different ($p < 0.05$). AFB1: aflatoxin B1); LYC: Lycopene; SOD: total superoxide dismutase; MDA: Malondialdehyde; GSH-Px: glutathione peroxidase; CAT: catalase, IL6: interleukin-6; and TNF-*a*: Tumor necrosis factor-alpha

Lycopene reversed AFB1-induced Inflammatory Response

As depicted in Fig. 2, birds exposed to AFB_1 showed a marked increase ($P < 0.05$) in the serum levels of IL-6 and TNF-α compared to the control group. Remarkably, supplementation of LYC reduced the elevation of IL-6 and TNF-α triggered by AFB_1. Hence, the results indicated that LYC could alleviate the negative influences evoked by AFB_1 on the inflammatory response.

Lycopene inhibited AFB1-induced oxidative injury

In order to assess the extent of oxidative damage, MDA,CAT,GSH-Px and SOD were measured in liver tissues. Compared to the control group, the activities of CAT, GSH-Px and SOD enzymes were significantly reduced in groups received the AFB_1-contaminated diet ($P< 0.05$), whereas the MDA levels increased ($P< 0.05$) in the AFB_1-fed group compared to those in birds fed the control diet. Notwithstanding, feeding a diet containing LYC (200 mg/kg) to AFB_1-fed quail could significantly mitigate the reduction in CAT, GSH-Px, and SOD levels and the increase in MDA level ($P< 0.05$). The results confirmed that LYC could prevent the oxidative injury of liver tissues provoked by AFB_1 as displayed in Fig. 2.

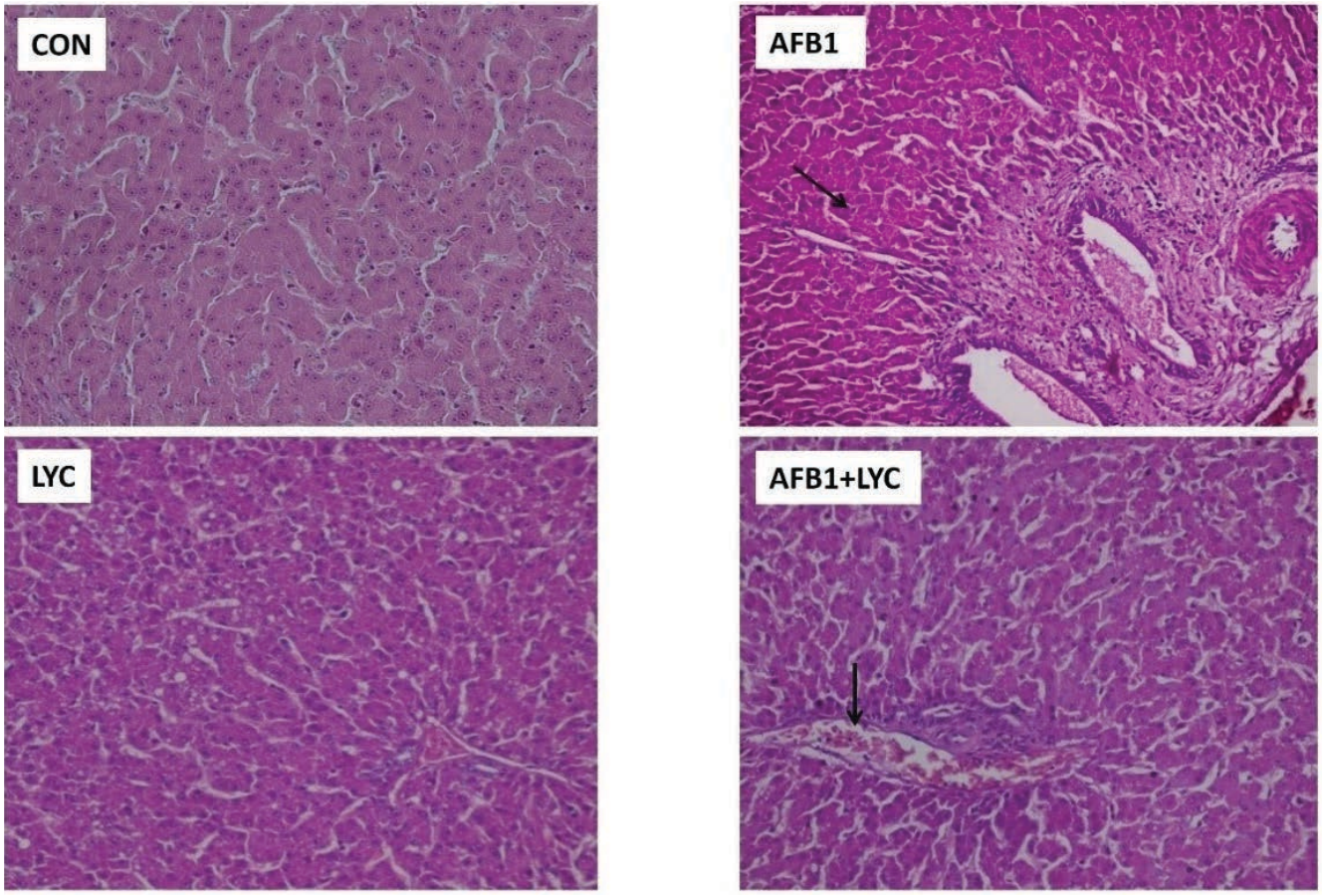


Figure 3: H&E-stained liver sections of CON Control quails showing normal histological appearance of the liver, AFB1 Hepatic tissues of quails treated with aflatoxin revealing marked fibrosis of portal traid area associated with focal dilation of hepatic sinusoids (arrow) and LYC+AFB1 aflatoxin -induced quails treated with Lycopene revealing almost normal portal traid area associated with moderate dilation of hepatic sinusoids (arrow). (H&E, X400)

Table 2: Effect of dietary supplementation of LYC and AFB1 on immunohistochemical staining of the liver sections

Item	Control	Lycopene	AFB1	AFB1+ Lycopene
Average number of positive cells ± SD	23 ± 4.5 3	27 ± 8.5 3	109± 20.5 5	53.3± 7.6 4
Intensity of staining	Weak 1	Weak 1	Strong 3	Moderate 2
Total score	3	3	> 5	4

Histopathological observation

As depicted in Fig. 3, the control and LYC (200 mg/kg) treated group showed no marked histopathological lesions in the liver of Japanese quail. Conversely, the liver sections from the AFB_1 group, showed severe histopathological alterations ($P< 0.05$), including marked fibrosis of the portal triad area associated with focal dilatation of hepatic sinusoids compared with those from quail fed the control diet. Noteworthy, the supplementation of LYC (200 mg/kg) to AFB_1 diet counteracted the hepatic injury evoked by AFB_1. Moreover, histological findings assured the beneficial effect of LYC on ameliorating the toxic impacts triggered by AFB_1 in Japanese quail.

Effect of Lycopene treatment on caspase-3 expression

To elucidate how LYC protects the liver from AFB_1 damage, the proapoptotic protein expression level of caspase-3 was estimated by the im-

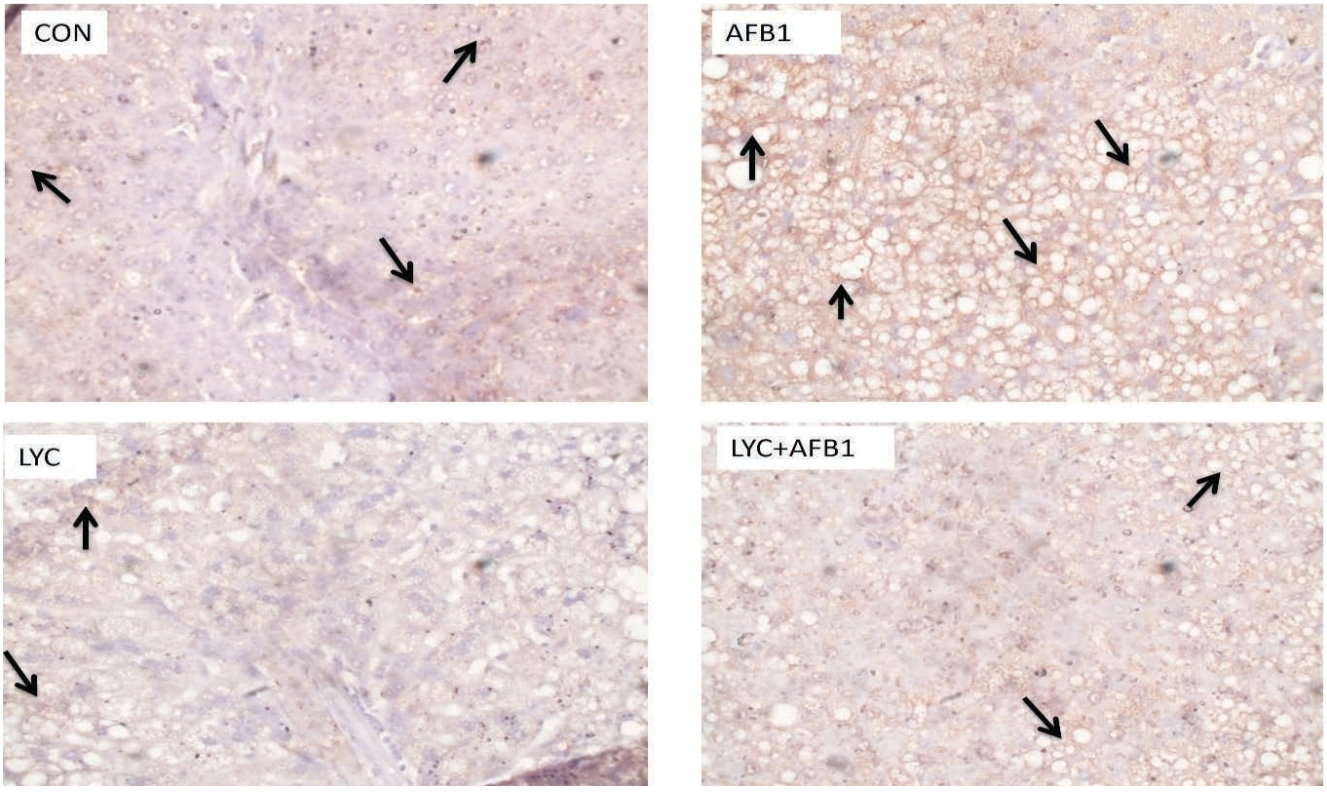


Figure 4: The immunohistochemical staining of the liver sections, positive cells appeared as brown cells (Black arrows). In CON group Scattered uniform hepatocytes show weak positive reaction to caspase 3 antibody, In AFB1 group hepatocytes show weak positive reaction to caspase 3 antibody, In LYC group hepatocytes show strong positive reaction to caspase 3 antibody and in LYC+AFB1 showed Significant reduction in number of cells and intensity of reaction to caspase 3

munohistochemical method. The results revealed a marked increase ($P < 0.05$) in caspase-3 expression level in the liver tissues in the AFB_1 group compared to that in the control group as shown in Fig. 4 and Table 2.

On the other hand, co-treatment of LYC with AFB_1 decreased the expression of caspase-3 than that in the AFB_1diet (Fig. 4).

Discussion

The dangers of mycotoxin contamination in feed resources are still an unavoidable problem, which should be taken into account to avoid its adverse effects on both humans and animals. The present study evidently demonstrated the influence of LYC on hepatotoxic and nephrotoxic implications of AFB_1, which were expressed as impaired growth rate, increased the activities of AST, ALP, and ALT; while the activities of CAT, GSH-Px, SOD as well as the activities of apoptotic protein caspase-3 were enhanced. Our data recorded a significant reduction in body weight (-6.05%) and BWG (-14.51%) in AFB_1-challenged quail against to the control group. The negative impact of AFB_1 on growth performance could be ascribed to the changes induced by AFB_1. AFB_1 could contribute to altering the intestinal morphology, absorbing function, decreasing the activity of digestive enzymes as (pancrelipase, amylase, and trypsin) and modifying the cellular energy metabolism by disturbing the gluconeogenesis and fatty acid synthesis (20). These findings match those observed in the previously published results of (21), which revealed that AFB_1 consumption has detrimental influences on the growth rate, evidenced by reduced WG and increased FCR in mice. Children exposed to aflatoxin toxicity not only suffered from serious health problems as growth impairment and stunted growth, but also died from infectious diseases, lower school achievements, decreased life-time earnings, and productivity (22).In our study, the inclusion of Lycopene (200 mg/kg) into the AFB_1 contaminated diets improved body weight (+14.72%) and BWG

(+25.66%) when compared with those in the AFB_1 group. Substantially, some authors have observed that dietary administration of LYC in chicken diets not only increased ADG but also could relieve the deleterious effects due to stress conditions on growth performance(23).The study of (24) showed that chronic aflatoxin exposure in African children is closely related to low birth weight, stunted growth, immunosuppression and impaired liver function. Moreover, LYC co-treatment in quail's diets improved the weight gain and FCR even under stress conditions (25). The observations of the current work are similar to those of (20) who reported that dietary LYC administration (200 and 400 mg/kg) significantly improved the intestinal digestive enzymes (amylase and lipase) activities in broiler chicks received AFB_1 diet. The possible explanation for the increased enzyme activities could be attributed to improving the intestinal morphology and integrity by dietary LYC supplementation, thereby, improving the growth performance of birds challenged with AFB_1. The increased activities of AST, ALT and ALP in serum have been known as crucial pointers for the diagnosis of hepatocellular damage or dysfunction, or bile duct obstruction (26). Therefore, in the current study, quail chicks fed AFB_1 contaminated diet had elevated levels of AST, ALP, and ALT, indicating liver injury. This could be attributed to the fact that AST and ALT, which are normally found in the cytoplasm, are only released into the bloodstream when the structural integrity of the liver is altered, resulting in enzyme leakage (27). In our study, the histopathological picture of the liver in AFB_1 challenged quail showed destroyed hepatic structure and hydropic/fatty degeneration which confirmed the increase in ALT, AST and ALP activities. However, these histopathological and biochemical alterations were reversed by the addition of LYC to the quail diet. These findings agree with the findings reported by (28) indicating that LYC supplementation provides significant protection against AFB_1-induced liver injury. Moreover, our findings revealed an increment in the levels of creatinine and uric acid, which act as indices of impaired renal functions. This result is similar to previous studies conducted on broilers (29)and quail (30)following ingestion of AFB_1 diets. Prolonged exposure to toxins poses potential risks to the body's health. AFB_1 toxicity causes hepatic injury, which in turn leads to liver cirrhosis and even cancer in severe cases (31). On the other hand, our data showed that co-supplementation of LYC to the AFB_1-treated diet significantly improved the negative impacts of AFB_1 on the liver and kidney. Supporting these results, the previous study of (34) stated that LYC inclusion substantially alleviated aflatoxin (AF) induced nephrotoxicity and hepatotoxicity. The CAT, GSH-Px and SOD enzymes have an essential role in the antioxidant defense system due to acting as scavengers for free radicals produced by oxidative stress, reducing oxidative damage, and maintaining cell structure (33). The MDA content acts as a key indicator for assessing the potential antioxidant capacity of the body (34). Therefore, the degree of oxidative damage can be evaluated by estimating the activity of antioxidant enzymes (CAT,GSH-Px and SOD) and the content of MDA. Our data revealed that ingestion of AFB_1 decreased the activities of CAT,GSH-Px and SOD, but elevated the MDA content. Our results are in line with those of previous study of(35) who concluded that AFB_1 administration provoked oxidative stress and prohibited the antioxidant enzymes activities in broilers. Our data recorded that LYC inclusion in the AFB_1-treated diet significantly lowered MDA concentration and increased the CAT,GSH-Px and SOD activities, demonstrating that AFB_1-induced oxidative stress could be overcome by LYC administration. In this regard, several studies strengthen the protective efficacy of LYC against oxidative stress caused by different toxic agents mainly through augmenting the antioxidant capacity of SOD and CAT, rather than the inhibiting ROS generation (11).

AFB_1 up regulated the activities and mRNA expression of caspase-3 and caspase-9. These findings implied that $AFB_1$is implicated in excessive oxidative damage to the liver in mice (36). However, these negative impacts could be alleviated by LYC supplementation. Therefore, one of the pivotal mechanisms of LYC against AFB_1 immunotoxicity is the anti-apoptotic effect (37). Consequently, Lycopene up regulated anti-apoptotic Bcl-2, lowered the expression of proapoptotic Bax, and suppressed the activation of caspase-3 (38).From the present study, it is interesting to note that AFB_1 treatment showed a significant increment in tumor necrosis factor-α (TNF- α) and serum IL-6 levels as previously mentioned by (39).Conversely, the enhancement of the serum pro-inflammatory cytokines was significantly relieved by co-administration with

Lycopene as compared with the AFB_1-treated group (39). Our data confirm previous findings suggested by (37)proving that LYC revealed the potent immunoenhancing activity and ability to decrease TNF-α and IL-6 expression. Furthermore, the ameliorative effect of LYC against AFB_1-induced immunotoxicity may be partially ascribed to the direct capturing of free radicals and stimulating the antioxidant capacity.

Conclusion

This study revealed that Lycopene supplementation could protect livers from AFB_1-triggered injury. Moreover, LYC counteracted hepatotoxicity by diminishing hepatic oxidative damage and suppressing apoptosis. Thus, Lycopene may be a promising candidate for alleviating the toxic effects of aflatoxin.

Acknowledgement

The authors declare that there is no conflict of interest.

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Original Research Article
Veterinary Medicine and The One Health Concept

MELOXICAM-INDUCED GASTROPATHY IN DOGS: CLINICAL, HEMATO-BIOCHEMICAL, ENDOSCOPIC FEATURES AND TRIALS FOR PREVENTION

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Abstract: This study was conducted to evaluate and to compare the clinical, hemato-biochemical and endoscopic aspects of gastropathy in dogs treated with meloxicam alone or incombination with esomeprazole and misoprostol. Twenty baladi healthy dogs were included in the experimental study. Dogs were divided into four groups each group consisting of five animals; Group I (control group), the group that does not receive any medication. Meloxicam treated groups divided into: Group II which received meloxicam at a dose of 0.2 mg/kg BWT per OS /24 hr. Group III animals received the same previous dose of meloxicam and esomeprazole at a dose of 1mg/kg BWT per OS /24 hr. Group IV animals received the same dose of meloxicam and misoprostol 3µg /kg BWT per OS tid. Upon drug administration, dogs were kept under observation for 14 consecutive days. Clinical and hemato-biochemical analysis were evaluated across time (T0, T3, T7, T10 and T14). The image analysis of the gastroscopic examination was evaluated across time (T0, T7 and T14), endoscopic examinations were applied to all animals in four groups at three time points (T0, T7, and T14), endoscopic lesions were scored by use of a 5-point scale. Clinically, the most common clinical sings in dogs with Meloxicam induced- gastropathy were inappetence to anorexia, hematemesis, melena, abdominal pain and weakness, the specific endoscopic lesions of gastropathy were gastric erosion, hemorrhage and ulcers. Serum gastrin concentration is a biochemically sensitive indicator of gastropathy. The overall results concluded that meloxicam-induced gastropathy was more severe in group II compared to groups III and group IV. The proton pump inhibitor (esomeprazole) was more effective and better tolerated than misoprostol.

Key words: endoscopy; esomeprazole; gastropathy; meloxicam; misoprostol

Introduction

There have been numerous reports of nonsteroidal anti-inflammatory drug (NSAID)-related gastric abnormalities in humans, known as NSAID-gastropathy. Gastritis and gastropathy are conditions that affect the gastric mucosa. In gastritis, the stomach lining is inflamed and in gastropathy, the stomach lining is damaged, but with little or no inflammation (1). NSAID-gastropathy is characterized by subepithelial hemorrhages, erosions, and ulcers (2). NSAID are commonly used in human and animal treatment. Its adverse effects widely involve the gastrointestinal tract, the most important adverse effect is development of ulcers especially in the stomach, and the presence of such lesions is often unpredictable because the clinical signs may be missed until the complication develops (3).

Meloxicam is a new and the most commonly prescribed NSAID for canine in Egypt. It is a potent inhibitor of prostaglandin synthesis that has anti-inflammatory, analgesic, and antipyretic properties (4, 5). Meloxicam has slightly greater activity against COX-2 than against COX-1(6). Because of preferential COX-2 inhibition, meloxicam was superior regarding postoperative pain control in dogs (7). Along with the desired effects of meloxicam, the systemic inhibition of

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PGE2 production inhibits the normal mechanisms of the gastric mucosa protection. In the absence of the protective mechanisms, the stomach acidic environment damages gastric mucosa leading to gastropathy (8). Many reports were recorded about gastric erosion, ulceration or even perforation associated with meloxicam administration in dogs (9,10,11).

The diagnosis of canine gastropathy is based on the history, clinical, hemato-biochemical, radiography, and ultrasonography (12). There is no correlation between NSAID gastropathy and upper abdominal symptoms frequently. So, medical endoscopy has emerged as a key technology for minimally-invasive examinations for gastropathy. Using endoscopy, gastroduodenal lesions are identified including subepithelial hemorrhages and erosions not only ulcers (10,13) Gastroprotectants are drugs used in veterinary medicine secondary to administration of NSAIDs including meloxicam. Because of the various benefits and good efficacy of NSAIDs, many drugs have been developed to decrease gastric acid secretion and/or promote mucosal protective defenses to prevent and treat ulcerations. The most commonly used drugs are the prostaglandin analogues such as misoprostol, which is an antiulcer drug, and proton pump inhibitors, as esomeprazole whose efficacy in preventing NSAID-associated ulcers has been recently demonstrated (14). Therefore, the aims of this study are to evaluate and compare the clinical, hemat-biochemical and endoscopic aspects of gastropathy in dogs treated by meloxicam alone or coadministered with esomeprazole and misoprostol.

Material and methods

Ethical statement

All the experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), Zagazig University (Approval No. ZU-IACUC/ 2/F/18/2022).

Experimental design, animal management and feeding regime.

The experiment was carried out at the experimental animal unit of the Faculty of Veterinary Medicine, Zagazig University, Egypt. This study was applied on 20 male baladi dogs their body weight (20-25 kg BWT) and age ranged from 8-12 months. On admission physical examination was conducted for all animals. The animals were housed for 15 days to acclimatize and were monitored during this period. All dogs were vaccinated and treated with an appropriate anthelmintic (praziquantel [Drontal], 5 mg/kg PO). All groups were reared in uniform nutritional and management conditions. They were supplied with adlibitum diet (meat and chicken extract) and had free access to water.

Drug used

- Meloxicam (tablets, 7.5 mg).

Commercial name; Medexaflam 7.5 mg (GPI pharma - EGYPT). Dose: (0.2 mg/kg BWT) per os /24 hr. (9).

- Esomeprazole (capsule, 40 mg), Proton pump inhibitor

Commercial name: Esmorap 40 mg (AUG pharma -EGYPT). Dose: (1mg/kg BWT)/24hr per os (15).

- Misoprostol (tablets, 200 µg).

Commercial name: Misotac tablet 200 µg (Sigma pharma -EGYPT). Dose: (3µg /kg BWT) 3 times daily orally (16).

Animals grouping

After passing the acclimatization period, the dogs divided randomly into four groups each consisting of five animals.

- Group I (control group).
- Group II (Meloxicam)
- Group III (Meloxicam+ Esomeprazole)
- Group IV (Meloxicam+ Misoprostol)

Clinical examination

Clinical examination and vital signs monitoring were thoughtfully performed for all animals in the four groups at different time periods (T0, T3, T7, T0 and T14) with closed daily observation to any clinical abnormalities.

Hemato-biochemical screening

Two blood samples were collected from each animal in the four groups at five different time

points (T0, T3, T7, T0 and T 14) via cephalic vein puncture. For hematological studies, 2 mL of blood was taken into (k2 EDTA) tubes, red blood cells (RBCs) count, hemoglobin (Hb) concentration, packed cell volume (PCV), platelets count, total leucocytic counts and neutrophil percent were measured using full version automatic cell counter (Sysmex KX-21N, Japan) according to the method of (17). The other blood sample of 5 ml was taken in plain tubes without anticoagulant for serum separation for making biochemical analysis. Serum total protein and albumin were determined spectrophotometrically by standard procedures using (Diagnostic Zrt. Commercial kits) provided by Biomerieux, Egypt. Serum gastrin was performed via the GASTRIN (125 I) Radioimmunoassay Kit (MP Biomedicals, USA). According to (18). Serum IL1 Beta was estimated by using a quantitative sandwich enzyme immunoassay technique according to the manufacture of CUSABIO.

Endoscopic examination Preparation of animals

A more updated protocol was applied; the food was withheld for 24 hrs and water was withheld 6 hrs before anesthesia (19). Atropine sulfate (MISR CO.EGYPT) at a dose 0.05mg/kg SC and 2% xylazine HCL (ADWIA, 10th of Ramadan City, Egypt) at a dose of 0.5 mg/kg BW I/M.

The endoscopy procedure

All dogs were examined by endoscopy at 3 time points (T0, T7 and T14). At T0 to assess the presence of any visible lesions before the experiment (19,20).The dogs were anaesthetized for endoscopic examination, general anesthesia was induced with I/V injection of thiopental sodium 2.5% (Thiopental Sodium, EIPICO, 10th of Ramadan city) at a dose of 25 mg/kg BW. The upper gastrointestinal tract was examined using a Porta scope endoscope (PVSM3M, Florida), the endoscopic examination being the same on all occasions starting by the esophagus, followed by the stomach body, the antrum/body junction and the antrum and then duodenum. The images of the mucosa at these sites were videotaped. Presence of any erosions, hemorrhage and/ or ulcerations were recorded (20). A subjective endoscopic severity scoring system has been established in which a stomach with no visible lesions is scored as 0; a stomach with a few submucosal petechia but no visible defects in the mucosa is scored as 1, a stomach with few erosions is scored as 2, stomach with extensive areas of erosions is scored as 3, and a stomach with an ulcer of any size is scored as 4 modified from (21).

Statistical analysis

Data of hemato-biochemical parameters were statistically analyzed with one-way analysis of variance (ANOVA) using a statistical software program (SPSS for Windows, version 16.0, SPSS Inc., Chicago, IL). Duncan's post-hoc test was used to determine the level of significance between the four groups. Results were expressed as means ± SE and were considered statistically significant when $p<0.05$.

Results

Clinical Findings:

Clinical findings were recorded daily during the experiment. The reported findings are presented in Table 1. In group II, the recorded findings include mild inappetence and anorexia, hematemesis, occult blood, melena, signs of colic and abdominal pain manifested as arching of the back and sitting on the hindquarters, congested mucous membrane, dullness and depression, weakness and emaciation, shock, distended abdomen and death in 1 dog. In group III, only inappetence is observed in 1 dog while in group IV, transient diarrhea, inappetence and occult blood were recorded. Regarding vital signs, no remarkable changes were recorded in body temperature, pulse, and respiratory rates and their values were within the reference range in the four groups at different time points except 1 dog, the dog was febrile, tachycardic and tachypneic and hypothermic. It is worthy to mentioned that 1 dog with a gastric ulcer showed mild clinical signs

Hemato-biochemical findings:

The results of hematological and biochemical parameters are shown in Table. 2, 3 there were a significant decrease ($P<0.01$) in Hb, RBCs and PCV were recorded in group II at T10 when compared with other groups, whilea significant decrease

Table 1: Clinical findings were recorded daily during the experiment in the control and the three experimental groups

Items		Control group Group I % (N/5)	Experimental groups %(N/T)		
			Group II (n=5)	Group III (n=5)	Group IV(n=5)
Diarrhea	%(N/T)	0% (0/5)	0% (0/5)	0% (0/5)	60 % (3/5)
	Onset	----------	----------	----------	4 th to 7 th day
Inappetence to anorexia	%(N/T)	0% (0/5)	60% (3/5)	20% (1/5)	40 % (2/5)
	Onset	----------	7 th to 14th day	14t th day	12 th day
Hematemesis	%(N/T)	0% (0/5)	20% (1/5)	0% (0/5)	0% (0/5)
	Onset	----------	8 th day	----------	---------
Occult blood* or melena	%(N/T)	0% (0/5)	60% (3/5)	0% (0/5)	20% (1/5)
	Onset	----------	8 th day	----------	12 th day
Congested mucous membrane	%(N/T)	0% (0/5)	20% (1/5)	0% (0/5)	0% (0/5)
	Onset	----------	9th day	--------	---------
Dehydration	%(N/T)	0% (0/5)	20% (1/5)	0% (0/5)	0% (0/5)
	Onset	----------	9 th day	----------	---------
Dullness and depression	%(N/T)	0% (0/5)	20% (1/5)	0% (0/5)	0% (0/5)
	Onset	----------	9 th day	---------	-------
Weakness &emaciation	%(N/T)	0% (0/5)	40% (2/5)	0% (0/5)	0% (0/5)
	Onset	----------	9 th day	----------	---------
Shock	%(N/T)	0% (0/5)	20% (1/5)	0% (0/5)	0% (0/5)
	Onset	----------	10th day	----------	----------
Colic signs	%(N/T)	0% (0/5)	40% (2/5)	0% (0/5)	20% (1/5)
	Onset	----------	9 th and 11th to 14 day	---------	11 th day
Distended abdomen	%(N/T)	0% (0/5)	20% (1/5)	0% (0/5)	0% (0/5)
	Onset	----------	10 th day	--------	---------
Death	%(N/T)	0% (0/5)	20% (1/5)	0% (0/5)	0% (0/5)
	Onset	----------	11 th day	---------	---------

($p<0.05$, $p<0.01$ and $p<0.01$) respectively at T14 was recorded in the three experimental groups in comparison with control group, with the lowest value in the meloxicam treated group. Platelets was significantly decreased ($P<0.05$) at T7 in group II in comparing with groups I, III and IV. While, at T10 and T14 a highly significant decrease ($P<0.01$) was recorded in the three experimental groups in comparison with control group with the lowest value in group II. Regarding WBCs, significantly increased ($P<0.01$) at T10 and T14 in the meloxicam treated groups with the highest value in group II, significant increase in WBCs ($P<0.05$) at T10 was recorded in group II in comparing with group I, III, IV. While a highly significant increase ($P<0.01$) at T 14 was recorded in the three experimental groups in comparison with control group with the highest value in group II.

Serum gastrin recorded a significant increase ($P<0.05$ and $P<0.01$) at T3 and T7 in group II in comparison with the remaining groups. A highly significant increase ($P<0.01$) in serum gastrin was recorded at T10 and T14 n the three experimental groups in comparison with control group with the highest value in the meloxicam treated group. Serum IL-1β was significantly increased ($P<0.05$ and $P<0.01$) respectively at T10 and T14 in the three experimental groups in comparison with control group with the highest value in group II. TNF-α recorded significant increase ($P<0.05$) at T3 in group II in comparison with group I, III and IV, while at T7, T10 and T 14 a highly significant increase in TNF-α ($P<0.01$) in the three experimental groups in comparison with control group. A significant decrease ($P<0.05$) in total protein was recorded at T4 in comparison

Table 2: Means and SE of hematological parameters in control and experimental group at five time points

Items		Group 1 (control)	Experimental groups			P-value
			group 2 (Meloxicam)	group 3 (Melox.+ Esmop.)	group 4 (Melox.+ Misop.)	
Hemoglobin (g/dl)	Day 0	15.17 ± 1.041	15.37 ± 0.651	15.04 ± 1.178	15.53 ± 1.301	**0.943**
	Day 3	15.23 ± 0.681	15.52 ± 0.912	15.02 ± 1.433	15.34 ± 1.634	**0.967**
	Day 7	15.37 ± 0.862	13.87 ± 1.629	14.99 ± 1.770	15.27 ± 1.966	**0.663**
	Day 10	15.20 ± 0.755^{a}	11.24 ± 0.966^{b}	14.81 ± 1.409^{a}	14.52 ± 0.951^{a}	**0.006**
	Day 14	15.00 ± 1.058^{a}	11.03 ± 1.750^{b}	12.53 ± 0.586^{b}	12.51 ± 1.115^{b}	**0.023**
RBCs (cell/ microliter)	Day 0	6.167 ± 0.404	6.100 ± 0.265	6.000 ± 0.500	5.967 ± 0.306	**0.910**
	Day 3	6.067 ± 0.551	5.967 ± 0.764	5.967 ± 0.351	5.767 ± 0.551	**0.930**
	Day 7	5.900 ± 0.819	5.367 ± 0.850	5.933 ± 0.551	5.667 ± 0.651	**0.765**
	Day 10	6.100 ± 0.361^{a}	4.040 ± 0.505^{b}	5.867 ± 0.351^{a}	5.400 ± 0.400^{a}	**0.001**
	Day 14	6.233 ± 0.709^{a}	4.167 ± 0.252^{b}	4.667 ± 0.231^{b}	4.467 ± 0.709^{b}	**0.006**
PCV (%)	Day 0	46.33 ± 4.163	45.33 ± 4.163	48.00 ± 3.606	47.33 ± 4.163	**0.858**
	Day 3	47.67 ± 3.512	44.67 ± 3.786	47.67 ± 7.095	47.00 ± 4.583	**0.860**
	Day 7	46.00 ± 6.557	39.67 ± 5.033	46.00 ± 3.606	46.67 ± 4.726	**0.353**
	Day 10	47.00 ± 4.359^{a}	29.67 ± 4.509^{b}	44.00 ± 2.646^{a}	46.00 ± 3.606^{a}	**0.002**
	Day 14	47.00 ± 3.606^{a}	29.33 ± 1.528^{c}	38.67 ± 3.055^{b}	36.67 ± 1.528^{b}	**0.000**
WBCs (cell/ microliter)	Day 0	7.667 ± 0.473	7.833 ± 1.097	7.610 ± 0.609	7.870 ± 0.814	0.970
	Day 3	7.867 ± 0.907	7.767 ± 1.290	7.700 ± 0.572	7.967 ± 0.764	0.985
	Day 7	7.833 ± 1.102	8.433 ± 0.808	8.000 ± 1.249	8.067 ± 0.586	0.891
	Day 10	7.933 ± 0.850^{b}	11.18 ± 1.301^{a}	9.167 ± 0.764^{b}	9.313 ± 0.697^{b}	0.018
	Day 14	7.767 ± 1.002^{c}	13.90 ± 0.715^{a}	10.87 ± 0.808^{b}	11.27 ± 1.102^{b}	0.000
Neutrophils (%)	Day 0	60.67 ± 7.638	61.33 ± 2.517	60.30 ± 7.292	62.33 ± 9.074	0.985
	Day 3	61.33 ± 8.083	63.00 ± 5.000	64.33 ± 10.02	64.04 ± 8.203	0.966
	Day 7	61.67 ± 11.59	62.50 ± 7.365	66.67 ± 8.737	65.10 ± 8.350	0.899
	Day 10	61.00 ± 10.15	77.67 ± 5.859	70.33 ± 5.686	66.94 ± 7.111	0.121
	Day 14	61.67 ± 8.327^{b}	83.33 ± 5.033^{a}	78.00 ± 6.000^{a}	80.33 ± 5.132^{a}	0.012

with group I, III, IV. and albumin and at T7, T10 a significant decrease (P<0.05) in albumin and at T7, T10 was recorded in group II when compared with group I, III and IV while at T 14 a significant decrease (P<0.01), was recorded in albumin in the three experimental groups in comparison with control group with the lowest value in group II.

Endoscopic findings:

Table 4. and Figure 1 (a-e) explain the endoscopic features in this study. In group I, a normal endoscopic image showed gastric rugal folds of the stomach, gastric mucosa thrown into folds, folds larger on the greater curvature than on the lesser curvature, and no visible lesions.. The same previously-described picture was observed in the stomachs of groups II, III &IV at T0 and also at T7 in groups III &IV. In our endoscopic evaluation gastric mucosal injuries including five endoscopic severity scores have been reported rapidly at T7 in group II, and maximal damage appeared within T7 to T14in group II including one perforated ulcer in pylorus that causing death of the at T11, also, in group II, the endoscopic severity scores of 3 and 4 at T14 were 50% and 25%. Both esomeprazole and misoprostol co-treatment recorded gastric protection in T7 in groups III and IV and the stomach mucosa was entirely normal, so esomeprazole with endoscopic severity score of 0% in both groups, while endoscopic severity scores of 3 and 4 at T 14 were 20% and 0% respectively in group III and 60% and 0% respectively in group IV. The site of gastric lesions in meloxicam treated groups (II, III, IV) were the antrum, and the lesser curvature and frequently the pylorus.

Table 3: Means and SE of biochemical parameters in control and experimental group at five time points

Items		Group I (control)	Experimental groups			P-value
			group II (Meloxicam)	group III (Melox.+ Esmop.)	group IV (Melo+ Misop.)	
Gastrin (ng/l)	Day 0	29.33 ± 2.517	28.00 ± 5.568	27.67 ± 3.512	28.33 ± 2.082	0.949
	Day 3	30.67 ± 2.082^{b}	39.00 ± 3.606^{a}	29.33 ± 4.041^{b}	33.33 ± 3.512^{ab}	0.034
	Day 7	30.67 ± 3.512^{b}	147.0 ± 18.73^{a}	37.90 ± 3.381^{b}	39.00 ± 3.606^{b}	0.000
	Day 10	30.00 ± 2.646^{c}	265.3 ± 46.44^{a}	74.33 ± 2.517^{b}	84.00 ± 4.000^{b}	0.000
	Day 14	28.67 ± 4.041^{c}	379.5 ± 53.50^{a}	106.7 ± 7.024^{b}	111.3 ± 13.38^{b}	0.000
IL1B (Pg/ml)	Day 0	401.7 ± 17.95	395.3 ± 68.19	407.3 ± 30.99	399.3 ± 60.93	0.992
	Day 3	397.0 ± 59.27	455.3 ± 87.65	420.3 ± 62.85	438.0 ± 66.34	0.769
	Day 7	405.0 ± 34.39	471.0 ± 72.63	441.3 ± 18.93	429.3 ± 35.92	0.399
	Day 10	393.3 ± 60.21^{b}	576.3 ± 46.80^{a}	495.3 ± 27.10^{a}	520.7 ± 51.73^{a}	0.010
	Day 14	406.7 ± 62.74^{b}	643.0 ± 44.00^{a}	569.3 ± 40.15^{a}	609.7 ± 34.82^{a}	0.001
TNF-α (Pg/ml)	Day 0	13.67 ± 2.517	13.37 ± 2.219	13.33 ± 1.528	13.73 ± 2.013	0.993
	Day 3	12.83 ± 1.041^{b}	19.67 ± 2.930^{a}	16.50 ± 2.179^{ab}	15.33 ± 2.082^{b}	0.028
	Day 7	13.00 ± 2.000^{c}	68.00 ± 5.568^{a}	31.00 ± 3.606^{b}	28.80 ± 1.992^{b}	0.000
	Day 10	13.83 ± 2.255^{c}	89.67 ± 8.505^{a}	53.67 ± 8.145^{b}	55.27 ± 9.296^{b}	0.000
	Day 14	13.50 ± 2.646^{c}	95.33 ± 15.50^{a}	69.00 ± 12.29^{b}	82.00 ± 8.718^{ab}	0.000
Total protein (gm/dl)	Day 0	6.693 ± 0.466	6.767 ± 0.451	6.667 ± 1.193	6.870 ± 0.365	0.984
	Day 3	6.650 ± 0.797	6.780 ± 0.381	6.557 ± 0.851	6.497 ± 0.592	0.959
	Day 7	6.557 ± 0.522	6.200 ± 0.346	6.300 ± 0.400	6.193 ± 0.681	0.796
	Day 10	6.563 ± 0.566	5.220 ± 0.697	5.540 ± 0.535	5.713 ± 0.738	0.142
	Day 14	6.870 ± 0.552^{a}	5.017 ± 0.401^{b}	5.310 ± 0.332^{b}	5.383 ± 0.798^{b}	0.013
Albumin (gm/dl)	Day 0	4.067 ± 0.462	4.137 ± 0.412	4.133 ± 0.451	4.083 ± 0.284	0.995
	Day 3	4.097 ± 0.663	3.967 ± 0.252	4.067 ± 0.232	4.167 ± 0.451	0.953
	Day 7	4.093 ± 0.388^{a}	3.033 ± 0.351^{b}	3.720 ± 0.464^{ab}	3.900 ± 0.361^{a}	0.048
	Day 10	4.037 ± 0.215^{a}	2.490 ± 0.429^{b}	3.233 ± 0.603^{ab}	3.367 ± 0.404^{a}	0.016
	Day 14	4.100 ± 0.339^{a}	2.533 ± 0.126^{b}	2.883 ± 0.340^{b}	3.063 ± 0.220^{b}	0.001

Table 4: Endoscopic severity scoring of gastropathy in examined dogs.

Time points	Grade *	Group I	Group II	Group III	Group IV
0 day	0	(5/5) 100%	(5/5)100%	(5/5)100%	(5/5)100%
	1	(0/5) 0%	(0/5)0%	(0/5)0%	(0/5)0%
	2	(0/5)0%	(0/5)0%	(0/5)0%	(0/5)0%
	3	(0/5)0%	(0/5)0%	(0/5) 0%	(0/5) 0%
	4	(0/5) 0%	(0/5) 0%	(0/5) 0%	(0/5) 0%
7 day	0	(5/5)100%	(1/5) 20%	(5/5) 100%	(5/5) 100%
	1	(0/5) 0%	(1/5) 20%	(0/5) 0%	(0/5) 0%
	2	(0/5) 0%	(1/5) 20%	(0/5) 0%	(0/5) 0%
	3	(0/5) 0%	(1/5) 20%	(0/5) 0%	(0/5) 0%
	4	(0/5) 0%	(1/5) 20%	(0/5) 0%	(0/5) 0%
14 day	0	(5/5) 100%	(0/4) 0%	(1/5) 20%	(0/5) 0%
	1	(0/5) 0%	(0/4) 0%	(2/5) 40%	(1/5) 20%
	2	(0/5) 0%	(1/4) 25%	(1/5) 20%	(1/5) 20%
	3	(0/5) 0%	(2/4) 50%	(1/5)20%	(3/5) 60%
	4	(0/5) 0%	(1/4) 25%	(0/5) 0%	(0/5) 0%

*Grade: endoscopic severity scoring, stomach with no visible lesions is scored as 0; a stomach with a few submucosal petechia but no visible defects in the mucosa is scored as 1, a stomach with few erosions is scored as 2, stomach with extensive areas of erosions is scored as 2, and stomach with ulcer of any size is scored as 4

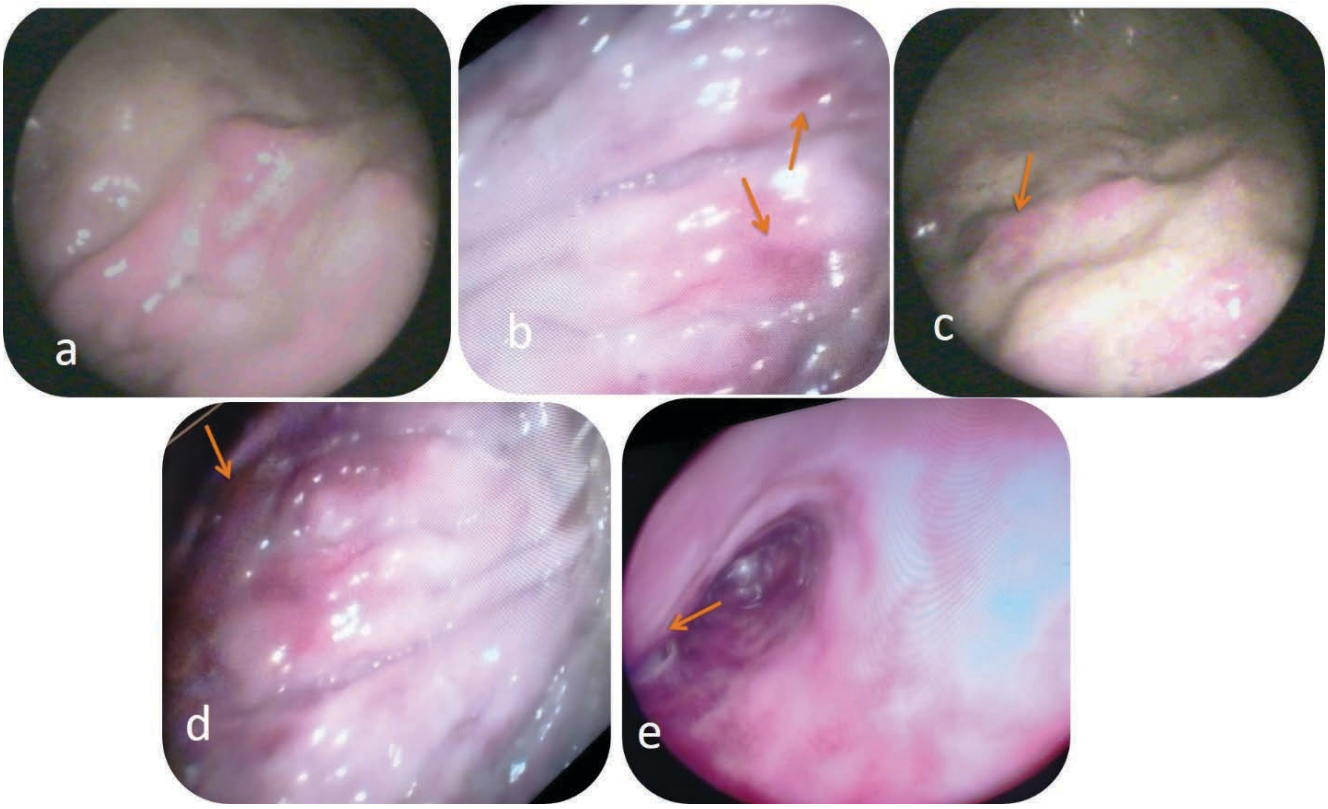


Figure 1: Endoscopic view of gastric mucosa, normal rugal folds of the stomach with no visible lesion (a, score 0), gastric mucosa with multiple petechie (b, score 1), gastric mucosa with few erosions (c, score 2), gastric mucosa with extensive erosions (d, score 3), endoscopic view of pyloric ulcer(e, score 4),

Discussion

NSAIDs are often prescribed only for short-term use in veterinary practice because of the incidence and severity of side effects with prolonged use. However, there is little information in the veterinary literature about long-term use of meloxicam (more than seven days). Gastroprotectants are widely used by veterinarians for the treatment of gastroduodenal mucosal injuries in dogs and cats. This study performed to diagnose gastropathy in dogs caused by 14-day administration, by different methods (clinical, haemato-biochemical, endoscopic, post mortem and histopathological examination).

Regarding vital signs, no remarkable changes were recorded in body temperature, pulse, and respiratory rates similarly (10,11). Except 1 dog in group II, the dog was febrile, tachycardic and tachypneic at T10 and hypothermic at T11 owing to peritonitis caused by perforated gastric ulcer similar to previous reports (9,24).

Inappetence to anorexia, abdominal pain, hematemesis, melena, weakness, emaciation which were recorded in some dogs in experimental groups were similar to previously reported studies (9-11) and in contrast to Eskafian et al. (23). Death in one dog in group II is due to peritonitis resulted from a perforated gastric ulcer (22).

The significant decrease in Hb conc., RBCs and PCV% in group II at T10and the significant decrease at T14 recorded in the three experimental groups (II, III &IV) could be attributed to the adverse effect of NSAID that causes ulceration in the gastric wall, blood loss and anemia, this result coincided with Enberg et al., (9,11).On the other hand, Dobre et al., (24) stated that the preparations of the non-steroidal anti-inflammatory drug used in dogs do not cause obvious hematological changes.

The significant increase in WBCs recorded at T10 and T14 in groups II, III and IV could be attributed to response to the inflammatory reaction in the gastric wall that occurred as a result of the destruction of the bicarbonate layer of the mucosa after inhibition of prostaglandin synthesis this result was in keeping with Elfadadny et al., (11) and disagreed with Enberg et al., (9,24). In group II, the significant increase in neutrophil% at T10

and T14 owing to sepsis resulting from ulcer formation, perforation and peritonitis. This result was agreed with Enberg et al., (9,25) who revealed that the main laboratory finding of piroxicam induced gastric ulceration was neutrophilic leukocytosis. Our results disagreed with Dobre et al., (24).

Gastrin is produced by the G-cells in the antrum of the stomach, and plays a central role in the regulation of gastric acid secretion in humans and animals (26).In our study, Serum gastrin levels in group II increased significantly at T7, T10, and T14, while serum gastrin levels in groups III and IV increased significantly at T10 and T14, consistent with previous studies (10,11,27). The marked increase in serum gastrin concentration in meloxicam treated groups is the result of impairment in the feedback mechanism between gastric acid and antral gastrin secretion. Or due to the direct relationship between the stomach lesions and G cell stimulation (27). So, serum gastrin concentration is used as a biomarker for prediction and monitoring of the severity of meloxicam-induced gastric ulcer (11).

In groups II, III and IV a significant increase in IL1B was recorded at T10 and T14 owing to the damage to the intestinal mucosa that causes microorganisms to enter the lamina propria from the lumen, which triggers an inflammatory response, thus leading to the overproduction of inflammatory cytokines (28).

The highly significant increase in TNFα in groups II, III and IV at T7, T10 and T14 may be owed to gastric damage caused by NSAIDs and leukocyte migration within the gastric microcirculation which triggers the inflammatory response, thus leading to the overproduction of inflammatory cytokines (29).

Endoscopy provides definitive diagnosis for upper gastrointestinal tract disorders, helps confirm diagnosis, and might have therapeutic use (30). Endoscopy can be used to see mucosa of digestive tract when radiography and ultrasonography imaging cannot confirm the diagnosis of such case (31).

Normal endoscopic images recorded in this study were similar to those recorded by Elfadadny et al. (10). In group II at T7 five endoscopic severity scores have been reported equally, This is due to the effect of short-term meloxicam use on gastric mucosa, particularly in the pyloric area.. this result was in agreement with previous reports (10,11,18).

In a previous study, among dogs receiving NSAIDs, six dogs developed gastric lesions on day 7 during endoscopic examination (18). In group II at T14 the endoscopic severity scores of grades 3 and 4 at T14 were 50% and 25% respectively. This result is due to the effect of long-term use of meloxicam on gastric mucosa and submucosa and in some cases musculosa (11).

In group III at T14, the endoscopic severity scores of grades 3 and 4 were 20% and 0% respectively. This is due to the protective and therapeutic action of esomeprazole (15). In group IV at T14, the endoscopic severity scores of grades 3 and 4 were 60% and 0% respectively. This result is due to the cytoprotective action of misoprostol as previous reports (16, 32). Misoprostol co-administered with meloxicam for 14 days prevented a grade 4 endoscopic severity score, but the endoscopic severity score of grades 3 was 60%. So, esomeprazole is superior and more potent to control meloxicam-inducing gastropathy than misoprostol.

Conclusion

This study provides further confirmation of the association between meloxicam use and gastropathy. Endoscopic findings of meloxicam-induced gastropathy are diverse, including gastric mucosal erosions, hemorrhage and ulcers. Giving either esomeprazole or misoprostol concurrently with meloxicam substantially reduces the risk of ulceration. Esomeprazole is more effective than misoprostol at controlling long-term meloxicam-induced gastropathy in dogs

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All of the authors contributed in the experiment design and in the writing of the manuscript. The samples were collected by GEN, SE, YHB, and, who also monitored the animals. The data was analyzed by HS and YHB, and the results were reviewed by HS. The final manuscript was read and approved by all authors.

The authors declare that they have no competing interests.

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Original Research Article
Veterinary Medicine and The One Health Concept

ANTI-CANCER ACTIVITY OF GRAVIOLA (*Annona muricata*) LEAVES EXTRACT ON INDUCED BREAST CANCER IN RATS' MODEL

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Abstract: Breast cancer is the most frequent type of invasive cancer in women. However, chemotherapy affects all cells that grow and divide quickly in the body, including cancer and normal cells. On the other hand, Graviola is commonly used as a source of food and has a wide range of bioactive components. In this study, out of 50 albino rats, breast cancer was induced in 40 rats using 7,12-dimethylbenanthracene (DMBA). These rats were subjected to the treatment using Graviola leaves ethanolic extract (GLEE) and 5-Fluorouracil (5-FU) to evaluate the anti-carcinogenic activity and the immunohistochemical changes. A single oral dose of DMBA led to the induction of rats' breast cancer. Induced mammary tumors were diagnosed, and all were malignant without any benign tumor. Among the malignant tumors, non-invasive, invasive, mixed (invasive and non-invasive), and unclassified malignant tumors were detected. The immunohistochemical analysis showed that estrogen and progesterone receptors revealed negative nuclear expression. However, HER2 receptors score was +3, and Ki-67 revealed 85-90% nuclear stainability, denoting a very high proliferative index. The morphometric analysis revealed that staining reactivities of HER2 and Ki-67 were 60.66 and 89.84%, respectively, and were significantly higher than ER (15.24 %) and PR (15.68%). Genetic studies revealed marked upregulation of P53 with GLEE more than 5-FU while Bcl2 showed down regulation with GLEE more than 5-FU. The quantitative analysis of GLEE phytochemical constituents showed the presence of some bioactive chemical compounds that exhibit many therapeutic activities. Therefore, GLEE improved histological appearance of DMBA induced breast cancer in female rats. Therefore, GLEE could be a promising natural alternative to chemotherapy agents as a potent anticancer.

Key words: breast cancer; graviola; phytochemical; DMBA; 5-Flurouracil; P53; Bcl2

Introduction

Breast cancer is considered one of the most frequent malignancies in women taking into consideration one-quarter of all malignancies diagnosed in women and could be a heterogeneous illness on the molecular level (1). This cancer is developed in breast tissues, including lobules and ducts. The worldwide deaths in women related to cancers represent a worldwide burden. Globally, breast cancer incidence has been steadily increasing over the last few decades (2). Breast cancer was assessed to be 30% of recently analyzed cancer cases and about 15% of cancer-related deaths in women (3).

Breast cancer can be categorized into non-invasive or invasive. The surrogate intrinsic subtypes are dependent on key proteins histological and immunohistochemical examination: progesterone receptor (PR), estrogen receptor (ER), the proliferation marker Ki67, as well as human epidermal growth factor receptor 2 (HER2) (4). The strongest predictors in detecting and determining the risk for metastasis are the tumor diameter, axillary lymph nodes metastasis, histological grade, HER2

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status and hormone receptors, beside the Ki-67 proliferation index (5).

Bioactive compounds derived from natural products have recently gained considerable attention due to their efficacy in treating inflammatory diseases such as cancer. Earlier studies, including epidemiological, preclinical, and clinical studies, have suggested that consuming polyphenols, which are abundant in fruits and vegetables, may help to limit the advancement of numerous diseases, including cancer (6). In addition, dietary phytochemicals have numerous inherent benefits over manufactured substances because of their known safety, low cost, and oral bioavailability. Many researchers are studying plant-derived drug modes of action at the molecular, cellular, as well as tissue levels (7).

Graviola (*Annona muricata*) is a plant in the *Annonaceae* family that is both a source of nutrition and an indigenous medicinal herb. It has been proven to have a wide range of bioactivities, including anti-cancer properties (8).

All aerial and subterranean parts of Graviola, such as leaves, fruits, seeds, as well as roots, have all been applied in traditional medicine (9). Therefore, several studies on various parts of Graviola have demonstrated the presence of different phytochemical substances, including cyclopeptides and essential oils (10), flavonoltriglycosides (11), phenolics (12), and alkaloids (13). Hence, phytochemical studies indicated that the Annonaceae family's major constituents were acetogenins. Graviola's phenolic compounds have been shown to recover free radicals from human breast cancer cells (14). The plant has long been used to produce chemically active metabolites (15). Extensive anti-cancer studies have been conducted on Graviola due to its ethnomedicinal applications against tumors and cancer. Graviola induced apoptosis, necrosis, and inhibition of proliferation (16) on a diversity of cancer cell lines, in between breast, colorectal, lung, prostate, pancreatic, leukemia, hepatic, renal, cervical, and ovarian cancers (17).

5-Fluorouracil (5-FU) is an antineoplastic antimetabolite drug effective in the palliative management of carcinomas of different organs, and treatment of head, and neck carcinomas (18). Anti-cancer drugs are frequently given systemically, affecting tumor cells as well as all other body organs, resulting in increased toxicity in normal cells.

A tumor suppressor gene called wild type p53 is essential for preserving a cell's genomic integrity because it stops cells with damaged DNA from proliferation. The most frequent genetic flaws seen in clinical cancer are p53 mutations and deletions. Immunohistochemistry has been used to identify significant quantities of stable, frequently mutant, p53 protein in the cells of about 40% of breast carcinomas. Similar cells lacking p53 activity continue to proliferate, perpetuating potentially cancerous alterations, while cells with functional p53 perish via apoptosis. In addition to possibly serving as a predictor of a tumor's biological aggressiveness and potential response to treatment, the restoration of normal p53 function is already one of the main objectives of cancer therapy (19).

The BCL2 family of proteins is essential for controlling apoptosis. New members of the BCL2 gene family were found to express themselves differently in many diverse types of cancer (20).

A set of proteins known as caspases conduct the evolutionarily conserved apoptosis mechanism. Caspases are cysteine proteases that break down their substrates after an Asp residue. They are created as latent zymogenes and are then activated by proteolytic cleavage; the BCL2 family proteins primarily control this process (20).

Consequently, this study conducted to evaluate the anticancer activity of Graviola leaves ethanolic extract (GLEE) in comparison with the commercial therapeutic drug (5-FU) on 7,12-dimethylbenz[a] anthracene (DMBA)–induced breast cancer in rats by investigating the histological and immunohistochemical changes.

Material and methods

Graviola leaves preparation and extraction

Fresh Graviola leaves were obtained from the Faculty of Agriculture, Zagazig University, Egypt. The leaves were harvested, rinsed with tap water, and air-dried for four weeks at 25 °C. A milling machine was used to grind the plant samples into a homogeneous powder. The plant tissue homogenization method was used to extract equal amounts (350 g) of powdered leaves using 96% ethanol for three days (21). The prepared extract was concentrated by a rotary evaporator then stored at -20 °C for further use.

Phytochemical screening of Graviola extract

Sample derivatizations:

The samples were extracted and resuspended in 50 µL of N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) incubated in a Dry Block Heater at 70 °C for 30 min.

Gas chromatography–mass spectrometry analysis (GC-MS):

The GC-MS system (Agilent Technologies) was equipped with gas chromatograph (7890B) and mass spectrometer detector (5977A) at Central Laboratories Network, National Research Centre, Cairo, Egypt. The GC was outfitted with an HP-5MS column that measured 30 m and had an internal diameter and film thickness of 0.25 mm. The following temperature program was used for the analyses, with hydrogen serving as the carrier gas, flowing at a rate of 1.0 ml/min at a splitless injection volume of 1 l: 50 °C for one minute, followed by a 20-minute hold at 300 °C after rising 10 °C/min. At 250 °C, the injector and detector were maintained. By employing a spectral range of m/z 30-700 and a solvent delay of 9 min, mass spectra were produced using electron ionization (EI) at 70 eV. The mass temperature was 230 °C and Quad 150 °C. Identification of different constituents was determined by comparing the spectrum fragmentation pattern with those stored in Wiley and NIST Mass Spectral Library data.

Experimental animals

This study was performed on fifty mature female albino rats weighing 100 - 120 g that were purchased from laboratory animal house at the Faculty of Veterinary Medicine, Zagazig University. Throughout the trial, the animals were housed at 23 ± 2 °C with a 12-hour light/dark cycle, and they had free access to standard food and water. Attempts were taken to reduce the pain and suffering of the animals during the research. They were removed from the trial and euthanized under deep anesthesia if they displayed aberrant symptoms. The ethical guidelines of dealing with laboratory animals were observed throughout the investigation, and this protocol was evaluated and approved by the ZU-IACUC committee with the permission number ZU-IACUC/2/F/63/2020, Zagazig University, Egypt.

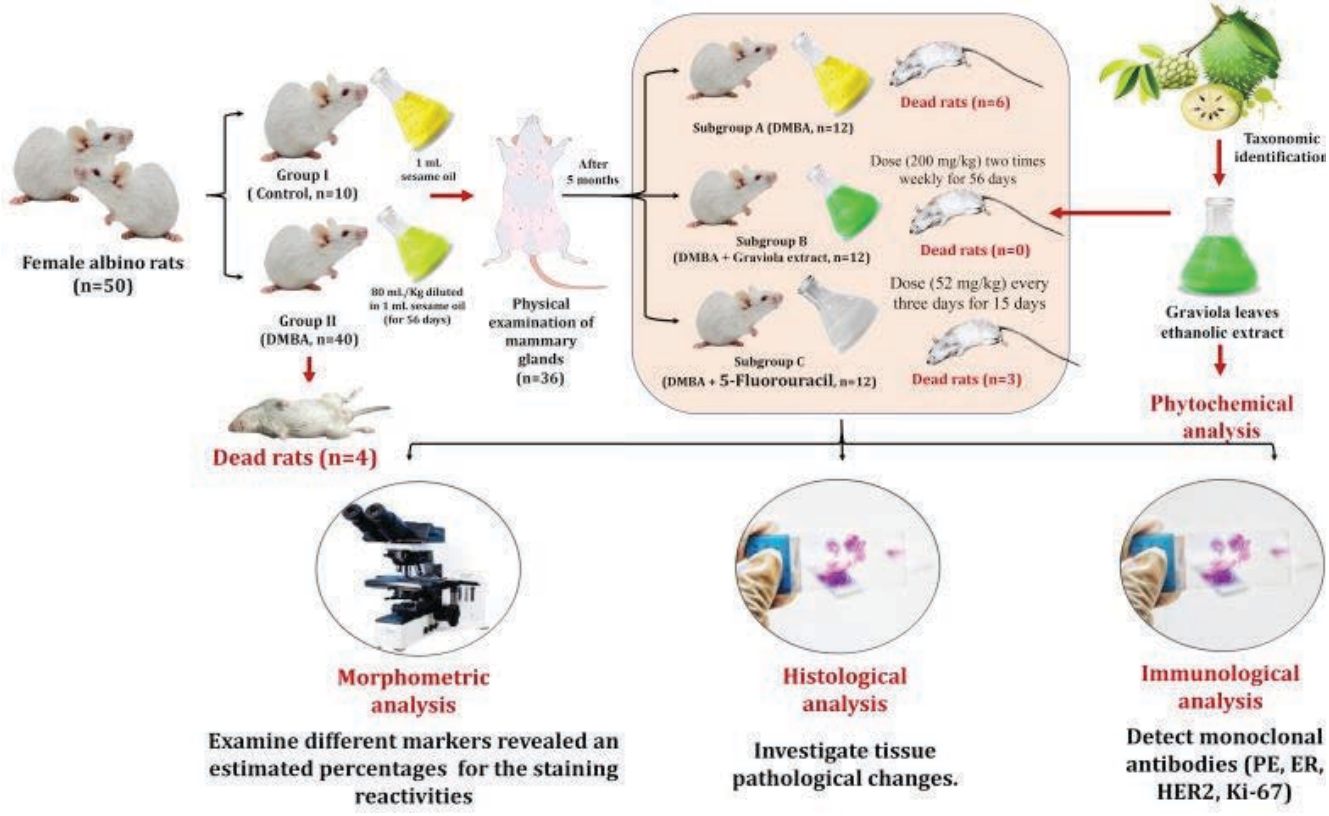


Figure 1: Experimental design of Breast cancer induction using DMBA in albino rats.

Experimental design

As shown in Fig. 1, after one week of acclimation, 50 female albino rats were divided into two experimental groups. Group I (control, n = 10) was given 1 mL sesame oil orally, while Group II (DMBA, n = 40) was given a single dose of DMBA (80 mg/kg, diluted in 1 mL sesame oil) by stomach tube (p.o.). Weekly physical examinations were conducted. Inspection, touching, and palpation were used to check each rat's six pairs of mammary glands. After 5 months and the confirmation of the tumor incidence by palpation and excluding the mortality ratio of investigated rats, as shown in Fig. 1, Group II was divided into three subgroups; subgroup A (DMBA, n = 12) is considered a positive control group, and subgroup B (DMBA + Graviola extract, n = 12) administrated GLEE to DMBA induced breast cancer in rats at a dose (200 mg/kg) two times weekly (p.o.) for eight weeks according to (22), subgroup C (DMBA + 5-FU, n = 12), administrated 5-FU at a dose (52 mg/kg) (i.p.) every three days (day 0, 3, 6, 9, 12, and 15). The applied dose was according to the previous method (23).

Rats of subgroup C were sacrificed at the 16th day post-administration of 5-FU, while rats of subgroup A and subgroup B were sacrificed at 7 months post-administration of DMBA, and animals were sacrificed by CO2 asphyxiation. And a section of the tumors were fixed in buffered formalin.

Histopathological and immunohistochemical analysis.

The formalin-preserved rat's mammary gland tissue specimens were impregnated with molten paraffin wax, then embedded and blocked out. The obtained paraffin sections were then stained with Hematoxylin and Eosin (H&E) based on the described method (24). Stained sections were examined for histopathological changes.

ER, PR and HER2 were detected by a specific primary monoclonal antibody. Paraffin was removed from the investigated sections (4 µm) using xylene and slowly rehydrated using ethanol. Antigen retrieval was performed using microwave in 10 mM citric acid monohydrate at 900 W for 1 × 5 min and at 600 W for 3 × 5 min. Treatment with H_2O_2 (0.5%) blocked the endogenous peroxidase activity. The prepared slides were kept overnight at 4 °C with the primary antibodies at appropriate dilutions. The negative controls were prepared by the same procedures without the overnight incubation; however, performed in PBS diluent without antibody.

For PR, the sections were subjected to dual colorimetric immunohistochemical (Envision G/2 Doublestain, Dako). However, For ER, the reaction was visualized using the Elite ABC Kit (Vectastain, CA, USA). The result was evaluated as the proportion of positively stained tumor cells (0–100%). The investigated samples were considered as positive for PR and ER when ≥1% of the tumor cells exhibited positive nuclear staining (25). For PR dual staining tumors, ≥1% Diaminobenzidine-stained nuclei or ≥1% Perm Red-stained cytoplasm were considered positive.

For HER2, the Envision kit (Dako, Denmark) was applied (24). HER2 was evaluated based on the intensity and percentage of positive cells on a scale of 0 to +3. The result was reported as negative (0) if no staining or membrane staining in less than 10% of invasive tumor cells was detected, or negative (+1) if faint/barely perceptive membrane staining was detected in more than 10% of invasive tumor cells. However, the result was considered positive (+2) when weak to moderate complete membrane staining in more than 10% tumor cells or <30% with strong complete membrane staining, or positive (+3) if strong complete membrane staining in >30% invasive tumor cells was detected (26). For Ki-67, tissue samples were considered positive for the tumor when ≥14% of the tumor cells exhibited positively stained nuclei (27). The Ki-67 index is defined as the percentage of the total number of tumor cells with nuclear staining (28). Immuno-stained cells were explored in different groups according to their numbers and nuclear staining intensity using the IMAGE J software analysis.

Morphometric analysis

Morphometric analysis was digitized using Olympus digital camera (Olympus LC20- Japan) installed on Olympus microscope (Olympus BX-50, Tokyo, Japan) with 1/2X photo adaptor, using 40X objective. The software Video Test Morphology 5.2 (Russia), which has a dedicated built-in method for immunohistostaining analysis, was used to evaluate the results photos. Average grayscale was used to express the immunoreactive

intensity. High values were <160, and medium values ranged from 160 to 170, and low values ranged from170 to180 (29).

Gene expression and reverse transcription polymerase chain reaction (RT PCR).

RT-PCR analysis was digitized using *Rotorgene RT- PCR system* and Qiagen RNA extraction/ BioRad syber green PCR MMX kit. Analyses were carried out using DMSO as the solvent. Primers used in this study were presented in Table 1.

Results

Phytochemical analysis of ethanolic leaves extract of Graviola

As shown in Fig. 2, the phytochemical analysis by GC-MS analysis of GLEE revealed the presence of different bioactive compounds. The details of the GC-MS analysis of the extracts are listed in Table 2.

Table 1: Primers used in the present study

Target	Nucleotide sequence
Bcl2	F: 5'-ATCGCCCTGTGGATGACTGAGT-3' R: 5'-GCCAGGAGAAATCAAACAGAGGC-3'
Casp3	F: 5'-TTCATTATTCAGGCCTGCCGAGG-3' R: 5'-TTCTGACAGGCCATGTCATCCTCA-3'

Target	Nucleotide sequence
P53	F: 5'-CCTCAGCATCTTATCCGAGTGG-3' R: 5'-TGGATGGTGGTACAGTCAGAGC -3'
β-actin	F:5'-ATCGTGGGGCGCCCCAGGCAC-3' R:5'-CTCCTTAATGTCACGCACGATTTC-3'

Table 2: Phyto-components obtained from the Annona muricata ethanolic leaves extract using GC-MS

Peak	RT	Name	Formula	Area	Area Sum %
1	12.812	D-(-)-Lactic acid, trimethylsilyl ether	C6H14O3Si	29550.77	0.24
2	13.161	Propanoic acid, 3-(trimethylsilyl)-	C6H14O2Si	204646.07	1.63
3	16.216	Butanoic acid, 4-[(trimethylsilyl)oxy]-, trimethylsilyl ester (CAS)	C10H24O3Si2	72449.35	0.58
4	20.502	3-(Bistrifluoromethylamino-oxy)norbornylene	C9H9F6NO	69062.06	0.55
5	20.817	Arachidonic acid, TMS derivative	C23H40O2Si	98415.71	0.78
6	21.738	Germacrene D	C15H24	54323.81	0.43
7	23.134	2-Hydroxyisocaproic acid, TMS derivative	C9H20O3Si	47663.68	0.38
8	23.815	Aromandendrene	C15H24	70628.83	0.56
9	24.159	9,12-Octadecadiynoic acid, trimethylsilyl ester	C21H38O2Si	89930.65	0.72
10	24.433	5-Hydroxymethyl-2,2,5-trimethyl-1,3-dioxane, TMS derivative	C11H24O3Si	233462.98	1.86
11	24.748	Methyl .alpha.-Arabinofuranoside, 3TMS derivative	C15H30O5Si3	359310.9	2.86
12	24.891	D-(-)-Ribofuranose, tetrakis(trimethylsilyl) ether (isomer 1)	C17H42O5Si4	512373.99	4.08
13	25.257	1,3-Dihydroxyacetone dimer, 4TMS derivative	C18H46O6Si4	1331645.5	10.6
14	25.532	D-(-)-Lyxofuranose, tetrakis(trimethylsilyl) ether	C17H42O5Si4	1121679.3	8.93
15	25.652	L-(-)-Sorbofuranose, pentakis(trimethylsilyl) ether	C21H52O6Si5	798000.22	6.35
16	26.012	D-(-)-Fructopyranose, pentakis(trimethylsilyl) ether (isomer 1)	C21H52O6Si5	732986.99	5.83
17	26.178	D-Psicofuranose, pentakis(trimethylsilyl) ether (isomer 1)	C21H52O6Si5	408524.64	3.25
18	26.287	D-Lyxose, 4TMS derivative	C17H42O5Si4	127728.01	1.02
19	26.459	D-Glucopyranose, 5TMS derivative	C21H52O6Si5	85661.53	0.68
20	26.791	beta.-D-(+)-Talopyranose, 5TMS derivative	C21H52O6Si5	519150.01	4.13
21	27.031	beta.-D-(+)-Xylopyranose, 4TMS derivative	C17H42O5Si4	555103.42	4.42
22	27.363	D-Arabinopyranose, 4TMS derivative (isomer 2)	C17H42O5Si4	1020398.9	8.12
23	27.695	Glucose, 5TMS derivative	C21H52O6Si5	895722.74	7.13
24	28.462	Scyllo-Inositol, 6TMS derivative	C24H60O6Si6	336907.67	2.68
25	28.627	Myo-Inositol, 6TMS derivative	C24H60O6Si6	1069880.3	8.52
26	28.885	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	C19H32O2	281593.1	2.24
27	29.108	Phosphonic acid, dioctadecyl ester	C36H75O4P	421307.1	3.35
28	29.926	Methyl galactoside (1R,2R,3S,4S,5R)-, 4TMS derivative	C19H46O6Si4	51776.68	0.41
29	31.088	1H-3a,7-Methanoazulene, octahydro-1,4,9,9-tetramethyl-	C15H26	53446.93	0.43
30	32.061	1,2-Benzenedicaboxylic acid, bis(2-ethylhexyl) ester	C24H38O4	38999.18	0.31
31	32.427	Adamantane-1-carboxamide, N-[2-(3,4-dimethylphenoxy)ethyl]-	C21H29NO2	36219.71	0.29
32	33.148	arachidonic acid tert-butyldimethylsilyl ester	C26H46O2Si	43174.55	0.34
33	33.875	Pinellic acid, 2TMS derivative	C13H28O4Si2	31202.22	0.25
34	36.055	dl-.alpha.-Tocopherol	C29H50O2	149904.45	1.19
35	36.633	(E)-5,10-secocholest-1(10)-en-3,5-dione	C27H44O2	98489.91	0.78
36	36.827	Stigmasterol	C29H48O	64887.82	0.52
37	36.93	3-Amino-2-carbomethoxy-4,4,6,6-tetramethyl-4,6-dihydro-5H-thieno[2,3-c]pyrrole-5-yloxyl radical	C12H17N2O3S	106029.52	0.84
38	37.182	gamma.-Sitosterol	C29H50O	277184.14	2.21
39	37.342	Cholest-5-en-3-ol (3β)-, 9-octadecenoate, (Z)-	C12H17N2O3S	65124.83	0.52

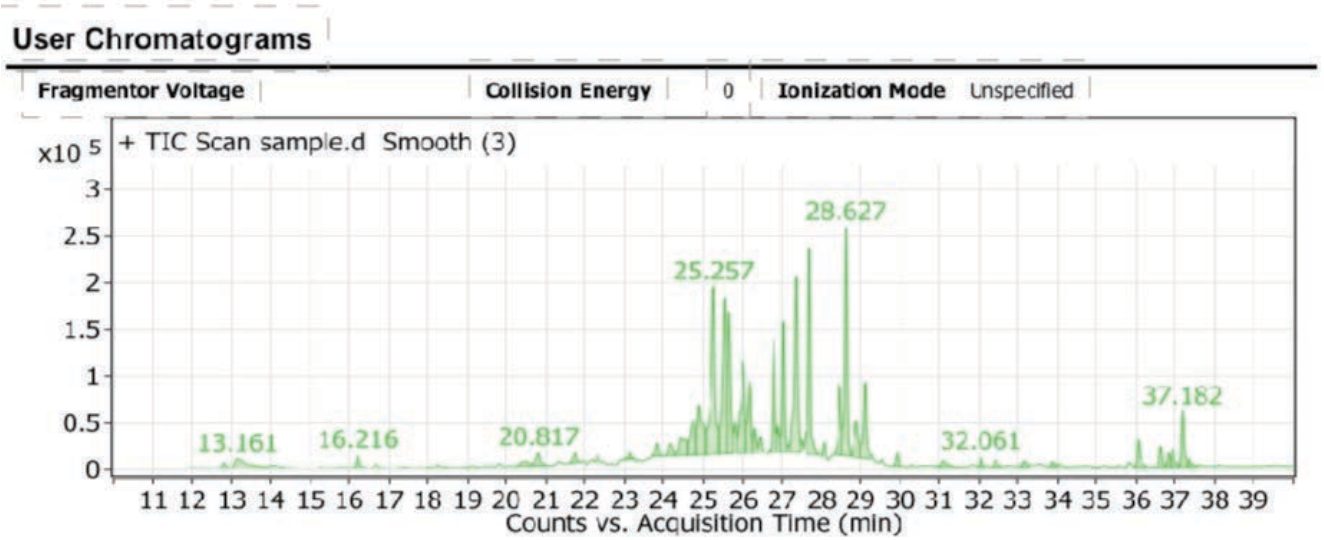


Figure 2: Phytochemical analysis by GC-MS analysis of GLEE

Histological Observations

Tumor induction and detection

Breast cancer was induced experimentally using a single oral dose of DMBA, as shown in Fig. 3.

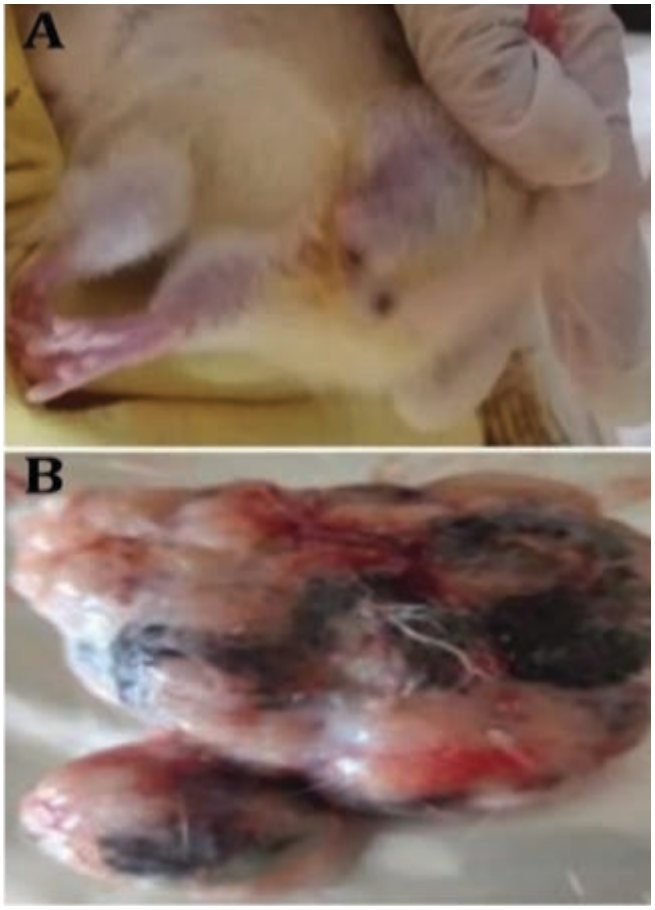


Figure 3: DMBA- induced breast cancer in albino rats

In our study, induced mammary tumors were diagnosed, in which 100% were malignant without any benign tumor. Among the malignant tumors, non-invasive, invasive, mixed invasive and non-invasive tumors, and unclassified malignant tumors were detected. Mammary gland of control adult female rats showed normal tissue architecture with normal secretory acini and ductules (Fig. 4A) that are lined with cuboidal or low columnar epithelial cells and surrounded by myoepithelial cells with loose connective tissue in between. As illustrated in Fig. 4B, the induction of rat's mammary gland tumor by DMBA shows sheets of tumor cells separated by tiny cystic gaps describe adenoid cystic carcinoma. Tumor cells appeared large, rounded with vesicular nuclei, enlarged nucleoli, and moderate mitotic activates in situ, and invasive papillary carcinoma was shown. Proliferating neoplastic cells were grouped in long finger-like extensions with a delicate fibrovascular core in the center and showed signs of malignancy (H&E). The scale bar is 50 µm. Figure 4C shows *in situ* and microinvasive papillary cancer. Proliferating neoplastic epithelial cells develop inward in the lumen to create finger-like papillary structures with little fibro-vascular core, which are restricted within the lobule by an intact basement membrane. A compact clump of malignant epithelial cells generated secondary projections (papillae). By breaching the foundation membrane, papillae from one lobule are visible entering the adjacent lobule (Fig. 4D).

As shown in Fig. 5, among all detected tumor types, invasive tubular adenocarcinoma (Fig.

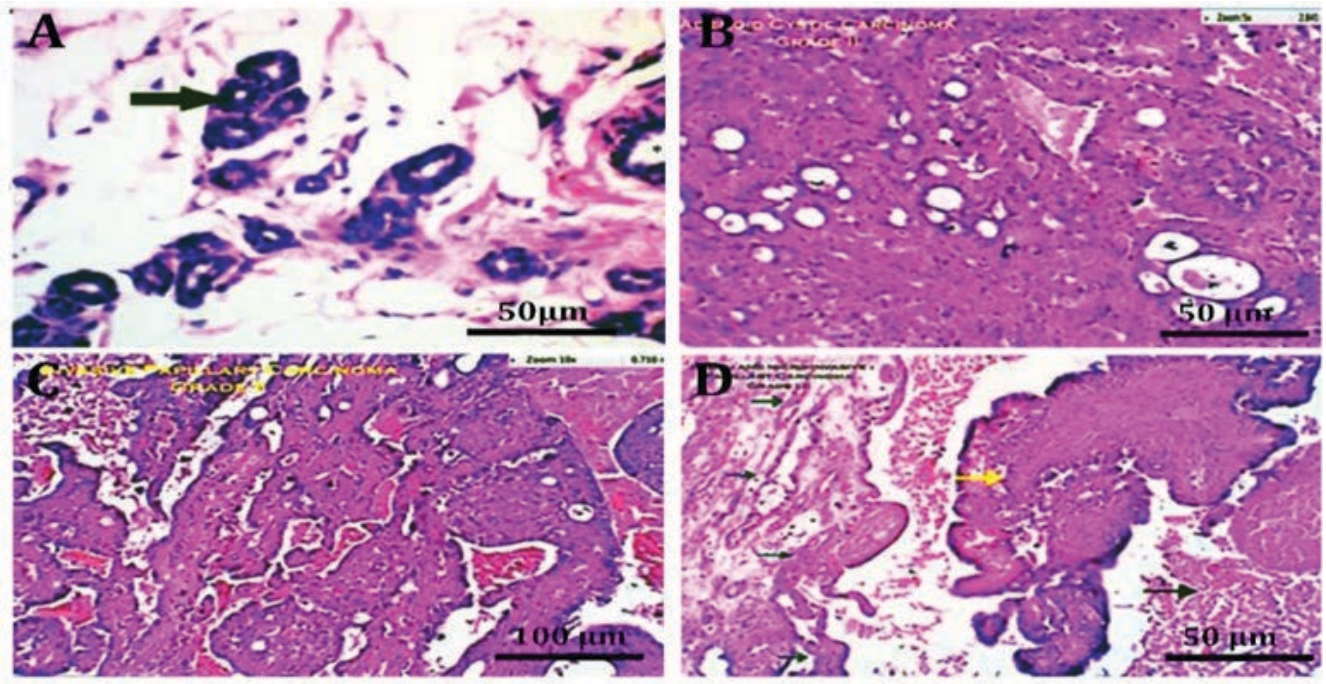


Figure 4: Mammary gland of control adult female rat (H&E) showing normal tissue architecture with normal acini (ar-row) and ductless with interlobular stroma inbetween (A). Mammary gland treated with DMBA showing ade-noid cystic carcinoma (B). induced rat's mammary gland tumor by (DMBA) showing invasive papillary carci-noma (C). Oncogenic epithelium cells that are proliferating, Secondary projections were generated by a com-pact cluster of neoplastic epithelial cells with moderate mitotic figures (3.210.04/hpf) (D)

5A) was characterized by clusters of ductal or alveolar structures proliferate, separated by a little quantity of connective tissue. The nuclei of the neoplastic cells ranged in size from circular to expanded oval, with conspicuous nucleoli. Mitotic figures were found in copious quantities (5.6 ± 0.24/hpf) and there was considerable tissue necrosis.

Solid cribriform carcinoma (in situ and invasive) was characterized by the appearance of the sieve is defined by proliferating neoplastic cells grouped in solid sheets with the frequent development of spherical or irregular-shaped secondary lumina of varying diameters. Neoplastic cells grew quickly and had a lot of pleomorphism. The neoplastic cells can be observed infecting the stroma around them (Fig. 5B).

Furthermore, as demonstrated in Fig. 5C, in situ and invasive comedocarcinoma was distinguished by dilated ductal structures lined by a multi-layered epithelium with necrotic material in the center. Desmoplastic reaction was seen in the stroma surrounding the separate ducts. Individual cancer cells were pleomorphic, with big hyperchromatic nuclei with conspicuous nucleoli.

Moreover, in situ and invasive ductal carcinoma (solid type), as shown in Fig. 5D**,** where mammary lobules were totally replaced by proliferating ductal cells, which completely obliterate and expand the main ducts with no evidence of microcystic or necrotic changes. Some tumor cells break the basement membrane, infiltrate and totally replace the adjacent tissue. Tumor cells were large hyperchromatic with the presence of moderate mitotic figures.

Furthermore, keratinized squamous cell carcinoma in rat mammary gland after DMBA induction, as shown in Fig. 5E**,** was detected and characterized by the presence of pathognomonic Epithelial Pearls showing invasive squamous cell carcinoma, characterized by many invasive malignant masses and columns invading the dermis and subcutaneous tissue. Tumor cells are hyperchromatic, pleomorphic with vesicular nuclei, and marked mitosis (Fig. 5F).

Immunohistochemical analysis

All examined tissue sections for estrogen receptors (Fig. 6A) progesterone receptors (Fig. 6B) revealed negative nuclear expression with a score of 0%. However, all examined tumor tissue sections treated by monoclonal antibodies against HER2 receptors revealed complete thick

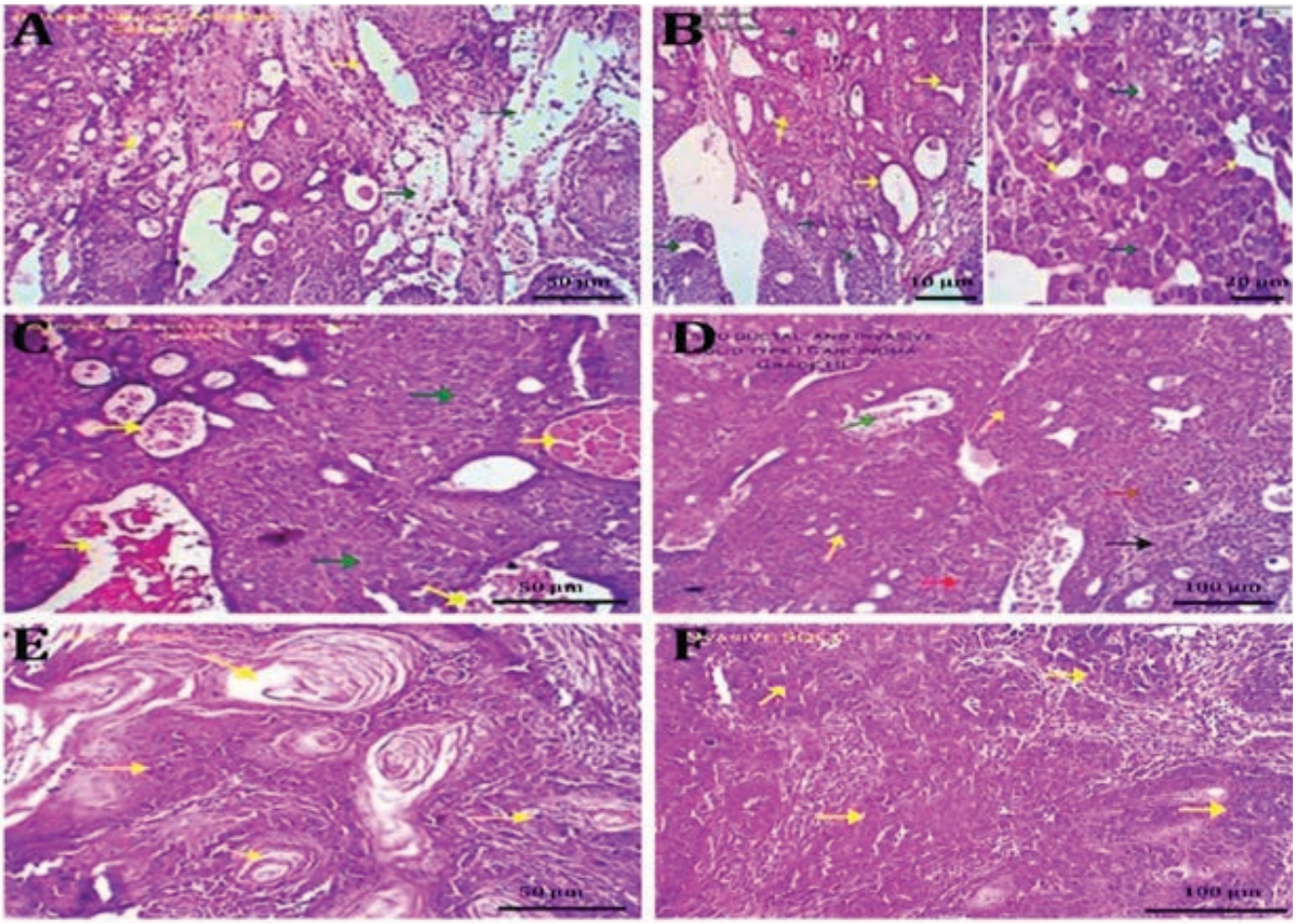


Figure 5: Experimentally induced rat's mammary gland tumor by DMBA (H&E) showing invasive tubular adenocarcinoma (A), in situ and invasive cribriform carcinoma (B), in situ and invasive comedo-carcinoma (C), in situ and invasive ductal carcinoma (D), keratinized squamous cell carcinoma (E), and invasive squamous cell carcinoma (F)

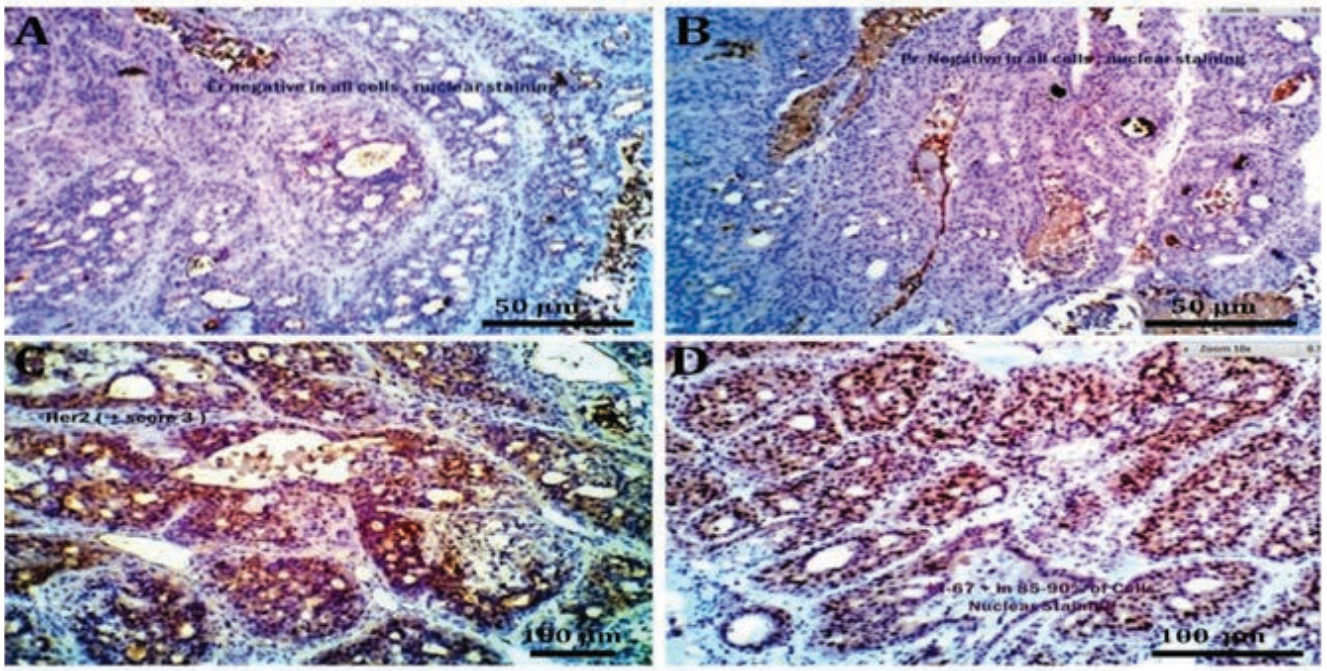


Figure 6: The immunohistochemical examination of induced rat's mammary gland tumor by DMBA showing negative nuclear expression of estrogen receptors in all tumor cells (A), a negative nuclear expression of progesterone receptors (B), HER2 immuno-stained cells with complete thick membrane staining reaction (C), and Ki-67 immuno-stained cells with high expression (D).

membrane staining reaction in more than 10% of the tumor cells with a score of +3 (Fig. 6C). Moreover, in breast cancer, Ki-67 identifies a high proliferative subset of patients. Treated tumor tissue sections by specific monoclonal antibodies against Ki-67 antigens revealed 85-90% nuclear stainability, denoting a very high proliferative index, as shown in Fig. 6D.

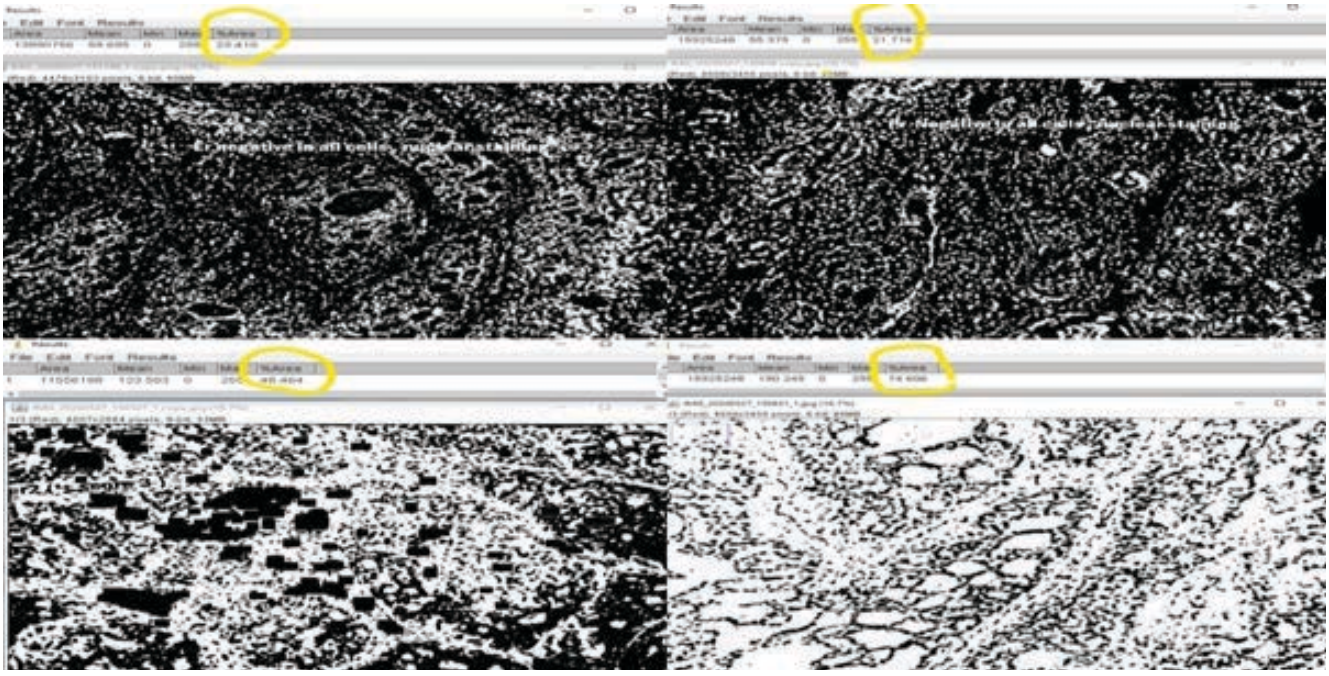


Figure 7: Morphometric analysis showing the morphometric analysis with an estimated percentage for the staining reac-tivates in the different used immunostaining markers.

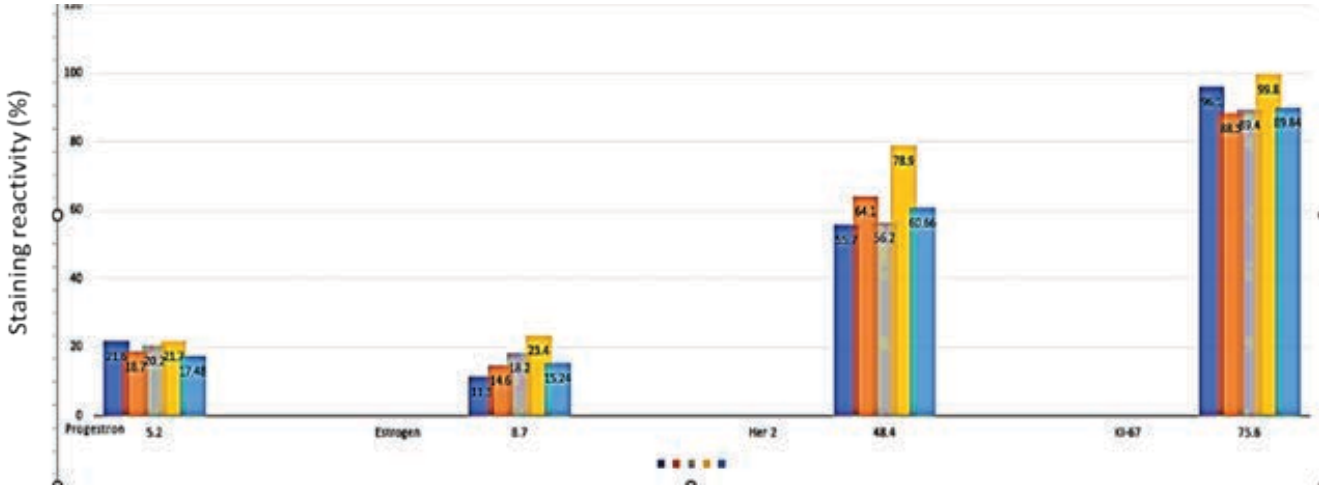


Figure 8: Illustrative statistical analysis chart for different immunostaining markers

Our obtained data of morphometric analysis (Fig. 7) revealed that staining reactivity of HER2 and Ki-67 was 60.66 and 89.84%, respectively, and were significantly (P <0.05) higher than ER and PR, which were estimated as 15.24 and 15.68%, respectively (Fig. 7B). Our statistical analysis showed that Ki-67 was significantly higher than HER2 (P 0.0023), ER (P <0.0001), and PR (P <0.0001). However, PR and ER were statistically insignificant (P 0.5956) (Fig. 8).

Treatment regimen using 5-FU

Based on our obtained results, we demonstrated three types of malignant tumors, including in situ ductal and comedo-carcinoma and invasive

tubular carcinoma. All of these tumoral tissues showed marked cytotoxic therapy response to 5-FU. Of those, 72-78 % of the tumor cells were necrotic; the remaining cells showed degenerative changes. Marked desmoplastic reactions were observed. The examined non-tumoral normal mammary tissue showed marked cytotoxic effect as most of the mammary acini were necrotic, as shown in Figure 9.

Treatment regimen with Graviola leaves extracts.

As shown in Fig. 10, the treatment using GLEE reduced tumor size compared to the positive control group. In the histological study, investigated sections of GLEE treated groups demonstrated a moderate to marked cytotoxic effect of the GLEE. Examined sections showed

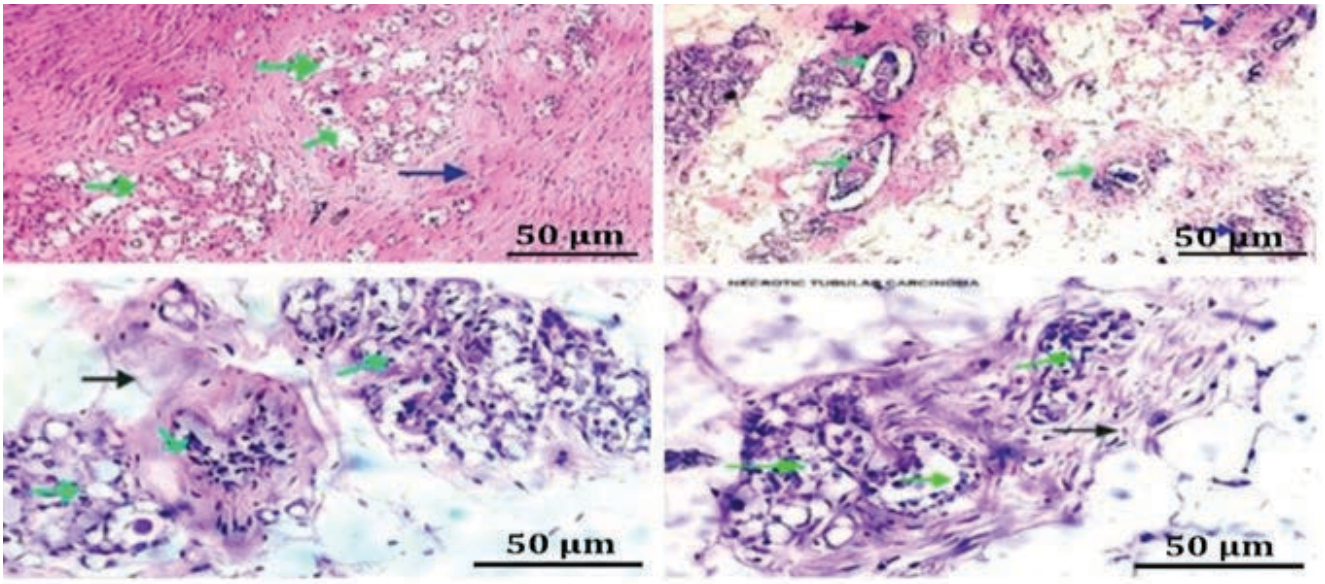


Figure 9: Induced rat's mammary gland tumor treated by 5-Fluorouracil, Of those, 72-78 % of the tumor cells were necrotic; the remaining cells showed degenerative changes. Marked desmoplastic reactions were observed. The examined non-tumoral normal mammary tissue showed marked cytotoxic effect as most of the mammary acini were necrotic

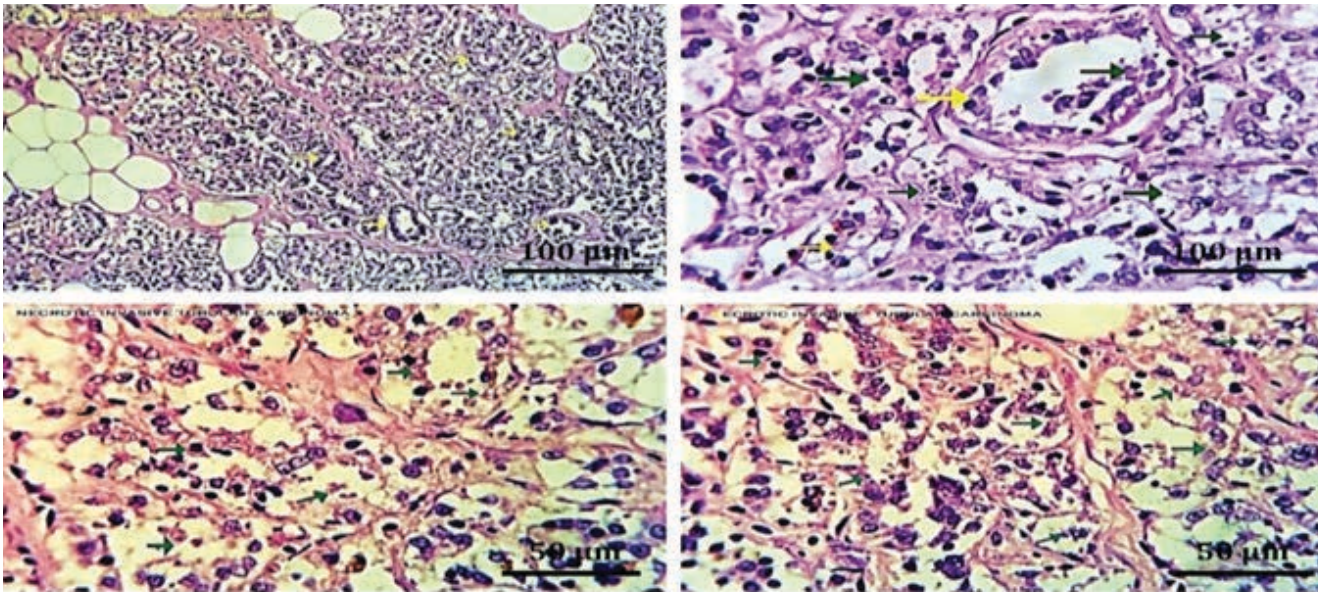


Figure 10: Induced rat's mammary gland tumor treated by Graviola L. extract, investigated sections of GLEE treated groups demonstrated a moderate to marked cytotoxic effect of the GLEE. Examined sections showed a marked cytotoxic effect of the plant extract, where a large number of the invasive tubular carcinoma cells were necrotic, as evidenced by pyknosis and karyorrhexis, and karyolysis of the tumor cells

a marked cytotoxic effect of the plant extract, where a large number of the invasive tubular carcinoma cells were necrotic, as evidenced by pyknosis and karyorrhexis, and karyolysis of the tumor cells. Moreover, a variable number of the invasive tumor cells showed apoptotic changes. The estimated therapeutic effect of the used extracts is ranged between 59-63%. The normal non-tumoral mammary tissue appeared healthy with an undetectable hazard effect of the used extracts.

Gene expression analysis

Genetic studies revealed marked up-regulation of P53 with GLEE more than 5-FU while Bcl2 showed down-regulation with GLEE more than 5-FU (Table 3).

Table 3: Genes expressions analysis of the experimental groups

Ser	Group	Fold Change	
		p53	Bcl2
1	DMBA group	4.01	0.51
2	DMBA + Graviola extract group	6.41	0.2
3	DMBA + 5-Fl Fluorouracil	3.28	0.35
4	Control group	1	1

Discussion

The most common invasive malignancy in women is breast carcinoma. Researchers have been working hard in recent years to discover a better treatment for breast carcinoma, and they are still working on it. It is also the second greatest cause of cancer-related death in women, after lung carcinoma (30).

In this study, DMBA was used for chemical induction of breast cancer which showed diverse types of breast cancer according to the histological subtypes (31).

In the present study, DMBA-induced breast cancer group revealed up regulation of P53, may be due to mutation of the p53 gene in DMBA-induced breast cancer cells led to cells lacking P53 function continue to proliferate, perpetuating potentially oncogenic mutations (32). Also, DMBA affect the microtubule dynamics through changing the expression of genes involved in cellular differentiation, proliferation/cell cycle regulation (33). The tumor incidence of our model was similar to other studies with the same chemical inductor (DMBA), which found tumor incidences of 82% (34), 92.3% (35), and 100% (36).

For the detection of Estrogen, Progesterone, and HER2 receptors in breast carcinoma cases for responsiveness to endocrine therapy in the management of breast cancer, immunohistochemistry is now a widely acknowledged approach. The immunohistochemistry investigation in our study demonstrated that the estrogen and progesterone receptors had negative nuclear expression. However, the HER2 receptors score was +3, and a nuclear stainability of between 85 and 90 percent on the Ki-67 test indicated a very high proliferative index. It has been shown that Ki-67 high expression enhances tumor growth in breast cancer rats by promoting breast cancer progression to higher histological grade in breast carcinoma rats (37). ER and PR were negatively correlated with HER2 overexpression (38). Also, the obtained results of HER2 in this study were similar to those reported before (26) where HER2 protein overexpression is found in tumors of around 25–30% of breast cancer patients, and this overexpression is associated with a poor clinical prognosis.

In our histological study, all of these tumoral tissues treated with 5-FU showed marked cytotoxic therapy response to 5-FU. Of those, 72-78 % of the tumor cells were necrotic; the remaining cells showed degenerative changes. Marked desmoplastic reactions were observed. The examined non-tumoral normal mammary tissue showed marked cytotoxic effect as most of the mammary acini were necrotic. By modifying cell death pathways, this medicine can decrease the spread of breast cancer in a variety of ways, including apoptosis. As a result, numerous treatment studies to overcome drug resistance caused by apoptosis modulation are now being done. 5-FU showed direct apoptotic action. 5-FU also has various modes of action, such as interfering with RNA processing and boosting p53 expression and our results in agreement with Prince *et al.* (18).

Natural products have been a target for cancer treatment because of their therapeutic properties for many years. In this study, the anti-cancer effect of the ethanolic leaf extract of Annona muricata was evaluated on induced HER2-positive breast cancer using DMBA in albino rats. Our study revealed that phytochemical analysis of the crude GLEE showed the presence of a complex

mixture of secondary metabolites that are found to have various therapeutic activities and GC-MS analysis showed bioactive compounds. Of these Arachidonic acid, Propanoic acid, Lactic acid, and Phytol. Our results agree with those of Ojezele *et al.* (39) and Magadi *et al.* (40). Beneficial anti-cancer activity of GLEE is due to its acetogenins contents (8). These *Annonaceous acetogenins* have the ability to block mitochondrial complex I, which can lead to cellular death by reducing intracellular ATP generation (41). Besides, Graviola extract improved glutathione S-transferase activity and glutathione levels, implying glutathione-dependent reactive oxygen species detoxification (ROS). Graviola's antioxidative properties can thus be related to its flavonoid concentration, which acts as a free radical scavenger and/or promotes the antioxidant system (40). Phytol also is known to have strong anticancer and immune-enhancing properties (42).

In the histological study, investigated sections of GLEE treated groups demonstrated that a large number of the invasive tubular carcinoma cells were necrotic as evidenced by pyknosis, karyorrhexis and Karryolysis of the tumor cells. Moreover, variable number of the invasive tumor cells showed apoptotic changes. Graviola, rather than apoptosis due to up-regulation of P53 and down regulation of Bcl2 in our genetic analysis, can destroy cancer cells by necrosis (43). Beside mitochondrial-dependent apoptotic pathway (44). This means that Gaviola's cytotoxic effect varies depending on the type of cancer cells, and that no single mechanism exists for all cancer cells.

Genetic analysis revealed upregulation of p53 and downregulation of Bcl2 indicating an increase in apoptotic index in graviola-treated group as compared to the carcinogen control and more than in 5-FU-treated group.

Apoptosis is induced as one of the most dramatic reactions to p53 activation. There are two main mechanisms through which apoptosis can occur: the intrinsic mitochondrial pathway and the extrinsic death receptor pathway (45). Bax and Bak oligomerization are induced in the mitochondria by p53, which also physically interacts with protective Bcl-XL and Bcl2 to counteract their antiapoptotic activities, disrupting the structure of the mitochondria (46). In this approach, it encourages the release of proteins that trigger apoptosis from the mitochondria, activating caspase-3 in the process. In the present study, Graviola treatment significantly decreased the Bcl2 expression whereas strong p53 expression was noted in breast cancer tissues.

Conclusions

In this study, we concluded that GLEE has several phytochemical components, which showed a moderate to marked anti-cancer effect on DMBA-induced breast cancer, and tumor cells showed apoptotic changes and up-regulation of P53 and down regulation of Bcl2 in comparison with traditional anticancer drug 5-FU. Moreover, it improved the histological appearance of DMBA-induced breast cancer in female rats. Therefore, Graviola could be a promising anticancer agent.

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A.I.S.: design of the experiment, animal work, methodology, data collection, writing the manuscript, and corresponding author. S.E. and A.S.A.: methodology and revision of the manuscript. H.A.E. and N.A.: methodology. The authors have read and approved the manuscript.

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The authors declare no conflict of interest.

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Original Research Article
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EFFECT OF ESSENTIAL OILS ON MULTIDRUG RESISTANT GRAM-NEGATIVE BACTERIA

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Abstract: Obtaining healthy food, free from chemical or synthetic additives, is a major challenge. In this study, we developed a preservation method using essential oils and evaluated their effect on multidrug resistant pathogenic Gram-negative bacteria. Different concentrations (1%,0.1%,0.17% and 0.35%) of Thyme oil and black seed oil were employed in this study against pathogenic *E. coli* and *A. hydrophila* in soft cheese. The used oils at a concentration of 0.1% through dipping method resulted in accepted color and odor, little effect on flavor and normal texture and appearance, while 0.1% during inoculation had the same effect as in dipping method except moderate odor. Thyme oil had the highest reduction rate in case of 1% dipping and 0.17% inoculation on *A. hydrophila,* while against *E. coli* it was found that 0.17% inoculation and 0.35% of the dipping method had the highest reduction effect. Thyme essential oil seems to be a suitable natural food preservative alternative.

Key words: essential oils; thyme oil; black seed oil; cheese; *E. coli*; *A. hydrophila*

Introduction

Annually, many consumers were subjected to foodborne diseases (1), which necessitate the orientation of food safety principles (2). Dairy products represent major sources for those pathogens. Cheese that contains important nutrients but may cause severe health hazards through carrying pathogenic bacteria (3) specially *E. coli* that indicates poor hygienic condition of manufacture processes (4). Multi drug resistant strains of *E. coli* cause severe symptoms start with diarrhea, urinary tract infection (UTI), meningitis and septicemia according to their virulence (5). *Aeromonas hydrophila* (*A. Hydrophila*) is an emerging pathogen that threat public health (6).

Synthetic and food chemical additives that are used to prolong food shelf life may cause intoxications, progressive diseases, that may reach to cancer. To overcome this problem, utilization of natural essential oils (EOs) extracted from aromatic plants to control the growth of pathogenic microorganisms (7, 8) may offer a solution.

EOs are mixtures of various chemical compounds that have been Generally recognized as safe extracts (GRAS) (9). They have antibacterial activities that used in food industry as a bio preservative to prevent spoilage and to extend food shelf life (10) due to presence of phenolic constituents and polypeptides (11) that inhibit spoilage and pathogenic bacteria (12). Additionally, they enhance food nutritive characteristics, sensory properties and shelf life (13).

Nowadays, EOs substitute synthetic flavors (14), but with limited concentrations that have no adverse effect on food due to their strong aroma (15, 16).

Thyme (*Thymus vulgaris* L.) is used in aromatic industrial food additives (17). It acts as an excellent

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natural antimicrobial agent due to presence of thymol which constitutes around 50 % of the components which have potent antimicrobial effect (18). It is considered one of the world's top ten essential oils (19). However, EOs have some selectivity on their action against microbial types (20), Gram-negative bacteria like *E. coli* 0157:H7 is more resistant to EOs (21) due to cell wall structure (22). *N. sativa* seed (Black seed) has pharmacological properties, including antimicrobial activity, so it is used in medicinal and cookery purposes (23).

These natural preservatives should meet the growing consumer demands for clean-label products that are free from chemical or synthetic additives (24). EOs action depends on permeability of cytoplasmic membrane, that cause cytoplasmic contents releasing, (25), or through inhibition of the ATPase enzyme (26).

Food inherent contents of protein and lipid may interfere with EOs antimicrobial action (27). Usage of natural EOs with emergent technologies like high pressure, ultrasound, and electric pulses result in using lower concentrations of EOs, because they damage cell membrane and facilitate cell penetration and antimicrobials action of EOs (28). Although, the sensory attributes of food may be negatively impacted even with low EOs concentration (29). So, we made our effort to obtain healthy natural acceptable product with high value by reducing some of multidrug resistance bacteria with keeping food quality.

Materials and methods

Preparation of Essential Oils:

EOs of Thyme and Black seed with concentrations of 0.1,0.17,0.35 and 1% for each were purchased from the oil extraction unit, National Research Center, Egypt. These oils were stored away from light at 4°C until use.

Preparation of bacterial strains:

Multi drug resistant *E. coli* and *A. hydrophila* strains (which carry virulence and resistance genes) that obtained from our previous study (30) were preserved in Tripticase Soya Broth (TSB, Oxoid) at 4°C. Strain inoculum was incubated in TSB at 37°C for 24 h. then serial dilution was made to provide 10^6 CFU/mL (31).

Preparation of white soft cheese:

Milk was pasteurized at 75°C for 15 seconds, then cooled to 43°C, salted and divided into 2 main groups (1st for inoculation, 2nd for dipping). For inoculation group, EOs (with 0.5 tween 80) with different concentrations and bacterial strain (mentioned later) were added. Following thorough stirring, milk was renneted (32) and left to curdled, cut and whey drained. Cheese was preserved at 4 ±1°C for 10 days (33). Samples were examined for sensorial evaluation and bacterial counts at 2 days interval (34). All tests were accomplished in three replicates and the mean values were then measured.

Preparation of treated samples (dipping):

Prepared white soft cheese samples were grouped into the following: control groups dipped in tested pathogens suspension only, and other groups dipped in the tested pathogens suspension *supplied with* 0.1, 0.17, 0.35 and 1% of thyme oil and Black seed oil separately. Dipping was done for 30 second (35).

Sensory evaluation of treated samples:

Cheese sensorial evaluation was done by nine-point hedonic scale and was performed by ten trained members of Animal Health Research Institute, where 1 unacceptable and 9 very acceptable, while the limit of acceptability was 5 (36). Cheeses were evaluated for their appearance and color, body and texture, odor and flavor. Cheese samples were cut into cubes (1.5 x 1.5 x 1.5 cm) and covered with plastic wrap to prevent dehydration. Each member was given three cubes of each sample. The maximum acceptable concentration of the studied essential oils in soft cheese samples was determined through sensory evaluation tests. For each of the examined essential oils (thyme and black seed), different soft cheese samples were made by adding different concentrations (0.1, 0.17, 0.35 and 1%) for each essential oil (by inoculation and dipping methods.

E. coli count:

Counting was done through a direct method by the spread surface technique on selective agar

media, eosin methylene blue (EMB) agar, and incubation for 24 hours at 37°C (37).

A. hydrophila count:

Aeromonas isolation medium base with the addition of Aeromonas Selective Supplement (FD039) was used (38). Cultured plates were incubated at 35-37°C for 18-24 hrs. Dark green and opaque with a dark center were considered *A. hydrophila.*

Statistical analysis:

Reduction rate was calculated as [reduced number-main number/main number] x 100. Results were statistically evaluated by application of analysis of variance (ANOVA) test (39).

Results and discussion:

Dairy products are suitable environment for bacterial contamination ruining products quality and making them unfit for human consumption which may lead to public health hazard (40). So, it is necessary to decrease bacterial count and improve product quality to obtain healthy food. One of preservative methods of food is using natural additives like EOs.

EOs had the ability to damage cell membrane of bacterial cell and increases its permeability and elimination of protein and ions (41), disturb bacterial cell membrane and decrease the intracellular ATP pool of the bacterial cell, causing loss of cytoplasmic membrane integrity and cell death that result in bactericidal effect (42). Difference in the structure of bioactive compounds may be affected by time of the part of harvesting and method of extraction that made variation in EOs inhibitory effect (43). Pathogenic *E. coli* is more resistant to EOs than other types (44). There is restriction in EOs usage as preservatives like negative effect on flavor meanwhile high concentrations are prerequisite to reach to satisfactory antimicrobial activity (45).

Most recently emerging foodborne pathogens are *A. hydrophila*, and *E. coli* (46). In this study, we used thyme and black seed oils to overcome harmful effect of multidrug resistant Gram-negative bacteria like *E. coli* and *A. hydrophila.* Thyme oil is used as a flavoring agent in the food industry (47).

Effect of essential oils on sensory evaluation:

In this study, thyme and black seed oils were added to manufactured cheese through inoculation and dipping. During dipping method 1% concentration of the 2 types was not accepted due to their very strong aroma, bitter taste with no clear effect on color and little effect on texture and appearance. While the same concentration through inoculation results in rejected cheese due to unpleasant color, odor, taste and unaccepted texture and appearance. On other hand, 0.1% concentration of both oils during dipping method was nearly accepted for color, odor and texture and appearance with little effect on flavor, while the same concentration during inoculation was accepted for color, appearance and texture with moderate effect on both odor and flavor. The most concentration was acceptable was 0.17% during dipping method, while the same concentration in inoculation method was acceptable except moderate effect on odor and flavor at 1st days that become normal with time. The concentration of 0.35% during dipping has the same effect as in case of 0.17% but 0.35% during inoculation was not accepted (table 1).

EL- Kholy et al. (48) reported that 0.1% of thyme oil was the most acceptable concentration according to sensory evaluation.

Effect of essential oils on bacterial count:

A. hydrophila:

A. hydrophila is an aquatic organism occurring naturally in freshwater like rivers and lakes (49). It is transmitted to man through the consumption of contaminated food or water (50). It is responsible for *Aeromonas* septicemia (MAS) epidemic outbreak (51).

In this study, the most reduction rate was resulted from adding thyme oil with 1% during dipping method and reached 100% from 2nd day and still along 10 days at refrigerator without deterioration in case of *A. hydrophila*. While, the same concentration of black seed oil had a moderate reduction effect on *A. hydrophila* not as in case of thyme oil, it was 14.29% at 2nd day, 32.86% at 4th day, gradually increased with time 38.57% at 6th day, 57.14% at 8th day, 61.43% at 10th day (table 2).

Table 1: Sensory evaluation of manufactured soft cheese treated with essential oils (Thyme oil +Black seed oil).

EO	Method	Color	Odor	Flavor	Appearance and texture
Thyme oil 1%	**Dipping**	No effect on color	Very strong aromatic odor	Not palatable (better taste)	Little effect on appearance and texture
Black seed oil 1%	**Dipping**	No effect on color	Very strong aromatic odor	Not palatable (better taste)	Little effect on appearance and texture
Thyme oil 1%	**(Inoculation)**	Not accepted (strong effect on color)	Very strong aromatic odor	Not palatable (Better taste).	Affect texture (No curd formation).
Black seed oil 1%	**(Inoculation)**	Not accepted (strong effect on color)	Very strong aromatic odor	Not palatable (better taste)	Affect texture(No curd formation)
Thyme oil 0.1%	**Dipping**	Accepted	Accepted	Little effect on Flavor(Slightly accepted)	No effect on appearance and texture
Black seed oil 0.1%	**Dipping**	Accepted	Accepted	Little effect on Flavor(Slightly accepted)	No effect on appearance and texture
Thyme oil 0.1%	**(Inoculation)**	Accepted	Moderate odor	Little effect on Flavor(Slightly accepted)	No effect on appearance and texture
Black seed oil 0.1%	**(Inoculation)**	Accepted	Moderate odor	Little effect on Flavor (Slightly accepted)	No effect on appearance and texture
Thyme oil (0.17%)	**Dipping**	Accepted	Accepted	Accepted	No effect on appearance and texture
Black seed oil (0.17%)	**Dipping**	Accepted	Accepted	Accepted	No effect on appearance and texture
Thyme oil (0.17%)	**(Inoculation)**	Accepted	Moderate effect	Moderate effect	No effect on appearance and texture
Black seed oil (0.17%)	**(Inoculation)**	Accepted	Moderate effect	Moderate effect	No effect on appearance and texture
Thyme oil (0.35%)	**Dipping**	Accepted	Moderate effect but decreased with time	Moderate effect but decreased with time until become accepted.	No effect on appearance and texture
Black seed oil (0.35%)	**Dipping**	Accepted	Moderate effect but decreased with time	Moderate effect but decreased with time until become accepted.	No effect on appearance and texture
Thyme oil (0.35%)	**(Inoculation)**	Little effect on color	Very strong aromatic odor	Not palatable (better taste)	No effect on appearance and texture
Black seed oil (0.35%)	**(Inoculation)**	Little effect on color	Very strong aromatic odor	Not palatable (better taste)	No effect on appearance and texture

Table 2: Reduction rate of *A. hydrophil*a with the addition of 1% EOs through dipping.

	2nd day	4th day	6th day	8th day	10 th day
Control	13.5%	-59.6%	-92.3%	-342.3%	-145.2%
***Aeromonas hydrophila* + Thyme oil**	100%	100%	100%	100%	100%
***Aeromonas hydrophila* + Black seed oil**	14.29%	32.86%	38.57%	57.14%	61.43%

Table 3: Reduction rate of *A. hydrophila* with the addition of 0.1% EOs through inoculation.

	2nd day	**4th day**	**6th day**	**8th day**	**10 th day**
Control	99.9%	98.6%	98.6%	99.5%	99.9
***Aeromonas hydrophila* + Thyme oil**	20%	26.67%	42%	55.33%	60%
***Aeromonas hydrophila* + Black seed oil**	-27%	-29.73%	-135%	13.5%	32.43%

Table 4: Reduction rate of *E. coli* with the addition of 0.17% EOs through inoculation.

	2nd day	**4th day**	**6th day**	**8th day**	**10 th day**
Control	53.13%	-62.5%	62.5%	-93.8%	28.13%
***E.coli* + Thyme oil**	100%	100%	100%	100%	100%
***E.coli* + Black seed oil**	46.67%	55%	60%	73.33%	80%

Table 5: Reduction rate of *E. coli* with the addition of 0.17% EOs through dipping.

	2nd day	**4th day**	**6thday**	**8th day**	**10 th**
Control	4.7%	41.9%	-32.6%	90%	77.4%
***E.coli* + Thyme oil**	-12.2%	92.45%	86.33%	75.5%	75.5%
***E.coli* + Black seed oil**	Zero	-142.86%	Zero	57.14%	57.14%

Table 6: Reduction rate of *E. coli* with the addition of 0.35% EOs through dipping.

	2nd day	**4th day**	**6th day**	**8th day**	**10 th day**
Control	4.5%	63.63%	28.8%	-112%	-400%
***E.coli* + Thyme oil**	100%	100%	100%	100%	100%
***E.coli* + Black seed oil**	89.55%	94.55%	94.55%	94.55%	96.82%

In case of 0.1% concentration during inoculation with thyme oil, reduction rate was 20% at 2nd day, 26.67% at 4th day, 42% at 6th day, and gradually increased till reach 55.33% at 8th day, 60% at 10th day. While in case of black seed oil with the same concentration, there was reduction in *A. hydrophila* count that noticed after 8th day with reduction rate 13.5%, till reach 32.43% at 10th day, after that cheese became deteriorated as in table (3).

Kirrella et al. (52) found that thyme oil had the most effective EO at concentration of 1% that extend shelf life of samples till 12 day and reduce *Aeromonas* count while 0.5% concentration keeps samples till 9th day only in healthy status.

E. coli:

Several *E. coli* isolates from raw milk and dairy products can cause severe foodborne illnesses in humans including hemolytic uremic syndrome, thrombotic thrombocytopenic purpura, hemorrhagic colitis, and bloody diarrhea (53), neonatal meningitis *E. coli* (NMEC), which is among the primary causes of meningitis in neonates worldwide (54) that may reach to critical illness and death (55).

In this study, we found that in case of 0.17% concentration during inoculation with thyme oil, reduction rate was 100% from 2nd day and still until 10th day in the same rate. On the other hand, in case of black seed oil the reduction rate was 46.67% at 2nd day, 55% at 4th day, 60% at 6th day, 73.33% at 8th day and reach 80% at 10th day. While the same concentration in dipping method, the count of *E. coli* starts to decrease at 4th day with reduction rate 92.45%, then reduction rate decreases slightly and became 86.33% at 6th day, 75.5% at 8th day and 75.5% at 10th day in case of thyme oil; but in case of dipping with black seed oil, the reduction of *E. coli* count started at 8th day with reduction rate 57.14% and still at the same rate at 10th day as in tables (4,5).

In case of 0.35 % concentration during dipping method, both thyme and black seed oil had high reduction rate and thyme oil had the highest

reduction rate that reach 100% from 2nd day and still at the same rate till 10th day, while reduction rate in case of black seed oil was 89.55% at 2nd day, 94.55% at 4th day, 94.55% at 6th day, 94.55% at 8th day and 96.82% at 10th day (table 6).

Control of pathogenic *E. coli* strains using thyme oil as a food additive with 1% thyme oil the reduction % reached 99.8% at 1st week and disappear at 2nd week of refrigerated storage (56). Salman et al. (23) reported that antibacterial effect of thyme oil against *E. coli* ranging from 0.02 to 1 % and Salem et al. (57) reported that thyme oil with concentration (1% and 1.5%) was acceptable till 8th day of storage, and (0.5%, 1% and 1.5%) decreased count of *E. coli* with reduction rate of 18.28% and 28.92% on 6th and 8th of storage, respectively.

Thyme oil acts as bacteriostatic and bactericidal against *E. coli* O157: H7 (58). Thymol, carvacrol and linalool in thyme are mainly the reason of the highest antimicrobial effects against bacteria (59). Hoel et al. (50) mentioned in their study that the inhibitory effect of thyme oil ranged from (0.063 to 1%) and it had the maximum antimicrobial activity against *E. coli*. Also Chandan (33) had similar report.

Black seed oil has antibacterial properties (60), but it had no inhibitory effect against *E. coli* O157:H7 while thyme oil considered the strongest EO (61). Reduction the inhibitory activity of EOs may be due to low temperatures that resulted in decreasing bacterial cell membrane permeability due to higher quantities of saturated fatty acids are contained in the membrane (62). Also, presence of fat, water, carbohydrates, proteins, salt, antioxidants, preservatives and some additives in food may decrease the inhibitory activity of EOs (63).

Silva et al. (44) reported that plant-based materials usage like thyme in food industry was necessary to inactivate *E. coli* O157:H7.

Oussalah et al. (64) and Al-Nabulsi et al. (61) reported that *E. coli* O157:H7 inhibited with 0.05% thyme oil. While Hossain et al. (65) found the effective concentration against *E. coli* O157:H7 was 0.01% while Selim (66) reported that the most effective thyme oil concentration was 0.25-0.5% on to reduce *E. coli* O157:H7 count. Alsawaf and Alnaemi (67) reported that black seed oil had an excellent effect on *E. coli* at concentration ranged from 0.3 to 1%, but Gill and Holley (26) and Al-Salman (68) had another aspect which said that black seed oil affect Gram +ve bacteria than Gram –ve bacteria and it was agree with our study.

Conclusion

Our study indicated that thyme oil is the most effective essential oil on Gram-negative bacterial count reduction while black seed oil had a moderate effect on reduction rate. In addition, some natural oils with strong aroma may be unacceptable from some consumers.

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Original Research Article
Veterinary Medicine and The One Health Concept

EFFECTS OF DIETARY NUCLEOTIDES SUPPLEMENTATION ON THE PERFORMANCE OF BROILER CHICKS

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Abstract: This study examined the effects of nucleotide supplementation on broiler chick growth performance, immunological response, carcass traits, meat quality, serum biochemical parameters, total antioxidant capacity, intestinal morphology, mortality rate, and economic efficiency measures of broiler chicks. A total of 180 one-day-old chicks (Ross 308) were distributed into 6 groups, each of which consisted of 30 chicks, and each group was divided into 3 replicates so that each replicate contained 10 chicks fed on six experimental diets as follows: basal diet without oil or nucleotide (T1), basal diet with 1% soybean oil and without nucleotide(T2), 50 grams nucleotide per 100-kilogram diet with different fat sources (no oil(T3), 1% soybean oil(T4), 1% linseed oil(T5) &1% fish oil(T6) respectively during the experimental period (5 weeks). Growth performance parameters were detected per stage period. Four chicks from each replicate were used at the termination of the experiment for analysis of the above mentioned measurements. Results revealed that supplementation of nucleotide in diets of broiler chicks improved feed conversion ratio, carcass traits, intestinal morphology, serum biochemical parameters, immunological response, bursa of Fabricius weight, and the best ratios were observed in groups fed nucleotide in combination with fish and linseed oil. Also, there was an increased economic efficiency in the SBO fed group(T2) then group fed nucleotides in mix with linseed oil (T5) and control (T1). In comparison to the control groups (T1 & T2), groups fed nucleotides and PUFA oil sources had significantly lower n-6: n-3 ratio in breast muscle, and mortality rate.

Key words: broiler; nucleotide; PUFA oil sources; growth performance; immunity; gut morphology and economic effi-ciency

Introduction

Production of poultry meat has improved frequently over the years and is expected to remain. Alternatively, advancement in genetic characters of poultry strains and more understanding of nutrition help chickens to achieve the market weight of 2 kg at 35 days old, and the efficiency of converting feed into poultry products moreover continues to progress (1). Most vegetable oils have a high omega-6 to omega-3 fatty acid ratio. Soybean oil is in an intermediate omega-6 to an omega-3 proportion (2). The very long chain polyunsaturated fatty acids (PUFAs) (C18–C22) and n-3 Omega PUFAs are evidently generally acknowledged as a piece of current nutrition as a result of their valuable impacts on metabolism (3). Also, dietary nucleotides are emerging as one of the potential feed additives because of their ability to enhance the villous growth of the intestine and production performance (4). In addition, nucleotide supplementation has numerous significant physiological, gastrointestinal, and immunological capabilities in the body through times of fast growth and development, disease tasks, injury or conditions of stress like high stocking density or dirty litters in addition to saving energy (5).

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Nucleotides of yeast source which supplemented in bird feed led to higher body weight, daily body weight gains, and better feed conversion ratios (5) but, they didn't have any effect on the feed intake of broiler chicken (6). Improvement in weight gain which particularly noted when nucleotides nourished at the first three weeks of life signifying ideal early bird development and will support performance later because the accessibility of nucleotides might be rate-limiting in rapidly dividing tissues, like in young chickens which have juvenile digestive system (7). The positive effect of a nucleotide preparation in broiler chickens with a dose of 500 mg/kg diet was demonstrated by Esteve-Garcia et al. (8). Exogenous nucleotides lower the animal's energy needs because de novo synthesis of nucleotides requires a lot of energy (5).

Improvements in villus height, crypt depth, and villus height to crypt depth are signs of a better gut in chickens fed nucleotides (5) & (9). Furthermore, Wu et al. (9) found that supplementing diets with nucleotides improved the gut flora, as seen by higher levels of lactic acid bacteria and a more varied intestinal microbiota. The immune system after dietary nucleotide supplementation has been boosted (10). Additionally, chickens have a quicker and more potent antibody response to standard immunizations (9).

Nucleotide-fed birds had higher body weights and, as a result, delivered more carcasses, including heavier drumsticks, thighs, wings, and breasts (7). Additionally, their meat had greater nutritional qualities, was redder in color and tender, and had more lipids with higher unsaturation degrees, all of which are good for humans' health and are aesthetically acceptable to consumers (11).

New researches are required to determine the impact of nucleotides added to broiler diets under conditions typical of commercial farms Pelcia et al. (12). As a result, the current study's goal was to examine the impact of dietary nucleotide supplementation when combined with various fat sources (soybean oil, linseed oil, and fish oil) on broiler performance, carcass traits, immune response, serum biochemical markers, intestinal morphological characteristics, mortalities, economic effectiveness, and their impact on breast lipid unsaturation levels.

Materials and methods

Place at which the study conducted

The study was conducted in the Department of Nutrition & Clinical Nutrition, Faculty of Veterinary Medicine, Zagazig University, Egypt.

Experimental birds, accommodation and management

A commercial hatchery provided 180 unsexed one-day-old broiler chicks (Ross 308) in total. Chicks were reared in brooder battery cages. They were then divided into 6 equal groups, and weighed individually upon entry (as an initial average body weight 43.47, 43.47, 34.33, 34.47, 34.44, 34.58 grams for groups from T1 to T6 respectively) each of which consisted of 30 chicks, and each group was divided into 3 replicates, so that each replicate contained 10 chicks. The feeding trial persisted for 5 weeks. All chicks were fed crumble diet in the starter and grower period, then pellet diet until the termination of the experimental period, where feeding was ad libitum. Chicks were vaccinated as stated by vaccination programs against Newcastle disease through Eye drops (Hitchner and La Sota vaccine at 7 and 17 days of age respectively). Six experimental (isocaloric and isonitrogenous) diets divided into 3 phases were formulated in accordance with Aviagen (13) to satisfy the nutritional needs of broiler chicks as follows: control T1 basal diet without oil or nucleotide, control T2 basal diet with 1% soybean oil and without nucleotide T3 basal diet with nucleotide 50 Gm/100 kg diet without oil addition T4 basal diet with nucleotide 50 Gm/100 kg diet plus 1% soybean oil T5 basal diet with nucleotide 50 Gm/100 kg diet plus 1% linseed oil T6 basal diet with nucleotide 50 Gm/100 kg diet plus 1% fish oil and shown in Table 1. A representative sample of each feed component was examined using SupNIR (FPI SupNIR-2700 SERIES, Hangzhou, Zhejiang, China). Metabolic energy was calculated according to Janssen (14). The diets were kept in a cold dry place to avoid oxidative rancidity.

The growth performance was determined by weighing the daily feed consumption and the body weight for each cage after each period until

5 weeks. Total feed intake, weight gain, and feed to gain ratio were calculated at the conclusion of the feeding trial.

Tested feed additive: Nucleoforce Poultry is a concentrated form of unlimited nucleotides made from dry yeast extracts and suited for broilers and layers developed by Bioiberica. The product is a creamy-colored powder with a nucleotide content of 26.4%.

Sampling

at 5-wk-old, four birds from each replicate were sampled, weighed, slaughtered, and eviscerated without a feed withdrawal period according to Brake et al. (15). Eviscerated carcass, Liver, heart, gizzard, spleen, bursa, thymus gland, breast, thigh, abdominal fat yields and whole evacuated intestine were weighed for calculating dressing percent. Weights of the three lymphoid organs (thymus, spleen, and bursa of Fabricius) from the slaughtered birds in each group were recorded in order to determine the relative organ weight.

Humoral immune response for Newcastle virus vaccine antibodies was measured by hemagglutination Inhibition test according to Anon (16). Also, the total leukocyte count of non-coagulated blood samples during slaughter was measured at the termination of the trial using an automatic blood analyzer (Diagon ® Ltd D-Cell 60 auto hematology analyzer). According to Wahlefeld and Bergmeyer (17), triglyceride levels in the serum were examined, total cholesterol depending on Naito and Kaplan (18), serum low-density lipoprotein-cholesterol, serum very low-density lipoprotein (Triglyceride/5) based on Friedewald et al. (19), serum high-density lipoprotein-cholesterol on the basis of Burstein and Scholnick (20), total serum protein as stated by Grant et al. (21), albumin as stated by Doumas and Biggs (22) and globulin on the basis of Doumas and Biggs (23). Serum levels of total antioxidant capacity and glutathione peroxidase were tested in line with the methods approved by Koracevic et al. (24). Homogenized freeze-dried meat from the breast was evaluated for fatty acid content on the basis of the method described by Folch et al. (25), and fatty acid methyl esters were prepared as designated by Ichihara and Fukubayashi (26) through gas chromatography.

According to Drury et al. (27), representative samples from the jejunum were used in the histopathological examination of the intestine to measure villous height (at 100X), crypts depth (at 100X), villous height: crypt depth ratio (at 100X), intestinal wall thickness (at 40X), goblet cell proliferation, villus width (at 100X), and villus perimeter and surface area. The perimeter of the villus was computed as (2π × (average villi width/2) × villous height) and the surface area of villus was computed as (villus perimeter× villous height). Throughout the trial, mortality rates for each treatment were determined.

According to El-Kerdawy (28), an economic analysis was calculated using the following equation: Y= (A-B)/B x 100, where A is the selling cost of the acquired gain and B is the feeding cost of this gain.

Statistical analysis

The mean standard error and the coefficient of variation for the prior data were determined using the standard statistical formula which has been given by Snedecor and Cochran (29). The obtained data will be studied by ANOVA one-way classification via a totally randomized plan to detect the significance of the distinction between assorted treatment groups.

Our findings in table 2 demonstrated that the main impact of dietary nucleotides on broiler chick growth performance was that there was no significant difference in final BW, BWG, and FI between all groups.

All treatments containing nucleotides significantly reduced FCR compared to control treatments which did not have it, and the best FCR was recorded in dietary nucleotides in combination with fish and linseed oil treated groups. According to the results of the carcass traits in table 3, the dressing %, breast yield, bursa of Fabricius, thymus, and intestinal weight were considerably ($P<0.05$) higher in the nucleotide-fed

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Table 1: Composition of the experimental diets' ingredients (%)

	Starter						grower						finisher					
Feeding period (days)	0-10 days						11-24 days						25-35 days					
Groups	T 1	T 2	T 3	T 4	T 5	T 6	T 1	T 2	T 3	T 4	T 5	T 6	T 1	T 2	T 3	T 4	T 5	T 6
Yellow corn	59.71	57.10	59.69	56.74	56.64	56.64	65.00	62.74	65.00	62.68	62.71	62.71	71.42	69.11	71.40	69.08	69.08	69.08
Corn gluten meal 60%	12.2	9.66	12.25	9.91	9.90	9.90	15.55	12.73	15.50	12.75	12.73	12.73	17.85	15.10	17.90	15.10	15.10	15.10
Soybean meal 46%	23.32	27.60	23.23	27.70	27.80	27.80	14.93	19.12	14.93	19.11	19.15	19.15	6.45	10.62	6.37	10.60	10.60	10.60
Oil	0.00	1.00[1]	0.00	1.00[1]	1.00[2]	1.00[3]	0.00	1.00[1]	0.00	1.00[1]	1.00[2]	1.00[3]	0.00	1.00[1]	0.00	1.00[1]	1.00[2]	1.00[3]
Ground limestone	1.87	1.85	1.87	1.86	1.86	1.86	1.70	1.65	1.70	1.65	1.65	1.65	1.59	1.59	1.59	1.59	1.59	1.59
mono-ca. phosphate	1.29	1.25	1.29	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.10	1.05	1.10	1.05	1.05	1.05
L-lysine	0.41	0.33	0.41	0.32	0.32	0.32	0.46	0.38	0.46	0.38	0.38	0.38	0.53	0.44	0.53	0.44	0.44	0.44
Dl- methionine	0.19	0.20	0.19	0.20	0.19	0.19	0.11	0.13	0.11	0.13	0.13	0.13	0.07	0.09	0.07	0.09	0.09	0.09
L-threonine	0.07	0.06	0.07	0.05	0.05	0.05	0.04	0.03	0.04	0.03	0.03	0.03	0.04	0.03	0.04	0.03	0.03	0.03
Nucleoforce ***	0.00	0.00	0.05	0.05	0.05	0.05	0.00	0.00	0.05	0.05	0.05	0.05	0.00	0.00	0.05	0.05	0.05	0.05
Vitamin mineral premix*	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Common salts	0.33	0.35	0.33	0.33	0.33	0.33	0.33	0.35	0.33	0.33	0.33	0.33	0.35	0.35	0.35	0.35	0.35	0.35
Sod. bicarbonate	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Phytase	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Antimycotoxins	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Sum	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Nutritive value																		
CP	23.00	23.16	23.00	23.31	23.35	23.35	21.52	21.50	21.50	21.52	21.52	21.52	19.52	19.53	19.52	19.52	19.52	19.53
EE	2.69	3.56	2.69	3.58	3.55	3.55	3.74	3.74	3.74	3.74	3.74	3.74	3.94	3.94	3.94	3.94	3.94	3.94
ME	3000.16	3000.38	3000.18	3000.24	3000.09	3000.09	3100.91	3100.32	3100.25	3100.05	3100.74	3100.74	3200.36	3200.30	3200.36	3200.36	3200.36	3200.84
Calcium	0.96	0.96	0.96	0.96	0.96	0.96	0.87	0.87	0.87	0.87	0.87	0.87	0.79	0.79	0.79	0.79	0.79	0.79
Av. Phosphorous	0.45	0.45	0.45	0.45	0.45	0.45	0.44	0.44	0.44	0.44	0.44	0.44	0.40	0.40	0.40	0.40	0.40	0.40
N-3: N-6 ratio**	1:31.81	1:14.96	1:31.85	1:14.94	1:2.33	1:3.55	1:37.65	1:16.31	1:37.65	1:16.31	1:2.47	1:3.86	1:45.06	1:17.79	1:45.06	1:17.79	1:2.68	1:4.18

* Premix contain vitamins and minerals according to requirement for broiler chicks as recommended in Aviagen (13) and was Produced by Multivita Company. ** According to Gunstone's (30) calculations, various n-3 to n-6 fatty acid ratios. *** Tested feed additives: Nucleoforce Poultry which is a concentrated version of unrestricted nucleotides derived from dried yeast extracts and intended for broilers and layers obtained from dried yeasts extracts were produced by company called Bioiberica. The product is creamy colored powder contain 26.4% nucleotides. [1]=soybean oil, [2]=linseed oil, [3]= fish oil

Results

Table 2: Effect of dietary nucleotides supplementation on overall growth performance of broiler chicks

	Dietary nucleotides supplementation%					
	T1 0% nucleotides without oil	**T2 0% nucleotides with 1% SBO**	**T3 0.05% Nucleotides without oil**	**T4 0.05% nucleotides with 1% SBO**	**T5 0.05 % nucleotides with 1% LO**	**T6 0.05 % nucleotides with 1% FO**
BW, g/bird	2253.15 ± 88.10	2355.89 ± 63.32	2241.42 ± 51.50	2286.63 ± 80.13	2365.00 ± 57.50	2412.08 ± 56.81
BWG, g/bird	2209.68 ± 88.02	2312.42 ± 63.33	2198.08 ± 51.58	2243.15 ± 80.06	2321.56 ± 57.62	2368.50 ± 56.95
FI, g/bird	3247.15 ± 143.90	3341.94 ± 88.50	3112.29 ± 59.69	3202.43 ± 65.12	3152.63 ± 64.53	3212.95 ± 103.36
FCR	1.47^{a} ± 0.03	1.45^{ab} ± 0.00	1.42^{abc} ± 0.04	1.43^{abc} ± 0.03	1.36^{bc} ± 0.02	1.36^{c} ± 0.01

$^{a, b, c}$ means ± standard Error values within the same row with various superscripts differ significantly (P<0.05).

BW, BWG, FI and FCR, stand for body weight, body weight gain, feed intake and feed conversion ratio, respectively.

Table 3: Effect of nucleotides supplementation in chicks' diets on carcass traits

	Dietary nucleotides supplementation%					
	T1 0% nucleotides without oil	**T2 0% nucleotides with 1% SBO**	**T3 0.05% Nucleotides without oil**	**T4 0.05% nucleotides with 1% SBO**	**T5 0.05% nucleotides with 1% LO**	**T6 0.05% nucleotides with 1% FO**
Body weight, g	2241.67^{b} ± 7.26	2358.33^{a} ± 4.41	2346.67^{ab} ± 52.07	2346.67^{ab} ± 42.06	2343.33^{ab} ± 18.56	2393.33^{a} ± 40.86
Carcass weight, g	1520.00^{d} ± 2.89	1614.33^{c} ± 2.33	1680.00^{bc} ± 33.29	1713.33^{ab} ± 36.09	1725.00^{ab} ± 10.41	1788.33^{a} ± 33.46
Carcass %	67.81^{d} ± 0.18	68.45^{d} ± 0.08	71.60^{c} ± 0.41	73.00^{b} ± 0.25	73.62^{b} ± 0.33	74.72^{a} ± 0.14
Liver%	2.06^{b} ± 0.05	2.36^{a} ± 0.04	2.15^{ab} ± 0.13	2.39^{a} ± 0.07	2.32^{a} ± 0.04	2.31^{a} ± 0.06
Heart%	0.44 ± 0.06	0.47 ± 0.03	0.57 ± 0.06	0.43 ± 0.03	0.44 ± 0.04	0.55 ± 0.03
Spleen %	0.14 ± 0.00	0.17 ± 0.06	0.11 ± 0.03	0.12 ± 0.01	0.10 ± 0.01	0.15 ± 0.00
Gizzard%	1.46 ± 0.10	1.15 ± 0.05	1.26 ± 0.12	1.27 ± 0.08	1.14 ± 0.17	1.30 ± 0.04
Total fat%	2.89 ± 0.12	2.80 ± 0.40	2.43 ± 0.36	2.89 ± 0.10	2.64 ± 0.20	2.17 ± 0.04
Bursa %	0.04^{b} ± 0.00	0.04^{b} ± 0.00	0.05^{ab} ± 0.01	0.05^{ab} ± 0.01	0.04^{ab} ± 0.00	0.06^{a} ± 0.00
Thymus %	0.26^{b} ± 0.04	0.28^{b} ± 0.01	0.42^{a} ± 0.07	0.34^{ab} ± 0.02	0.31^{ab} ± 0.02	0.37^{ab} ± 0.02
Intestine %	2.57^{b} ± 0.19	2.50^{b} ± 0.08	3.14^{a} ± 0.05	3.14^{a} ± 0.05	3.24^{a} ± 0.03	3.33^{a} ± 0.10
Pectoral muscle %	29.07^{b} ± 0.23	29.82^{b} ± 0.70	30.01^{b} ± 0.93	29.79^{b} ± 1.00	30.02^{b} ± 0.42	32.67^{a} ± 1.08
Thigh weight %	27.51^{ab} ± 0.12	27.91^{a} ± 0.35	26.33^{c} ± 0.45	26.53^{bc} ± 0.29	27.10^{abc} ± 0.30	27.26^{abc} ± 0.16

a, b, c, d Means ± standard Error values within the same row with various superscripts differ significantly (P <0.05).

Table 4: Effect of nucleotides supplementation in chicks' diets on Serum biochemical parameters and total Antioxidant Capacity

	Dietary nucleotides supplementation%					
	T1 0% nucleotides without oil	**T2 0% nucleotides with 1% SBO**	**T3 0.05% nucleotides without oil**	**T4 0.05% nucleotides with 1% SBO**	**T5 0.05 % nucleotides with 1% LO**	**T6 0.05 % nucleotides with 1% FO**
Triglycerides (mg/dl)	$57.16^{a} \pm 1.96$	$48.74^{abc} \pm 1.40$	$53.41^{ab} \pm 2.76$	$46.82^{bc} \pm 5.19$	$41.78^{cd} \pm 1.93$	$37.61^{d} \pm 0.95$
Total cholesterol (mg/dl)	$141.71^{a} \pm 6.98$	$131.92^{ab} \pm 7.93$	$135.46^{ab} \pm 2.28$	$125.25^{b} \pm 0.08$	$105.63^{c} \pm 3.89$	$100.29^{c} \pm 0.63$
HDL (mg/dl)	56.26 ± 0.67	58.99 ± 0.75	53.58 ± 0.87	54.82 ± 2.38	56.28 ± 1.59	58.02 ± 3.34
LDL (mg/dl)	$71.45^{a} \pm 7.60$	$60.93^{a} \pm 7.24$	$68.61^{a} \pm 2.93$	$58.75^{a} \pm 2.77$	$39.10^{b} \pm 3.61$	$33.00^{b} \pm 3.69$
VLDL (mg/dl)	$11.43^{a} \pm 0.39$	$9.75^{abc} \pm 0.28$	$10.68^{ab} \pm 0.55$	$9.36^{bc} \pm 1.04$	$8.36^{cd} \pm 0.39$	$7.52^{d} \pm 0.19$
Cholesterol ester (mg/dl)	$2.57^{a} \pm 0.16$	$2.25^{a} \pm 0.12$	$2.58^{a} \pm 0.07$	$2.31^{a} \pm 0.11$	$1.89^{b} \pm 0.09$	$1.75^{b} \pm 0.11$
Total protein (mg/dl)	$3.24^{b} \pm 0.10$	$3.28^{b} \pm 0.09$	$3.51^{ab} \pm 0.12$	$3.47^{ab} \pm 0.06$	$3.72^{a} \pm 0.04$	$3.66^{a} \pm 0.11$
Albumin (g/dl)	1.89 ± 0.07	1.93 ± 0.04	1.83 ± 0.13	1.75 ± 0.01	1.72 ± 0.07	1.73 ± 0.08
Globulin (g/dl)	$1.35^{c} \pm 0.03$	$1.35^{c} \pm 0.09$	$1.68^{b} \pm 0.01$	$1.72^{b} \pm 0.07$	$2.00^{a} \pm 0.11$	$1.93^{ab} \pm 0.11$
TAC (mM/l)	$0.33^{a} \pm 0.01$	$0.29^{ab} \pm 0.04$	$0.27^{ab} \pm 0.03$	$0.24^{bc} \pm 0.01$	$0.18^{cd} \pm 0.02$	$0.14^{d} \pm 0.01$
GSH-Px (IU/mg)	$48.99^{c} \pm 1.89$	$50.98^{c} \pm 4.87$	$66.50^{b} \pm 3.62$	$68.50^{b} \pm 4.07$	$74.83^{ab} \pm 2.62$	$79.76^{a} \pm 2.80$

a, b, c, d Means ± standard error in the same row with different superscripts are significantly different (P<0.05).The abbreviations HDL, LDL, VLDL, TAC and GSH-Px stand for high-density lipoprotein, low-density lipoprotein, very low-density lipoprotein, total antioxidant capacity and glutathione peroxidase respectively.

Table 5: Effect of nucleotides supplementation in chicks' diets on Newcastle vaccine antibody titter, total leucocyte count and mortality rate

	Dietary nucleotides supplementation%					
Exp. period (weeks)	**T1 0% nucleotides without oil**	**T2 0% nucleotides with 1% SBO**	**T3 0.05% nucleotides without oil**	**T4 0.05% nucleotides with 1% SBO**	**T5 0.05 % nucleotides with 1% LO**	**T6 0.05 % nucleotides with 1% FO**
Antibody titer1st day	9.67 ± 0.33	9.67 ± 0.33	9.67 ± 0.33	9.67 ± 0.33	9.67 ± 0.33	9.67 ± 0.33
Antibody titer at 14-day age	3.33 ± 0.33	3.67 ± 0.67	4.33 ± 0.88	4.33 ± 1.20	5.67 ± 0.33	5.67 ± 0.33
Antibody titer at 26-day age	3.00 ± 0.00	3.33 ± 0.88	4.00 ± 1.00	4.33 ± 0.88	5.00 ± 0.00	5.00 ± 0.58
Antibody titer at 35-day age	2.33 ± 0.88	3.33 ± 0.33	2.67 ± 0.33	3.33 ± 0.33	4.00 ± 0.58	4.33 ± 0.88
Total leucocyte count	$166.07^{b} \pm 2.46$	$170.87^{b} \pm 3.01$	$188.83^{a} \pm 5.60$	$185.57^{a} \pm 6.37$	$193.17^{a} \pm 2.68$	$193.03^{a} \pm 3.33$
Mortality rate	13.87 ± 2.79	8.32 ± 4.81	11.11 ± 7.35	2.77 ± 2.77	2.77 ± 2.77	0.00 ± 0.00

a, b, means ± standard Error values within the same row with various superscripts differ significantly (P<0.05)

Table 6: Effect of nucleotides supplementation in chicks' diets on intestinal morphology

Intestinal morphology	Dietary nucleotides supplementation%					
	T1 0% nucleotides without oil	T2 0% nucleotides with 1% SBO	T3 0.05% nucleotides without oil	T4 0.05% nucleotides with 1% SBO	T5 0.05 % Nucleotides with 1% LO	T6 0.05 % nucleotides with 1% FO
VH	$823.00^{f} \pm 7.51$	$896.00^{e} \pm 4.04$	$1091.33^{d} \pm 3.76$	$1112.33^{c} \pm 4.33$	$1178.00^{b} \pm 1.15$	$1222.00^{a} \pm 5.03$
CD	$84.40^{e} \pm 2.83$	$93.60^{d} \pm 1.85$	$114.10^{c} \pm 2.48$	$122.67^{b} \pm 2.62$	$133.73^{a} \pm 3.12$	$130.43^{ab} \pm 2.24$
VH/CD	$9.78^{a} \pm 0.42$	$9.58^{ab} \pm 0.15$	$9.57^{ab} \pm 0.17$	$9.07^{ab} \pm 0.16$	$8.82^{b} \pm 0.20$	$9.37^{ab} \pm 0.12$
Mucosa thickness	$418.50^{f} \pm 4.05$	$431.00^{e} \pm 2.16$	$724.13^{d} \pm 3.35$	$973.87^{b} \pm 3.10$	$1390.00^{a} \pm 2.89$	$846.77^{c} \pm 3.45$
wall thickness	$492.67^{e} \pm 2.90$	$472.47^{f} \pm 1.55$	$1037.83^{d} \pm 2.52$	$1200.13^{b} \pm 2.89$	$1516.20^{a} \pm 2.72$	$1083.90^{c} \pm 2.48$
VW	$47.83^{d} \pm 1.17$d	$73.28^{c} \pm 1.09$	$75.03^{bc} \pm 2.07$	$77.43^{bc} \pm 1.37$	$83.02^{a} \pm 2.13$	$79.73^{ab} \pm 1.54$
villus perimeter x 10^4	$12.36^{d} \pm 0.19$	$20.63^{c} \pm 0.23$	$25.73^{b} \pm 0.75$	$27.06^{b} \pm 0.57$	$30.73^{a} \pm 0.79$	$30.61^{a} \pm 0.53$
villus surface area x 10^6	$10.17^{a} \pm 0.08$	$18.48^{c} \pm 0.15$	$28.08^{b} \pm 0.88$	$30.11^{b} \pm 0.75$	$36.19^{a} \pm 0.94$	$37.40^{a} \pm 0.61$

a, b, c, d, e, f Means ± standard Error values within the same row with various superscripts differ significantly (P<0.05). The abbreviations VH, CD, VH/CD and VW stand for villus height, crypt depth, Villous height to Crypt depth and Villous width respectively.

Table 7: Effect of nucleotides supplementation in chicks' diets on breast muscle content of fatty acids (%)

fatty acids percent of breast muscle	Dietary nucleotides supplementation%					
	T1 0% nucleotides without oil	T2 0% nucleotides with 1% SBO	T3 0.05% nucleotides without oil	T4 0.05% nucleotides with 1% SBO	T5 0.05 % nucleotides with 1% LO	T6 0.05 % nucleotides with 1% FO
SFA	$29.31^{e} \pm 0.14$	$54.23^{a} \pm 0.25$	$27.83^{f} \pm 0.13$	$51.36^{b} \pm 0.24$	$34.36^{c} \pm 0.10$	$32.06^{d} \pm 0.15$
USFA	$65.71^{b} \pm 0.31$	$45.47^{e} \pm 0.22$	$69.57^{a} \pm 0.33$	$47.82^{d} \pm 0.22$	$62.65^{c} \pm 0.30$	$66.43^{b} \pm 0.31$
Total n-3 fatty acids	$1.69^{e} \pm 0.14$	$2.19^{de} \pm 0.17$	$2.80^{c} \pm 0.23$	$2.59^{cd} \pm 0.21$	$9.18^{b} \pm 0.04$	$22.93^{a} \pm 0.11$
Total n-6 fatty acids	$36.55^{a} \pm 3.01$	$14.14^{b} \pm 1.09$	$38.67^{a} \pm 0.18$	$15.37^{b} \pm 1.27$	$18.24^{b} \pm 0.09$	$15.07^{b} \pm 0.07$
n-6:n-3 ratio	$22.94^{a} \pm 1.89$	$6.97^{c} \pm 0.53$	$15.38^{b} \pm 1.27$	$6.87^{c} \pm 0.52$	$2.09^{d} \pm 0.17$	$0.69^{d} \pm 0.06$

a, b, c, d, e, f Means ± standard Error values within the same row with various superscripts differ significantly (P<0.05). The abbreviations USFA = unsaturated fatty acid, SFA =saturated fatty acid; n-6=omega 6, n-3=omega 3

Table 8: Effect of nucleotides supplementation on the economic efficiency of the experimental diets

	Dietary nucleotides supplementation%					
	T1 0% nucleotides without oil	T2 0% nucleotides with 1% SBO	T3 0.05% nucleotides without oil	T4 0.05% Nucleotides with 1% SBO	T5 0.05 % Nucleotides with 1% LO	T6 0.05 % nucleotides with 1% FO
Feeding cost of the obtained gain (LE)	18.52	18.93	18.94	19.43	19.42	20.27
Selling price of the obtained gain (LE/kg live weight)	23	23	23	23	23	23
Selling cost of obtained gain (LE)	50.82	53.19	50.56	51.59	53.40	54.48
Economic efficiency (EE) %	174.41	180.92	166.90	165.55	174.94	168.73

(LE)= Egyptian pound date of experiment= 1/3//2019

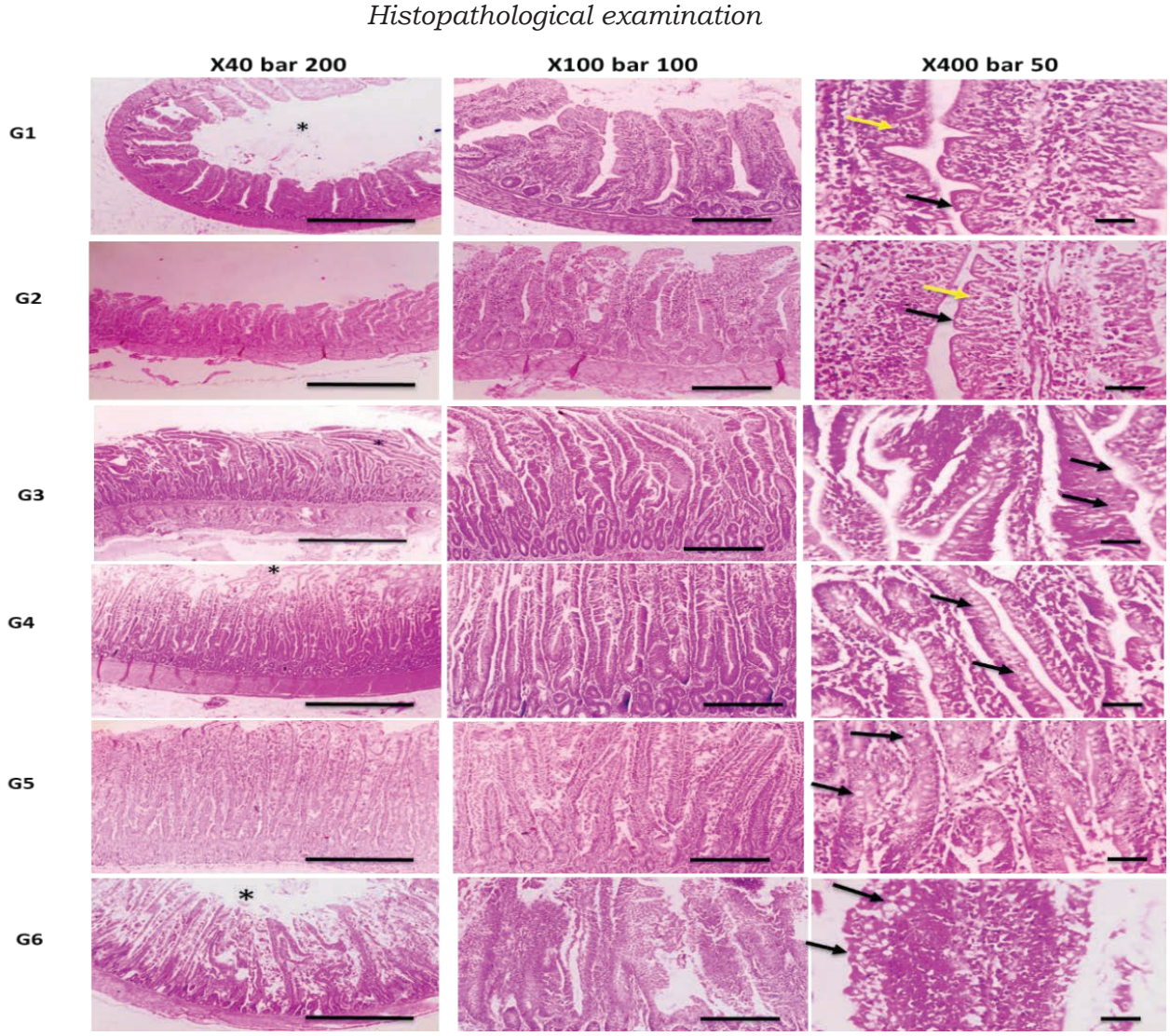


Figure 1: microscopic images of the jejunal sections stained with H&E

Microscopic images of H&E-stained jejunal slices from the control group show normal villi, lamina propria, and muscular coat. Higher magnification X: 400 show presence of few goblet cells (black arrow) along with epithelial vacuolization (yellow arrows). Meanwhile, groups supplemented with mixture of different oils and nucleotide showing gradual increase in villous height, numbers and size of goblet cells (black arrow) and decrease epithelial vacuolization (yellow arrows). (Asterisks point to lumen of jejunum).

table 3, the dressing %, breast yield, bursa of Fabricius, thymus, and intestinal weight were considerably ($P<0.05$) higher in the nucleotide-fed groups than in the control groups. The group administered nucleotides together with fish and linseed oil showed the best carcass characteristic, bursa of Fabricius, and intestinal weight.

Our findings in table 4 demonstrated that serum concentrations of triglycerides, cholesterol, LDL, VLDL, TAC, and albumin (A) were significantly ($P<0.05$) decreased with increased serum content of total protein (TP), globulin, and GSH-Px values with nucleotide supplementation, particularly in combination with PUFA oil sources.

According to the results in table 5, there was no significant difference in antibody titer against Newcastle virus vaccine between all groups but TLC significantly ($P<0.05$) increased with nucleotides fed than control groups and the best value was recorded in group fed nucleotides in combination with fish and linseed oil. Also, significant ($P<0.05$) decreases mortality ratio with nucleotides fed groups and the best value was observed in in group fed nucleotides in combination with fish oil.

As shown in table 6 significant ($P<0.05$) improvement in villus height, crypt depth, mucosal thickness, wall thickness, villus width, villus

perimeter, and villus surface area in nucleotide fed groups compared to control groups, especially when combined with PUFA oil sources (FO, LO, and then SBO).

As shown in table 7, there was a substantial difference between each group. With T1, T3, T5, and T6 compared to T2 and T4, there were significantly ($P<0.05$) higher levels of USFA and lower levels of SFA in the breast muscle. N-3 fatty acids are present in greater percentages in T3, T4, T5, and T6 than in T1 and T2. In comparison to other groups, T1 and T3 had significantly ($P<0.05$) greater levels of N-6 fatty acids. As a result, the n-6: n-3 ratio gradually dropped with the addition of nucleotides to the diet and USFA oil sources.

Our results showed in table 8 that the feeding cost of the obtained gain (LE) and the selling cost of the obtained gain (LE) had increased with nucleotide use and the highest economic efficiency was recorded in the control group (T2), which was supplemented with SBO then group fed nucleotides in combination with linseed oil and control group(T1) than other groups.

Discussion

Effect of nucleotides dietary supplement on growth performance

The chief conclusions of the positive effect of dietary nucleotides on the growth response of broiler chicks are presented in Table 2, where the best *growth performance* was recorded in dietary nucleotides in combination with fish and linseed oil treated groups. These findings were in line with Jung & Batal (5) and Salah et al. (6) who discovered that adding yeast nucleotides to bird diet increased body weight, daily weight gains, and feed conversion ratios without affecting feed consumption. However, Pelcia et al. (12) discovered that the 0.05, 0.06, and 0.07% additions of nucleotides to broiler feed had no effect on the broiler's performance. The encouraging outcomes could be attributed to the easier access to nucleotides for the growth of intestinal cells and subsequently improved activity of the digestive enzymes, which lead to improved digestion and nutritional absorption (6) Additionally, according to Jung and Batal (5) supplementing broiler feed with nucleotides increased villus height and villus height-to-crypt depth ratio while lowering the animal's energy consumption because de novo synthesis of nucleotides requires a lot of energy. Oil sources like fish and linseed oil, which are well known as important nutrients for health and are essential for many regular bodily activities as well as stimulating growth, also have a dual effect with omega-3 fatty acids (31). Additionally, the involvement of n-3 PUFA in the enhancement of bile production, which enhances fat digestion in the colon, enhances the effectiveness of feed digestion and absorption (32).

Effect of nucleotides dietary supplement on carcass composition

According to our findings in Table 3, nucleotide supplementation improved carcass characteristics and intestinal weight and decreased the fat content of the breast muscles. The group administered nucleotides together with fish and linseed oil showed the best carcass characteristics and intestinal weight. The liver, gizzard, and lean meat production showed the maximum performance in the group treated with 0.03% of nucleotides, according to Fonia et al (33) findings that dietary nucleotide supplementation improves the weight of different organ cut up sections. However, Pelcia et al (12) findings were different, showing that adding nucleotides to broiler feed in amounts of 0.05, 0.06, and 0.07% had no impact on carcass output. On the other hand, nutritional augmentation with omega-3 fatty acids enhances the carcass by lowering broiler abdominal fat distribution (34).

Effect of nucleotides dietary supplement on serum biochemical parameters

Our findings in table 4 demonstrated that nucleotide supplementation improved serum biochemical parameters, particularly in combination with PUFA oil sources. These outcomes were consistent with studies that observed decreased serum cholesterol and LDL cholesterol and increased HDL cholesterol with higher levels of nucleotide fed groups in addition to a significant improvement in total protein, albumin, and globulin, but A/G ratio showed no effect (35) and (36). A heightened immunological response and the proteinic character of the antibodies may be the cause of the increase in serum concentra-

tion of total protein with omega-3 fatty acid (37). However, adopting diets including fish oil as an omega-3 fatty acid source considerably increased blood HDL-c, total protein (TP), globulin (GL), and GSH-Px values while significantly lowering serum triglycerides, cholesterol, LDL, VLDL, albumin (A), and TAC concentrations (P<0.05) (38). Additionally, Qi et al. (39) and Ibrahim et al. (40) discovered that adding n-3 PUFA to the diets clearly improved antioxidative status.

The effect of nucleotides dietary supplement on avian immune function

According to our findings in Table 5, the groups fed nucleotides in conjunction with fish and linseed oil had the best outcomes, with antibody titers against the Newcastle disease vaccination, TLC and bursa of Fabricius weight. These findings support the work of Raheel et al. (10), who demonstrated that the immune system is strengthened by nucleotides when they are added to the diet. increased bird immunoglobulin production is another factor (41). in order to lessen the effects of pathogens (42). Likewise, adding nucleotides at a dosage of 0.5 g/kg raised bursa of Fabricius weight in comparison to the control group but had no impact on spleen weight (6). This outcome could have occurred because the percentage of cell turnover in tissues like the Fabricius bursa was increased during stresses. They need enough extra nucleotides to synthesize DNA and RNA for maintenance and growth in this way (41). The intestinal lumen is where foreign nucleotides are absorbed, and they subsequently go to immunological organs like the bursa (43). In addition, Hassanein et al. (38) discovered that TLC substantially (P< 0.05) provided the best ratio in fish oil-fed groups as opposed to control groups.

Effects of nucleotides dietary supplement on mortality ratio

According to our findings in Table 5, nucleotide supplementation decreased the mortality ratio, and the lowest value were observed when mixed with fish oil. This outcome was consistent with the findings of Daneshmand et al. (41), who discovered that broilers given nucleotides had a decreased mortality rate. It could be because, in addition to storing energy, nucleotides perform several vital physiological, gastrointestinal, and immune roles in the organism during rapid growth and development, disease problems, injury or stress situations like high stocking density or unclean litters (5). In addition, Hassanein et al. (38) shown that supplementing with fish oil considerably (P 0.05) reduces the mortality ratio.

Effects of nucleotides dietary supplement on intestinal morphology

By significantly, Table 6 demonstrates that intestinal morphology in nucleotide-fed groups is significantly improved when compared to control groups, especially when combined with PUFA oil sources (FO, LO, then SBO). These findings were in agreement with those of Khedr et al. (44), who demonstrated that, in the jejunum region of the small intestine, broiler groups fed nucleotides showed a substantial increase in intestinal villi length when compared to control groups. And yet, Hassanein et al. (38) found that fish oil-based diets significantly improved intestinal morphology compared to control groups in terms of villus height, crypt depth, mucosal thickness, wall thickness, villus width, villus perimeter, and villus surface area. However, Aziza et al. (45) found that when compared to fish oil and camelina meal holding diets, control and camelina meal diets as a source of n-3 PUFA increased villus height, VH: CD, and villus perimeter of the jejunum, and there was no significant difference in villus width, surface area, or muscularis thickness between different groups. These effects might be caused by fish oil, which enhances the intestine's absorption capacity. As a result, broilers fed these diets should thrive and have more robust immune systems (37).

Effect of nucleotides dietary supplement on meat quality

Significant changes were found between the groups, as shown in Shawn's table 7, where the addition of nucleotides and USFA oil sources to the diet gradually reduced the n-6: n-3 ratio in the breast muscle. These findings corroborated those of Chiofalo et al. (11), who concluded that meat had better nutritional qualities because it included more lipids with greater unsaturation degrees, which had positive impacts on human health when consumed with nucleotides.

The economic evaluation of nucleotides dietary supplement

According to our results in Table 8 nucleotide supplementation increased the feeding cost of obtained gain (LE) and selling cost of obtained gain and had the highest economic efficiency only when used in conjunction with linseed oil. But as demonstrated by Zahran et al. (46), Ahiwe et al. (47), and Fathi et al. (48), broiler groups given a diet containing nucleotides performed significantly better economically than the control group. However, Hassanein et al. (38) report that the group given 1% FO had the best economic efficiency, followed by 1% SBO, 2% FO, 3% FO, and 0% oil, in that order.

Conclusion

The performance of growth, dressing percentage, meat quality, serum biochemical parameters, immunological response, and intestinal morphology in chicks is considerably enhanced by the addition of nucleotides to their diets. It also lowers mortality, but it's only cost-effective when used in conjunction with linseed oil.

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Original Research Article
Veterinary Medicine and The One Health Concept

MONITORING THE INFLAMMATORY PROCESS OF FELINE LOWER URINARY TRACT DISEASE

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Abstract: This study aimed to compare the hemato-biochemical parameters in cats suffered from FLUTD with those of healthy cats, and to evaluate the inflammatory process by measuring serum amyloid A, alpha-1-Acid glycoprotein, and plasma fibrinogen concentrations. Cats frequently suffer from feline lower urinary tract disease (FLUTD), which causes acute renal failure, electrolyte buildup, and acid-base imbalance. Acute-phase proteins can be used to monitor the inflammatory processes of feline lower urinary tract disease.

The present study included thirty cats of both sexes, nine cats defined as clinically healthy cats and 21 cats with signs of idiopathic cystitis and/ or urethral obstruction for up to 24 hours were defined as cat with FLUTD group. Blood samples were collected from cephalic vein for hematological, biochemical assays and the measurement of acute-phase protein concentrations, including SAA, AGP, and fibrinogen. Serum SAA and AGP were measured using commercial ELISA kits.

In cats with FLUTD, the total white blood cell count, neutrophils, and platelets count increased significantly, as did the mean values of hematological and biochemical indices. However, there was a significant decrease in the mean value of red blood cells, hemoglobin, and PCV. Blood urea nitrogen, creatinine, total proteins, albumin, potassium, and phosphorus levels all increased significantly, while sodium and chloride levels decreased significantly. When compared to clinically healthy cats, plasma fibrinogen, serum alpha-1- acid glycoprotein, and serum amyloid A levels were significantly higher in cats with FLUTD. There was a significant positive correlation between the concentrations of acute phase proteins biomarkers (SAA, AGP, and fibrinogen) and blood urea nitrogen and creatinine. It is concluded that, in cats with FLUTD, serum amyloid A, AGP, and fibrinogen could be employed as an indicator of inflammatory processes.

Key words: FLUTD; serum smyloid A; AGP; fibrinogen; feline

Introduction

Feline lower urinary tract disease (FLUTD) is a common disease of feline that causes urinary bladder and urethral dysfunction (1-3). FLUTD is a multifactorial condition that includes feline idiopathic cystitis, urolithiasis, urinary tract infection (2). It is characterized by several changes in the structure and function of the urinary bladder and urethra that obstruct urine flow (4). It could be related to sterile inflammation, which causes acid-base imbalance, electrolyte accumulation, and acute renal impairment (3, 5).

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Feline lower urinary tract disease primarily affects inactive, obese male cats that eat dry cat diet and drink little water (6). Owner of cats with FLUTD commonly observe signs of bloody urine (hematuria), straining during urination (stranguria), difficult in urination (dysuria), urinary house soiling (periuria) which are common clinical manifestations of the disease. As well, animal exhibits clinical symptoms of azotemia, such as anorexia, emesis, weakness, dehydration, depression, stupor, hypothermia, acidosis with hyperventilation, bradycardia, and finally death, if the obstruction is not removed within 36–48 hours. (1, 7, 8).

Lower urinary tract disease is diagnosed based on the physical examinations, blood and

urine tests, radiography, and abdominal ultrasonography of the urinary bladder and urethra. If no specific cause is found, the disease is classified as idiopathic cystitis (1, 9-11).

In veterinary medicine, acute phase proteins (APP), which are biomarkers of tissue damage caused by inflammation, assist with therapy monitoring and prognosis (3) and can be used to identify a wide range of illnesses, including infectious, immune-mediated, and inflammatory diseases (12).

In response to inflammation, the APP concentrations can either increase (positive APP) or decrease (negative APP) (13). Positive APP can be divided into two categories: the first category includes APP that increases 10–1000 times in humans or 10–100 times in domestic animals when there is inflammation, such as C-reactive protein (CRP) and serum amyloid A (SAA), and the second category includes APP that increases 2–10 times when there is an inflammatory response, such as haptoglobin and alpha-globulins. Fibrinogen, on the other hand, is a minor acute-phase protein. In healthy animals, plasma fibrinogen is constant, but when an inflammatory condition is acute, hepatocytes release large amounts of fibrinogen. While, negative APP concentration decreases in response to inflammation, such as albumin (14). In cats, the primary APPs are SAA, alpha-1-acid glycoprotein (AGP), and fibrinogen. SAA, alpha-1-acid glycoprotein and fibrinogen concentrations have been linked to inflammatory diseases (15, 16).

Therefore, the goal of this study was to compare the hemato-biochemical parameters in cats suffered from FLUTD with those of healthy cats. Additionally, to evaluate the inflammatory process in cat with FLUTD by measuring the serum amyloid A, alpha-1-Acid glycoprotein, and plasma fibrinogen concentrations in healthy and FLUTD cats.

Material and methods

Animals and selection criteria

The present study was performed on cats admitted to a private pet's clinic and shelters in Sharkia Governorate, Egypt. The data collected from cats that were used in the current study have been permitted by the owner through signing a consent form. Because this study did not include any experimental work, approval from the Zagazig University Institutional Animal Care and Use Committee (ZU-IACUC) was not required.

A total of 30 cats were selected to participate in this research. Of which, nine cats were selected as a control group based on absence of urinary tract diseases, normal physical examination, CBC, and serum biochemistry results. Inclusion criteria for cats with FLUTD comprised clinical findings of hematuria, stranguria, periuria and dysuria, idiopathic cystitis and/ or urethral obstruction for up to 24 hours. Diseased cats must not receive any prior medical treatments. All cats were subjected to physical and ultrasound examinations upon admission. Age, breed, sex, urination abnormalities are the signalment that were collected from cats included in the present study.

Samples collection and analysis

Blood samples were collected from cephalic vein for hematological, biochemical assays and the measurement of acute-phase protein concentrations, including SAA, AGP, and fibrinogen. The first blood sample (5 ml) was collected on an ethylenediaminetetraacetic acid (EDTA) tube for determination of hematological parameters. After that plasma harvested and used for fibrinogen assessments. A second blood sample (5 ml) was collected without anticoagulant, clotted at room temperature for 20 min, centrifuged at 3000 rpm for 10 min using Megafuge 3.0R (*Thermo Fisher Scientific GmbH, 63303 Dreieich, Germany*), and then the clear serum samples were harvested for determining the biochemical parameters, AGP and SAA concentrations.

Hematological parameters were measured by hematological analyzer (Sysmex XN1000 analyzer, *USA*) as previously described by standard methods (17).

The biochemical measurements were applied using Beckman AU5800 analyzer (*Beckman Coulter, California, USA*). The method was performed according to the manufacturer's instructions using standard methods (17). Results were compared with normal reference values according to Latimer (18).

Plasma fibrinogen was determined using an automated coagulation analyzer (*STA Compact; Roche Diagnostics*) (19). While serum SAA and AGP

were measured using commercial kits Cat Serum Amyloid A ELISA (*Life Diagnostics®, USA*), with 1:400, 1:800, and 1:2500 dilutions to determine serum concentration of SAA; and Cat Alpha-1-Acid Glycoprotein ELISA (*Life Diagnostics, West Chester, PA, USA*), with 1:40 000 dilution to determine serum AGP concentrations and the measurements were applied according to the manufacturer's instructions. Results were compared with normal reference values according to Ceron, Eckersall (15).

Ultrasonography examination

Ultrasonographic examination of the kidneys and urinary bladder was performed in sagittal and transverse planes using a 3.5 MHz convex transducer (DUS60VET, Edan®, EDANUSA Co., China).

Statistical analysis

All data were statistically analyzed using SPSS Statistics® 22.0 (*Version 22.0, Armonk, NY: IBM Corp*). Descriptive statistics (mean and standard error) were calculated. The data were tested for normal distribution using *Shapiro Wilks W Test* and were found normally distributed. The obtained results were analyzed using one-way ANOVA test, all data are listed as mean ± SE. Differences between parameters were tested for significance at probability level of $p<0.05$. Pearson correlation was used for estimating the relationship between the concentration of total protein, albumin, urea and creatinine and SAA, AGP and plasma fibrinogen concentrations in healthy and FLUTD cats.

Results

Signalment

The current study comprised a total of 30 cats, from which 21 cats of different ages (7 to 12 years old) and breeds (14 Baladi, 5 Shirazi and 2 cross breed) met the inclusion criteria and diagnosed as FLUTD. While, the remaining 9 were healthy cats with an age range from 7 to 12 years old and breeds (3 Baladi, 3 Shirazi and 3 cross breed) met the inclusion criteria where it showed no signs of urinary tract disorders and the physical examination was normal. FLUTD was most commonly detected in male cats (85.5 %), while, female cats (14.5 %) (Table 1).

Clinical findings

The most common clinical indicators in cats with FLUTD, based on clinical assessment, are anorexia (85.7 %), lethargy (61.9 %), vomiting (28.5 %), dehydration (66.6 %), dysuria and strangulation (100 %) (Table 1), dehydration status was based on sunken eyes and skin fold test.

Cats with FLUTD had a significant increase in the mean body temperature of 39.6 ± 0.32 °C, respiratory rate of 46 ± 4 breaths per minute and heart rate of 200 ± 20 per minute ($p<0.05$) compared to clinically healthy cats (38.7 ± 0.6 °C, 29 ± 3 breaths per minute, 130 ± 10 per minute), respectively (Table 2).

Hematological and biochemical findings

The mean values of hematological and biochemical indices in Cats with FLUTD are summarized in Table (3 and 4). Complete blood count in cats with FLUTD showed a significant increase in the total white blood cell count (12.6 ± 0.15), neutrophils (69.59 ± 0.12) and platelets count (243.2 ± 16.08). While, a significant decrease in the mean value of red blood cells (5.37 ± 0.08), hemoglobin (8.3 ± 0.48) and PCV (28.6 ± 0.85) compared to clinically healthy cats (Table 3).

A highly significant increase ($p<0.01$) in the serum activities of AST (60.8 ± 8.44), ALT (49.8 ± 4.6), blood glucose (122.2 ± 10.5), blood urea nitrogen (38.33 ± 0.26) and blood creatinine (13.07 ± 2.41) in cats with FLUTD compared to clinically healthy cats. In addition, there was a significant increase ($p<0.05$) in the mean values of serum total proteins (9.23 ± 0.26), albumin (4.51 ± 0.33) and creatine kinase (387 ± 11.5) in cats with FLUTD compared to clinically healthy cats (Table 4).

Blood electrolytes showed significant decrease ($p<0.05$) in sodium (135.02 ± 0.07), chloride (101 ± 6.3) while there was a significant increase in the mean values of potassium (5.73 ± 0.37) and phosphorus (15.42 ± 0.79) in cats with FLUTD compared to clinically healthy cats (Table 4).

The measurements of serum alpha-1- acid glycoprotein and serum amyloid A levels as

well as plasma fibrinogen concentration were significantly increased (1.42 ± 0.17, 34.14 ± 16.12 and 283.42±12.1, respectively) in cats with FLUTD compared to clinically healthy cats (Table 5).

The relationship between the acute phase proteins concentrations (AGP, SAA and Fibrinogen) and total protein, albumin, BUN and creatinine in cats with FLUTD were recorded in Table (6). The results showed significant positive correlation between AGP, SAA and fibrinogen concentration (r = 0.726, r = 0.618, respectively) as well the correlation between SAA and AGP showed moderate positive correlation (r = 0.580) (Figure 1).

Total protein, albumin, blood urea nitrogen and creatinine showed a strong positive correlation with AGP (r = 0.843, r = 0.690, r = 0.881, r = 0.778, respectively) (Figure 2). As well a strong positive correlation with plasma fibrinogen (r = 0.857, r = 0.793, r = 0.938, r = 0.905, respectively) (Figure 3).

Total protein, albumin, creatinine showed a moderate positive correlation with SAA (r =0.490, r = 0.267, r = 0.368, respectively) whereas a strong positive correlation between blood urea nitrogen and SAA (r = 0.653) (Figure 4).

Ultrasonographic findings:

The ultrasonographic examination revealed a distended urinary bladder with the presence of anechoic fluid inside it, the wall was severely distended and an acoustic shadowing is observed next to the bladder (Figure 5). Kidneys in some cases revealed a polycystic kidney appeared severely distended with anechoic fluid (Figure 6).

Table 1: Signalment and clinical findings of healthy and FLUTD cats included in the present study, the results are ex-pressed as absolute number and their percentages [n (%)]

	Healthy cats (n = 9)	**Cats with FLUTD (n = 21)**
Age (years)		
<7	---	---
7-9	5 (55.6 %)	13 (61.9 %)
10-12	4 (44.4 %)	8 (38.1 %)
Sex (n)		
Intact male	3 (33 %)	15 (71.5 %)
Castrated male	3 (33 %)	3 (14 %)
Intact female	2 (22 %)	2 (9.5 %)
Spayed female	1 (11 %)	1 (5 %)
Breed (n)		
Baladi Cats	3 (33 %)	14 (66.5 %)
Shirazi Cats	3 (33 %)	5 (24 %)
Cross breed	3 (33 %)	2 (9.5 %)
Clinical Signs		
Anorexia	None	18 (85.7 %)
Lethargy	None	13 (61.9 %)
Vomiting	None	6 (28.5 %)
Dehydration	None	14 (66.6 %)
Dysuria, Stranguria	None	21 (100 %)
Urethral obstruction	None	17 (80.9 %)
Distended bladder	None	17 (80.9 %)

Table 2: Clinical observations in healthy and FLUTD cats included in the present study, the results are expressed as Mean±S.E.

Parameters	Healthy cats(n = 9)	Cats with FLUTD(n = 21)
Rectal temperature (°C)	38.7±0.6[b]	39.6±0.3[a]
Respiratory rate (breaths/min)	29±3[b]	46±4[a]
Heart rate (beats/min)	130±10[b]	200±20[a]

a, b: means within the same column with different superscripts differ significantly (P<0.05).

Table 3: Hematological findings in healthy and FLUTD cats included in the present study. The results are expressed as Mean±S.E.

	Healthy cats (n=9)	Cats with FLUTD (n=21)
Hb (g/dl)	12.1±0.14[a]	8.3±0.48[b]
PCV (%)	38.43±0.26[a]	28.6±0.85[b]
RBCs (10^6 /µl)	8.54±0.27[a]	5.37±0.08[b]
WBCs ($10^3/mm^3$)	6.61±0.32[b]	12.6±0.15[a]
Neutrophil (%)	56.98±1.27[b]	69.59±0.12[a]
Lymphocyte (%)	38.27±0.07[a]	35.02±0.24[a]
Eosinophil (%)	6.39±0.03[a]	8.42±0.12[a]
Monocyte (%)	1.71±0.02[a]	1.52±0.01[a]
Platelets ($10^3/mm^3$)	158.33±3.5[b]	243.2±16.08[a]

a, b: means within the same column with different superscripts differ significantly (P<0.05).

Table 4: Biochemical findings in healthy and FLUTD cats included in the present study. The results are expressed as Mean ± S.E.

	Healthy cats (n=9)	Cats with FLUTD (n=21)
Total protein (g/dl)	6.51±0.09[b]	9.23±0.26[a]
Albumin (g/dl)	3.3±0.14[b]	4.51±0.33[a]
Globulin (g/dl)	3.21±0.18[a]	4.72±0.39[a]
BUN (mmol/l)	8.63±0.03[b]	38.33±0.26[a]
Creatinine (mg/dl)	1.48±0.08[b]	13.07±2.41[a]
CK (U/l)	93.44±4.35[b]	387±11.5[a]
ALT (U/l)	29.11±0.53[b]	49.8±4.6[a]
AST (U/l)	22.1±1.4[b]	60.8±8.44[a]
Na (mmol/L)	152.7±0.46[a]	135.02±0.07[b]
K (mmol/L)	4.35±0.01[b]	5.73±0.37[a]
Cl (mmol/L)	121.1±0.97[a]	101±6.3[b]
Glucose (mg/dl)	74.7±2.5 [b]	122.2±10.5[a]
Phosphorous (mg/dl)	4.3±0.29 [b]	15.42±0.79[a]

a, b: means within the same column with different superscripts differ significantly (P<0.05).

Table 5: Serum SAA, AGP and Fibrinogen concentrations in healthy and FLUTD cats included in the present study. The results are expressed as Mean ± S.E.

	Healthy cats(n = 9)	Cats with FLUTD(n = 21)
AGP (mg/ml)	0.61 ± 0.02[b]	1.42 ± 0.17[a]
SAA (µg/ml)	0.42 ± 0.05[b]	34.14 ± 16.12[a]
Fibrinogen (g/l)	199.8 ± 0.56[b]	283.42 ± 12.1[a]

a, b: means within the same column with different superscripts differ significantly (P<0.05).

Table 6: Correlation results between blood variables; total protein, albumin, urea and creatinine and SAA, AGP and plasma fibrinogen concentrations in healthy and FLUTD cats included in the present study (Pearson correlation analysis)

Parameters	AGP	SAA	Fibrinogen	TP	Albumin	BUN	Creatinine
AGP		0.580*	0.726**	0.843**	0.690**	0.881**	0.778**
SAA			0.618*	0.490	0.267	0.653*	0.368
Fibrinogen			1	0.857**	0.793**	0.938**	0.905**
TP				1	0.728**	0.959**	0.832**
Albumin					1	0.753**	0.804**
BUN						1	0.881**
Creatinine							1

SAA, serum amyloid A; AGP, alpha-1-acid glycoprotein; TP, total protein; BUN, blood urea nitrogen; * P < 0.05 and **P < 0.01

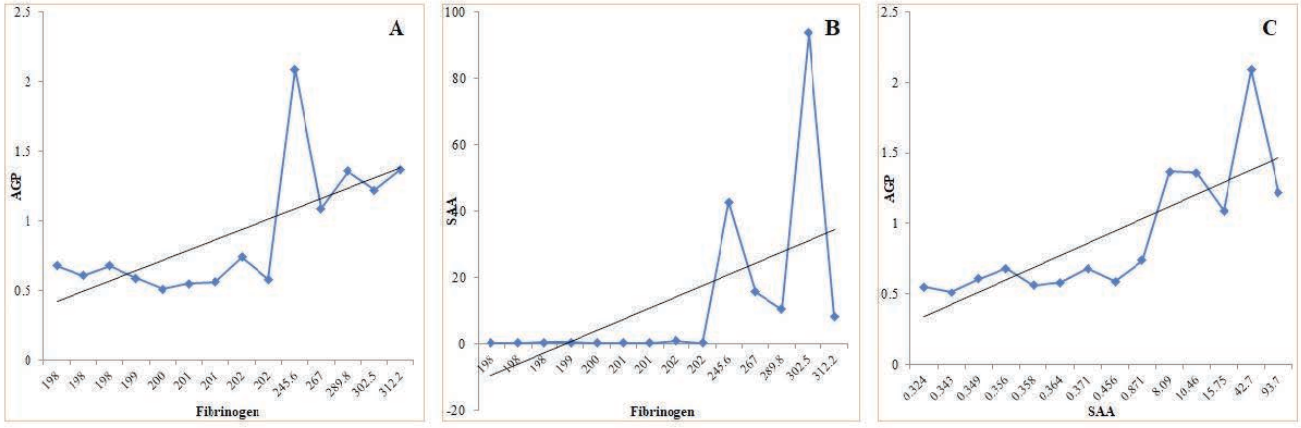


Figure 1: Correlation analysis graph between acute phase proteins (AGP – SAA and fibrinogen). (A) positive correlation between AGP and fibrinogen (r = 0.726). (B) positive correlation between SAA and fibrinogen (r = 0.618). (C) positive correlation between SAA and AGP (r = 0.580).

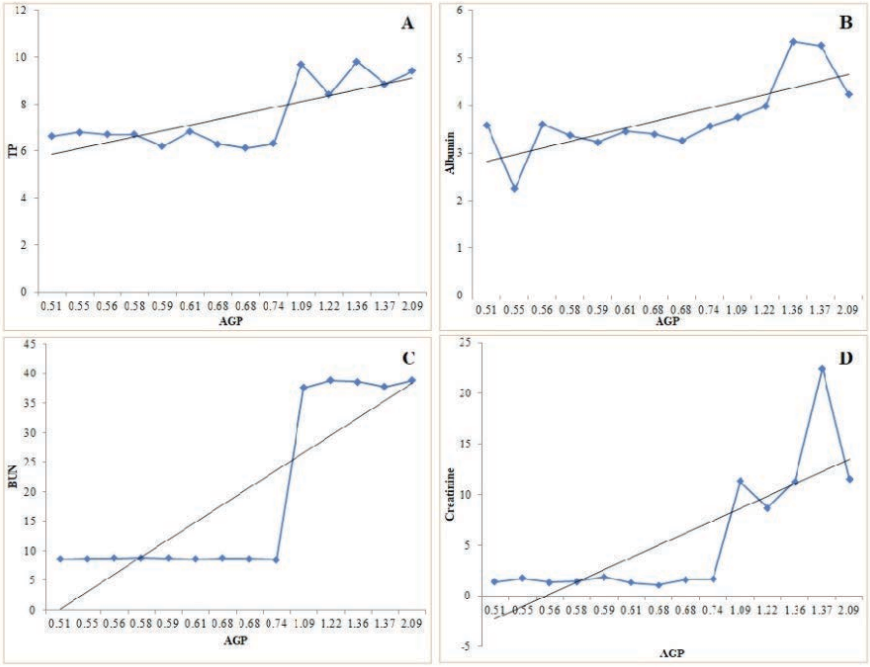


Figure 2: Correlation analysis graph between total protein, albumin, blood urea nitrogen, creatinine and AGP. (A) strong positive correlation between AGP and total protein (r = 0.843). (B) strong positive correlation between AGP and albumin (r = 0.690). (C) strong positive correlation between AGP and blood urea nitrogen (r = 0.881). (D) strong positive correlation between AGP and creatinine (r = 0.778)

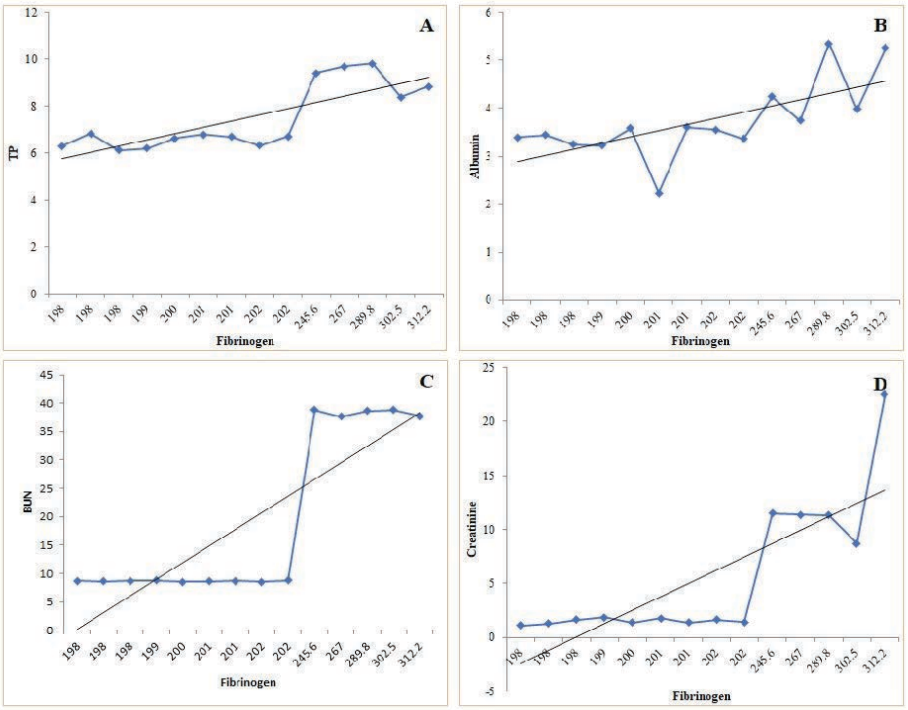


Figure 3: Correlation analysis graph between total protein, albumin, blood urea nitrogen, creatinine and fibrinogen. (A) strong positive correlation between fibrinogen and total protein (r = 0.857). (B) strong positive correlation between fibrinogen and albumin (r = 0.793). (C) strong positive correlation between fibrinogen and blood urea nitrogen (r = 0.938). (D) strong positive correlation between fibrinogen and creatinine (r = 0.905)

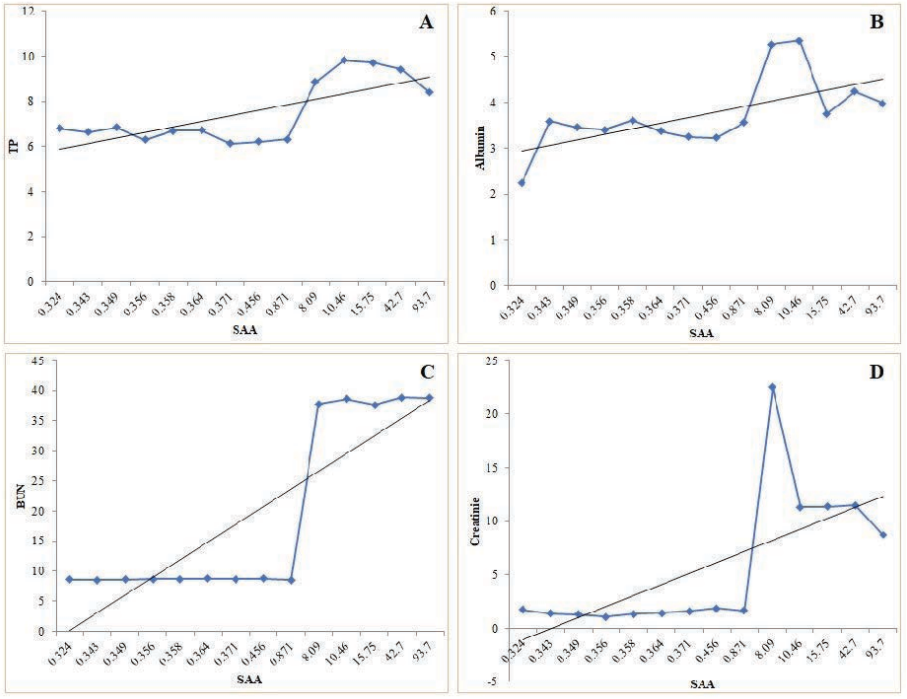


Figure 4: Correlation analysis graph between total protein, albumin, blood urea nitrogen, creatinine and SAA. (A) moder-ate positive correlation between SAA and total protein (r = 0.490). (B) moderate positive correlation between SAA and albumin (r = 0.267). (C) strong positive correlation between SAA and blood urea nitrogen (r = 0.653). (D) moderate positive correlation between SAA and creatinine (r = 0.368)

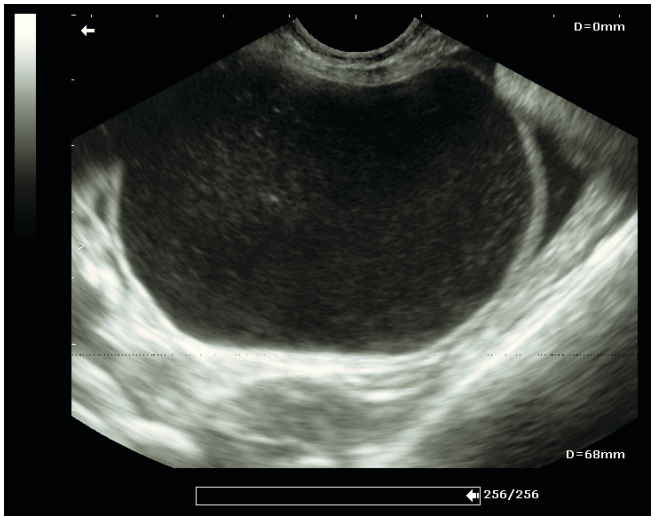


Figure 5: ultrasonography of the urinary bladder in a cat suffered from distended urinary bladder with signs of FLUTD. The image was obtained from the most caudo-ventral part of the abdomen

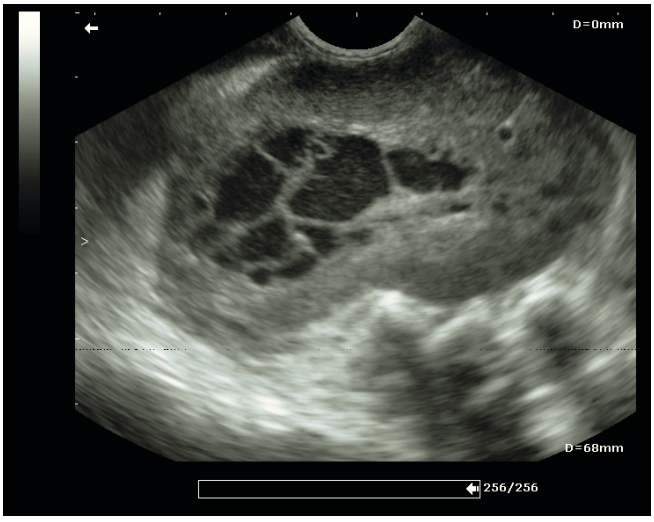


Figure 6: ultrasonography of the kidney in a cat suffered from distended urinary bladder with signs of FLUTD. The image was obtained from behind the lumbar vertebrae. The image showed polycystic kidney lobules due to the stasis of urine in the urinary bladder.

Discussion

Feline lower urinary tract disease is a common feline disease that characterized by several changes in the structure and function of the urinary bladder and urethra (2, 3). For daily use in the clinic, there is an increased need for rapid diagnostic, easily accessible, reliable, and low-cost assays for monitoring and early detection of renal inflammation during FLUTD. Several biomarkers have proven to be useful in the diagnosis of FLUTD in cats. The true power of these biomarkers is found in their ability to detect kidney damage or injury, as well as active pathological changes, at an early stage. Based on data collected from private pet clinics and shelters in Sharkia governorate, 21 cats were diagnosed with FLUTD in the current study based on ultrasonographic examination. FLUTD was more commonly found in male cats (85.5 %) and was associated with significant increases in mean body temperature, mean respiratory rate, and mean heart rate. Clinical examination revealed the following clinical findings; anorexia, lethargy, vomiting, dehydration, dysuria, and strangulation.

The obtained results were in agreement with previous findings reported by Kochan and Simsek (20)**,** who observed that the most clinical signs appeared as pain (100 %), stranguria (81.57 %), pollakiuria (73.68 %), obstruction (60.52 %), hematuria (50.0 %) and periuria (28.9%). Whereas, Nururrozi, Yanuartono (2) reported that the clinical signs associated with the disease were stranguria 45.3 %, hematuria 40.4 %, pollakiuria 11.9 %, dysuria 6.0 % and periuria 3.2 %.

The mean values of hematological and biochemical indices in healthy group and Cats with FLUTD group were tested for statistically difference. Mean values of hematological parameters showed a significant increase in the total white blood cell count, neutrophils and platelets count, while, a significant decrease in the mean of red blood cell counts, hemoglobin and PCV compared to clinically healthy cats. The findings are consistent with a previous study by Paraš, Paraš (21), who observed a significant decrease in erythrocytes in a group of cats suffering from renal failure especially in complete urinary obstruction. With the explanation that kidney load affected the poor stimulation of erythropoietin, the number of erythrocytes in this group of cats is lower than in the control group. Authors provide similar data, demonstrating that renal failure affects erythropoiesis and decreases erythrocyte-related hematological parameters such as PCV and hemoglobin (22).

The mean value of leukocytes and neutrophils, on the other hand, increased in cats with FLUTD compared to clinically healthy cats, due to intoxication and inflammatory kidney processes caused by renal insufficiency (23).

In fact, the rise in pressure within the renal pelvis and ureter reduces renal blood flow and

glomerular filtration rate, resulting in acute kidney damage. As a result, FLUTD is the most common and significant cause of acute kidney damage. The main biochemical changes in the current study were significant increase in the mean values of blood urea nitrogen and blood creatinine, total proteins, albumin, creatine kinase, blood glucose, serum AST, ALT, potassium and phosphorus concentrations in cats with FLUTD, while a significant decrease in sodium and chloride values compared to clinically healthy cats. The biochemical alterations in cats with FLUTD is related to the systemic inflammation induced by the lower urinary tract obstruction which supported by a previous literature by Chiu, Adler (24), Chen, Avital (25), Ceren, Gülten (26). Additionally, electrolytic and acid-base imbalances result from reduced glomerular filtration rates caused by a blockage in the flow of urine (3).

Phosphorus is freely filtered by the glomeruli, its concentration is heavily influenced by the glomerular filtration rate (GFR), and a drop in GFR will result in a proportional increase in serum phosphorous concentration (27). Hyperphosphatemia may cause soft tissue mineralization (including the kidneys), potentially contributing to the progression of chronic kidney disease and influencing the likelihood of survival (24, 26).

Blood urea nitrogen and blood creatinine revealed highly significant increase in cats with FLUTD and the obtained results were in agreement with a previous study by Chen, Avital (25), who reported that serum creatinine and blood urea nitrogen concentrations were associated with a worse short-term outcome, as expected given that they all reflect the severity of the injury. Additionally, blood creatinine and blood urea nitrogen levels increased five-fold in cats with renal disease, indicating impaired kidney function and poor blood filtration in a previous study by Mizutani, Takeuchi (28), Ceren, Gülten (26).

The primary acute-phase proteins that are positive in felines are SAA and AGP, which can help with the monitoring of inflammation in cats with FLUTD (4). The measurements of serum alpha-1- acid glycoprotein and serum amyloid A levels were significantly increased; as well plasma fibrinogen concentration was a highly significant increase in cats with FLUTD compared to clinically healthy cats. The obtained results were in line with the observations of Dinallo, Giuffrida (3), who observed that serum amyloid A, AGP and plasma fibrinogen concentrations significantly higher in FLUTD cats compared with clinically healthy cats at the time of examination, which were consistent with earlier research by Paltrinieri (13), Ceron, Eckersall (15), who argued that feline fibrinogen is a positive acute-phase protein that responds to inflammation in cats with FLUTD. As well, Sasaki, Ma (29) observed increased SAA concentrations in cats with urethral obstruction which peaked 24 hours after obstruction. Additionally, cats undergoing surgery for urinary diversion had higher serum AGP concentrations, according to Kajikawa, Furuta (30). Therefore, assessing the inflammatory profiles and clinical recoveries of cats with FLUTD based on these acute phase protein responses.

In the current study the results showed that total protein, albumin, creatinine showed a significant positive correlation with SAA, AGP and plasma fibrinogen, supporting the findings of Lannergard, Friman (31), Dinallo, Giuffrida (3) who found high APPs concentrations in cats with FLUTD. APPs concentrations rise as a result of severe systemic inflammatory reactions in which blood flow is reduced and inflammatory mediators (proinflammatory cytokines) are released, which is enough to activate hepatocytes and cause muscle spasms, edema, and pain (4).

Conclusions

It is concluded that, FLUTD is a most common disease affecting cats especially male cats. Diseased cats usually presented with signs of bloody urine, dysuria, stranguria and periuria. In addition, acute-phase proteins like SAA, AGP, and fibrinogen can be used as biological biomarkers to evaluate systemic inflammatory processes and aid in the monitoring of cats with FLUTD.

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Author contributions: Basma Mustafa, Ahmed Shehta and Heba Gouda have collected the sam-

ples and shared in study design. Tarek Shety, developed the idea and collected the literature, analyzed data and wrote and revised the manuscript. All authors read and approved the final manuscript.

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Original Research Article
Veterinary Medicine and The One Health Concept

ENHANCEMENT OF THE IMMUNE RESPONSE OF CHICKENS VACCINATED WITH ADJUVANTED LIVE NEWCASTLE DISEASE VIRUS VACCINE

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Abstract: The poultry industry depends heavily on immunization, particularly with live attenuated vaccines. These vaccines are commonly not adjuvanted and can be either injected or delivered in birds' mucosa. In the current study we evaluated the protective efficacy of adjuvanted and non-adjuvanted live Newcastle disease virus (LaSota strain) vaccines. Three non-adjuvanted live NDV vaccines were used to vaccinate three groups of chickens. The same immunizations were administered to three additional groups employing adjuvant technology where a mucosal adjuvant (Montanide TM IMS 1313 nanoparticles) was used. Under experimental conditions, humoral and cellular immune responses were assessed, and challenge test was done for evaluation of the vaccine efficacy in the vaccinated chickens. RT qPCR was used for determination of viral shedding in oropharyngeal swabs of challenged chickens. Mucosal nanoparticles adjuvanted live NDV vaccines significantly improved the antibody titer and the cell mediated immune response in comparison with the non-adjuvanted ones. In the challenged chicken groups with highly virulent NDV sub-genotype VIId at the third week post vaccination, the Montanide adjuvanted vaccines were fully protective and prevention of virus shedding was also noticed while the protection rate of non-adjuvanted vaccines ranged from 90% to 100% and the virus shedding was reduced. The study indicated that, efficacy of live vaccines could be improved by using Montanide™ IMS 1313 nanoparticles adjuvant in a model of mucosal delivery of live NDV vaccine in chickens.

Key words: LaSota; NDV; vaccine; nanoparticles; adjuvant; challenge

Introduction

Newcastle disease (ND) is considered one of the most important infectious diseases of poultry, where migratory birds act as natural reservoir (1). Newcastle disease virus (NDV) caused by Avian Orthoavulavirus 1 (AOaV-1) which associated with current outbreaks of poultry with highly variable clinical signs (2). The first line defense to control ND is a proper administration of the poultry with effective vaccines and preventing the virus from contacting the poultry through strict biosecurity (3). Evaluation of live vaccines have shown 100% efficacy that could be attributed to the initiation of both humoral and cellular protective response after one delivery (4). Vaccination against ND aims to three objectives which include elimination or decreasing the clinical disease, reducing the virulent virus shed amount and increasing the infectious dose of the challenge virus (5). Unfortunately, decreasing the clinical disease is considered the main objective for the current preventive strategy since veterinarians cannot evaluate the vaccine efficacy on the accomplishment of the second and third objectives

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(6). There has been extensive research conducted with the goal of production of an effective ND vaccine. However, there is a great need toward further improvements (3). The development of novel, safe and strong adjuvants is very important to enhance the immune response in chickens for maximizing the efficacy of the available vaccine administered through the mucosal route. The addition of adjuvants to live vaccines can have several benefits as lowering the cost, improved vaccine safety and better control of the risk related to the vaccine production which could be attributed to the reduction of the delivered antigen dose (7). Montanide™ range of adjuvants is an established well-known brand of vaccine adjuvants that has been used at industrial level with many types of antigens in all farm animal models (8). The Montanide™ IMS 1313 is one of nanoparticles adjuvants that can be used in intensive poultry industry for mass vaccination (9). When Montanide™ IMS 1313 was used as a live vaccine adjuvant, it significantly improved the antibody titer and showed protection to challenge compared to a commercial non-adjuvanted vaccine (8).Therefore, the current investigation aimed to evaluate the effectiveness of Montanide™ IMS 1313 nanoparticles as an adjuvant for mucosal live NDV vaccine versus the non-adjuvanted one.

Material and methods

Ethical approval

Institutional, national and international guidelines for animal care have been followed. The current protocol is reviewed and approved by ZU-IACUC committee under the number; ZU-IACUC/2/F/66/2021.

Vaccines

The live LaSota NDV vaccines with three different stabilizers [dried skimmed milk (DSM), Lactalbumin hydrolysate-sucrose (LS), and Polyvinylpyrrolidone (PVP)] were locally prepared in VSVRI to use in the current study. The vaccines were prepared using NDV LaSota strain seed virus that kindly supplied by the Central Veterinary Laboratory, Weybridge, England, the seed virus titer was 10^{10}EID50/0.1ml, and their HA units were 2^{11}.

Adjuvant

Montanide™ IMS 1313 VG NST was obtained from SEPPIC S.A, Paris La Defense, to use as a adjuvant diluent for the live LaSota NDV vaccines. It is a ready-to-dilute and consists of nanoparticles (10-500 nm) dispersed in an aqueous phase.

Challenge virus

Virulent NDV genotype VII (NDV-B7-RLQP-CH-EG-12) was used for challenge test. It was kindly provided by VSVRI with a titer of 108 EID50/0.1ml.

Specific pathogens free Embryonated Chicken Eggs (SPF–ECEs)

These were purchased from the SPF eggs project, Kom Oshim, Fayoum Governorate, Egypt and were utilized in propagation and titration of the NDV.

Experimental chicks

Two hundred-and forty Sasso broiler chicks at day one of age were provided from a local hatchery in Giza, Egypt, for in vivo evaluation of NDV live LaSota adjuvanted and non-adjuvanted vaccines

Whole blood Samples

The blood samples were collected from chicks at 3, 7, 10, 14 and 21 day post vaccination (DPV) for estimation of cellular immunity.

Serum samples

Blood samples were collected weekly from chicks starting from day one till the end of the experiment (8 weeks post vaccination) from all vaccinated and negative control groups. Sera were separated and inactivated at 56°C for 30 min then stored at −20°C till be used for immunological monitoring.

Kits

It was used for lymphocyte proliferation assay.

Cell Proliferation Kit II (XTT) was used for lymphocyte proliferation assay. Pure Link®

(Invitrogen, USA) RNA Mini Kit. It was used for extraction of shedding viral RNA according to its manual instructions. TOP real™ One-step - SYBR Green with low ROX - RT qPCR Kit (Enzynomics, Korea), it was used for RT-qPCR of NDV detection using CFX96 Touch real-time PCR detection system (Bio-Rad Laboratories, USA). Pair of specific primers for a conserved region of the matrix (M) gene of APMV-1were used as described earlier (10).

Virus preparation

Challenge virus strain was propagated and titrated in SPF-ECEs (ten-day-old). Calculation of EID_{50} was done according to Reed and Muench (11).

Experimental design

240-day-old Sasso chicks were randomly divided into 8 groups (1- 8), as shown in Table 1, and housed in clean and sterile cages. Pre-vaccination sera samples were prepared weekly for detection of ND HI antibodies that was found to be zero. Groups (1- 6) at 40 days-of-age at which groups (1- 6) were vaccinated with a dose of $10^{7.5}EID_{50}$, while groups (7 and 8) were kept non-vaccinated and used as controls.

Serology

The collected serum samples were subjected for hemagglutination inhibition (HI) to measure the specific antibodies against NDV in all chickens' groups (12)

Cell proliferation assay

Heparinized blood samples were collected at 3rd, 7th, 10th, 14th, and 21st DPV from vaccinated and negative control groups. Separation of blood lymphocyte was done according to Lee (13). Peripheral blood mononuclear cells were adjusted to 5-20 × 10^6 cells/mL in phosphate-buffered saline. Commercial cell proliferation kit II (XTT, Sigma-Aldrich, St. Louis, MO, USA) was used according to the manufacturer's instructions. The results were expressed as optical density that measured by ELISA reader (14).

Challenge test

At the 3rd week post vaccination (WPV), a dose of $10^{4.5}$ EID_{50} of sub-genotype VIId of NDV was injected I/M into 10 birds of vaccinated groups (groups 1 to 6) and positive control group (groups 1 to 7). Another 10 chickens of group 8 (negative

Table 1: Experimental design for evaluation of Montanide TM IMS 1313 nanoparticles as an adjuvant for mucosal live NDV vaccines in chickens

Experimental Groups[#]	Live LaSota Newcastle disease virus Vaccines[♐]						Newcastle disease virus challenge[☮]
	DSM stabilizer*		LS stabilizer**		PVP stabilizer***		
	Non adjuvant	Adjuvant[♦]	Non adjuvant	Adjuvant[♦]	Non adjuvant	Adjuvant[♦]	
Group 1	+	-	-	-	-	-	+
Group 2	-	+	-	-	-	-	+
Group 3	-	-	+	-	-	-	+
Group 4	-	-	-	+	-	-	+
Group 5	-	-	-	-	+	-	+
Group 6	-	-	-	-	-	+	+
Group 7	-	-	-	-	-	-	+
Group 8	-	-	-	-	-	-	-

[#]Each group contain 30 chicks, [♐]Live LaSota Newcastle disease virus Vaccines administered to 40 days old chicks by intraocular route at a dose of $10^{7.5}$ EID_{50}, [♦]Montanide™ IMS 1313 nanoparticles used as adjuvant for live LaSota Newcastle disease virus Vaccines, *DSM Stabilizer (dried skimmed milk), **LS (Lactalbumin hydrolysate-sucrose), ***PVP (Polyvinylpyrrolidone), [☮]Newcastle disease virus challenge: Virulent NDV genotype VII at a dose of $10^{4.5}$ EID_{50}/bird AEach group included 30 chicks,.O Live LaSota NDV vaccines were administered at 40 days old chicks by intraocular route in a dose of 107.5 EID50/bird, MMontanide™ IMS 1313 was used as an adjuvant for live LaSota NDV vaccines, *DSM Stabilizer (dried skimmed milk), **LS (Lactalbumin hydrolysate-sucrose), ***PVP (Polyvinylpyrrolidone),>Challenge: Challenge with virulent NDV genotype VII at a dose of 104.5 EID50/bird

control group) were kept without challenge. All challenged and non-challenged birds of all groups were kept for 15 days under observation with recording of clinical signs and deaths daily (12).

NDV Shedding

Three oropharyngeal swabs were collected from each group at the 1st, 3rd, 5th, 7th, 10th and 15th day post challenge (DPC). The swabs were immersed in Phosphate-Buffered Saline (PBS) supplemented with gentamycin (50 µg/ml) and Mycostatin (1000 units/mL) then pools of swabs from the same group were clarified via centrifugation at 2500 rpm for 15 minutes and kept at -80C until tested for virus shedding using real-time reverse transcription polymerase chain reaction (RT-qPCR). The obtained results were interpreted versus the standard curve to $EID_{50.}$

Statistical analysis

The results of HI and cell mediated immune response tests were analyzed with a statistical software program SPSS (Version 21, IBM. USA). Data are presented as mean titers ± standard deviation. Statistically significant differences between different vaccinated and control chicken groups were evaluated by ANOVA test. P-value <0.05 were considered significant.

Results

Serology

Specific NDV antibody titers for maternally derived immunity and those induced in sera of vaccinated chicks were measured by HI. Maternally derived ND HI antibody titers were gradually decreased till reach zero at 40 days of age then specific ND HI antibody titers followed for 8 WPV where it started to increase from 1st WPV till reaching the highest level at 3rd WPV. All groups immunized with adjuvanted live vaccine exhibited a significantly higher antibody titer than others receiving non-adjuvanted vaccines at 3rd and 4th WPV then the HI antibody titers started to decrease gradually in all vaccinated groups. However, at the 7th and 8th WPV, the HI antibody titers of non-adjuvanted groups decreased significantly compared with the adjuvanted ones (Table 2).

Cell proliferation assay

A lymphocyte proliferation test was carried out on the whole blood collected from vaccinated and non-vaccinated chicks. The results were expressed as OD readings determined by ELISA reader. The OD gradually increased in all vaccinated groups till reach the peak at 14th DPV however, the results of the lymphocyte proliferation test of groups vaccinated with NDV-adjuvanted nanoparticles vaccines at 3rd, 7th, 10th, 14th and and 21 DPV, were significantly higher than that of chickens vaccinated with NDV non-adjuvanted vaccines. These findings indicated that groups 2, 4 and 6 induced the best result of lymphocyte proliferation among all immunized groups of chickens (Table 3).

The challenge experiment was conducted using a virulent strain of NDV sub-genotype VIId ($10^{4.5}$ EID_{50}). Typical clinical signs of ND were observed in challenged unvaccinated chickens (control) starting at 3rd DPC, where birds suffered from dullness, severe depression, off food, severe respiratory distress with mucoid nasal discharge and greenish diarrhea. All challenged chickens in this group died within 5 days revealing P/M lesions of ND including petechial hemorrhages of the proventricular mucosa, congestion and mucoid exudates in the respiratory tract with opacity and thickening of the air sacs and button like ulcers on the intestinal mucosa in contrast to all challenged chickens vaccinated with adjuvanted live vaccines that showed 100% protections without observed any clinical signs or mortalities. However, the protection rate induced by the non-adjuvanted vaccines ranged from 90% to 100%, where 1 out of 10 chickens vaccinated with DSM NDV vaccine and another one chicken vaccinated with PVP NDV vaccine showed clinical sign of ND at 7th & 8th DPC and died at 8th and 9th DPC respectively (Figure 1).

Viral shedding consequences

Three oropharyngeal swabs were collected from each group at 1st, 3rd, 5th, 7th, 10th and 15th DPC to detect virus shedding using RT-qPCR (Table 6), and it was found that NDV shedding was detected in swabs from positive control group in 1st, 3rd and 5th DPC (100 % of birds died at the 5th DPC) while in groups (1) and (3) the viral shedding was detected in 3rd, 5th and 7th DPC. In the case of groups (2) and (6), the virus shedding was positive in the 5th DPC. While in group (5), the shedding results

Table 2: Effect of live LaSota Newcastle disease vaccines with Montanide TM IMS 1313 nanoparticles adjuvant on antibody titers against Newcastle disease virus by haemagglutination inhibition test (expressed as log 2)

Groups	Antibody titers against Newcastle disease virus (log 2) in Sera of examined birds/ WPV							
	1ST WPV	2nd WPV	3rd WPV	4th WPV	5th WPV	6th WPV	7th WPV	8th WPV
Group (1) DSM	3.33±0.5^{b}	5.62±0.7^{b}	6.37±0.7^{b}	6±0.6^{b}	5.28±0.9^{bc}	4.42±0.5^{b}	3±0.6^{b}	1.83±0.7^{a}
Group (2) DSM+1313	4.14±0.6^{bc}	6.5±0.83^{bc}	7.71±0.4^{cd}	7.5±0.5^{cd}	6.57±0.9^{bc}	5.66±1.03^{bc}	4.42±0.9^{cd}	3.5±0.5b^{c}
Group (3) LS	3.8±0.83^{bc}	6±1^{bc}	6.71±0.4^{bc}	6.16±0.7^{bc}	5.85±0.8^{bc}	4.71±0.7^{bc}	3.57±0.5^{bc}	2.2±0.8^{ab}
Group (4) LS+1313	4.71±0.9^{c}	7.14±0.8^{c}	8±1.0^{d}	7.71±1.2^{d}	7±0.7^{c}	6±0.8^{c}	5±0.8^{d}	3.85±0.8^{c}
Group (5) PVP	3.71±0.7^{bc}	6.28±0.7^{bc}	6.57±0.5^{bc}	5.71±0.7^{b}	5.16±0.7^{b}	4.33±0.8^{b}	3±0.8^{b}	2.12±0.8^{a}
Group (6) PVP+1313	4.66±0.5^{bc}	7.33±0.8^{c}	8±1.1^{d}	7.14±0.8^{cd}	6.2±0.8^{bc}	5.42±0.9^{bc}	4.5±0.5^{cd}	3.57±0.7^{c}
Negative Control	1.33±0.8^{a}	1.5±0.8^{a}	1.83±0.7^{a}	1.71±0.7^{a}	1.42±0.7^{a}	1.66±1.03^{a}	1.62±0.9^{a}	1.57±0.5^{a}

Means with different superscript letters (a, b, c, d) within the same column are significantly different at P value < 0.05 between chicken groups. WPV: weeks post vaccination - DSM: Dried skimmed milk - LS: Lactalbumin hydrolysate-sucrose - PVP: Polyvinylpyrrolidone -1313: Montanide™ IMS 1313 VG NST

Table 3: Effect of live LaSota Newcastle disease vaccines with Montanide TM IMS 1313 nanoparticles adjuvant on Cell mediated immune response of chickens by lymphocyte proliferation test (expressed as optic density)

Groups	Lymphocyte proliferation (optical density) of examined birds / DPV				
	3rd DPV	7th DPV	10th DPV	14th DPV	21st DPV
G1(DSM)	0.995±0.17^{b}	1.175±0.13^{b}	1.477±0.14^{b}	1.602±0.27^{b}	1.296±0.09^{b}
G 2 (DSM+1313)	1.424±0.06^{d}	1.545±0.17^{cd}	1.924±0.06^{d}	2.091±0.09^{cd}	1.809±0.06^{c}
G3 (LS)	1.028±0.11^{b}	1.223±0.08^{bc}	1.576±0.11^{bc}	1.624±0.16^{bc}	1.317±0.08^{b}
G 4 (LS+1313)	1.327±0.02^{cd}	1.631±0.04^{d}	1.853±0.04^{d}	2.138±0.24^{d}	1.885±0.15^{c}
G5 (PVP)	1.123±0.05 ^{bc}	1.205±0.18^{bc}	1.493±0.02^{b}	1.530±0.14^{b}	1.154±0.14^{b}
G6 (PVP+1313)	1.453±0.12^{d}	1.570±0.12^{d}	1.784±0.07^{cd}	2.155±0.16^{d}	1.766±0.07^{c}
Negative control	0.329±0.08^{a}	0.376±0.08^{a}	0.454±0.12^{a}	0.307±0.06^{a}	0.381±0.04^{a}

Means with different superscript letters (a, b, c, d) within the same column are significantly different at P value < 0.05 between chicken groups. G: group -DPV: days post vaccination - DSM: Dried skimmed milk - LS: Lactalbumin hydrolysate-sucrose - PVP: Polyvinylpyrrolidone -1313: Montanide™ IMS 1313 VG NST

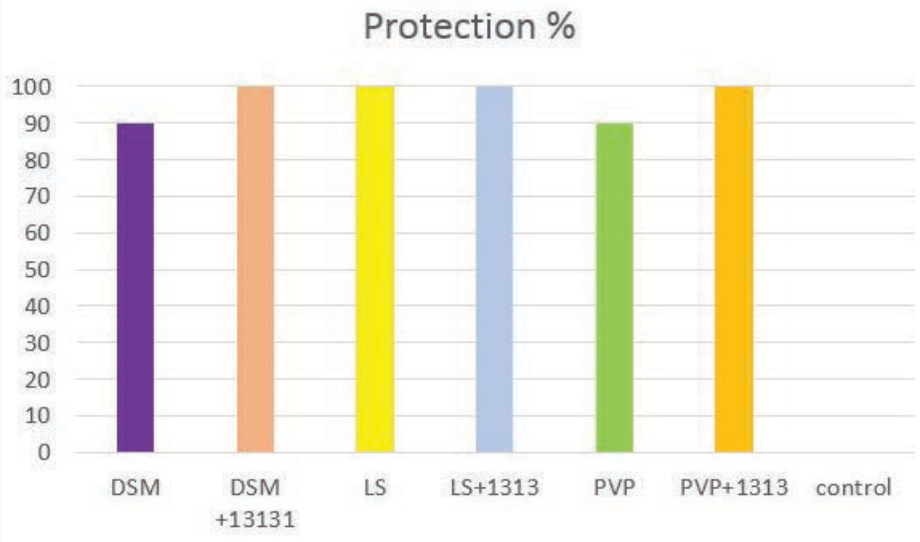


Figure 1: Protection percentage of challenged vaccinated and non-vaccinated chickens against NDV genotype VII

Table 4: Effect of live LaSota Newcastle disease vaccines with Montanide TM IMS 1313 nanoparticles adjuvant on Newcastle disease virus shedding by Real time RT- PCR

Groups	Days post challenge					
	1st	3rd	5th	7th	10th	15th
	Virus shedding titer					
G1 (DSM)	N/D	< 10	< 10	< 10	N/D	N/D
G2(DSM+1313)	N/D	N/D	< 10	N/D	N/D	N/D
G3 (LS)	N/D	< 10	< 10	< 10	N/D	N/D
G4 (LS+1313)	N/D	N/D	N/D	N/D	N/D	N/D
G5 (PVP)	N/D	< 10	< 10	N/D	N/D	N/D
G6 (PVP+1313)	N/D	N/D	< 10	N/D	N/D	N/D
Negative control	N/D	N/D	N/D	N/D	N/D	N/D
Positive control	< 10	$10^{1.7}$	$10^{3.7}$	NS	NS	NS

NDV shedding titer was calculated versus standard curve to EID50; G: group – DSM: Dried skimmed milk - LS: Lactalbumin hydrolysate-sucrose – PVP: Polyvinylpyrrolidone - 1313: Montanide™ IMS 1313 VG NST – N/D: non-detectable - NS: non-survival

revealed positive in samples collected at 3rd and 5th DPC, but no virus shedding was detected in group 4 (Figure 2).

Discussion

ND remains a hazard to the poultry industry as it causes great economical losses in many countries including Egypt where ND is endemic (15). One of the very useful solutions to protect poultry flocks against viral infections efficiently is a mucosal vaccination (16) that increases protection against viruses infecting birds through mucosal membranes by enhancing cellular immunity and reducing viral replication at the portal of entry (17, 18). Nanoparticles adjuvants are generated to improve mucosal vaccination efficiency against avian viral diseases (19). Now, it is well known that Montanide adjuvants are immuno-enhancer that increased the immune response of vaccinated birds (8, 19-21). In this regard, three live attenuated NDV LaSota strain vaccines were used once alone without adjuvant and another time with Montanide™ IMS 1313 nanoparticles adjuvants for birds' vaccination.

Sera of vaccinated chickens were examined for specific NDV HI antibody titer weekly for 8 WPV. Both humoral and cellular immunity are very important for protecting birds against NDV infection (22, 23). Specific HI antibodies against NDV appeared within 4–6 days post-vaccination with Live NDV vaccines (24), similar results were presented in our study where specific NDV HI antibody titers at 1st WPV ranged from 3.33 to 4.71 $\log_2$. At 3rd WPV, the level of HI antibodies increased and reached the peak where HI antibody titers ranged from 6.37- 6.71 $\log_2$ in non-adjuvanted groups to 7.71-8 $\log_2$ in adjuvanted groups with Montanide™ IMS 1313 and statistically it was found that adjuvanted live vaccine groups exhibited a significantly higher antibody titer than other non-adjuvanted ones and this confirms the results of Deville et al., who reported that adjuvanted live infectious bronchitis (IB) vaccine with IMS 1313 could enhance humoral immune response significantly stronger than the non-adjuvanted vaccines (8).

DSM, LS and PVP LaSota live vaccines adjuvanted with Montanide™ IMS 1313 nanoparticles compared to the same NDV vaccines without adjuvant had a higher HI antibody titer and the same results were obtained by Zhao *et al.,* who found that chickens immunized with live LaSota vaccine containing nanoparticles had higher HI antibody titers than the chickens immunized with the same vaccine but without nanoparticles (18).

Live NDV vaccines can stimulate cell-mediated immunity as early as 2 to 3 DPV (23) and this agrees with our results where the use of intra-ocular route as vaccination delivery method reflected on vaccinated birds that increase the cellular immunity and protection percent in challenge test by inhibiting replication of the virus at the site of infection (25).

The results of the lymphocyte proliferation assay in our study revealed gradual increases in optical density in all vaccinated groups and reached the highest stimulation of lymphocyte proliferation at 14th DPV and these results were nearly similar to El-Dabae et al.,(26) who prepared

Saponinadjuvanted ND vaccine and found that the Lymphocyte proliferative responses of vaccinated chickens reached a peak 15th DPV and also Radwan and Mikhael (27) who evaluated the cellular immunity of pigeons vaccinated by live pigeon pox vaccine and the result showed that the peak of lymphocyte proliferation assay was at 12th DPV.

Chickens in groups vaccinated with NDV-adjuvanted vaccines had lymphocyte proliferation response significantly higher than that of chickens vaccinated with non-adjuvanted vaccines and this result is comparable with Ma et al., who used also mucosal adjuvant (ginseng stem-leaf saponins) with live bivalent vaccine of NDV and IBV and found that cell-mediated immune responses were significantly higher in such group than in the group with the non-adjuvanted vaccine (28) and also Naggar et al., & Ismail et al., when used Montanide™ IMS 1313 nanoparticles as an adjuvant of mucosal inactivated vaccines, cellular immunity was significantly increased (9, 19).

Regarding the challenge test, full protection against sub-genotype VIId NDV challenge was achieved in vaccinated groups 2, 3, 4 and 6, while in vaccinated groups 1 and 5, 90% protection was achieved. A high percent of protection could be explained by previous studies that reported that the efficiency of live ND vaccines depends on the viral titer of the vaccinal dose where a dose of 10^4 –10^5 EID_{50} is able to protect the birds against mortality and achieves 100% protection but could not prevent virus replication (29-31).

In this study, three oropharyngeal swabs were collected from each group at 1st, 3rd, 5th, 7th, 10th and 15th DPC to detect virus shedding using RT-qPCR. The amount of virus shedding in saliva and feces are mostly decreased in vaccinated birds compared to non-vaccinated ones (32). Our results suggested that the Montanide™ IMS 1313 adjuvanted NDV vaccines induced a strong immune response and were able to reduce virus shedding (in group 2 and 6) and prevent it completely (in group 4) in comparison with the non-adjuvanted NDV vaccines (groups 1, 3 and 5), which indicates not only the safety of the vaccine but also its ability to induce sterile immunity and this agree with data reported by El-Dabae *et al.,* who found that no virus shedding in chickens vaccinated with saponin adjuvanted vaccine after challenge that means preventing virus shedding and infection of vaccinated birds with vNDV is a feature of highly potent NDV vaccines (26).

Vaccine efficacy depends on virus shedding, which is affected by species, immunity of the host, type of the vaccine, virulence and titer of challenge virus as well as, the time between immunization and challenge and the dose of the vaccine (5).

In our trials, the shedding was in very low concentration, and this may be attributed to our vaccinal dose ($10^{7.5}$ EID_{50}) which was higher than the NDV live vaccine minimum titer ($10^{5.5}$ EID_{50}) required by OIE, (2021) where Cornax et al., concluded that vaccinal doses (10^6 EID^{50} or higher) of LaSota vaccine induced strong humoral immunity leading to little or no replication of the challenge virus consequently reduce or prevent viral shedding (12, 30).

Conclusion

From our results, we could conclude the following, non-adjuvanted DSM, LS and PVP LaSota live vaccine could be improved by using Montanide™ IMS 1313 nanoparticles which improved cellular and humoral immunity in vaccinated chickens in addition to full protection against vNDV challenge and reduction of virus shedding.

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Original Research Article
Veterinary Medicine and The One Health Concept

PREVALENCE AND GENETIC CHARACTERIZATION OF *Sarcocystis fusiformis* IN WATER BUFFALOES (*Bubalus bubalis*) IN TWO NORTHERN PROVINCES OF EGYPT

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Abstract: Sarcocystosis considerably occurs in a wide host range including animals, reptiles, humans, and birds. This study was conducted to determine the prevalence of *Sarcocystis* spp. using abattoir inspection, genetic characterization, as well as histopathology in water buffaloes in two provinces, Elbehera and Kafrelsheikh, Egypt. Specimens were collected from esophagus, tongue, and masseters of 400 slaughtered buffaloes in Elbehera (*n*= 215) and Kafrelsheikh (*n*= 185). Samples were examined macroscopically and histopathologically. Furthermore, genetic characterization of *Sarcocystis* spp. was performed using the 18SrRNA gene-based PCR. The total prevalence was 71.0% (75.3% and 65.9% in Elbehera and Kafrelsheikh, respectively). Aged buffaloes had a higher prevalence than young ones. Females had a higher prevalence than males. The esophagus was the most infected organ. Molecular analysis revealed that the recovered species was *S. fusiformis*. This is the first genetic characterization of *S. fusiformis* in water buffaloes from Elbehera and Kafrelsheikh, Egypt. Higher prevalence proposed the potential role of cats in the transmission of *S. fusiformis*, which, in turn, requires strict hygienic measures to protect animals and humans from infection.

Key words: *Sarcocystis fusiformis*; prevalence; genetic characterization; Buffalo; Elbehera; Kafrelsheikh

Introduction

Sarcocystis is an intracellular cyst-forming protozoan parasite belonging to the phylum Apicomplexa. It is an obligatory heteroxenous parasite causing sarcocystosis. Carnivores are the final hosts harboring sexual stages, while herbivores are the intermediate hosts with asexual stages (1, 2). Hence, each host can be infected with multiple *Sarcocystis* species (1). Sarcocystosis is an important parasitic disease that has a global distribution in humans and various animal species (3). These food-borne zoonotic parasites infect humans through raw or insufficiently cooked infected meat consumption (3,4). Some *Sarcocystis* species are economically important due to their ability to induce anorexia, anemia, muscular weakness, gastrointestinal problems, fever, and decreased weight gain and milk production (5). A few species can induce abortion and mortality in intermediate hosts, such as *S. tenella* in sheep, *S. cruzi* in cattle, *S. miescheriana* in swine, and *S. capricanis* in goats (1). Macroscopic cysts like *S. buffalonis, S. fusiformis,* and *S. gigantea* make meat unsuitable for human consumption, resulting in the condemnation of infected carcasses (6). Water

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buffaloes are intermediate hosts for *Sarcocystis* species, including *S. fusiformis* and *S. buffalonis*, which form visible cysts, with cats as final hosts, and dogs serving as final hosts for *S. levinei*. *S. dubeyi*'s final host is unknown (7). Macroscopic sarcocysts, which almost always occur in skeletal or esophageal muscles, appear filamentous in *S. muris*, rice grain-like in *S. rileyi*, fusiform in *S. fusiformis*, globular in *S. gigantea* (1), and white thread-like in *S. buffalonis* (8).

Several investigations were conducted to measure the prevalence of *Sarcocystis* spp. infections with varying prevalence rates, including Northern Vietnam (9), Iran (10, 11), India (12), China (13), and Ethiopia (14). In Egypt, infection was detected in water buffalos from a few provinces such as Beni-Suef (2), El-Gharbia (8), Dakahlia (15), Menoufia (16), and Sharkia (17). The serological diagnosis was conducted using ELISA in water buffaloes from Iran (12). Molecular characterization is important for distinguishing the cyst of *Sarcocystis* species with similar morphology. Several prior investigations used the 18SrRNA gene as a target gene for *Sarcocystis* species because it has hypervariable areas that might give useful information for identifying and describing various species within the same genus (18). It was employed either alone or in combination with other molecular markers. Except for a few studies that utilized the Cox I gene alone (19) or the Cox I gene with another genetic marker (20). Some studies in Egypt used molecular diagnosis using the 18SrRNA gene for instance in Beni-Suef (2) and El-Gharbia (8). In addition, there was no epidemiological or molecular data on *Sarcocystis* infection in water buffaloes in Elbehera or Kafrelsheikh provinces. This study aimed to provide morphology, histopathology, epidemiology, and molecular characterization of *Sarcocystis* spp. in water buffaloes in Elbehera and Kafrelsheikh provinces, Egypt.

Materials and methods

Study design and samples

This study was assigned to detect the *S. fusiformis* in slaughtered water buffaloes from Elbehera and Kafrelsheikh governorates in northern Egypt. The study animals were designated using a simple random sampling method that took age and gender into account. The sample size required for this study was estimated consistent with the following formula $N= 1.96^2 [Pexp-(1-Pexp)]/d^2$ where N represents the required sample size, Pexp represents the expected prevalence, and d represents the desired absolute precision (21). To increase the degree of precision, an expected prevalence of 50% was utilized, and 384 sample sizes were calculated using a 5% absolute precision and a 95% confidence level. The esophagus, tongue, and masseter muscles were collected from 400 slaughtered buffaloes from Elbehera (n = 215) and Kafrelsheikh (n = 185) provinces in northern Egypt during the period from May 2020 to April 2021. The age of animals was determined by tooth examination (22). Also, sex was recorded. The esophagus, tongue, and masseter muscles were examined during routine meat inspection. Then samples were sent to the laboratory of Parasitology at the Faculty of Veterinary Medicine, Damanhour University, Egypt for morphological and molecular examination.

Gross examination

Muscle samples from the esophagus, tongue, and masseter muscles were grossly examined during normal meat inspection at abattoirs to detect macroscopic *Sarcocystis* cysts.

Histopathology

Tissue specimens from the esophagus infected with macroscopic cysts and noninfected normal esophagus were fixed in 10% neutral-buffered formalin for 2–5 days. Then it was processed for paraffin embedding and sectioned into 5-7 µm thick sections. The sections were stained with hematoxylin and eosin (H&E) and examined microscopically (23).

DNA extraction and PCR amplification

Cysts were separated from the surrounding muscles and genomic DNA was extracted independently from each of *Sarcocystis* cysts from the esophagus (five cysts from each province) using the Genomic DNA Extraction Kit (Thermo Fisher Scientific Inc., MA, USA) (24). The concentration of DNA was measured using a spectrophotometer (Thermo Fisher Scientific, USA). PCR reactions were applied using the primer set: forward (5´-GGCCCTTTTAGTGAGGGTGT-3´) and reverse (5´-TACGAATGCCCCCAACTGTC-3´) of the 18SrR-

NA gene of *S. fusiformis* targeting 270bp fragment (24). PCR reactions were performed in a final reaction volume of 25 µL using 2× PCR Master Mix Solution (i-Taq™) (iNtRON Biotechnology, Seoul, Korea). Negative control Double-distilled water was performed with all the reactions. The PCR reaction conditions were similar to previous research (24). Five µL of PCR products were tested by running on a 2.5% agarose gel (Sigma-Aldrich, USA), stained with ethidium bromide (Sigma-Aldrich, USA), using a 100-bp genetic marker (Sigma-Aldrich, USA), then visualized using a UV and photographed. PCR products were purified using MEGAquick-spin™ Plus Total Fragment DNA Purification Kit (iNtRON Biotechnology, Seoul, Korea) according to the recommendations of the manufacturer.

Sequencing analysis and phylogenic tree

Purified PCR products were submitted for sequencing at Animal Health Research Institute, Giza, Egypt. The Basic Local Alignment Search Tool (BLAST) was used to align and compare DNA sequences with the GenBank database. The sequences were submitted to GenBank. The Mega 5 software was used to generate the phylogenetic tree by the Neighbor-Joining method. Sequences from Elbehera (OP388862) and Kafrelsheikh (OP388863) and other sequences from GenBank were used, including *S. fusiformis* (KR186117, KR186119, KR186122, MF327256, MN986970, MN986971, KX574317, MF595830, MF595833, MF595837, MF595838, MF595839, and MF595840), *S. cafferi* (KJ778010, KJ778011, and KJ778019), *S. buffalonis* (KU247902, KU247908, KU247909, KU247910, KU247911, KU247912, KU247913, MF595842, MF595843, and MF595844), *S. hirsute* (AF017122, AF176940, AF176941, KC209741, KT901160, MN121568, MN121569, MN121570, and MN121571), and *Plasmodium Vivax* (AY598140) as out-group.

Statistical analysis

Locality, age, and sex variables were analyzed by the Chi-square ($x2$) test using SPSS statistical software (Version 22, SPSS Inc, Chicago, MI, USA) to evaluate the relationship between these variables and *S. fusiformis* infection. P value ≤ 0.05 was considered statistically significant.

Results

The infection rate of Sarcocystis cysts in water buffaloes

The total prevalence of *Sarcocystis* cysts among water buffaloes was 71.0%. The infection rate was 75.3% in the Elbehera western and 65.9% in the Kafrelsheikh middle delta, Egypt (Table 1). Locality significantly affected the infection (x^2 = 4.2699, P = 0.038) (Table 1). According to age, the prevalence was higher (75.1%) in aged animals > 3 years than (49.2%) in young animals < 3 years. Age significantly affected the infection (x^2 = 17.2494, P = 0.0001) (Table 1). Female buffaloes had a greater prevalence of 75.9% than males (30.2%). (Table 1). Sex significantly affected the infection of *Sarcocystis* (x^2 = 38.8894, P = 0.0001) (Table 1). The esophageal muscles were the most infected organ during meat inspection, with a prevalence of 96.12% (74.4% and 61.1%), followed by the tongue at 3.87% (0.9% and 4.8%), whereas the masseter muscles were uninfected in Elbehera and Kafrelsheikh, respectively.

Table 1: Risk factors affecting the prevalence of Sarcocystis fusiformis in water buffaloes in Elbehera and Kafrelsheikh, Egypt

Variable		No. examined	No. positive	%	x2	P Value
Locality	Elbehera	215	162	75.3	4.27	0.038*
	Kafrelsheikh	185	122	65.9		
Age	< 3 years	63	31	49.2	17.25	0.0001****
	≥ 3 years	337	253	75.1		
Sex	Male	43	13	30.2	38.89	0.0001****
	Female	357	271	75.9		

* Significant at $P \leq 0.05$

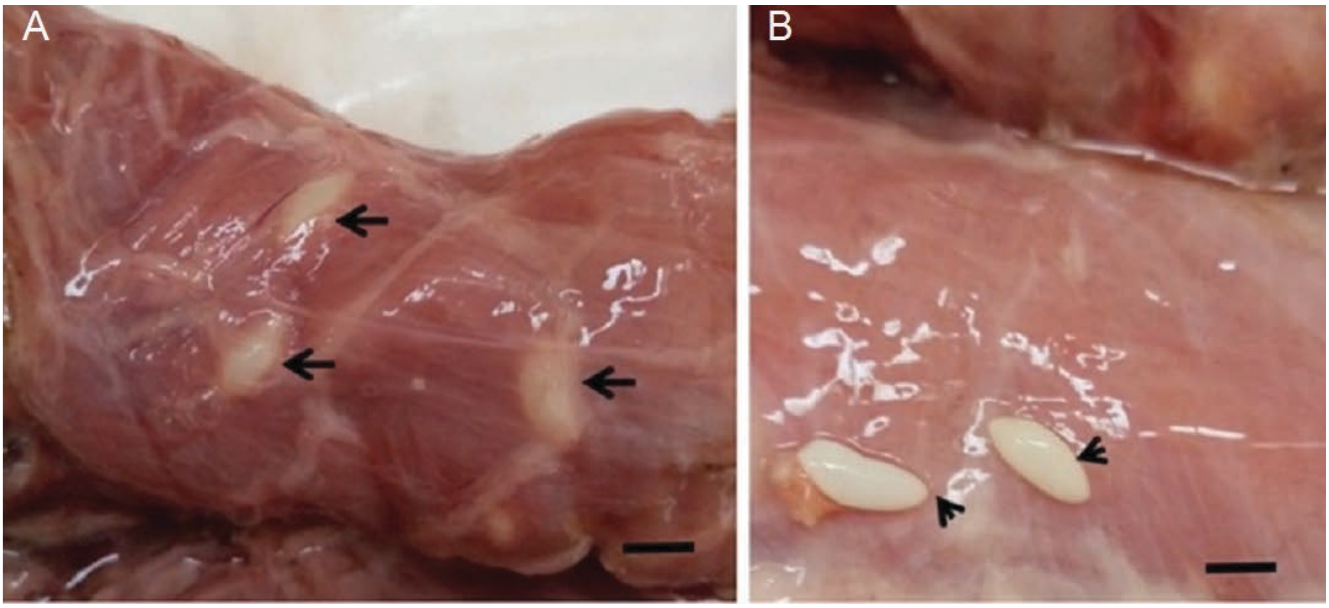


Figure 1: An esophagus of a water buffalo infected with macroscopic S. fusiformis cysts. A) Cysts are distributed beneath the serosal membrane (arrows) and B) the isolated macroscopic cysts from esophageal muscles (arrowheads), Scale bar = 1 cm

Gross examination

The species of *Sarcocystis* cyst was identified morphologically as *S. fusiformis* in esophageal and tongue muscles. The cyst was macroscopically fusiform in shape, with opaque white milky bodies in color, lying between muscle bundles in the same direction as the muscle mass and beneath the serosal membrane. It was found in different sizes, measuring about 0.5-3.0 cm (Figure 1).

Molecular characterization

The PCR primers revealed specific bands of 270bp for the 18SrRNA gene. *Sarcocystis* species sequences in buffalo from Elbehera (OP388862) and Kafrelsheikh (OP388863), Egypt, belonged to *S. fusiformis*. The 18SrRNA gene sequences of our tested isolates from Elbehera and Kafrelsheikh showed 97% similarity, according to BLAST analysis. The sequence identity between Elbehera isolate and published *S. fusiformis* (MF327256 and KR186119) was 99.5%. The Elbehera sequence shared 99% and 98% similarities with previously released *S. fusiformis* (KR186117 and KR186122), respectively. The Kafrelsheikh 18S rRNA sequence was found to have 97% sequence identity with *S. fusiformis* (MF327256 and KR186119). It exhibited sequence similarity of 96% with *S. fusiformis* (KR186117) and 95% with *S. fusiformis* (KR1861122). The cladogram showed that the Kafrelsheikh isolate was placed in a group with the *S. fusiformis* sequences in GenBank (KR186117, KR186122, MF327256, and KR186119), which are closely related to the Elbehera strain (Figure 3). The cladogram revealed that the sequences of *S. fusiformis* were genetically related to *S. cafferi*, followed by *S. buffalonis*, and then *S. hirsuta* (Figure 3).

Discussion

Sarcocystis species are intracellular zoonotic parasites that infect both animals and humans. We investigated them because they are common coccidian protozoan parasites of veterinary and economic importance. The total infection rate of *S. fusiformis* cyst among slaughtered water buffaloes was 71.0%. This high prevalence of *S. fusiformis* infection may be because of continuous exposure to infection via infected cats, the definitive host, shedding numerous sporocysts in their faces that contaminate the environment and serve as the primary source of infection (2). The locality had a significant effect on the infection which may be due to different husbandry management systems (25). Therefore, the difference in the prevalence may be due to geographic distribution (26) and the extent of exposure to infection. The prevalence was similar to 65% in the Philippines (27) and 68.2%,

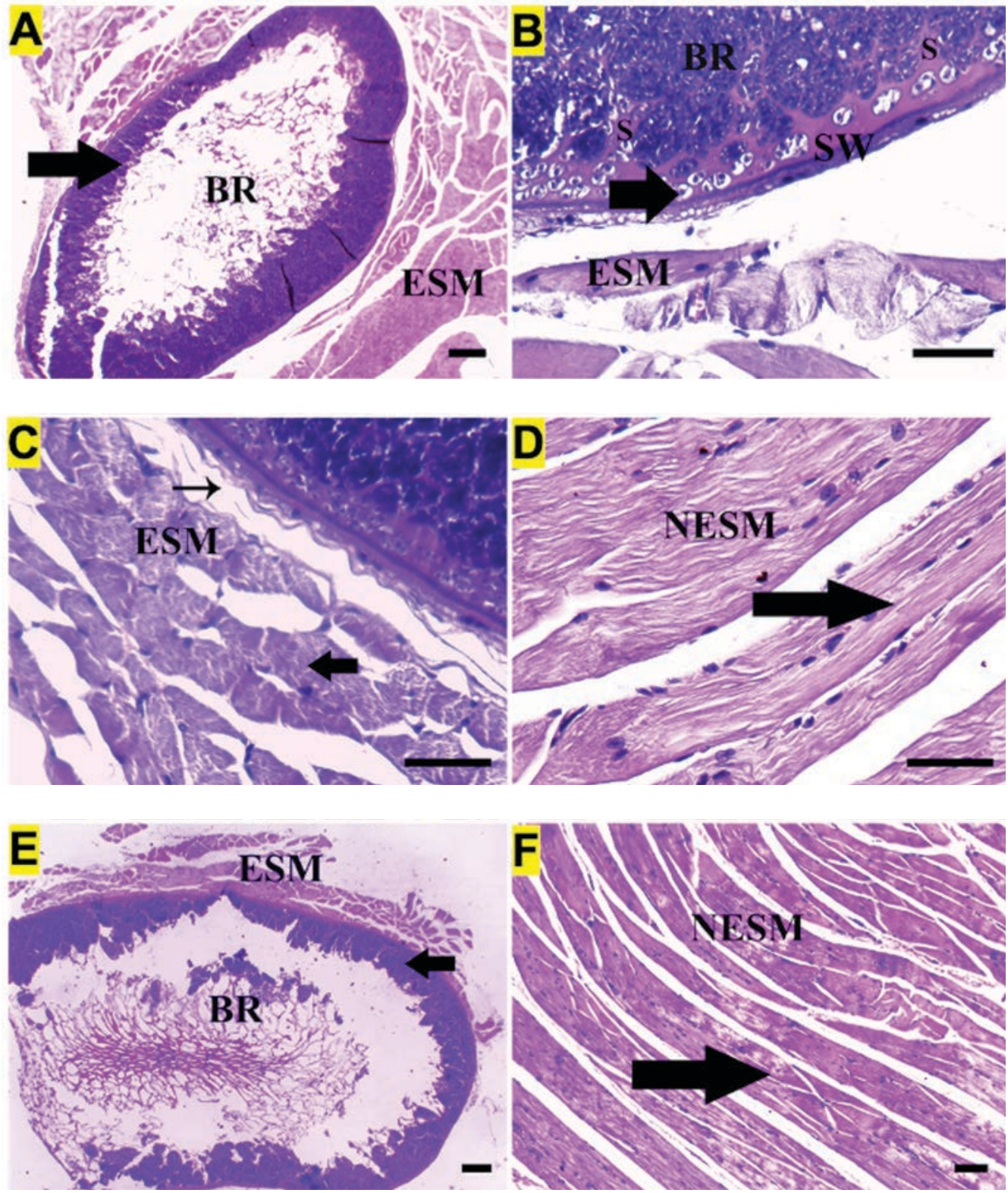


Figure 2: Photomicrograph of *S. fusiformis* from buffalo esophageal muscle in panels A, B, C, and E. Normal esophageal muscle without *Sarcocystis* in panels D and F. A) Esophageal skeletal muscle (ESM) containing *S. fusiformis* surrounded by a thin capsule (arrow) Scale bar = 200 µm. B) lightly stained metrocytes (arrow), bradyzoite (BR), scale bar = 50 µm. C) Muscle loss near the cyst (thin arrow), nucleus loss, and regular shape of striated cytoplasm (thick arrow), scale bar = 50 µm. D) Normal esophageal muscle (NEM) is shown, Scale bar= 50 µm. E) *S. fusiformis* cross-section (arrow) within detached esophageal skeletal muscle (ESM), scale bar= 200 µm. F) NEM with clear striation and arrangement (arrow), scale bar= 200 µm

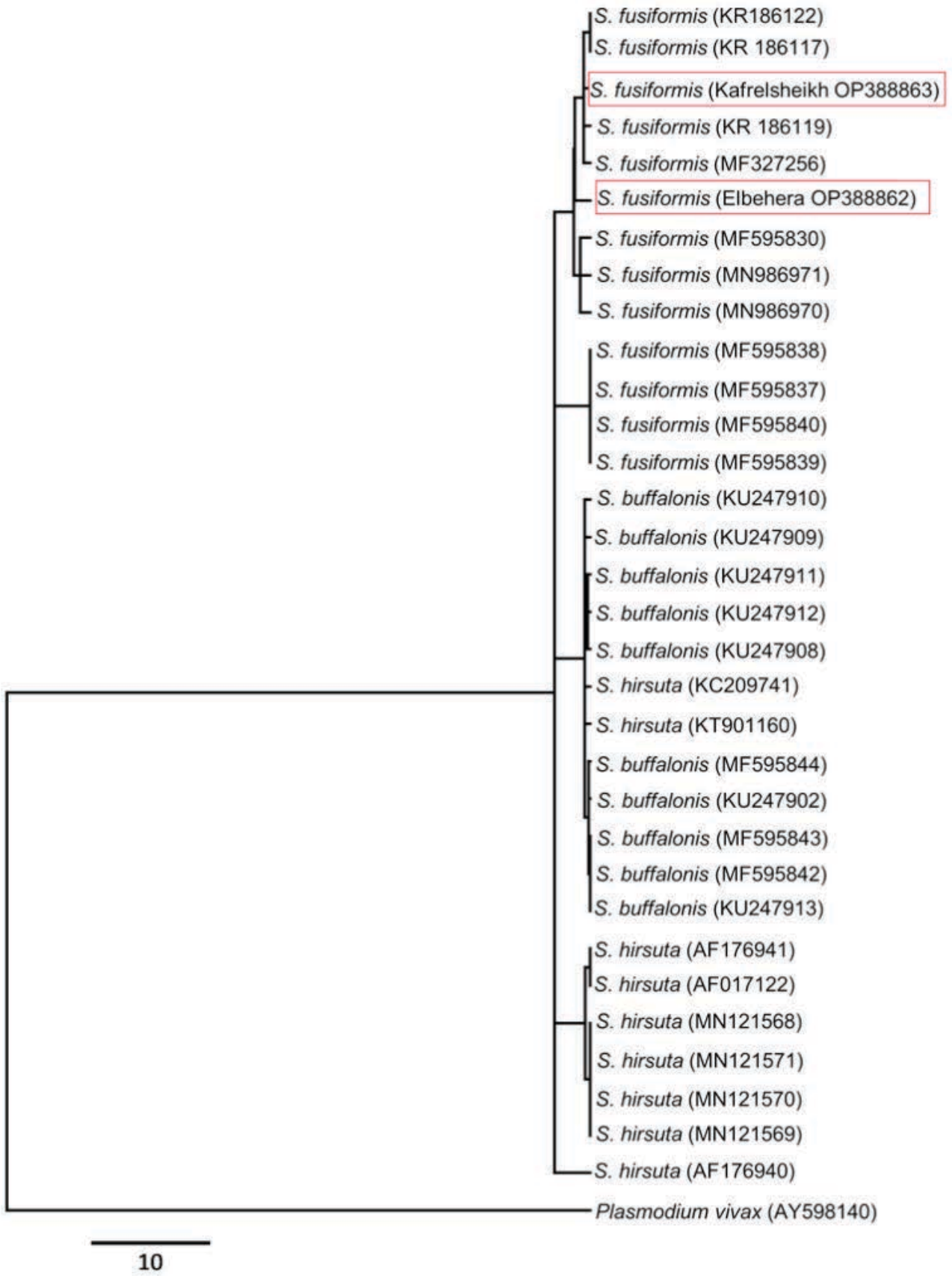


Figure 3: Phylogenetic tree of *Sarcocystis fusiformis* 18SrRNA gene from buffaloes in Elbehera and Kafrelsheikh provinces, Egypt. The tree was drawn using the Neighbor-Joining method and provided with a scale bar

69%, and 74% in Egypt (8, 28, 29). Our prevalence was higher than 41% in Vietnam (30), 22.62% in India (11), 15.6% in Iraq (31), and 8.33%, 28%, 41.5%, 42%, and 58.72% in Egypt (32, 33, 34, 17, 15). Higher prevalence of 87% in India (35) and 94%, 85.96%, and 82.3% in Egypt (36, 16, 2) were detected. Older buffaloes had a significantly higher prevalence than younger ones similar to the previous reports in Vietnam (30) and Egypt (16,17). This may be due to prolonged exposure to infections (17). Other authors reported a slight variation in prevalence in India (12) and Egypt (2). However, some owners preferred indoor rearing of young animals, so young buffaloes may be less susceptible to infection than older ones. Female buffaloes demonstrated a higher prevalence than males. This was consistent with the results reported in Egypt (32,34) and may be due to prolonged exposure to infection because of aging and exposure to stress factors like pregnancy and lactation that suppress the immune system (29). While the prevalence in males was higher than in females in Egypt (17), this may be due to the increased number of investigated males. Interestingly, gender did not affect the prevalence of *Sarcocystis* spp. infection in Iran (10, 11).

The esophageal muscles were the most infected organ, followed by the tongue. Our findings were consistent with previous studies that recorded a high prevalence of *S. fusiformis* infection in the esophagus (12, 16, 37, 17). There was no *Sarcocystis* spp. infection in the masseter muscles; in contrast with a report in Iran (10).

Histopathologically, *S. fusiformis* was embedded in the tunica muscularis of skeletal esophageal muscle and appeared elongated and large. Similar findings were reported by discovering macroscopic cysts in the esophageal muscles of water buffaloes that had thin walls filled with crescent-shaped bradyzoites (8, 28). Similarly, researchers in Malaysia (38) and Egypt (39) observed no inflammatory responses in the tissue around the cysts. The absence of an inflammatory reaction may be owing to the cysts' placement within muscle fibers, which are protected by a membrane from host immunity (40). The present study revealed normal striated muscle cells without *Sarcocystis* infection as illustrated by clear striated muscle cells and multiple peripheries located nuclei compared with infected muscle, which demonstrated detached fibers around the cyst, low number of nuclei, and disappearance of striation. This agreed with the fiber type composition of the striated muscle layer of the esophagus of the cow, sheep, donkey, dog, and cat examined with standard histochemical methods and immunohistochemical staining using type-specific anti-myosin sera (41).

18S rRNA sequencing indicated a close relationship between the locally tested isolates of Elbehera and Kafrelsheikh, with 97% identity. Elbehera and Kafr El Sheikh isolates showed sequence identity of 99.5% and 95%, and 94%, respectively, with *S. fusiformis* sequences in the GenBank, indicating that the discovered species is *S. fusiformis*. Phylogenetic analyses indicated that the two isolates were closely related to each other and the other previously registered *Sarcocystis* species in the GenBank database and were classified in the same clade. This might be related to variations in the nucleotide sequences of the *Sarcocystis* species. Furthermore, because they share the same intermediate and final host (cat), most *Sarcocystis* species are phenotypically identical (1). This finding is similar to the earlier one from Egypt (8). In conclusion, this study presented the prevalence, morphology, histopathology, and molecular characterization of *S. fusiformis* in slaughtered buffaloes in the Egyptian provinces of Elbehera and Kafrelsheikh. High *S. fusiformis* prevalence highlights the significant role of cats in *S. fusiformis* transmission and the need to adopt sanitary measures to protect animals and humans from infection such as preventing cat feces to contaminate animal feed and water and hygienic disposal of infected carcasses; furthermore, proper cleaning of the cooking utensils used to handle infected buffalo meat to protect humans.

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Original Research Article
Veterinary Medicine and The One Health Concept

MOLECULAR CHARACTERIZATION SOME BACTERIAL PATHOGENS CAUSING BOVINE MASTITIS WITH SPECIAL REFERENCE TO *Mycoplasma bovis*

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Abstract: In dairy industry, bovine mastitis is the most prevalent disease, which reduces milk production and causes economic losses. This study was conducted to estimate the prevalence of *Mycoplasma bovis* and some bacteria causing mastitis in dairy farms and partial sequencing of 16SrRNA target genes and Quinolones Resistance Determining Regions (QRDRs) (*gyr*A and *par*C) in *M. bovis* isolates. 370 milk samples were obtained from farms located in villages in Fayoum governorate, Egypt. The examined milk samples (8,91%) were positive for the California mastitis test (CMT). Multiplex RT-PCR was used for the recognition of microorganisms causing mastitis (*Staphylococcus (S.) aureus, Streptococcus* species (spp.), *Escherichia (E.) coli*, and *Mycoplasma (M.) bovis*) from mastitic milk. The results revealed that *E. coli* was the most predominant (84.8%) followed by *S. aureus* (81.8%) while *M. bovis* was the lowest one (51.5%). Mixed infection with two or more mastitic bacterial agents was also identified. All 33 examined mastitic milk samples were diagnosed with mixed infection with *E. coli*, *S. aureus, Streptococcus* spp. and *M. bovis* (36.36%), *E. coli* and *S. aureus* (21.21%), and rephrase *E. coli*, *M. bovis,* and *Streptococcus* spp. (6.06%). The sequence analysis of *M. bovis* 16SrRNA genes illustrated a high similarity of examined isolates to strains previously deposited in the GenBank recovered from the same locality. The *gyr*A amino acids showed no substitution but showed 100% similarity with *M. bovis* isolates worldwide. However, the amino acid sequence of *par*C, showed substitution at positions 2 (Gln to Arg) (CAG >>CGT), 75 (Ile to Ser) (ATT>>AGC), and 79 (Asn to Asp) (AAC>>GAT). Sequence results can lead to the creation of appropriate treatment and control measures for *M. bovis*, while multiplex RT-PCR, can be exploited as a standard diagnostic method for major mastitis pathogens.

Key words: mastitis; multiplex RT- PCR; *M. bovis*; sequence; molecular diagnostics

Introduction

Mastitis is a complicated issue that affects both the quantity and grade of milk produced by milk-producing cattle [1]. In addition to decreased milk production, treatment cost, and disposal of milk, mastitis impacts the sale price of milk and causes animals to be culled [2,3]. Mastitis has various etiologies and is commonly caused by bacterial species [2] such as *S. aureus*, *Streptococcus agalactiae*, *Mycoplasma* spp., *E. coli*, *Klebsiella* spp., Enterobacter spp., *Streptococcus uberis*, *Serratia* spp., *Pseudomonas* spp., and *Proteus* spp. [4]. The dominant bacterial species are *E. coli*, *S. aureus*, and *Streptococcus* species [5]. *E. coli* was recently detected in many mastitis cases in dairy farms [6]. *S. aureus* is a facultative anaerobic gram-positive Staphylococcus bacterium of the udder [7]. It was mentioned that *S. aureus* mastitis is extremely costly for dairy farms and is known for being widespread and chronic [8]. *Streptococcus uberis* is a major mastitis-causing pathogen that is typically categorized as an organism with an

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environmental origin, implying that infection arises from organisms in cows' surroundings [9,10].

M. bovis can cause not only mastitis but also, can be the cause of several disorders in cattle, including pneumonia, arthritis, genital problems, keratoconjunctivitis, and otitis media. It is regarded to be a growing, underappreciated cow pathogen [11]. Mycoplasma mastitis frequency has increased recently, particularly in association with dairy herds [12]. *M. bovis* is the predominant species and also the one that results in the most serious clinical cases [13]. Mastitis costs each cow $159 in annual losses. To reduce these costs promptly, and early diagnosis of single and multiple infection by a suitable test is crucial for the adoption of appropriate treatment and control measures. In comparison to conventional tests, the PCR assay is the best method for detecting infection at suboptimal amounts and at a faster rate [14]. By RT-PCR, the time for the recognition of bacteria directly from mastitis samples could be further reduced. These PCR methods allow the recognition of bacteria within hours. Multiplex PCR is advantageous in the simultaneous recognition of many pathogens with cost-effectiveness and thus could be used for quick diagnosis [15].

In this study, multiplex RT-PCR assays were used to identify microorganisms that cause mastitis in dairy farms (*M. bovis* and other bacteria). The target genes (*M. bovis* 16S rRNA) and QRDRs (*gyr*A and *Par*C) of *M. bovis* were partially sequenced to determine whether they are genetically related to other *M. bovis* isolates, particularly those that cause mastitis in dairy farms.

Material and methods

Study design

370 milk samples (300 cows' milk and 70 buffaloes' milk) during three years in 2019 (100 cows and 30 buffaloes), 2020 (100 cows and 20 buffaloes and 2021 (100 cows and 20 buffaloes) were obtained from three private dairy cattle farms and farmers located in villages in Fayoum governorate, Egypt. The examined animals with a history of being suffered from mastitis and reluctance to antibiotic therapy or the recent replacement of newly purchased animals from local markets without previous examination to *M. bovis* infection. The individual farmers had cows or buffaloes or both. The farmers are mainly keeping their animals in the back-yard of their houses. The back-yard units are connected directly to the owner's home. From a separate quarter of animals, 25 ml of milk samples were obtained in sterile bags under sterile conditions. The samples were transported directly at 4°C to the laboratory for bacteriological examination.

California mastitis test (CMT)

The collected Cows and buffalos' milk were tested for subclinical mastitis by the California test according to [17].

Multiplex RT-PCR

The CMT-positive samples were subjected to multiplex RT-PCR for bacterial-causing mastitis *E. coli, S. aureus, Streptococcus* spp, and *M. bovis.*

Table 1: Nucleotide sequence of primers and probes used in the multiplex RT-PCR assay

Primer/ probe	Sequence 5´- 3´	Reference
M. bovis. F	GAGAATGCTTCAGTATTTTGACGG	[18]
M. *bovis.* R	CAAAAGCAAAATGTTAAATTCAGG	
M. *bovis*-Prob ROX	CAL Fluor Red 610- -CATATATAAGTGAGACTA ACTTATT-BHQ2	
E. coli. F	CGCCTAATCCGCAACGTAAT	[19]
E. coli. R	CGCAGCGTGATCCTGTTTAT	
E. coli-Prob FAM	FAM-TGGCGCAGATGACTGATAAAGCCA-BHQ1	
Strept. F	GTACAGTTGCTTCAGGACGTATC	
Strept. R	ACGTTCGATTTCATCACGTTG	
Strept-Prob HEX	CAL Fluor Orange 560 -ACAATTGGACGAAGGTCTTGCTGGA-BHQ1	
S. aureus. F	TCGAAATTAAATGTTGTCGTGTCTTC	[20]
S. aureus. R	TCATTTTTGACATGRAGAGAAACATC	
S. aureus-Prob FAM	FAM- TCGCGACATTCATTATGCCCAAATTTTTAA-BHQ1	

Table 2: Nucleotides sequence of primers and amplified products for four target genes of *M. bovis*.

(Target Gene)	Primer Sequence (5'-3')	PCR Amplified Product (bp)	Reference
16S ribosomal RNA for ruminant *Mycoplasma*	F: AGACTCCTACGGGAGGCAGCA R: ACTAGCGAT TCCGACTTCATG	1000bp	[22]
M. bovis (16S rRNA)	F: CCTTTTAGATTGGGATAGCGGATG R: CCGTCAAGGTAGCATCATTTCCTAT	360bp	[23]
QRDRs (gyrA)	F: GACGAATCATCTAGCGAG R: GCCTTCTAGCATCAAAGTAGC	531bp	[24]
QRDRs (parC)	F: GAGCAACAGTTAAACGATTTG R: GGCATAACAACTGGCTCTT	488bp	[24]

DNA Extraction:

25ml of milk sample was centrifuged at 4000 g/20 min. Then the supernatant was discarded, and the pellet was cleaned twice with sterile phosphate-buffered saline (PBS). After that, re-suspend the pellet in 200µl of sterile PBS. Consequently, the nucleic acid was extracted using a QIAamp DNA mini kit (Qiagen, Germany, GmbH) according to the manufacturer's instructions. Finally, the purified DNA was stored at −20 °C until the amplification.

PCR amplification:

PCR amplifications were done on the ABI 7500 (Applied Biosystems, Paisley, UK). RT-PCR assays were executed in 20µl containing 10 µl of 2x Sensifast probe No-ROX buffer (Bioline, UK), 3.75 µl water, 0.25µl of each primer (50pmol conc.), and 0.125 µl of each probe (30pmol conc.) (Table 1) and 5µl of DNA template. Reaction mixtures containing water substituted for DNA templates were used as negative controls. thermocycling conditions were set as per the following parameters: 95°C /15 min, 40 cycles of 95 °C/15 s, 50 °C /30 s, and 72 °C/30s.

Mycoplasma bovis isolation and identification

Mycoplasma PPLO agar and broth media were used for the isolation and identification of *M. bovis* as previously described [21]. Colonies were seen using a stereo microscope.

Detection of M. bovis using conventional polymerase chain reaction (PCR)

DNA from a *Mycoplasma* suspension was extracted using a DNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions and was conducted to detect members of the Class Mollicutes (16S ribosomal RNA for ruminant Mycoplasma), one specific for *Mycoplasma bovis* used for the identifying of *M. bovis* isolates (16S rRNA *M. bovis*), and the other two, specific for the detection of QRDRs genes (*gyr*A, *par*C) (Table 2).

Gene sequencing for M. bovis

The PCR product of randomly selected one isolate for 16S ribosomal RNA for ruminant *Mycoplasma*, 2 isolates for *M. bovis* 16S, and one isolate for gyrA, and *par*C genes were sequenced by the GATC Company using an Applied Biosystems 3130 genetic analyzer (ABI, USA) by employing forward and reverse primers and combining the old Sanger method with the new 454 technology. The NCBI Blast search was used to verify the sequencing data, and BioEdit software version 7.1.5 was used to compile and edit chromate graphs. Edited sequences of the *M. bovis* isolate were characterized using BLAST n (http://www.ncbi.nlm.nih.gov/BLAST/) for nucleotide analysis or BLAST p for protein analysis. The homology between the isolates and other chosen reference isolates was assessed using the amino acid sequence identity matrix. The phylogenetic tree was created to determine the genetic relatedness of the *Mycoplasma bovis*. By MEGA version 11, the neighbor-joining (NJ) method generated distance-based [25, 26].

Results

Subclinical mastitis by CMT:

The prevalence of mastitic cows' milk in examined samples was 8%, 6%, and 5% during the

years 2019, 2020, and 2021, respectively. While it was 6.7%, 10%, and 10% during the years 2019, 2020, and 2021 respectively in the Buffaloes' milk.

In 2019, 2020, and 2021, subclinical mastitic cows' milk prevalence was 2%, 2%, and 1%, respectively. In contrast, subclinical mastitis in Buffaloes' milk was 6.7%, 5%, and 0%, respectively (Fig.1). However, overall mastitis in cattle was 8%, while in buffaloes it was 12.8%. From all examined milk samples by CMT 33/370 (8.91%) milk samples were mastitic (25 clinical mastitic and 8 subclinical mastitic)

The recovery rate of Mastitis causing Pathogens using multiplex RT-PCR

The most common pathogen found in examined mastitic milk samples was *E. coli* 28/33 (84.8%), followed by *S. aureus* 27/33 (81.8%), then *Streptococcus* 23/33 (69.6%) and *M. bovis* 17/33 (51.5%) (Fig. 2). Interestingly, the incidences of *E. coli* and *M. bovis* in 2019 were higher than 2020 and 2021 (Fig. 3). The mixed infection by *E. coli, S. aureus, Streptococcus* spp., and *M. bovis* recorded the maximum occurrence rate (36.36%) but mixed infection by *E. coli, Streptococcus spp., and M. bovis* was the lowest occurrence rate (6.06%) (Fig. 4).

M. bovis conventional PCR:

All examined samples were confirmed by both 16S rRNA for ruminant *Mycoplasma* which gave a specific band at 1000bp and 16S rRNA specific *for M. bovis* gave 360bp. However, 20 % positive for *gyr*A, and *par*C each gave specific bands at 531bp and 488bp, respectively.

Sequencing of M. bovis 16SrRNA genes and gyrA and Par C genes:

The sequenced genes were uploaded to GenBank under accession no. OP268410 for

16S rRNA for ruminant Mycoplasma, OP268399, and OP268400 for *M. bovis* 16S, OP270479, and OP270480 for *gyr*A, and *par*C.

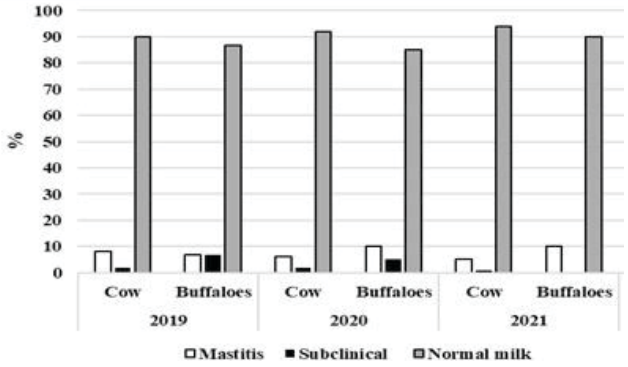


Figure 1: The prevalence of subclinical mastitis by CMT

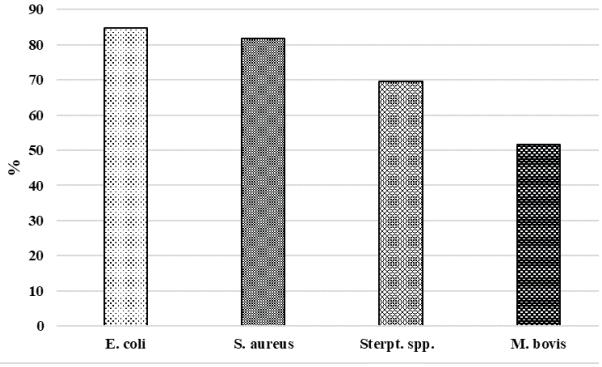
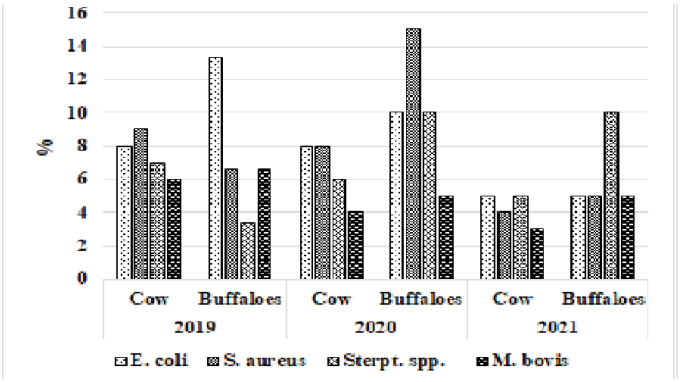


Figure 2: The recovery rate of Mastitis causing Pathogens (*E. coli, S. aureus, Streptococcus* spp., and *M. bovis*) using multiplex RT- PCR. % According to the detected mastitic milk sample (n=33)



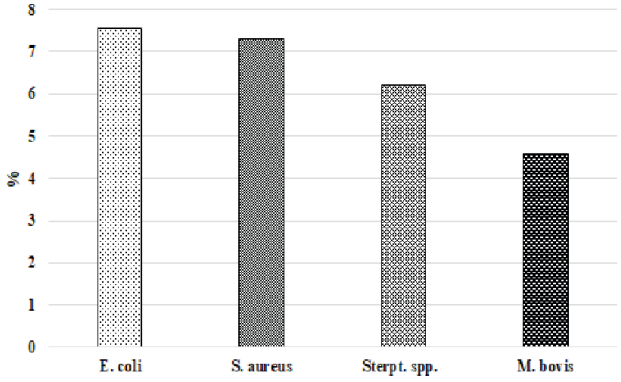


Figure 3: The recovery rate of Mastitis causing Pathogens (*E. coli, S. aureus, Streptococcus* spp., and *M. bovis*) using multiplex RT- PCR; the left panel details result in each year; the right panel the total recovery rate during the study. % According to the total examined milk sample(n=370)

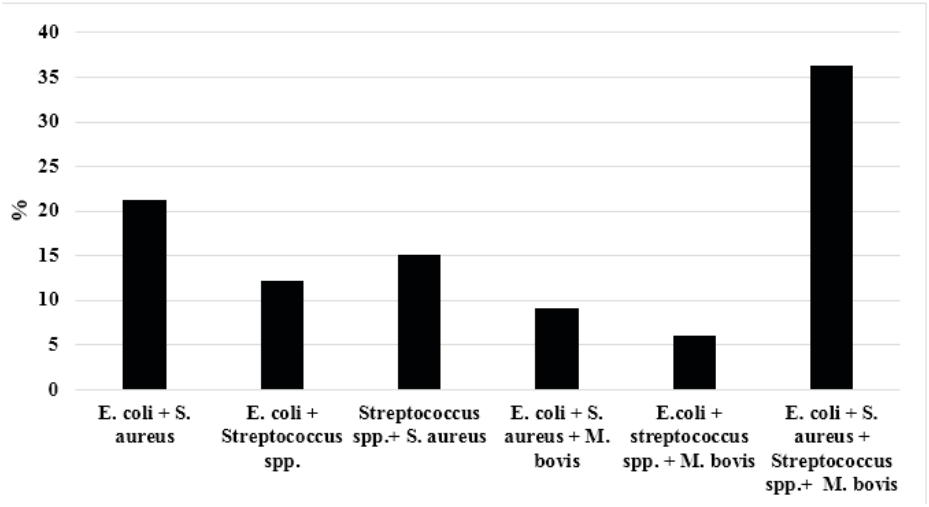


Figure 4: Occurrence of mixed infection and type of organisms detected in examined mastitic milk by RT-PCR. % According to the total examined mastitic milk sample

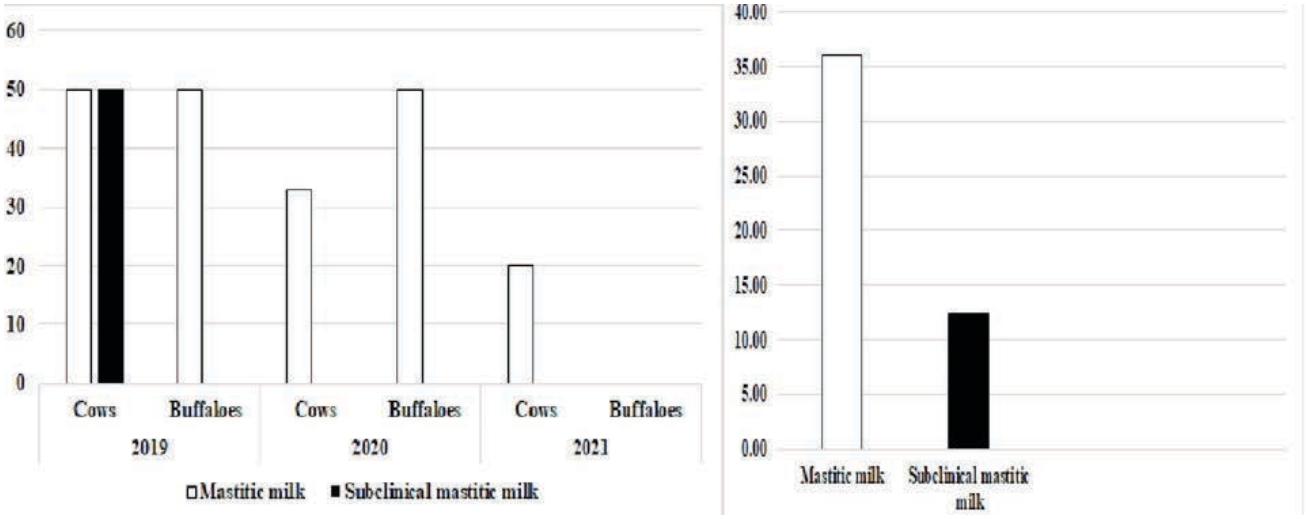


Figure 5: Isolation rate of *M. bovis* in examined mastitic and subclinical mastitic milk samples the left panel showed results each year; the right panel the total recovery rate during the study

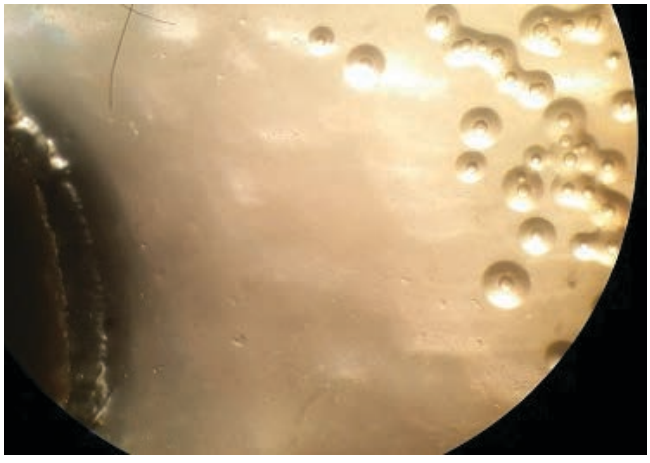


Photo 1: *M.bovis* under stereo microscope

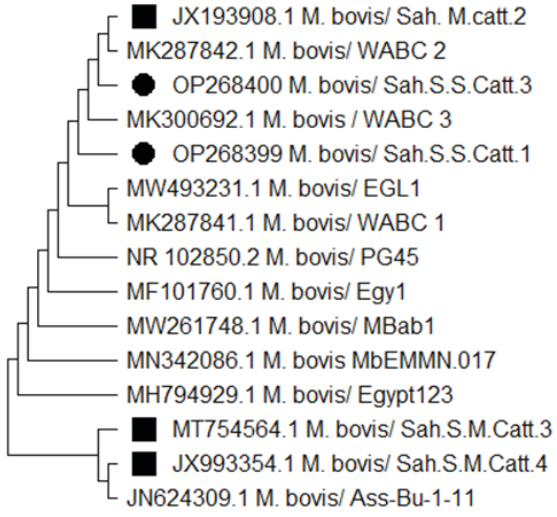


Figure 6: Phylogenetic tree of *Mycoplasma bovis*. 16s gene black circle: this research isolate; black square: previous *Mycoplasma bovis* isolates from the same locality

M. bovis 16S isolates OP268399 and OP268400 showed high nucleotide identity (99% and 97% respectively) with another JX193908 isolate which was isolated from the same locality (Fig. 6).

*M. bovis gyr*A (OP270479.1) showed high nucleotide identity (99%) with other isolates such M. bovis/ CP042939.1, M.bovis/ CP042938.1, and M. bovis/ CP045797.1 (Fig 7 and Table 3). In the current study, *Gyr*A showed no amino acid substitution than *E. coli* K12 (according to *E. coli* numbering).

Mycoplasma bovis *Par*C (OP270480.1 M. bovis/Sah.S.S.par.1) showed high nucleotide identity (100%) with other isolates such M. bovis/ CP042938.1, M. bovis/ CP022596.1, and M. bovis/CP022599.1, M. bovis/ CP045797.1, and M. bovis/ CP045798 (Fig.8 and Table 4). Moreover, *par*C amino acid showed substitutions at positions 2 (Gln to Arg) (CAG >>CGT), 75 (Ile to Ser) (ATT>>AGC), and 79 (Asn to Asp) (AAC>>GAT) (according to E. coli numbering)

Discussion

In milk production, mastitis continues to be one of the most prevalent and expensive illnesses, where the vast majority of clinical and subclinical bovine mastitis occurs. Mastitis caused by *S. aureus*, *S. agalactiae*, and *M. bovis* is now regarded as a serious infectious issue and makes up about 3% of clinical milk submissions. Animal culling remains to be the recommended technique to reduce *M. bovis* mastitis because there are no effective medications or vaccines for the treatment or prevention of this illness, which results in considerable animal replacement expenses for the producer [27].

It was interesting to notice that the prevalence of mastitis in the current study by CMT in cows was (8%) lower than that in buffalo (12.8%) which was in contrast to the previous findings which showed that mastitis in cows' milk more prevalent (32%) than in buffalo milk (22%) [28].

Table 3: Nucleotide identity between *M. bovis gyrA* and other GenBank isolates

Seq->	OP270479.1 M.bovis/ Sah.S.S.Catt.gyr.1	CP042939.1 M. bovis/NADC59	CP042938.1 M. bovis/MJ1	CP045797.1 M. bovis/XBY01	CP022588.1 M. bovis/MJ4	CP022587.1 M. bovis/MJ3	CP019639.1 M. bovis/08M	KR493100.1 M. bovis/MYC22	CP092775.1 M. bovis/L15527	KR493099.1 M. bovis/MYC2	CP092776.1 M. bovis/L15762	CP076229.1 M. bovis/Mb191	CP005933.1 M. bovis/CQ-W70	CP069101.1 M. bovis/Mb304	CP069100.1 M. bovis/Mb300	CP058464.1 M. bovis/TO VK	CP058515.1 M. bovis/VK9	CP062195.1 M. bovis/Tibet-10	E. coli K12	NC_014760.1 M. bovis/PG45
OP270479.1	ID	100%	100%	100%	100%	100%	100%	100%	100%	100%	99%	100%	100%	100%	100%	100%	100%	100%	95%	95%
CP042939.1	100%	ID	100%	100%	100%	100%	100%	100%	100%	100%	99%	100%	100%	100%	100%	100%	100%	100%	95%	95%
CP042938.1	100%	100%	ID	100%	100%	100%	100%	100%	100%	100%	99%	100%	100%	100%	100%	100%	100%	100%	95%	95%
CP045797.1	100%	100%	100%	ID	100%	100%	100%	100%	100%	100%	99%	100%	100%	100%	100%	100%	100%	100%	95%	95%
CP022588.1	100%	100%	100%	100%	ID	100%	100%	100%	100%	100%	99%	100%	100%	100%	100%	100%	100%	100%	95%	95%
CP022587.1	100%	100%	100%	100%	100%	ID	100%	100%	100%	100%	99%	100%	100%	100%	100%	100%	100%	100%	95%	95%
CP019639.1	100%	100%	100%	100%	100%	100%	ID	100%	100%	100%	99%	100%	100%	100%	100%	100%	100%	100%	95%	95%
KR493100.1	100%	100%	100%	100%	100%	100%	100%	ID	100%	100%	99%	100%	100%	100%	100%	100%	100%	100%	95%	95%
CP092775.1	100%	100%	100%	100%	100%	100%	100%	100%	ID	100%	99%	100%	100%	100%	100%	100%	100%	100%	95%	95%
KR493099.1	100%	100%	100%	100%	100%	100%	100%	100%	100%	ID	99%	100%	100%	100%	100%	100%	100%	100%	95%	95%
CP092776.1	99%	99%	99%	99%	99%	99%	99%	99%	99%	99%	ID	99%	99%	99%	99%	99%	99%	99%	94%	94%
CP076229.1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99%	ID	100%	100%	100%	100%	100%	100%	95%	95%
CP005933.1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99%	100%	ID	100%	100%	100%	100%	100%	95%	95%
CP069101.1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99%	100%	100%	ID	100%	100%	100%	100%	95%	95%
CP069100.1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99%	100%	100%	100%	ID	100%	100%	100%	95%	95%
CP058464.1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99%	100%	100%	100%	100%	ID	100%	100%	95%	95%
CP058515.1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99%	100%	100%	100%	100%	100%	ID	100%	95%	95%
CP062195.1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99%	100%	100%	100%	100%	100%	100%	ID	95%	95%
E. coli K12	95%	95%	95%	95%	95%	95%	95%	95%	95%	95%	94%	95%	95%	95%	95%	95%	95%	95%	ID	100%
M. bovis/PG45	95%	95%	95%	95%	95%	95%	95%	95%	95%	95%	94%	95%	95%	95%	95%	95%	95%	95%	100%	ID

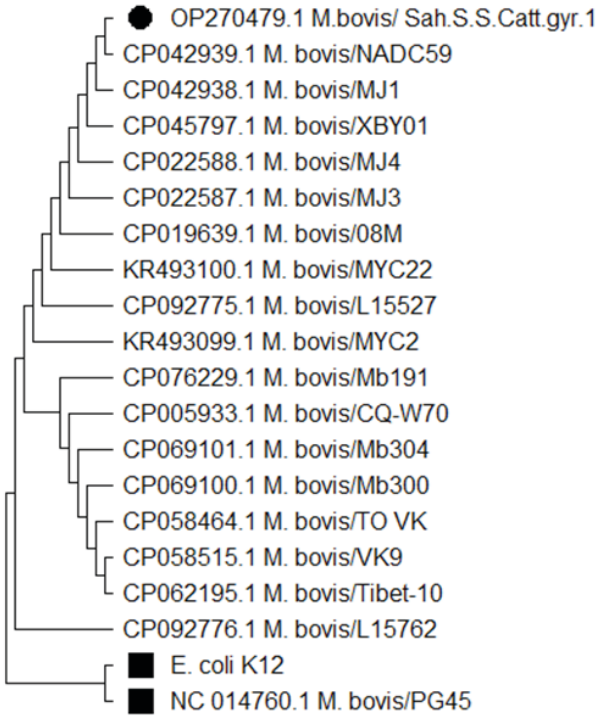


Figure 7: Phylogenetic tree of *M. bovis gyr.* A black circle: this research isolate; black square reference strains

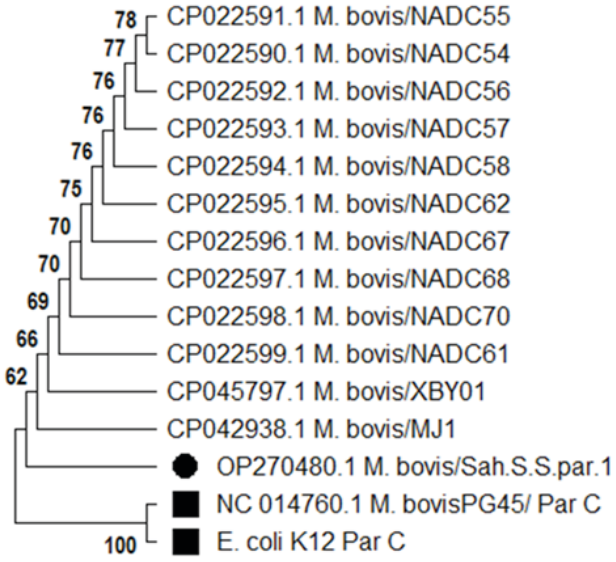


Figure 8: Phylogenetic tree of *Mycoplasma bovis parC*, black circle: this research isolate; black square reference strains

Table 4: Nucleotide identity between *M. bovis gyrA* and other GenBank isolates

Seq->	OP270480.1 M. bovis/Sah.S.S.par.1	CP042938.1 M. bovis/MJ1	CP045797.1 M. bovis/XBY01	CP022599.1 M. bovis/NADC61	CP022598.1 M. bovis/NADC70	CP022597.1 M. bovis/NADC68	CP022596.1 M. bovis/NADC67	CP022595.1 M. bovis/NADC62	CP022594.1 M. bovis/NADC58	CP022593.1 M. bovis/NADC57	CP022592.1 M. bovis/NADC56	CP022591.1 M. bovis/NADC55	CP022590.1 M. bovis/NADC54	NC_014760.1 M. bovisPG45/ Par C	E. coli K12 Par C
OP270480.1	ID	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	74%	73%
CP042938.1	100%	ID	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	74%	73%
CP045797.1	100%	100%	ID	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	74%	73%
CP022599.1	100%	100%	100%	ID	100%	100%	100%	100%	100%	100%	100%	100%	100%	74%	73%
CP022598.1	100%	100%	100%	100%	ID	100%	100%	100%	100%	100%	100%	100%	100%	74%	73%
CP022597.1	100%	100%	100%	100%	100%	ID	100%	100%	100%	100%	100%	100%	100%	74%	73%
CP022596.1	100%	100%	100%	100%	100%	100%	ID	100%	100%	100%	100%	100%	100%	74%	73%
CP022595.1	100%	100%	100%	100%	100%	100%	100%	ID	100%	100%	100%	100%	100%	74%	73%
CP022594.1	100%	100%	100%	100%	100%	100%	100%	100%	ID	100%	100%	100%	100%	74%	73%
CP022593.1	100%	100%	100%	100%	100%	100%	100%	100%	100%	ID	100%	100%	100%	74%	73%
CP022592.1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	ID	100%	100%	74%	73%
CP022591.1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	ID	100%	74%	73%
CP022590.1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	ID	74%	73%
NC_014760.1	74%	74%	74%	74%	74%	74%	74%	74%	74%	74%	74%	74%	74%	ID	99%
E. coli K12 P	73%	73%	73%	73%	73%	73%	73%	73%	73%	73%	73%	73%	73%	99%	ID

The RT-PCR has been demonstrated to be an effective diagnostic method for enhancing bacterial identification and other benefits of reducing throughput time, to enable objective interpretation of data [29]. Additionally, prior research showed that, as compared to bacterial cultures, the real-time PCR technique offers higher sensitivity and specificity for the detection of mastitis pathogens [15]. In this study, the detection rates of *E. coli*, *S. aureus*, *Streptococcus* spp., and *M. bovis* in examined mastitic milk were 84.8%, 81.8%, 69.6%, and 51.5%, respectively. However, the detection rate from Fayoum farms in a prior study was the most frequently identified microbes after isolating *S. aureus* at 37.8% while *S. agalactiae, E. coli, C. pyogenes, E. faecalis,* and *M. bovis* were 13.2%, 12.6%, 8.4%, 5.6 %, and 1.4%, respectively [30].

Moreover, this study's results differed from Katholm *et al.* [31] who mentioned that the detection rate of *Staphylococcus* spp. and *E. coli* was 61% each from all bulk tank milk (BTM) using rt-PCR. Additionally, the recovery rate of *S. aureus* in the current study (81.8%) was higher than reported previously in Egypt 29.1%, 35.9% [19,32], 24.4% [33**]**, Turkey 26.1% [34], but lower than those reported in Denmark 91% [35].

Furthermore, RT-PCR results showed that the most predominant microbial genera were *E. coli* (84.8%) in the investigated mastitic milk samples. While the relatively high frequency of *E. coli* (86.6%) was also reported in Egypt [19]. Based on the results of this study, the incidences of *E. coli*, *S. aureus*, *Streptococcus spp.* and *M. bovis* in examined bovine milk samples in 2019 were higher than recorded in 2021. These may be attributed to insufficient hygienic circumstances, inadequate managemental routines, bad prevention and control measures, contaminated milking equipment and deficient supervision.

In the meantime, *E. coli* was identified in a mixed infection together with *S. aureus* in 21.21% of investigated samples, with *Streptococcus* spp. in 12.12% of investigated samples, together with *S. aureus*, *M. bovis*, and *Streptococcus* spp. in 36.36% of examined samples. These findings were higher than those recorded previously [36]. On the other hand, the occurrence rate of *E. coli* mixed with *S. aureus* and *E. coli* mixed with *Streptococcus* spp. in examined samples was near to the previous study [16].

Comparatively, the recovery rate of *M. bovis* recorded in the present study was 51.5% which was higher than that found in BTM in both Cyprus at 6.25% and Canada at 0.53% [35].

A three-year study on *M. bovis* mastitic infection was carried out at dairy farms in the Fayum Governorate between 2019 and 2021. These herds had previously been certified as *M. bovis* positive, and the prevalence of *M. bovis* in mastitic milk ranged from 50% in 2019 to 20% in 2021. This may be due to the various stages of infection, the variable *M. bovis* strains, the intermittent shedding of the microbe by the infected cows, or the low concentration of *M. bovis* in the tested milk samples, as mentioned by [37] or the application of strict biosafety and biosecurity measures in these farms.

It is known that molecular typing of *Mycoplasma* is extremely helpful for testing dairy farms, clinical samples, and for identifying mollicutes that are difficult to cultivate or that grow slowly. The 16SrRNA group-specific PCR assay amplifies *Mycoplasma* of ruminants and also all other mollicutes species that belong to the genera *Mycoplasma*, *Acholeplasma*, *Ureaplasma*, and *Spiroplasma* but does not amplify sequences of any other prokaryote [38, 39]. In this study we used16srRNA common for Ruminant according to [22] and another one more specific for *Mycoplasma bovis* according to [23].

The molecular typing elucidates the phylogenetic analysis and detects any mutation in the circulating strains. molecular typing and sequencing of *M. bovis* isolates can give additional information about their relationships and evolution [37]. From the sequence of 16sRNAgenes common and specific for *M. bovis* the isolates OP268399 and OP268400 showed high nucleotide identity (99% and 97% respectively) with another JX193908 isolate which was isolated from the aforementioned locality. The *gyr*A and *par*C genes of *M. bovis*, which are linked to Enrofloxacin resistance, were examined for mutations. The phylogenetic tree of *Mycoplasma bovis gyr*A (OP270479.1) and its nucleotides identity showed 100% similarity between our *M. bovis gyr*A and other GenBank isolates circulating worldwide and 99% similarity with CP092776 *M. bovis* strain L15762 which circulates in France.

According to the *E. coli* numbering system, Mycoplasma bovis *Par*C (OP270480.1 M. bovis/ Sah.S.S.par.1) in our investigation displayed

amino acid transpositions at positions 2 (Gln to Arg) (CAG >>CGT), 75 (Ile to Ser) (ATT>>AGC), and 79 (Asn to Asp) (AAC>>GAT), while [40] found four potential mutations (Asp79Asn, Ser80Ile, Ser81Pro, and Asp84Asn/Tyr/Val/ Gly). The result of amino acid transpositions at positions 2 (Gln to Arg) in Mycoplasma bovis *Par*C is the second time to be recorded in Egypt similar to [41] who stated that routine molecular techniques such as DNA sequencing can help in detecting mutation sites As amino acid positions are identified using the traditional *E. coli* numbering system, the newly proposed Epidemiological Cutoff determents (ECOFF) a single mutation in either *Gyr*A (Ser83) or *Par*C (Asp79) [40], *M. bovis* strains were divided into (non-)WT or susceptible/resistant isolates.

Conclusion

Major mastitis pathogens (*M. bovis* and other bacteria) could be detected using multiplex RT-PCR, which can be exploited as a standard diagnostic method.

The phylogenetic analysis of the target genes (*M. bovis* 16S rRNA) showed high genetic relation to isolate from the aforementioned locality; while that of Mycoplasma bovis *gyr*A (OP270479.1) showed 100% similarity with other GenBank isolates circulating worldwide and 99% similarity with *M. bovis* strain circulates in France.

Mycoplasma bovis *Par*C (OP270480.1 M. bovis/Sah.S.S.par.1) displayed, amino acid transpositions at positions 2 (Gln to Arg) (CAG >>CGT) for the second time to be detected in Egypt, and at position 79 (Asn to Asp) (AAC>>GAT) similar to the newly proposed Epidemiological Cutoff determents (ECOFF). We believe that the findings presented in our research may appeal to farmers, veterinary doctors, and researchers and can lead to the creation of appropriate treatment and control measures.

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Original Research Article
Veterinary Medicine and The One Health Concept

CHICKEN AIR SACS AND MESENTERY: A HISTOMORPHOMETRICAL AND IMMUNOLOGICAL STUDY

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Abstract: Fat-associated lymphoid clusters (FALCs) are novel lymphoid tissues that have been reported in the mesenteric and mediastinal fat tissue of mouse and human. It plays role in the progression of respiratory and intestinal inflammation and parasitic infestations. However, their occurrence in the chicken air sacs and mesenteric adipose tissue has not yet been identified. Here we investigated the occurrence and distribution of FALCs in the air sacs (cervical, clavicular, thoracic, and abdominal) and mesenteric adipose tissue of healthy chicken. The latter air sacs and mesentery were immediately harvested after anesthesia and cutting the chicken heads then fixed in 4% paraformaldehyde fixative solution for histopathological examination. The degree of FALCs development among different specimens was measured and statistically analyzed. Our results revealed lymphoid clusters associating with the adipose tissues in mesentery, and air sacs (clavicular, thoracic, and abdominal), but not the cervical one. Interestingly, the thoracic air sacs showed significant higher FALCs size in comparison to that of other air sac types and the mesentery. Our findings suggested other possible immunological role of the air sacs and mesentery that could have impact on the progression of air sacculitis and mesenteritis- associate diseases. However, further investigations are required for clarification of air sacs and mesenteric FALCs in the progressions of respiratory and digestive tract diseases.

Key words: air sacs; chicken; fat-associated lymphoid clusters; air sacculitis; mesenteritis

Introduction

Fat-associated lymphoid clusters (FALCs) are unique lymphoid clusters (LCs) that have been reported to be associated with the gonadal, mesenteric, pericardial and mediastinal adipose tissue of healthy mouse and human (1-3). It is consisted mainly of lymphocytes, some macrophages, and granulocytes that were in direct contact with the surrounding adipocytes. Furthermore, novel innate lymphoid cells "natural helper cells" were detected in such clusters. Unlike other immune organs including lymph nodes, and spleen, no fibrous capsule was observed around such clusters, and no zonation for B- and T- lymphocytes (1, 3).

Interestingly, it has been reported that inflammation induces the development of FALCs (4, 5). Moreover, we documented strain difference in the degree of mediastinal FALCs development among healthy mice strains with well and less developed clusters in Th1-biased C57BL/6N (B6), and Th2-biased DBA/2Cr (DBA) mice,

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respectively (3). Interestingly, the role of FALCs in the pathogenesis of various respiratory and digestive tract diseases in mammals has been recently clarified. FALCs have been reported to control local IgM secretion during pleural infection and lung inflammation (6). Furthermore, the role of FALCs in controlling the *Nippostrongylus brasiliensis* helminth infection in mice has been revealed via B-1 B cell proliferation and promotes goblet cell hyperplasia (1, 7). Furthermore, our previous research revealed that mice strain difference in the susceptibility to Mycoplasma pulmonis infection correlates with degree of FALCs development (8).

The respiratory system differs in mammals than that in birds due to the facts that the birds required more efficient system than that of mammals for transferring more oxygen. The birds lack diaphragm and have relatively smaller lung (9, 10), but they have a complex system of well-developed air sacs which represent a much larger percentage of the respiratory system (11). In chickens and ducks, the air sacs volume has been reported to larger than the lung volume by approximately 10× (12, 13).

The air sacs in birds consisted of thin-walled bubble-like pockets connected with the lung, usually eight or nine in number (11, 14). Air sacculitis is an inflammatory disease of the air sac in birds, that is frequently associated with infection, mostly bacteria (15). Our previous report revealed the occurrence of mediastinal FALCs in the mice mediastinal adipose tissue extending from diaphragm to the thymus. However, no reports concerning the occurrence of FALCs in the bird's air sacs and mesentery and its possible role in the development of various respiratory and digestive diseases.

Here we investigated the occurrence of a novel lymphoid clusters in the air sacs (cervical, clavicular, thoracic, and abdominal) and mesenteric adipose tissue of healthy chicken. Except for the cervical air sacs, our results revealed the presence of FALCs in other types of air sacs as well as in the mesenteric adipose tissue. Interestingly, dramatic differences in the development of FALCs among different types of air sacs and the mesentery were highest development in the thoracic air sacs. Our data suggested an additional immunological role of air sacs and mesentery in chicken. However, further investigations are required to clarify its significant contribution in inflammatory conditions of the respiratory and digestive tract.

Materials and methods

Animals

Adult healthy Rhode Island red (RIR) chickens were used in the current experiment at age of one years old. All experiment procedures were approved in accordance with the Institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University, Japan (approval number 20–0171).

Experimental design

Four RIR chickens of both sexes were employed at 12 months of age. The chickens were anaesthetized by intravenous injection of pentobarbital sodium (25 mg/kg) through the ulnar cutaneous wing vein. After confirming the disappearance of immobilization, pain sensation, and corneal reflex, the chickens were euthanized by exsanguination and cutting the head. Then the chest and abdomen were opened, and both air sacs (cervical, clavicular, thoracic, and abdominal) and mesentery were immediately harvested and fixed in 4% paraformaldehyde fixative solution at 4°C. After overnight fixation, the air sacs and mesentery were washed in distilled water, then subjected to histopathological procedures.

Tissue preparation for histopathological observations

Following washing of the fixed specimens, the air sacs and mesentery were subjected to gradual dehydration in ascending graded alcohol (70%, 80%, 90%, absolute alcohol I, II, and III), cleared in xylene (I, II, and III, 30 min/ each), embedded in paraffin, paraffin blocking, and 3 μm paraffin sections were prepared and were used for both routine histopathological observations following staining with hematoxylin and eosin (H&E) staining.

Histomorphometrical measurements

Morphometrical analysis of the degree of FALCs development among different studied air sacs and

mesentery in H&E- stained sections according to our previous report (16). Briefly, the H&E- stained sections of various air sac types and mesentery were converted to virtual slides using Nano Zoomer 2.0 RS (Hamamatsu Photonics Co.; Hamamatsu, Japan). Then, we measured the area of both LCs, and total adipose tissue (AT) by the NDP. view2 (Hamamatsu Photonics Co., Ltd.) software, and the percentage of area ratio for LCs /total AT was calculated and compared among different studied air sac types and mesentery.

Statistical analysis

The values were presented as mean± standard error (SE). Kruskal-Wallis test was conducted to assess the significant differences among studied groups, followed by Scheffé's test for multiple comparisons among the studied groups when significant differences (*P*-values < 0.05) observed among different groups.

Results

Grossly, the chicken had nine air sacs, one of which was single (interclavicular "saccus clavicularis" air sac) and four were paired (cervical "Sacuus cervicalis", cranial thoracic "Saccus thoracicus cranialis", caudal thoracic "Saccus thoracicus caudalis", and abdominal "Saccus abdominalis" air sacs). The air sacs have direct connection with the lung secondary bronchi and indirectly with parabronchi except the abdominal air sac which connected with the primary bronchi. The medullary cavities of pneumatic bones in fowl skeleton are included and aerated by diverticula of the air sacs (Figures 1A, 1B).

Histologically, the air sacs lined by simple squamous to cuboidal cells, supported by connective tissue containing numerous white adipose tissues. The adipose tissue associating with the cervical air sacs lacked lymphoid clusters, however, scattered lymphoid cells could be observed among the adipocytes (Figures 1C, 1D).

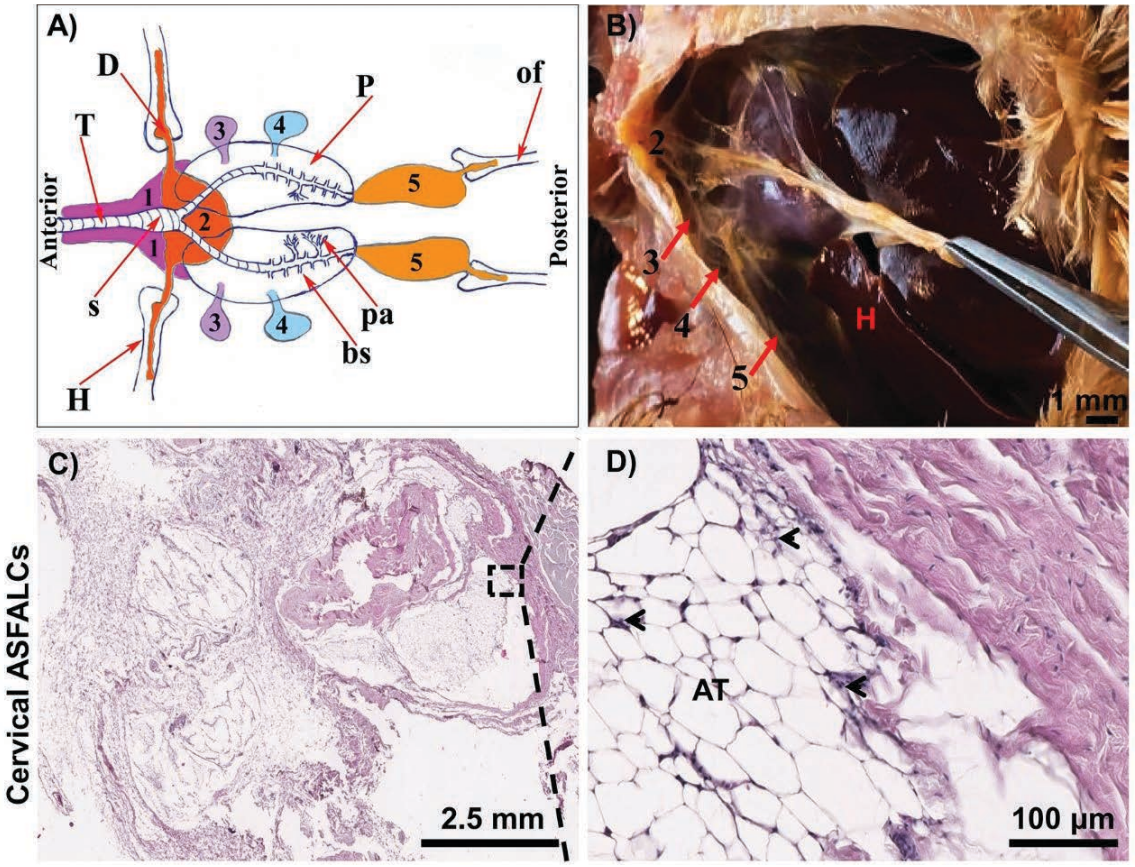


Figure 1: A schematic diagram of fowl air sacs (A), Macroscopic localization of air sacs after opening the chest and abdominal cavities of chicken (B). Notice Trachea (T), Syrinx (S), Pulmo (P), Bronchi secundarii (bs), Parabronchi (pa), Humerus (H), Os femoris (Of), Sacuus cervicalis (1), Saccus clavicularis (2), Saccus thoracicus cranialis (3), Saccus thoracicus caudalis (4), Saccus abdominalis (5), Divericula of air sac (D) and hepar (h). (C, D) Light micrographs of H&E-stained section of the cervical air sac. Notice scattered lymphoid cells (arrow heads), adipose tissue (AT)

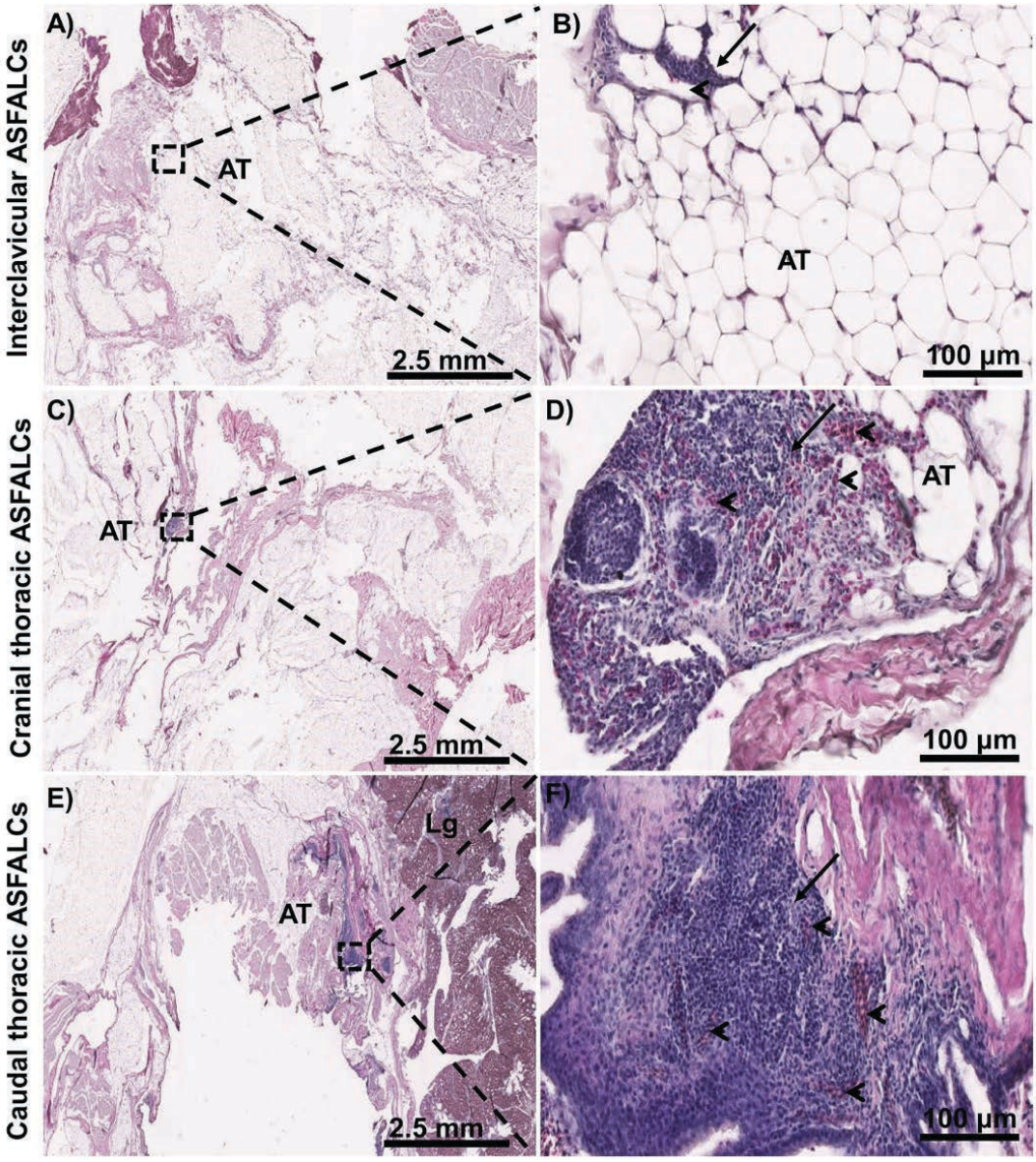


Figure 2: Light micrographs of H&E-stained section of the interclavicular (A), cranial thoracic (C), and caudal thoracic (E) air sacs. (B, D, F) higher magnification to the boxed area in (A, C, E), respectively. Notice blood vessels (arrow heads), lung (Lg), less- and well-developed lymphoid clusters (arrows) in the adipose tissue (AT) of interclavicular and thoracic air sacs, respectively

As shown in Figure 2, the interclavicular air sac showed small sized lymphoid clusters among the adipose tissue and we named it as interclavicular air sac FALCs "interclavicular ASFALCs" (Figures 2A, 2B). On the other hand, well developed lymphoid clusters were observed in the adipose tissue of both cranial and caudal thoracic air sacs especially in the adipose tissue close to the lung tissue and we termed such clusters as thoracic ASFALCs (Figures 2C, 2F).

Similar to the mammalian FALCs, the ASFALCs and mesenteric FALCs in chicken were highly vascularized and lack surrounding connective tissue capsule and their immune cells showed direct contact with the surrounding adipocytes. Interestingly, numerous blood vessels were observed in both cranial and caudal thoracic ASFALCs than that of the abdominal one (Figures 2B, 2D, 2F). Furthermore, the caudal thoracic ASFALCs were frequently observed in numerous number the nearby to the lung tissue (Figure 2E).

As shown in Figure 3, less developed lymphoid clusters were observed in the adipose tissue of abdominal air sacs especially close to the liver

tissue and we termed such clusters as abdominal ASFALCs (Figures 3A, 3B). Interestingly, moderate sized lymphoid clusters were noticed to be associated with the adipose tissue of the mesentery and we termed it as mesenteric FALCs (Figures 3C, 3D).

To analyze the degree of development of the FALCs observed in the adipose tissue in different types of air sacs and mesentery, we calculated the ratios of total lymphoid clusters areas to that of the total adipose tissue areas and compared such values among studied air sacs and mesenteric adipose tissue. Interestingly, the statistical analysis revealed significant higher value for the thoracic ASFALCs than other ASFALCs (Figure 3E).

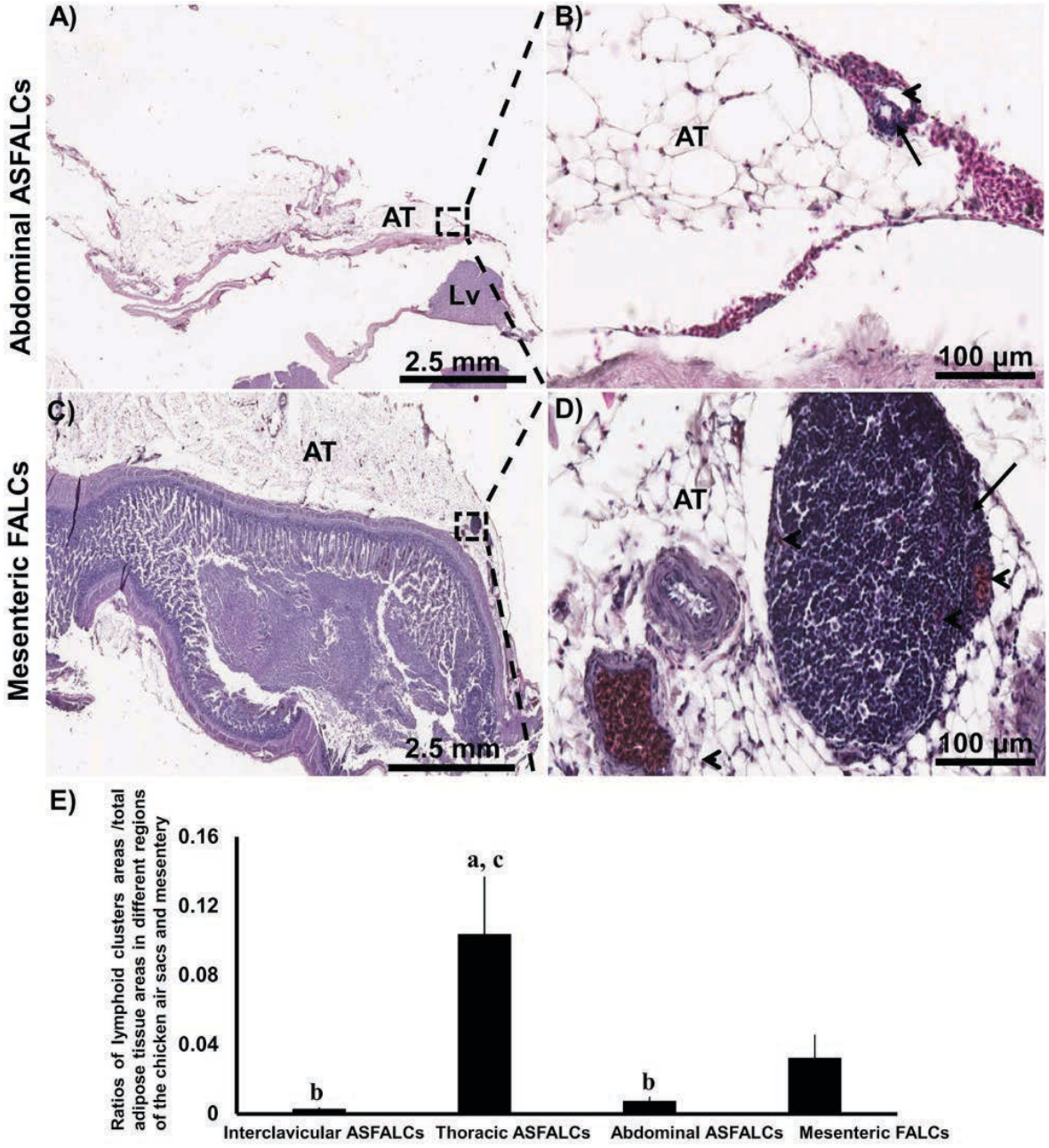


Figure 3: Light micrographs of H&E-stained section of the abdominal air sacs (A), and mesentery (C). (B, D) higher magnification to the boxed area in (A, C), respectively. Notice blood vessels (arrow heads), liver (Lv), lymphoid clusters (arrows) in the adipose tissue (AT) of abdominal air sacs (A, B), and mesentery (C, D). Graph showing the morphometrical data of the ratios of the lymphoid clusters area/ total adipose tissue area in the chicken air sacs and mesentery (E). The letters a, b, c, indicate significant differences between interclavicular air sacs (a), thoracic air sacs (b), and abdominal air sacs (c), with $P < 0.05$, n= 4/experimental tissue group, analyzed by the Kruskal-Wallis test, followed by Scheffé's method. Data are presented as mean values ± SEM

Discussion

The functional activity of the respiratory system in mammals varied extensively from that in birds due to structural variation. The lung in mammals is homogenously partitioned and performs both ventilation and gas exchange simultaneously via its common structures including the respiratory bronchioles, alveolar ducts and alveoli. On the other hand, the respiratory system in birds is heterogeneously partitioned and therefore the functions of ventilation and gas exchange were completely separated (17, 18). The birds characterized by having a unique structure, the air sacs, that serve as bellows to store extra air and allows continuous stream of air to pass through the lungs in one direction (19, 20). Furthermore, other function of the avian air sacs is to remove excess heat as the bird breaths through its connection with the pneumatic bones (21, 22). In birds, it has been reported that the lung is relatively smaller than that of mammals and comprised 10% of the total respiratory system and consisted of parabronchi rather than alveoli. On the other hand, the remaining 90% is occupied by the air sacs (18).

Air sacculitis is common inflammatory condition of the air sacs in birds that is often observed in many infectious diseases including Mycoplasma (23), Newcastle disease (24), Escherichia coli (25), and infectious laryngotracheitis , avian influenza (26, 27). Previously, we reported novel lymphoid clusters associating with the mediastinal fat tissue of healthy mice strains (3). Furthermore, we revealed their role in the development of numerous respiratory diseases including septic condition such as bleomycin induced pneumonitis (5), as well as aseptic infection with Mycoplasma pulmonis (8). Interestingly, in birds more close contact of air carrying infectious agent could lead to the development of air sacculitis due to its functional attribution in allowing continuous flow of the air in one way direction. However, no reports concerning the immunological role of air sacs. Therefore, we histologically examined different types of chicken air sacs for the occurrence of ASFALCs that could be similar structure to that of mammals.

The present investigation revealed less developed ASFALCs in the adipose tissue associated with interclavicular and abdominal air sacs. But well developed ASFALCs were observed in that of both cranial and caudal thoracic air sacs suggesting their possible role in the development of lung associated diseases.

Interestingly, the air sacs of broiler chickens experimentally infected with E.coli revealed the presence of numerous aggregated inflammatory cells replacing the normal air sacs structure (28). This could suggest that the inflammation induce the development of ASFALCs similar to that observed in mammalian FALCs (4, 5). Previous reports indicated that the air sacs communicate with the lung tissues via five perforations on the lung ventral surface of each side (29). Our results revealed well developed thoracic ASFALCs in the vicinity of lung tissue, suggesting their possible role in inflammatory lung progression.

Additionally to the latter, It is known that all air sacs give aeration to specific pneumatic bone of bird except cranial and caudal thoracic air sacs which never aerate any bone and not have any diverticula (30), that may explained the immunological role of thoracic air sacs by presence of higher percentage ASFALCs than other air sacs which have role in ventilation.

Additionally, our results revealed the occurrence of FALCs in chicken mesentery. Similar lymphoid clusters were reported in the mesentery of both human and mice (1). The chicken ASFALCs and mesenteric FALCs showed similar characteristic features to that reported in human and mice where a close contact of immune cells with the surrounding adipocytes and well-developed vasculature were observed (1, 3).

Inconclusions, the current investigations suggest an immunological role of the air sacs and mesenteric adipose tissue in chicken. However, further investigations are required to examine the role of ASFALCs and mesenteric FALCs in the pathogenesis of various avian respiratory and digestive tract diseases.

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Original Research Article
Veterinary Medicine and The One Health Concept

CAMEL MILK MODULATES LIPID METABOLISM, EXPRESSION OF ENZYMATIC ANTIOXIDANTS GENES AND PARAOXONASE ACTIVITY IN RATS FED HIGH CHOLESTEROL DIET

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Abstract: Current findings aimed to find out camel milk's effects on lipid metabolism, expression of the genes for antioxidant enzymes and paraoxonase-1 (PON-1) activity in rats fed diet-containing cholesterol 1%. Therefore, 30 rats were divided into three groups (10 rats per each). Rats of the first group, which acted as a control group, fed a basic diet. Rats of the second group fed a basal diet that included 1% cholesterol however, rats of the third group fed cholesterol 1% accompanied by oral administration of camel's milk (100mL/24h/cage/5 rats) as the only source of water for them. Diet of cholesterol 1% induced significant increase in serum total cholesterol, triacylglycerol (TAG), low-density lipoprotein cholesterol (LDL-c), alanine aminotransferase (ALT), aspartate aminotransferase (AST) activities whereas lower serum PON-1 activity was observed when compared to control. Diet of cholesterol 1% induced significant increase in hepatic Thiobarbituric acid reactive substance (TBARS) however, compared to the control, a significant decrease in the activity and gene expression of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione-S transferase (GST), and reduced glutathione (GSH) were detected. These detrimental effects were ameliorated into accepted range in camel milk treated group. Fatty degeneration and fatty cysts in liver tissues were detected in rats fed cholesterol diet but the affected liver showed acceptable degree of recovery in camel milk treated group. Conclusively, camel milk was potential for the treatment of hyperlipidemia and hypercholesterolemia in rats.

Key words: camel milk; biochemistry; paraoxonase; antioxidants; lipids

Introduction

Hyperlipidemia and hypercholesterolemia are the main risk factors for cardiovascular illnesses (1). The most well-known barriers to employing synthetic medications are their high cost and known negative effects (2). The best options are natural products because they have few side effects and are reasonably priced. An essential source of protein in tropical and subtropical regions is camel milk (3). Low carbohydrate, low cholesterol and higher level of vitamin C minerals are the key distinctions between camel milk and milk from other ruminants (4, 5). The most frequent applications for camel's milk are anti-autoimmune (6, 7), antimicrobial (8), antitoxic (9-14), antioxidant (14) and antidiabetic (15, 16). Camel milk has been claimed to be useful in the treatment of cardiovascular disorders because it contains high concentrations of essential fatty acids, which are known to have a hypolipidemic impact (17). Additionally, camel milk's hypocholesterolemic impact has been attributed to its high ascorbic acid concentration, which is important for bile acids production from cholesterol (18). There are conflicting reports on the hypolipidemic and hypocholesterolemic effects of camel milk, though. Although some studies (15, 19, 20) demonstrated the insignificant influence

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of camel milk on the plasma lipid profiles, other studies (21) showed that it can lower cholesterol and triglycerides. An antioxidant enzyme called paraoxonase guards' lipoproteins against oxidative damage (22). The main prognosis if lipoprotein oxidation has taken place is arteriosclerosis (23). Hypercholesterolemia has been associated with low serum paraoxonase activity (24). The literatures regarding the control of higher level of TG and lower HDL-c level by camel milk still requires additional research. Furthermore, there is a lack of evidence reported the influence of camel milk on paraoxonase activity. The oxidative stress of high cholesterol diet and its protection by camel milk still not fully studied. The objective of the current work was to find out the influence of camel milk on lipid metabolism, expression of the genes for antioxidant enzymes and paraoxonase-1 (PON-1) activity in rats fed diet containing cholesterol 1%.

Materials and methods

Reagents of the experiment

The commercial kits for the detection of blood total proteins, albumin, total lipid, triglyceride, total cholesterol, HDL-c, LDL-c, and VLDL-c were given by ELIPSE, United Diagnostic Industry, UDI, Dammam, Saudi Arabia. Sigma-Aldrich, USA, sold pure cholesterol (Cat# C3045). The common reagents employed in the current investigation were of the highest degree and were readily accessible from retailers. According to the instructions, an ELISA kit (USCN, Life Science Inc; Cat #. E90243Ra) was used to measure the activity of paraoxonase. The following items were purchased from Sigma Chemical Co.: EGTA, EDTA, butanol, EDTA, and H2O2 (St. Louis, MO, USA). The remaining compounds were all of an analytical grade.

Camel's milk samples

Daily samples of camel milk were taken at morning from the camel farm at the King Faisal University in Al-Ahsa, Saudi Arabia. Manual labor was used to obtain camel milk. Prior to being delivered to the lab, sterile screw-top vials were used to collect the samples, which were then kept in cool boxes for storage. Without any additional care, fresh milk was offered to the rats (100 ml/24 hours/cage (25).

Animals and treatment

Prior to the experiment, 30 albino rats (230±20 g) were adapted for 10 days in the laboratory of the King Faisal University's College of Veterinary Medicine in Al-Ahsa, Saudi Arabia. All animals were kept in typical cages (5 rats per cage), given a regular laboratory meal, and given access to unlimited amounts of tap water. The experimental animals were kept on a 12-hour light/12-hour dark cycle in air-conditioned rooms that ranged in temperature from 21 to 23°C and relative humidity levels between 60 and 65°C. The King Faisal University in Saudi Arabia's ethics of scientific research committee published the instructions for use and care of Laboratory Animals, and the animals were handled humanely in compliance with its guidelines (permission # AN000456; GRANT1486).

Experimental hypercholesterolemia

A total of 1g of cholesterol powder should be added to each 99 grams of basal diet (1%), with the exception of the control, in order to generate hypercholesterolemia (26, 27, 28).

Experimental groups and protocol

After receiving a standard diet, rats were randomized into one of three groups, with ten rats in each. Group 1: Rats used as a control were fed a basic meal without any extra ingredients. Rats receiving a 1% cholesterol diet (1g/99g of a basal diet; Sharma et al., 1984) (27) comprise the second group. Group 3 includes rats who are fed a high-cholesterol diet and receive their only water from camel's milk (100 mL/24 hours/cage/5 rats (14, 25).

Samples collection

After two weeks of treatment, blood samples were taken to verify the development of hypercholesterolemia. After overnight fasting, all experimental animals were slaughtered at the end of the experiment while being lightly sedated with diethyl ether. Before making an abdominal incision, blood samples were taken by cardiac

puncture. The collected serum stored frozen at 30°C to be analyzed for liver function enzymes and paraoxonase activities by commercial kits in accordance with manufacturer's instructions. For a molecular and biochemical investigation of antioxidant enzymes, tissues of liver were excised, and liver pieces were quickly frozen by liquid nitrogen and preserved at -80°C. For a histological investigation, a portion of liver tissues was cut into small pieces and placed in neutral buffered formalin for 24 hours.

Analysis of biochemical parameters

Commercial diagnostic kits (United Diagnostic Industry, UDI, Dammam, Saudi (Rome, Italy) were used to determine serum total proteins, albumin, glucose, ALT, AST, CK, BUN, uric acid, creatinine, total cholesterol, TAG, HDL-c. The production instructions were followed while calculating the concentration of the biological ingredients. LDL-c and VLDL-c were calculated as described earlier (29). Paraoxonase activity was estimated by using commercial ELISA enzyme immunoassay (Cat #. E90243Ra).

Detection of antioxidant enzymes activities, TBARS and GSH concentrations in liver tissue

The process of homogenization of liver tissues and estimation of TBRAS concentrations and antioxidant enzymes activities were discussed in our previous published work (14). The commercial ELISA kits (Cayman Chemical Company, USA) were used for detection CAT, GPX, SOD, GST and concentrations of GSH and TBARS by using The ELISA reader (BioTek®, USA).

Analysis of RNA expression for enzymatic antioxidants

The processes started from homogenization of liver tissues until RNA expression of antioxidants enzymes are illustrated previously (14). Table 1 showed primer sequences.

Histopathology of liver tissues

After fixation, liver tissues underwent regular processing, including paraffin embedding, sectioning, deparaffinizing, and rehydrating (30). H & E stains were used to examine the impact of diet containing cholesterol 1% and camel milk.

Statistical analysis

Using one way analysis of variance, the data were showed as mean standard error of mean (ANOVA). The statistical analysis system's computer was used to conduct each test (31).

Results

Determination of biochemical parameters and paraoxonase activity

TAG, total cholesterol and LDL-c were increased in rat fed diet containing cholesterol 1% compared to control (Table 2). Administration of oral camel milk to rats fed a diet containing cholesterol 1% significantly lowered the values of serum cholesterol compared to rats fed a diet containing cholesterol 1% alone but still higher than that of the control. Insignificant changes were observed in all groups' biochemical measurements of remaining biochemical parameters (Table 2).

Table 1: The sequences of primers used in the current study (14)

Gene	Forward primer sequence	Reverse primer sequence
ß-actin	5/-AGC CAT GTA CGT AGC CAT CC-3/	5/- CTC TCA GCT GTG GTG GTG AA-3/
SOD	5/- AGG ATT AAC TGA AGG CGA GCA T-3/	5/- TCT ACA GTT AGC AGG CCA GCA G-3/
CAT	5/-ACG AGA TGG CAC ACT TTG ACA G -3/	5/-TGG GTT TCT CTT CTG GCT ATG G-3/
GPx	5/-AAG GTG CTG CTC ATT GAG AAT G-3/	5/-CGT CTG GAC CTA CCA GGA ACT T-3/
GST	5/- GCT GGA GTG GAG TTT GAA GAA-3/	5/- GTC CTG ACC ACG TCA ACA TAG-3/

SOD: Superoxide dismutase; CAT: Catalase; GPX: Glutathione peroxidase; GST: Glutathione-S transferase

Table 2: Protein and lipid profile and glucose concentration in rats fed diet-containing cholesterol 1% (group 2) and treated with camel milk (group 3) compared to the control (group 1)

Parameters	Group 1	Group 2	Group 3
Glucose (mg/dl)	130.2 ± 2.01	135.1 ± 4.02	131.0 ± 4.02
Total proteins (g/dl)	5.0 ± 0.34	5.1 ± 0.40	5.2 ± 0.51
Albumin (g/dl)	4.3 ± 0.29	4.4 ± 0.49	4.3 ± 0.19
Globulins (g/dl)	1.2 ± 0.09	1.1 ± 0.19	1.1 ± 0.19
Triglycerides (mg/dl)	35.9 ± 2.60	60.1 ± 2.40*	40.0 ± 2.22*
Total cholesterol (mg/dl)	25.2 ± 0.09	45.0 ± 0.19*	31.0 ± 0.21*
HDL-c (mg/dl)	12.4 ± 0.77	11.87 ± 0.90	12.0 ± 0.91
LDL-c (mg/dl)	5.4 ± 0.99	24.0 ± 0.99*	11.1 ± 0.89*
VLDL-c (mg/dl)	7.1 ± 0.9	9.1 ± 0.90	6.4 ± 0.90

Every number corresponds to the mean ± standard deviation of 10 rats. HDL-c: High density lipoprotein cholesterol; LDL-c: Low density lipoprotein cholesterol; VLDL-c: Very low density lipoprotein cholesterol. *When compared to the control, mean values are significantly (P<0.05) different.

Table 3: Liver and kidney function Biomarkers in rats fed diet-containing cholesterol 1% (group 2) and treated with camel milk (group 3) compared to the control (group 1)

Parameters	Group 1	Group 2	Group 3
ALT (IU/L)	22.14 ± 0.39	40.11 ± 0.59*	30.0 ± 0.68**
AST (IU/L)	80.13 ± 0.70	110.11 ± 0.91*	90.1 ± 0.81**
CK (IU/L)	509.98 ± 5.12	493.20 ± 8.21	500.30 ± 7.11
BUN (mg/dl)	7.99 ± 0.29	6.98 ± 0.95	7.97 ± 0.96
Creatinine (mg/dl)	0.19 ± 0.94	0.18 ± 0.89	0.17 ± 0.76
Uric acid (mg/dl)	1.00 ± 0.09	1.10 ± 0.08	1.10 ± 0.09

Every number corresponds to the mean ± standard deviation of 10 rats. ALT: Alanine transaminase; AST: Aspartate transaminase; ALP: Alkaline phosphatase; ACP: Acid phosphatase; CK: Creatine kinase; BUN: Blood urea nitrogen* When compared to the control, mean values are significantly (P<0.05) different. ** When compared to the cholesterol treated rat (group 2), mean values are significantly (P<0.05) different.

ALT and AST activities were increased significantly in rat a diet containing cholesterol 1% compared to control groups (Table 3). However, administration of camel milk to rats fed a diet containing cholesterol 1% corrected the serum levels of both ALT and AST activities towards the normal control values (Table 3). Activities of CK and the values of BUN, uric acid, and creatinine remained unaltered (Table 3).

Rats fed diets containing 1% cholesterol had lower paraoxonase activity than the controls (Figure 1). When these rats were given camel milk, the paraoxonase activity was almost fully recovered.

Analysis of hepatic lipid peroxidation

Rats fed diet containing 1% cholesterol induced a significant (P<0.05) higher levels of hepatic TBARS than the control group (Fig. 2A). After administration of camel milk, liver tissue TBARS levels significantly (P<0.05) reduced; nevertheless, this decline did not reach the normal control value (Fig.2A).

Determination of GSH concentration and activities enzymatic antioxidants in liver tissues

Significant (P<0.05) reduction of GSH concentration was observed in rats fed diets containing

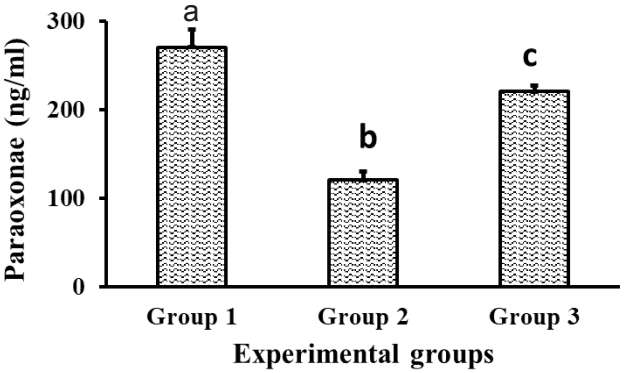


Figure 1: Paraoxonase activities of rats fed diet-containing cholesterol 1% (group 2) and treated with camel milk (group 3; 100ml/24h/cage) compared to the con-trol (group 1)

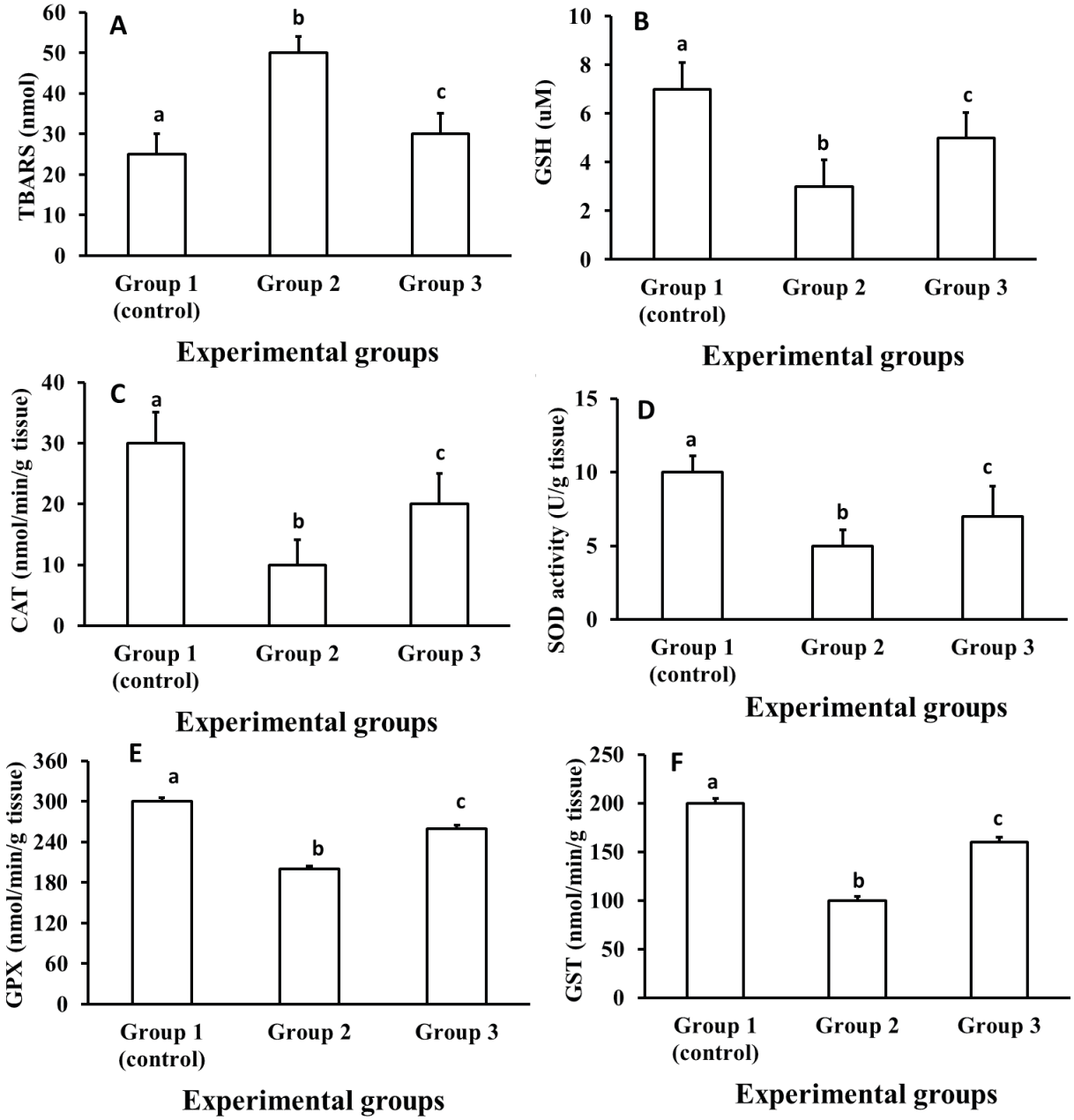


Figure 2: Colorimetric estimation of (A) TBARS, (B) GSH, (C) CAT, (D) SOD, (E) GPX, (F) GST liver of rats received diet containing cholesterol 1% (Group2) and treated with camel milk (group 3) compared to the control (group 1)

cholesterol 1% compared to the control (Fig. 2B). Although, providing of camel milk restored GSH concentration in these rats, the normal control values have not been achieved (Fig. 2B). Significant reduction ($P<0.05$) of CAT (Fig. 2C), SOD (Fig. 2D), GPX (Fig. 2E) and GST (Fig. 2F) activities were observed in rats fed cholesterol diet 1% in comparison to the control. While camel milk delivery induced a considerable ($P<0.05$) recovery in the activity of these enzymes, the normal control values have not been reached (Fig. 2C, D, E, F).

Determination of expression of the genes for antioxidant enzymes

The amount of mRNA expressed was measured using real-time PCR (Fig. 3). According to the current research, rats fed a 1% cholesterol diet had significantly ($P<0.05$) lower levels of CAT (Fig. 3A), SOD (Fig. 3B), GPX (Fig. 3C), and GST (Fig. 3D) expression than control rats. Although camel milk delivery resulted in a considerable ($P<0.05$) up-regulation of these enzymes' activity, the normal control values have not been reached (Fig. 3).

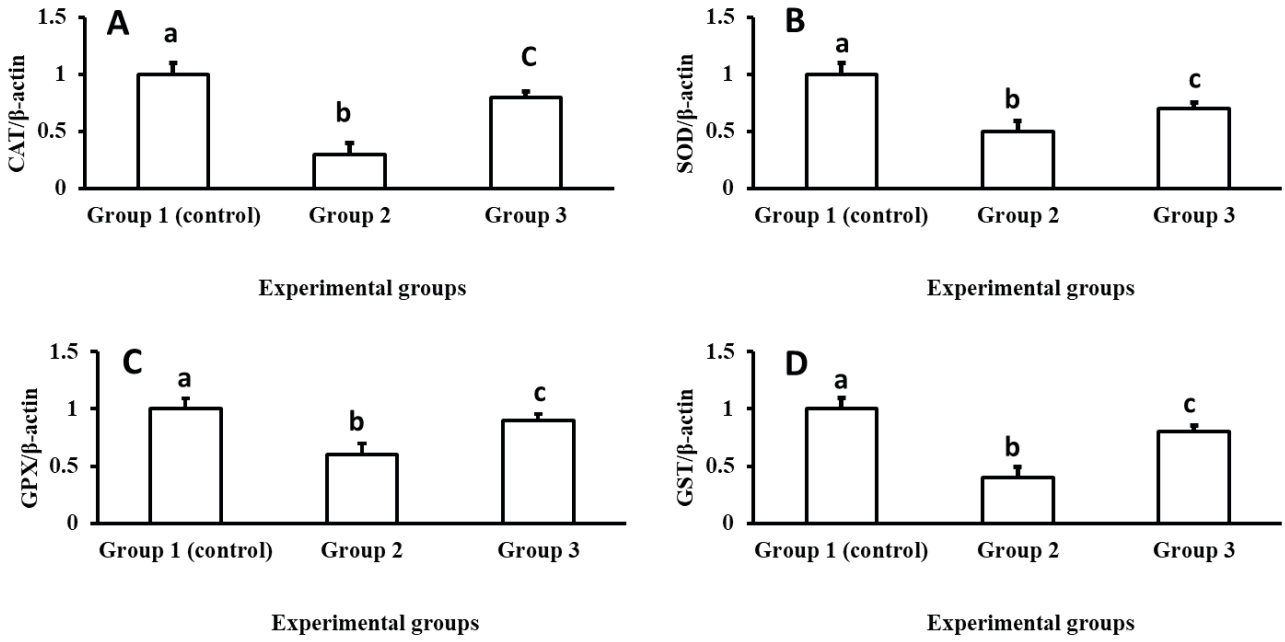


Figure 3: mRNA gene expression of enzymatic antioxidants, CAT (A), SOD (B), GPX (C) and GST (D) in liver tissues of all studied group

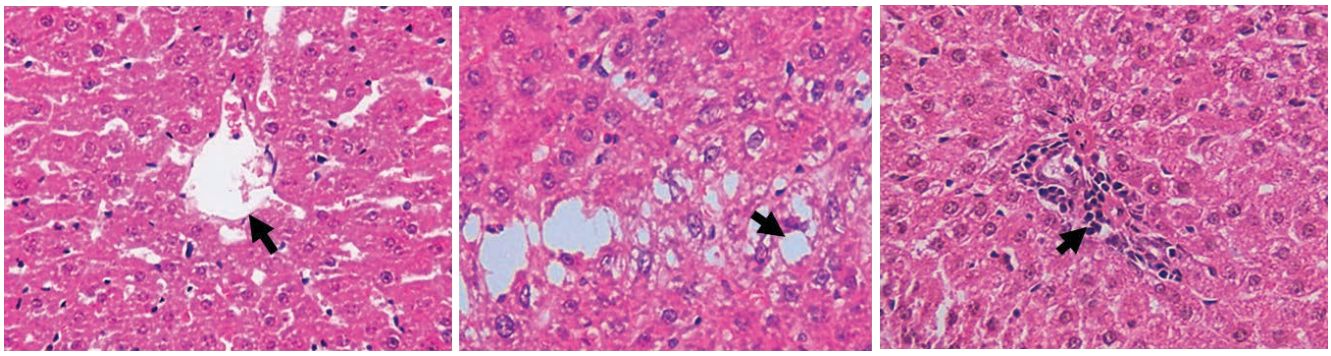


Figure 4: Histopathology of liver of (A) control rats (group 1) showed normal central vein and hepatic cords (ar-row), (B) liver of cholesterol treated rats (group 2) showed fatty changes (arrowhead) and cysts of fats (arrow), (C) liver of camel milk treated rats (group 3) showed normal architecture (arrowhead) of portal and hepatic cords (ar-row). HE bar= 40.00

Histopathological examination

The control rats' livers had sinusoids separating the principal veins from the polygonal cells grouped in regular cords (Fig. 4A). Rats given cholesterol revealed different levels of vacuolar degeneration and variations in sizes, shapes, and staining preferences in their liver (Fig. 4B). Rats fed a high-cholesterol diet and given camel milk showed complete degrees of healing in their livers. The liver cell plates simultaneously recovered their usual structure (Fig. 4C).

Discussion

Hypercholesterolemia is a significant risk factor for cardiac diseases which diagnosed by rise of LDL-c and decrease of HDL-c levels in serum of affected patients or animals (32). Chemical antihyperlipidemic and antihypercholesterolemic medications are widely utilized, but their main drawbacks are their high costs and side effects (16). There is increasing interest concerning the role of natural products in health and medicine (33). Camel milk is gaining great importance in

control and prevention of many diseases as steatohepatitis, insulin resistance and lipid peroxidation (33). However, results of hypolipidemic and hypolcholesterolemic effect of camel milk still contradictory (15, 19, 20, 21, 28, 33, 34). The data concerning the management of high Triglyceride (TG) levels and low HDL cholesterol levels by camel milk remains inconclusive. Furthermore, there is a lack of evidences reported the impact of camel milk on paraoxonase activity. There is still much to learn about the oxidative stress caused by a high cholesterol diet and the involvement of camel milk to protect against it. Therefore, the present study aimed to find out camel milk's effects on lipid metabolism, expression of the genes for antioxidant enzymes and paraoxonase-1 (PON-1) activity in rats fed a diet containing cholesterol 1%. According to the current research, there were insignificant changes in any of the experimental groups' serum total proteins, albumin, globulins, or glucose levels. The current study supports earlier research (34) that showed that treatment of camel milk had insignificant effect on the level of glucose in female albino rats. The current results, however, do not correlate with other studies (33, 35), which showed that rats fed a high fat and high-cholesterol diet showed a considerable rise in serum glucose concentration. The results of the current investigation showed that rats' TAG concentration was increased by a diet high in cholesterol. These results support earlier studies (33, 35, 36, 37) showing that a high-fat, high-cholesterol diet causes hyperlipidemia, which is linked to increased de-esterification of the ample free fatty acids and lower lipoprotein levels (38). The significant reduction of TAG made by administration of camel milk come in concurrent of previous work (34) in female rats. The current study's observation of a significant rise in total cholesterol in rats fed a diet containing cholesterol 1% compared to the control group is consistent with earlier studies (33, 35, 36, 37, 39) in rats fed a high fat and high-cholesterol diet. Higher de-esterification of ample free fatty acids and the reduced lipoproteins may be responsible for this hyperlipidemia (38). Parallel to the current study, earlier investigations (33, 35, 37) showed that rats fed a high fat and cholesterol diet experienced large increases in serum LDL-c and significant decreases in HDL-c levels in comparison to control. The significant effect of camel milk on total cholesterol and LDL-c come in harmony with previous reports in rats (15, 19, 20) and disagree with other reports (21) that indicated the non-significant effect of camel milk the same parameters. The presence of vitamin C, an antioxidant, is thought to be responsible for camel milk's impact on cholesterol. Stress was induced on liver function through biochemical changes and changes in enzyme activity. Rats fed a diet containing cholesterol 1% in the current research had higher ALT and AST activity than the control group. These findings agree with early study in rats fed high fat and cholesterol diet (35, 37). The treatment of rats fed a diet containing cholesterol 1% with camel milk induced a significant decrease in these enzyme activities, which suggests a sort of membrane stabilizing activity of camel milk (40). The current study might claim that camel milk may have a hepatoprotective effect based on the current results. Several reports (12, 13, 14, 25, 41) demonstrated the protection of hepatocytes by camel milk treatment. Previous report (34) demonstrated that camel milk did not affect ALT and AST activities of healthy female albino rats. Current histopathological observations that showed fatty alterations in the liver of rats fed diet containing cholesterol 1% and a considerable recovery after camel milk administration provided evidence of the hepatoprotective action of camel milk. Current histopathological findings about the impact of a diet containing cholesterol 1% on liver tissues are consistent with earlier research (33) in humans. As far as the author is aware, the current work provided the first histological report demonstrating the preventive impact of camel milk on hepatic fatty alterations brought on by administration of a high cholesterol diet to rats. However, camel milk has been shown to protect rats' livers against alcohol-induced hepatotoxicity (42). Cholesterol and camel milk were safe to rats at the level of renal and mineral metabolism. This was demonstrated by BUN, creatinine, and uric acid levels that were unchanged when compared to control in all experimental groups. Current results reported the reduced paraoxonase activity in rats fed a diet containing cholesterol 1% come in accordance with previous report (43) demonstrating that, hyperlipidemia and hypercholesterolemia are best media for lipid peroxidation and subsequent inhibition of paraoxonase activity. The current study's demonstration of the stimulation of paraoxonase activity in rats may have validated

the antioxidant effect of camel milk that was recently identified at the gene expression level as proven earlier (14) and confirmed in the current investigation. Rats fed a diet containing cholesterol 1% had significantly higher levels of TBARS and significantly lower levels of enzymatic antioxidants like CAT, SOD, GST, and GSH in their livers compared to the control group, indicating that the high-cholesterol diet caused lipid peroxidation and oxidative stress (28). SOD protects cells from oxidative damage by converting free radical superoxide to H_2O_2 and O2. The H_2O_2 produced will decompose enzymatically by CAT (44). Serum from rats fed a diet containing cholesterol 1% showed a significant increase in lipid peroxidation product and significant decreases in CAT, SOD, and GST (28). Camel milk may have a protective effect against cholesterol-induced oxidative stress, as demonstrated by the significant reduction in TBARS in the liver of rats given 1% cholesterol and treated with the milk. Due to the presence of vital vitamins and minerals, camel milk has a protective impact (45). The body's antioxidant system depends heavily on GSH. Through redox and detoxifying reactions, it keeps the cells' natural structure and function. In the present investigation, rats fed a diet containing 1% cholesterol showed a significant decline in GSH values. The reduction in GSH may be caused by GPX's urgent need for GSH to scavenge free radicals. The potential of camel milk to remove free radicals and restore the antioxidant state may be responsible for the ameliorative effect of camel milk as evidenced by the lowering of GSH levels. As shown in the current study, this mechanism was strengthened by an increase in the activity and gene expression of antioxidant enzymes. After intoxication with alcohol (42), cadmium chloride (9), aluminum chloride (9), and CCL4 (14), camel milk increased the GSH levels in rat liver. The present findings demonstrated that all studied antioxidant enzymes (SOD, CAT, GPX, and GST) had their gene expression downregulated by a diet containing 1% cholesterol. The current research revealed that camel milk increased the state of antioxidant by increasing genes expression of CAT, SOD, GPX, and GST. Similar findings (10, 14) showed that camel milk controlled gene expression of these enzymes in rats given cisplatin or CCl4 intoxication, respectively. The current study demonstrated that rats fed a diet containing 1% cholesterol had lower paraoxonase activity and lower levels of antioxidant enzymes. These indicators both increased when camel milk was received. This suggests that these values could be employed as hyperlipidemia and hypercholesterolemia diagnostic biomarkers.

Conclusion

Diet of cholesterol 1% induced significant increases in serum values of total cholesterol, TAG, LDL-c, ALT, AST and TBARS. The same diet induced significant reduction in activities and expression of CAT, SOD, GPX and GST compared to control. The activity of PON-1 in serum of these rats was significantly reduced as well. Fatty degeneration and fatty cysts in liver tissues were observed in histopathological picture of rats fed cholesterol diet. All these detrimental effects were ameliorated into accepted range in camel milk treated group. Conclusively, camel milk was potential for the treatment of hyperlipidemia and hypercholesterolemia in rats.

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Original Research Article
Veterinary Medicine and The One Health Concept

CARCASSES AND OFFAL CONDEMNATION AT KOM-ELNOUR ABATTOIR IN DAKAHLIA PROVINCE, EGYPT: MAJOR CAUSES AND ECONOMIC LOSS

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Abstract: An abattoir survey was conducted from October 2020 to September 2021 to identify the primary reasons for offal and carcass condemnation of slaughtered animals in Dakahlia province, Egypt, as well as to estimate their economic loss. During the survey, 643 animals (441 cattle, 178 buffalos, 19 camels, 4 sheep, and one goat) were submitted for antemortem and postmortem inspection. The results of both inspections were collected, analyzed, and the total economic loss was estimated by adding the condemnation of offal and carcasses over a twelve-month period. Retail prices for offal (liver, lung, heart, and tongue) and carcass prices per kg were obtained from local markets. Out of 643 animals slaughtered, 2 (0.31%) were totally condemned. In addition, postmortem inspection revealed that 217 (33.74%) livers, 158 (24.57%) lungs, 5 (0.78%) hearts, 14 (2.18%) heads, and 11(1.71%) tongues were condemned. Offal were condemned mainly due to pneumonia, fascioliasis, telangiectiasis, necrosis, and hydatidosis, whereas carcasses were rejected due to generalized tuberculosis. According to this study, the financial loss at the abattoir owing to carcass and organ condemnations was 244066 Egyptian Pounds over a twelve-month period (15746 USD). The incidence of meat rejection and subsequent financial loss was high compared to the amount of local revenue. The current study concluded that bacterial and parasitic diseases are still prevalent and cause significant economic damage in Dakahlia province, Egypt. This abattoir survey offered regional information on the principal reasons for carcass and organ condemnation in slaughtered animals, as well as an estimate of the direct economic consequences. Furthermore, the findings of the current work underlined the importance of developing an efficient monitoring system for meat condemnation and enforcing animal health strategies in Egypt.

Key words: abattoir; carcass; condemnation; economic loss; meat inspection; offal

Introduction

Meat inspection is an important approach for detecting diseased animals and avoiding the spread of unhealthy meat. Information provided by slaughterhouse inspections of live and slaughtered meat-producing animals is useful epidemiological data for disease evaluation and establishing the efficiency of protective actions. Therefore, slaughterhouses play an important role in differentiating meat with various affections and preventing the sale of infected meat to customers, as well as providing an information source and a reference point for disease occurrence (1, 2, 3).

Cattle, camels, sheep, and goats are the most predominant animal species in Egypt, according to the Egyptian ministry of agriculture and land reclamation in 2019, with around 9.5 million heads and 484 abattoirs. Meat produced from these animal species is the primary source of high-quality protein as well as a high level of unsaturated fat, phosphorous, vitamin B, and a low content of cholesterol and sodium, all of which are beneficial to human health. However,

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some parasitic, bacterial, and viral infections restrict the production of meat (2,4,5). These infections cause massive economic losses varying from morality of livestock, weight loss, and meat rejection at slaughterhouses (6, 7).

Numerous pathogenic bacteria are prevalent in food animals; these bacteria could be transmitted to humans through contaminated meat handling, preparation, and consumption, as well as during meat condemnation at slaughterhouses (8). However, bovine tuberculosis is a chronic lung condition that can damage other organs like the liver and possibly spread to the entire body, causing weight loss and decreasing animal productivity (9, 10). In addition, severe parasitic infestations such as cysticercosis, hydatidosis, and fascioliasis have a substantial influence on animal health and meat safety (2, 11, 12). The consumption of raw or undercooked meat harboring Cysticercus bovis can directly transmit the disease to humans (13). Furthermore, in extensively infested instances, bovine cysticercosis is responsible for carcass condemnation, but in the case of hydatidosis, meat-producing animals serve as intermediate hosts to complete the life cycle (14).

Although there is insufficient data about different animal diseases and affections that cause meat and organ condemnations inside Egyptian abattoirs, routine postmortem examination should be performed as soon as possible after carcass dressing is done. Thus, the aim of this research was to determine the major reasons of meat condemnation in Kom-Elnour abattoir in Mit-Ghamr city, Dakahlia province, Egypt and estimate the resulting financial loss. The outcomes of this research may help in establishing an appropriate strategy in Egypt for the monitoring and prevention of animal diseases, particularly in Dakahlia province.

Materials and methods

Research area

The research was conducted in Kom-Elnour slaughterhouse in Mit-Ghamr city, Dakahlia province, Egypt (Fig. 1). Dakahlia is an Egyptian governorate located northeast of Cairo. It covers around 3,500 km². Its capital is Mansoura and it has a population of approximately 6 million.

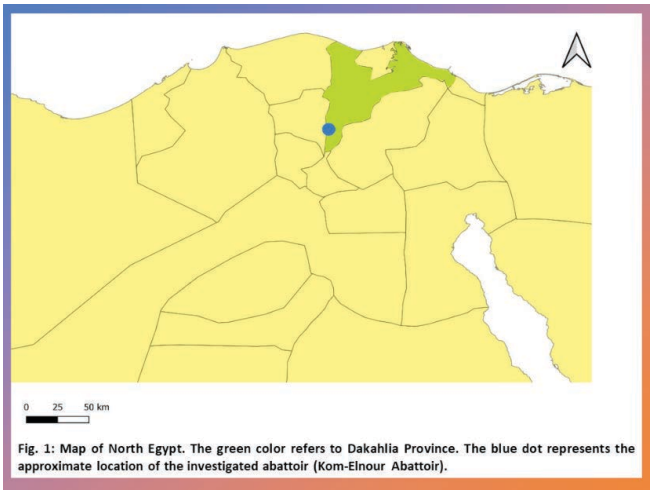


Figure 1: Map of North Egypt. The green color refers to Da-kahlia Provimce. The blue dot represents the approx-imate location if the investigated abattoir (Kom-Elnour Abattoir)

Duration of research and animal population

The research was carried out over a twelve-month period from October 2020 to September 2021. During the research period, 441 cattle, 178 buffalos, 19 camels, 4 sheep, and one goat were slaughtered (n=643). Animals were transported to the abattoirs from the surrounding areas and evaluated to identify the predominant causes of offal and carcass condemnation. A convenient sample procedure was used, and the number, sex, and breed of the animals were recorded.

Research methodology

The inspection procedures, including antemortem and postmortem examinations, were performed by appointed meat inspectors in accordance with the Ministerial decision No. 517/1986 of the Egyptian Ministry of Agriculture and Land Reclamation (MALR) (15). During antemortem inspection, individual animals in the lairage were examined at rest and in motion. Animal movements, behavior, disease symptoms, and any other abnormalities were all observed and registered. Postmortem inspection involves visual inspection, palpation of visceral organs, and incision of lymph nodes and organs. Pathological changes, including alterations in the size, color, texture, and presence of parasites or their related lesions, were identified and

recorded. The decisions and judgments on the findings were classified as completely or partially acceptable for human consumption. While in case of generalized disease such as miliary tuberculosis, a total condemnation decision was taken.

Table 1: Average price of carcasses and their offal in Egypt during 2020-2021

Item	Average weight (kg)	Average price (Egyptian pound /kg)	Total pric (Egyptian pound)
Cow	210	120	25200
Buffalo	240	120	28800
Camel	220	120	26400
Sheep	40	150	6000
Goat	30	150	4500
Bovine liver	8	120	960
Camel liver	8	120	960
Ovine liver	1.2	150	180
Bovine lung	8	45	360
Camel lung	8	45	360
Ovine lung	1.2	45	54
Bovine heart	2.5	45	112.5
Camel heart	2.5	45	112.5
Ovine heart	0.5	45	22.5
Bovine head	9	60	540
Bovine tongue & esophagus	0.5	60	30

1 USD was equal to 15.50 Egyptian pounds

Estimation of economic loss

The direct economic loss was calculated using the formula established by Khanjari et al. (16). DEL= N × W × P

DEL: Direct economic loss; N: number of condemned carcases / and offal; W: average weight of carcases / and offal (Kg); P: Average carcases / and offal price (Egyptian pound/Kg). The average selling price of the different carcases and their offal was estimated based on their marketable cost in Kom-Elnour, Mit-Ghamr, Dakahlia during 2020-2021 (Table 1)

Results

During the course of this investigation, 643 animals were slaughtered at Kom-Elnour slaughterhouse, with cattle (441) being the most popular, followed by buffalo (178), camel (19), sheep (4), and lastly goat (1). According to the findings of the current study, the greatest slaughtering rates were observed during March (76), April (80), June (65) and July (74) with percentages of 11.82%, 12.44%, 10.11%, and 11.51%, respectively (Table 2). The lowest percentages were reported after Eid Al-Adha ended in August and September 2021, with 0.62% and 5.29%, respectively. The fluctuation in the number of animals slaughtered between the months might be linked to the feast season (Eid Al-Adha) and people habits. In March 2021, the percentage of affection among slaughtered animals was the highest (20.00%). Furthermore, the number of affections (82) documented during this month surpassed the number of slaughtered animals, as many animals had two or more separate affections in their organs.

According to the current study, no condemnations happened during the antemortem examinations of slaughtered animals, but all condemnations were related to diseases discovered after the postmortem examination. The postmortem findings of animals slaughtered and inspected at Kom-Elnour slaughterhouse in 2020 and 2021 are summarized in Table (3). The results revealed that 217 (33.74%) livers, 158 (24.57%) lungs, 5 (0.78%) hearts, 14 (2.18%) heads, and 11 (1.71%) tongues were rejected. Cows were mostly infected by fascioliasis (109) (Fig. 3B), which predominantly affected female animals (82), whereas pneumo-

nia was the most common infection in buffalo (59) (Fig. 2D), which largely affected male animals (34). The most frequent parasitic infection in camels was *Cephalopina titillator* larvae (Fig. 3E) (10, with 8 males and 2 females). In addition, two buffalo (male and female) and one male sheep were among the emergency slaughtered cases that arrived at the slaughterhouse. They arrived broken and the only option was to slaughter them.

Table 2: Numbers and species of animals slaughtered at Kom-Elnour slaughterhouse during 2020 and 2021

Study period	Animal species					Numbers	
	Cow	Buffalo	Camel	Sheep	Goat	Slaughtered * (%)	Affections ** (%)
October 2020	30	9	0	0	0	39 (6.07%)	25 (6.10%)
November 2020	37	14	0	0	0	51 (7.93%)	31 (7.56%)
December 2020	43	8	5	0	0	56 (8.71%)	38 (9.27%)
January 2021	40	13	6	1	0	60 (9.33%)	48 (11.71%)
February 2021	37	16	3	0	0	56 (8.71%)	52 (12.68%)
March 2021	51	21	4	0	0	76 (11.82%)	82 (20.00%)
April 2021	53	26	1	0	0	80 (12.44%)	62 (15.12%)
May 2021	34	14	0	0	0	48 (7.47%)	28 (6.83%)
June 2021	41	24	0	0	0	65 (10.11%)	23 (5.61%)
July 2021	47	23	0	3	1	74 (11.51%)	13 (3.17%)
August 2021	3	1	0	0	0	4 (0.62%)	1 (0.24%)
September 2021	25	9	0	0	0	34 (5.29%)	7 (1.71%)
Total	**441**	**178**	**19**	**4**	**1**	**643**	**410**

* Numbers and percentages of slaughtered animals per month. ** Numbers and percentages of affections among slaughtered animals per month.

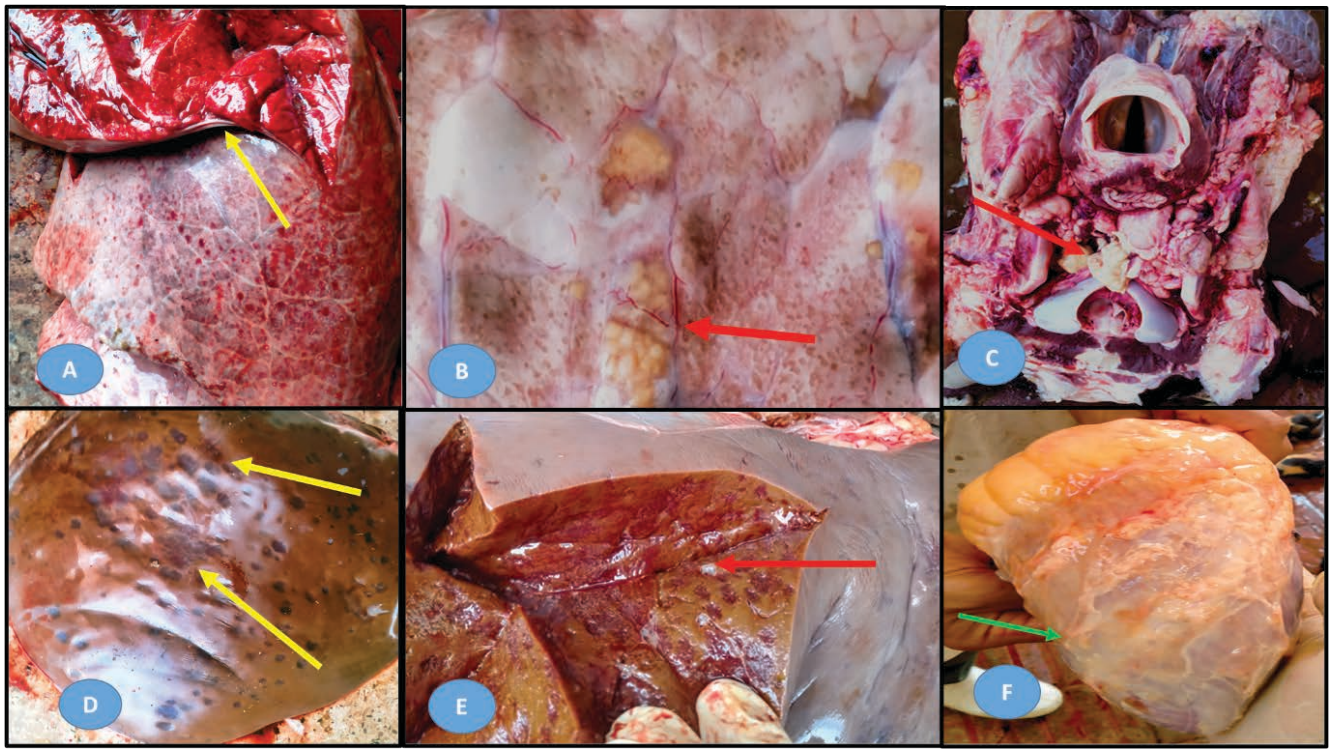


Figure 2: The post-mortem pathological findings of animals slaughtered and inspected at Kom-Elnour slaughterhouse. **(A)** Pneumonia in lung (Yellow arrow); **(B)** Tuberculosis in lung (Red arrow); **(C)** Actinobacillosis in cattle head with oozing pus (Red arrow); **(D)** Telangiectasis in liver (Yellow arrow); **(E)** Telangiectasis in liver (Red arrow); **(F)** Pericardial adhesion (Green arrow)

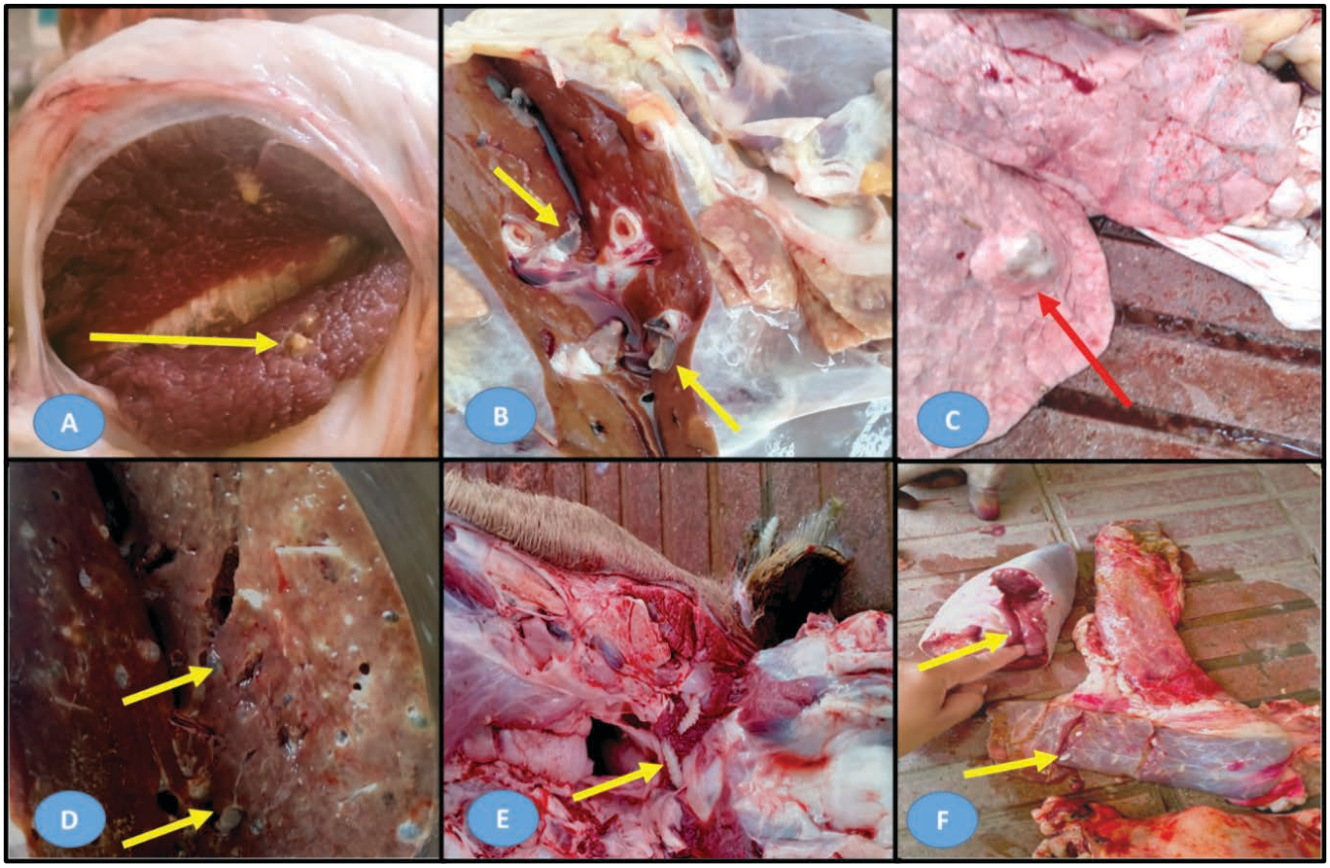


Figure 3: The post-mortem parasitic findings of animals slaughtered and inspected at Kom-Elnour slaughterhouse. (A) Cysticercus bovis in masseter muscle (Yellow arrow); (B) Adult Fasciola sp. in liver bile duct (Yellow arrow); (C) Hydatid cyst in lung tissue (Red arrow); (D) Multiple small hydatid cysts in liver (Yellow arrows); (E) Ceph-alopina titillator larvae in nasopharynx of camel (Yellow arrow); (F) Macroscopic white Sarcocystis in tongue and esophagus (Yellow arrow)

Table (4) displays the principal reasons for the partial and total condemnation of slaughtered animals. Two (0.31%) cows with generalized TB were totally condemned, whereas 408 animals were judged with partial approval for consumption after condemnation of various infected parts. The postmortem examination indicated that the lung was mostly condemned due to pneumonia (138), followed by hydatidosis (14) (Fig. 3C) and localized TB (6) (Fig. 2B). However, the most frequent cause of liver condemnation was fascioliasis (129), followed by telangiectiasis (46) (Fig. 2D and E), necrosis (40), and hydatidosis (2) (Fig. 3D). Five hearts were condemned owing to pericarditis and pericardial adhesion (Fig. 2F). Sarcocystis infection caused the condemnation of (11) tongue and esophagus (Fig. 3F). *Cephalopina titillator* larvae in camels (10), Actinobacillosis in cows (Fig. 2C) (3), and *Cysticercus bovis* in cow (1) were the causes of 14 heads condemned (Fig. 3A). Table 5 also depicts the economic loss caused by carcass and organ condemnations at the Kom-Elnour slaughterhouse. Fasciolosis was clearly the leading cause of financial loss, followed by generalized tuberculosis, telangiectasis, pneumonia, and hepatic necrosis. The total economic loss due to carcass and organ condemnation over a year was estimated to be 244066 Egyptian Pounds (15746 USD).

Discussion

The fundamental purpose of abattoirs is to safeguard public health. Abattoirs provide valuable information and are an excellent indicator of the real incidence of disease among slaughtered animals, which endangers people through the consumption of infected meat. Meat inspection plays a vital role in disease monitoring in the national livestock by offering insights to the veterinary authority in order to manage or eliminate disease, provide wholesome

Table 3: Postmortem findings among animals slaughtered and inspected at Kom-Elnour slaughterhouse during 2020 and 2021

Sex	Number	Lung				Liver					Heart	Tongue & Esophagus	Head		Emergency Slaughter (Fracture)
		Pneumonia		Partial Hydatidosis	Localized TB	Telangiectasis		Partial Hydatidosis	Partial Necrosis	Fasciolliasis	Pericarditis	Sarcocystosis	Actinobacillosis	Parasitic affections**	
		Whole	Parts			Whole	Parts								
Cow															
Male	168	0	17	2	0	0	4	0	5	27	3	0	0	1	0
Female	273	2	54	6	3	8	22	0	25	82	1	0	3	0	0
Total	441	73		8	3	34		0	30	109	4	0	3	1	0
Overall *		**84**				**173**					**4**	**0**	**4**		**0**
Buffalo															
Male	123	3	31	2	1	0	4	0	5	4	0	2	0	0	1
Female	55	0	25	2	2	0	6	0	3	16	1	9	0	0	1
Total	178	59		4	3	10		0	8	20	1	11	0	0	2
Overall *		**66**				**38**					**1**	**11**	**0**		**2**
Camel															
Male	16	1	4	2	0	0	2	2	2	0	0	0	0	8	0
Female	3	0	0	0	0	0	0	0	0	0	0	0	0	2	0
Total	19	5		2	0	2		2	2	0	0	0	0	10	0
Overall *		**7**				**6**					**0**	**0**	**10**		**0**
Sheep															
Male	4	1	0	0	0	0	0	0	0	0	0	0	0	0	1
Female	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	4	1		0	0	0		0	0	0	0	0	0	0	1
Overall *		**1**				**0**					**0**	**0**	**0**		**1**

During postmortem inspection, two cows were totally condemned, and only one male goat was slaughtered and approved for human consumption. * Total number of lesions per organ of each animal species. ** Parasitic affections include *Cysticercus bovis* and *Cephalopina titillator* larvae

products, and protect the population from zoonotic risks (17).

Causes of whole carcass condemnation

Out of 643 animals slaughtered in Kom-Elnour abattoir, 2 (0.31%) were totally condemned due to generalized tuberculosis. Similar results of 0.27% were detected by Mummed & Webb (18), where tuberculosis was noted in 0.03% of cattle slaughtered at private abattoirs in Ethiopia. Lower results of about 0.026% were obtained in Tanzania at Morogoro Municipality abattoir (19), but the cause was also detection of bovine tuberculosis. Furthermore, Tembo and Nonga (20) reported that the percentage of overall condemnation among cattle assessed at the Dodoma slaughterhouse in Tanzania was 0.05%, with 0.002% of TB.

Partial condemnation of the carcass

Disease conditions in different organs were found in 312 (48.52%) males and 331 (51.48%) females. Among 312 male animals slaughtered, 55 (8.56%) livers, 64 (9.95%) lungs, 3 (0.47%) hearts, 9 (1.40%) heads, and 2 (0.31%) tongues with esophagus were condemned; and out of 331 female animals slaughtered, 162 (25.19%) livers, 94 (14.61%) lungs, 2 (0.31%) hearts, 5 (0.78%) heads, and 9 (1.39%) tongues with esophagus were condemned. The most prevalent cause of partial condemnation was localized liver affections, followed by lung affections. Fascioliasis and telangiectiasis were the major causes of liver condemnation, whereas pneumonia and hydatid cysts were the major causes of lung condemnation. Based on gross pathological findings, 217 (33.75%) livers, 158 (24.57%) lungs, 5 (0.78%) hearts, 14 (2.18) heads, and 11(1.71%) tongues with esophagus were rejected as unfit for

Table 4: Major causes of partial and total condemnation of animals slaughtered at Kom-Elnour slaughterhouse during 2020 and 2021

Study period	Total condemnation	Partial condemnation												
	Generalized TB	Lung			Liver				Heart	Tongue & Esophagus	Head			Emergency Slaughter
		Pneumonia	Hydatidosis	Localized TB	Telangiectasis	Hydatidosis	Necrosis	Fasciolasis	Pericarditis	Sarcocystosis	Actinobacillosis	*Cephalopina titillator*	*Cysticercus bovis*	Fracture
October 2020	0	8	0	0	1	0	0	16	0	0	0	0	0	0
November 2020	0	10	0	1	0	0	2	16	0	1	1	0	0	0
December 2020	0	11	0	1	2	0	1	17	1	1	1	3	0	0
January 2021	0	13	2	1	3	0	4	20	0	1	0	3	0	1
February 2021	0	15	2	0	9	0	10	9	1	2	0	2	1	1
March 2021	0	24	3	0	14	2	17	19	0	1	0	2	0	0
April 2021	1	25	5	0	8	0	4	19	0	0	0	0	0	0
May 2021	1	7	2	0	3	0	2	8	2	2	1	0	0	0
June 2021	0	9	0	1	5	0	0	5	0	2	0	0	0	1
July 2021	0	9	0	1	1	0	0	0	1	1	0	0	0	0
August 2021	0	1	0	0	0	0	0	0	0	0	0	0	0	0
September 2021	0	6	0	1	0	0	0	0	0	0	0	0	0	0
Total	**2**	**138**	**14**	**6**	**46**	**2**	**40**	**129**	**5**	**11**	**3**	**10**	**1**	**3**

human consumption. Lower results were recorded by Tembo and Nonga (20), with a total of (10.5%) lungs and (6.3%) livers condemned, and Komba et al. (19) who reported condemnation rates of lungs, livers, hearts, and heads were 1.96%, 1.61%, 0.02%, and 0.21%, respectively.

Causes of liver condemnation

In the current study, the livers were found to have the highest abnormal conditions (217). Cows, buffaloes, camels, sheep, and goats had 173, 38, 6, 0, and 0 liver lesions, respectively. The primary causes of liver condemnation were fasciolosis (20.06%), telangiectiasis (7.15%), necrosis (6.22%), and hydatid cyst (0.31%). Pezeshki et al. (11) in Iran (0.25%) and Tembo and Nonga (20) in Tanzania (4.5%) found lower percentages of fascioliasis in cattle. Furthermore, according to Jaja et al. (21), the rate of fascioliasis in cattle was 5.95%, 4.48%, and 2.7% for the years 2010, 2011, and 2012 in South Africa, respectively. While Nzalawahe and Komba (22) found a higher prevalence of fascioliasis in cattle in Tanzania (76%), Quevedo et al. (23) in southern Brazil (37.06%), Mohammed et al. (24) in Ethiopia (36.06%), Mummed and Webb (18) in Ethiopia (29.74%), Raji et al. (25) in Nigeria (23.41%), Amuamuta et al. (26) in Gondar, Ethiopia (22.9%), and Souza et al. (27) in the state of Minas Gerais (21.95%). Also, a higher distribution of bovine fascioliasis in northern Côte d'Ivoire was revealed by Traoré et al. (28) at the regional (18.0% to 52.3%) and departmental level (14.3% to 64.0%). According to Alizadeh and Amniattalab (29), fascioliasis is crucial in the development of several hepatic disorders in buffaloes, such as bile duct hyperplasia, hepatitis, and fatty change. The data obtained revealed that fascioliasis is common in Egyptian slaughterhouses. The high prevalence of fascioliasis in this study may be attributed to favorable climatic circumstances and the presence of snails. Fasciola infection is common and pervasive in several African nations. It seldom causes mortality in farm animals, and its consequences have resulted in a drop in

Table 5: Economic loss due to carcass and organ condemnations in Kom-Elnour slaughterhouse during 2020-2021

Items	Cause of condemnation	Number of animals	Weight (Kg) *	Economic loss (Egyptian pound)
Whole carcass	Generalized TB	2 cows	420 kg	**50400**
Liver	Telangiectiasis	34 cows 10 buffalos 2 camels	142 Kg 30 Kg 6 Kg	**21360**
	Fascioliasis	109 cows 20 buffalos	872 Kg 160 Kg	**123840**
	Necrosis	30 cows 8 buffalos 2 camels	90 Kg 24 Kg 6 Kg	**14400**
	Hydatidosis	2 camels	6 Kg	**720**
Lung	Pneumonia	73 cows 59 buffalos 5 camels 1 sheep	229 Kg 192 Kg 20 Kg 1.2 Kg	**19899**
	Hydatidosis	8 cows 4 buffalos 2 camels	24 Kg 12 Kg 6 Kg	**1890**
	Localized TB	3 cows 3 buffalos	24 Kg 24 Kg	**2160**
Heart	Pericarditis	4 cows 1 buffalo	10 Kg 2.5 Kg	**562**
Head	Actinobacillosis	3 cows	27 Kg	**1620**
	Cysticercus bovis	1 cow	9 Kg	**540**
	Cephalopina titillator	10 camels	90 Kg	**5400**
Tongue and Esophagus	Sarcocystosis	11 buffalos	5.5 Kg	**330**
Emergency slaughter	Fracture **	2 buffalos 1 sheep	6 Kg 1.5 Kg	**945**
Total amount of financial loss due to condemnations				**244066**

*Weight of Condemned carcasses / organs (Kg), **Condemned area around fracture

animal productivity and the rejection of livers at slaughterhouses (30). Regarding telangiectiasis, Souza et al. (27) in Minas Gerais and Ngwu et al. (31) in Nigeria discovered a higher percentage of telangiectiasis that led to the condemnation of bovine liver (29.20% and 10%, respectively). While Rezac et al. (32) found a lower incidence of 1% in cows in the United States. Because hepatic lesions can reduce animal production and have a negative impact on animal reproduction, it is critical to pay closer attention to the accuracy of anti-parasitic programmes as well as the assessment of the potential role of mycotoxins, poisonous plants, and heavy metals in the development of hepatic lesions in various animals (29).

Causes of lung condemnation

The total number of lesions found in the lungs was 158. Cows, buffalo, camels, and sheep had 84, 66, 7, and 1 lung lesions, respectively. The major causes of lung condemnation were pneumonia (21.46%), hydatid cysts (2.18%), and TB (0.93%). The results coincided with Mummed and Webb (18), who found that pneumonia cases in cattle in Ethiopia were 22.68%. Lower rates of pneumonia were reported in Nigeria by Raji et al. (25) and Ngwu et al. (31) (8.79% and 6.67%, respectively). According to Jaja et al. (33), the percentage of condemned cases caused by pneumonia at three Namibian abattoirs was 1.09%, 2.21%, and 0.77%, respectively. On the other hand, Opara (34) demonstrated a higher percentage of 39.2% in Nigeria.

In terms of hydatidosis, Pezeshki et al. (11) in Iran and Tembo and Nonga (20) in Tanzania reported comparable results (2.25% and 3.1%, respectively). High prevalence rates were observed by Amuamuta et al. (26) in northwest Ethiopia (35.7%), Mulatu et al. (35) in eastern part of

Ethiopia (20.05%), Mummed and Webb (18) (23.01%) in Ethiopia, Ghasemian et al. (36) in Iran (15-20%), and Kere et al. (37) in Kenya (5.3%). In contrast, Komba et al. (19) detected a lower rate of about 0.046% in Tanzania. The variation in hydatidosis occurrence among countries can be attributed to some factors such as country location, the presence of dogs, and the level of animal hygiene (38).

A higher rate of tuberculosis was manifested (1.87%) in Tanzania by Komba et al. (19), (9.1%) in Nigeria by Okeke et al. (39), and in Ethiopia by Shitaye et al. (40), who indicated that the prevalence rate of bovine tuberculosis in slaughterhouses in various places of the country ranged from 3.5% to 5.2%. According to Woldemariyam et al. (41), the overall lesions of bovine tuberculosis among cattle carcasses slaughtered at Debre Birhan municipality abattoir, Ethiopia were 4.7%. However, the routine meat inspection only detected 0.5% of the examined carcasses as having TB lesions. Anatomically, 66% of the lesions were diagnosed in the lungs and associated lymph nodes. In countries where bovine TB in cattle is still highly prevalent and/or insufficient milk hygiene, 10% to 15% of human tuberculosis is caused by bovine TB (42). According to Ameni et al. (43), the primary route of Mycobacterium bovis (M. bovis) infection in humans is drinking raw milk. Hence, human tuberculosis caused by M. bovis is mainly extra-pulmonary, particularly in cervical lymphadenitis. With respect to this, Kiros (44) demonstrated that a significant number of patients with extrapulmonary TB were in intimate contact with animals, in particular those who frequently used to drink raw milk. Regassa (45) illustrated the link between M. bovis and M. tuberculosis in the transmission of TB in humans and cattle. Cattle belonging to tuberculous patients had a greater incidence (24.3%) than that of cattle held by non-tuberculous owners (8.6%). This highlighted the critical involvement of M. bovis in the development of human TB.

Causes of heart condemnation

The overall heart lesions (5) in cows, buffaloes, and camels were 4, 1, and 0 respectively. Pericarditis was the only cause of heart condemnation. This is consistent with EDO et al. (46), 2014; Mummed and Webb (18), who revealed that the main cause of heart condemnations was pericarditis (1.6%, 4.59%, respectively). The findings of this study corresponded with those of Ahmed et al. (47), who reported that 8% of tested hearts in the Ismailia slaughterhouse, Egypt, were rejected due to traumatic pericarditis and heart adhesions, and Raji et al. (25), who reported a pericarditis percentage of around 17.06%.

Causes of head condemnation

The total head lesions (14) in cows, buffaloes, camels, sheep, and goats were 4, 0, 10, 0, and 0 respectively. The primary causes of heads condemnation in the present research were *Cephalopina titillator* (71.4%), Actinobacillosis (21.4%), and cysticercosis (7.1%). Camel heads were the most frequently rejected among the other slaughtered animals. With postmortem examination, *Cephalopina titillator* larvae were mainly detected in the nasal cavities, turbinate bones, and frontal sinuses of infested camels, and those remained parasitizing the animal for a considerable period of time, causing a significant loss in animal production. Out of 19 camel heads examined, 10 were infested by C. titillator larvae, which gives an overall prevalence of 52.6% among camels. Higher infestation percentages (80%) were found in Cairo, Egypt by Attia et al. (48), (82.6%) in Ethiopia by Kissi and Assen (49), while lower results (26.27%) were noted in Sharkia Province, Egypt by Rahman and Mohammed (50), 42.43% in Iraq by Atiyah et al. (51), and 48.3% in Iran by Rad et al. (52).

Cysticercus bovis was identified in 0.16% of cow masseter muscles. Cueto González et al. (53) found closer findings (0.21%) in Northwest México. Higher prevalence (5.5%) was recorded in Ethiopia by Abdella (54) where the masseter muscle had the highest rate (11.5%), 20.57% by Shafi and Elemo (55) in Ethiopia with 20.40% of cysts observed in the masseter, 2.89% in Western Ethiopia by Shuramo et al. (56), where cysts were present in 6.7% of the examined masseter muscles, and 2.0% in Northwest Ethiopia by Ezeddin and Tewodros (57) with a rate of infestation of 33.33% in the masseter muscles, and 4.8% in Nigeria by Karshima et al. (58).

Causes of tongues and esophagus condemnations

There were 11 tongue and esophageal lesions in buffaloes, all of them were caused by Sarcocystis infection. Approximately 6.18% of the buffaloes evaluated were infected. Gerab et al. (59) found a higher incidence (26.5%) at Tanta slaughterhouse, Egypt, 12% in El-Minia Governorate Abattoirs, Egypt by Dyab et al. (60), 20% in Malaysia by NA and WOB (61), and 66.42% in Andhra Pradesh, India by JyothiSree et al. (62).

Causes of emergency slaughter

The only emergency slaughter cases recorded in Kom-Elnour abattoir were fracture-related, accounting for 0.47% of all cases. Higher percentages were observed by Raji et al. (25) in Zango-Zaria, Nigeria (6.50%) and by Oziegbe et al. (63) at Jos Abattoir, Plateau State, Nigeria (1.4%).

Financial losses due to condemnations

Another goal of this research was to estimate the financial losses caused by meat condemnation at the Kom-Elnour slaughterhouse. The economic losses at the abattoir were calculated for the years 2020-2021 (Table 5). Fasciolosis was the major cause of financial loss, followed by generalized tuberculosis, telangiectiasis, pneumonia, and hepatic necrosis. The entire economic loss caused by meat condemnation over a year was calculated to be 244066 Egyptian pounds (15746 USD). Several research studies have been conducted in Egypt to investigate the financial consequences of diseased meat-producing animals. Mohammed and Maky (6) conducted research in the North and South of Egypt for two years (2017-2018). They discovered that the financial loss due to the rejection of meat at three abattoirs was 4529010 Egyptian pounds (383063 USD), with TB, icterus, pneumonia, hydronephrosis, parasitic cysts, and fascioliasis being the main causes of meat condemnation.

The economic loss in the current study was greater than the findings published in Egypt by Ahmed et al. (47), who estimated an annual economic loss of 36480 Egyptian Pounds due to organs condemnation from slaughtered male cattle at Ismailia abattoir. Furthermore, Yibar et al. (64) conducted a slaughterhouse survey in Bursa Province, Turkey, where the monetary loss owing to organ and carcass condemnations at two abattoirs over a six-month period was 245,483 USD. While hydatidosis and fasciolosis were the leading causes of organ condemnation, TB and jaundice were the leading reasons of carcass condemnation. The variation in economic loss between studies could be attributed to differences in livestock populations, disease prevalence, incidence, estimation methodologies, loss items included in the analyses, and differences in animal productivity and prices.

Conclusion

During the current investigation, a significant number of offal was condemned from animals slaughtered at Kom-Elnour Abattoir in Dakahlia due to various pathological abnormalities observed in different organs, such as pneumonia and telangiectiasis. Affected meats were rejected as unsuitable for human consumption. However, one of the drawbacks of this study was the use of gross lesions in disease diagnosis; consequently, only diseases having pathognomonic gross pathological abnormalities were likely to be identified. The postmortem inspection's magnificent role in safeguarding the health of the public cannot be overemphasized. A significant amount of money, over 244066 Egyptian Pounds, was lost as a result of pathological conditions discovered in the investigated slaughterhouse. According to this research, the three most frequent affections that had the biggest impact on the Egyptian budget were liver diseases, fascioliasis, and TB. It is apparent that sufficient preventive and monitoring programmes, as well as frequent use of anti-parasitic medications, are required in Egypt, particularly in Dakahlia province, to reduce the risk of infectious diseases and financial losses.

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Original Research Article
Veterinary Medicine and The One Health Concept

COMPARISON BETWEEN RAPID AND SLOW CRYOPRESERVATION PROTOCOLS FOR RAM SEMEN

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Abstract: Information regarding adequate freezing protocols of ram semen considering freezing distance and time during cryopreservation has not been adequately reported. Therefore, this study aimed to compare two freezing protocols for Najdi ram's semen. In the rapid freezing protocol, the straws were frozen at 5 cm over the surface of liquid nitrogen for 15 minutes. While in the slow freezing protocol, the straws were frozen at 8 cm over the surface of liquid nitrogen for 20 minutes. The semen was collected from five rams and extended with tris egg yolk glycerol cryodiluent. The extended semen was chilled slowly to 5^0C within two hours and equilibrated for two hours before being frozen on the liquid nitrogen vapor and cryopreserved at -196^0C. There was no significant (P >0.005) effect of the freezing protocol on the sperm's total motility, plasma membrane integrity, DNA integrity, and abnormalities. However, the vitality, fast progressive motility, straight-line velocity, average pathway velocity, linearity, and wobble were significantly higher in rapid freezing than in slow freezing protocol. In conclusion, cryopreservation of ram semen using rapid freezing (5 cm for 15 mins) protocol was better than slow freezing (8 cm for 20 mins) protocol regarding post-thawing semen quality.

Key words: freezing protocol; cryopreservation; egg yolk; ram; semen

Introduction

Cryopreservation of semen is one of the essential technologies in animal research for enhancing productivity and reproductive efficiency (1). Semen cryopreservation helps to prolong sperm livability and vitality by slowing metabolism and preventing bacterial development, reducing the accumulation of metabolic byproducts (2), (3). Cryopreservation of mammalian sperm is a complex procedure involving balancing several elements to produce the best results. Diluents, dilution, cooling, freezing, and thawing procedures all have a role in the success of ram semen cryopreservation (4), (5). Compared to other species, ram spermatozoa have a low intramembrane cholesterol-to-phospholipid ratio. Therefore, cold-shock sensitivity in ram spermatozoa is higher than in other species (6).

The freezing protocol aims to gradually lower the semen temperature from 5 to −196°C to avoid sperm damage. The freezing rate regulates the extent and rate of sperm dehydration and ice crystal formation. These two main variables affect sperm freezing success; ice crystal formation is more dangerous than sperm dehydration. In the

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slow freezing protocol, sperm exposes to damage because of high solute concentrations for a long period which results in cell dehydration and volume contraction; however, minimum intracellular ice crystal formation has occurred. In the rapid freezing protocol, sperm exposes to damage because of intracellular ice crystal formation; however, minimum dehydration has occurred. Therefore, the optimum freezing protocol must be slow enough to minimize intracellular ice crystal formation and rapid enough to minimize sperm dehydration (7).

The acrosome, nucleus, mitochondria, axoneme, and plasma membrane are affected by rapid temperature variations because of the creation and dissolution of ice during the freezing and thawing processes (8), (9). Temperature changes during cooling and freezing cause stress on sperm membranes, resulting in lipid phase shifts and a difference in the functional condition of sperm membranes. Cold shock damage occurs when sperm is rapidly cooled from 30 to 4 °C (10). The phase of supercooling (0°C to −5°C) and the development of ice crystals (−6°C to −15°C) are the two main temperature ranges where sperm are damaged during freezing (11). Temperatures between 5° and −15°C are known to cause a significant phase change (12), and this could be the perfect temperature range for temperature-dependent damage. The sperm contents remain unfrozen at −10°C; while, the exterior surrounding media freezes between −5 and −10°C. Therefore, water flows out of the sperms osmotically and freezes outside (13). For the first time, Polge (14) and Mazur (15) reported that the crucial temperature range in which the most sperm destruction occurs is between −15 and −30°C and extended to −60°C and −80°C. Transferring the semen from +5°C to −100°C within 7 min at a rate of −15°C/min is recommended (16). Sperm freezing at a rate of 15–60°C/min is recommended and resulting in a good survival rate. A freezing rate of ≥ 20°C/min for ram is effective and recommended.

Semen is diluted with a cryoprotectant extender to minimize ice crystal formation and to avoid sperm damage (17). The proper freezing protocol aims to minimize intracellular ice crystal formation; therefore, it must be slow enough to allow water to leave the cells yet fast enough to prevent severe cell dehydration and the solu-tion effect (18). Therefore, this study aimed to know the best freezing protocol for Najdi ram's semen according to the freezing distance and time using the liquid nitrogen vapor (rapid freez-ing of 5 cm for 15 mins and slow freezing of 8 cm for 20 mins).

Materials and methods

Management of Animals

The experiment was conducted in the fall using five mature Najdi rams aged 2-4 years with an average body condition score of 3 at the Experimental Farm, Department of Animal Production, King Saud University, Riyadh, Saudi Arabia (latitude 24° 48′ N and longitude 46° 31′ E). The rams were sheltered in a covered yard within an open-sided barn. The ram's daily energy and protein needs were met with commercial mixed pellets (14.5 percent CP; 2.78 Mcal ME kg-1DM). All rams were found to be viable, and assessment of their ejaculates using computer-assisted sperm analysis (CASA) revealed normal parameters. The King Saud University Research Ethics Committee (REC) authorized the current project with Ethical Reference No: KSU-SE-21-33. This authorization was based on the advice of the Research Ethics Sub-Committee (minute number 7 and date 22/04/2021), as well as a suitable risk-to-benefit ratio and a study design that minimizes risks.

Preparation of extender

Unless otherwise stated, all chemicals were obtained from Sigma (Sigma-Aldrich Corp., St. Louis, MO, USA). This study prepared and utilized a Tris-hydroxymethyl-aminomethane, monohydrated citric acid, and D-fructose extender reported in previous studies (19)pigeon (P. Briefly, 250 mM of Tris, 88.5 mM of citric acid, and 69.38 mM of fructose were dissolved in 100 mL of distilled water to create the buffer. The buffer was then supplemented with 18 % chicken egg yolk and 8 % glycerol. Gentamicin was administered at a dosage of 13.3 mg mL^{-1}.

Collection and evaluation of semen

The artificial vagina was used to collect semen samples twice weekly from each ram (20). The semen samples were assessed macroscopically and microscopically using a sperm class analyzer (SCA®; version 4.0.0.5, Microptic S.L., Barcelona,

Spain). For further processing, only ejaculates that met the following parameters were used: 1.5 ml volume, score 4 on mass activity grade scores, 80% progressive motility, and sperm concentration of 2×10^9 ml^{-1}). The ejaculates were pooled to exclude individual ram differences. Seven pooled ejaculates were used in this study.

Semen Extension and Freezing

The pooled ejaculates were gradually diluted 1:4 with tris egg yolk glycerol diluent. The diluted semen was gradually chilled for less than two hours, from 30 to 5°C. The diluted and cooled semen was placed into 0.25 ml straws using a semiautomatic filling and capping machine (minitube GmBH, Tiefenbach, Germany) and left at 5^0C for 2 hours to equilibrate the glycerol according to (21). After reaching equilibrium, the straws were frozen in liquid nitrogen vapor using two different protocols. On the rapid freezing protocol, the straws were hung at 5 cm over the surface of liquid nitrogen for 15 minutes. While on the slow freezing protocol, the straws were hung at 8 cm over the surface of liquid nitrogen for 20 minutes. The straws were then preserved at -196 °C after submerging in liquid nitrogen.

Semen Evaluation

The semen samples were evaluated on two occasions. The first was after achieving equilibrium (before freezing) and the second was at least 48 hours after cryopreservation. The frozen straws were thawed for thirty seconds in a 37 °C water bath. The following parameters of the sperm were evaluated (16), (21)

Sperm cell motility

SCA® was used to estimate the speed of each spermatozoon in three ways including the average pathway velocity (VAP), straight-line velocity (VSL), and curvilinear velocity (VCL). The sperms were classified as rapidly progressive, moderately progressive, sluggish, or static. For evaluating progressiveness on a relative scale, the straightness (STR=VSL/VAP), linearity (LIN=VSL/VCL), and wobble (WOB=VAP/VCL) metrics were calculated and reported as percentages. The amplitude of the lateral head displacement (ALH) and the transverse beat frequency (BCF) were also measured. The sperms velocities were categorized to rapid (VCL >75 µm/s), medium (45 < VCL < 75 µm/s), slow (10 < VCL < 45 µm/s) or static (VCL < 10 µm/s). The sperm presenting movement with a STR index ≥80% was considered progressive motile. The results were categorized as rapid progressive (A), slow progressive (B), non-progressive (C) or static (D) according to WHO 4^{th} edition.

Livability (Vitality)

Using a FluoVit kit, fluorescence microscope, and SCA vitality software (Microptic S.L., Barcelona, Spain), the percentages of live/dead spermatozoa were determined.

Sperm morphology assessment and morphometry

The dry semen smears were stained for 1–2 min with New Rapid SpermBlue® fixative/stain (Microptic S.L., Barcelona, Spain). The stained smears were viewed with SCA® under a microscope at 600x magnification (33). Morphometries of sperms were measured including head length (µm), head width (µm), head area (μm^2), head perimeter (µm), acrosome (%), elongation, ellipticity, regularity, rugosity, midpiece width (µm), midpiece area (μm^2), distance (µm), and angle (°).

Plasma membrane integrity of spermatozoa

A hypo-osmotic swelling test was used to determine the functional integrity of the plasma membrane of spermatozoa via incubating 10 µl of semen with 100 µl of a 190 mOsM hypo-osmotic solution for 40 minutes at 37 °C in a 1.5-ml Eppendorf tube. On a warm slide, 100 µL of the liquid was distributed with a cover slide and examined under a microscope at 400x magnification. At least 200 sperms were evaluated. The percentage of spermatozoa with inflated and curled tails was calculated.

Sperm DNA fragmentation

The Halotech DNA kit was used to determine the degree of DNA fragmentation in ram sperm.

The samples of sperm were diluted to a concentration between 5 and $10x10^6$ mL-1. The agarose gel from the kit was incubated at 90-100°C for 5 minutes to fuse the agarose and then at 37°C for 5 minutes in an adjustable water bath. 25µ L of semen sample was added to the Eppendorf tube, along with the gel, and thoroughly mixed. Twenty microliters of the solution were deposited on a super-coated slide from the package, then placed on a cool surface and covered with a 22x22-mm coverslip. Slides were refrigerated for 5 minutes at 4 °C to produce a micro-gel containing an implanted sperm. Carefully removing the coverslips, the slides were immersed for 7 minutes in the previously prepared acid solution (80 L HCl in 10 mL of distilled water), according to (22). The slides were then transferred to a tray containing a lysing solution from the kit and incubated for 25 minutes, followed by moistening with distilled water and dehydrating in increasing ethanol concentrations for two minutes (70, 90, and 100 percent). Slides were stained with Giemsa or Wright's stain, washed with tap water, and dried at 25°C. Each slide was viewed at 100x magnification using a light microscope, and 200 spermatozoa were counted. A spermatozoon without fragmented DNA was placed in an agarose matrix and treated with lysing solutions to deproteinize the nucleus and form dispersed DNA halos. Halos refer to DNA loops loosely attached to the remaining nuclear structure (nucleus). Sperm nuclei with fragmented DNA produced small or no halos of scattered DNA, while nuclei without fragmented DNA shed DNA, forming large halos.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) to compare between two freezing protocols. The data were presented as mean ± standard error. Differences were considered significant at $P < 0.05$.

Results

The effects of using different freezing protocols on the motility grades of post-thawed Najdi ram sperms post thawing is presented in figure 2. The grade A (fast progressive) motility was significantly (P 0.05) higher in the rapid freezing protocol than in the slow freezing protocol. While grades B, C, and D motilities were not significantly ($P > 0.05$) different in the two protocols.

Effects of different freezing protocols on the vitality, plasma membrane integrity, and DNA integrity of the post-thawed Najdi ram sperms are presented in Figure 3. Plasma membrane integrity and DNA integrity of the post-thawed Najdi ram sperm were not significantly ($P > 0.05$) different in the two protocols. While, vitality was significantly ($P \leq 0.05$) higher in the rapid freezing protocol than in the slow freezing protocol.

The effects of the freezing protocol on post-thawed abnormalities and morphometry of Najdi ram sperms are presented in Table 2. The all-sperm morphometric parameters and sperm abnormalities of post-thawed Najdi ram semen were not significantly ($P > 0.05$) differed between the two protocols, rapid (5 cm in 15 mins) and slow (8 cm in 20 mins).

Discussion

Our results revealed that the percentages of fast-progressive motility, VSL, VAP, LIN, WOB, and vitality were significantly higher in the rapid freezing protocol than in the slow freezing protocol. However, the ALH was significantly higher in the slow freezing protocol than in the rapid freezing protocol. The velocities (rapid, medium, slow and static), grades B, C, and D motilities, all-sperm morphometric parameters, sperm abnormalities, plasma membrane integrity and DNA integrity of post-thawed Najdi ram semen were not significantly differed between the two protocols. These results agree with previous researches who concluded that slow freezing appears to be the most crucial aspect of the ram semen preservation procedure (23, 24, 25). Our results agree with previous researches which reported that Freezing semen straws 4–5 cm above liquid nitrogen for 4–5 min resulted in satisfactory post-thawing quality (26, 27).

The decreasing of post-thawed semen quality after using slow freezing protocol in our experiment can be explained by the sever sperm dehydration. While, in rapid freezing rates acceptable sperm dehydration and intracellular ice crystals were obtained. Our explanation agrees

Table 1: Effects of using different freezing protocols on the degree of motility and kinetic parameters of post-thawed Najdi ram sperms

parameter	Freezing protocol	
	Rapid*	Slow#
Total motility (%)	65.07±1.09	64.10±1.06
Total progressive (%)	24.15±0.96	22.79±0.94
Fast progressive (%)	12.63±0.60[a]	10.66±0.59[b]
Slow progressive (%)	11.52±0.81	12.14±0.79
Curvilinear velocity (VCL; µm s−1)	50.75±0.34	50.05±0.32
Straight-line velocity (VSL; µm s−1)	30.05±0.29[a]	28.49±0.27[b]
Average pathway velocity VAP (µm s−1)	39.19±0.31[a]	37.84±0.29[b]
Lateral head displacement (ALH; µm)	2.49±0.01[b]	2.56±0.01[a]
Beat frequency (BCF; Hz)	3.67±0.02	3.64±0.02
Linearity (LIN=VSL/VCL) (%)	48.01±0.22[a]	46.98±0.20[b]
Straightness (STR=VSL/VAP) (%)	63.49±0.22	63.12±0.20
Wobble (WOB=VAP/VCL). (%)	70.23±0.15[a]	69.13±0.14[b]

a, b mean ± the standard error that carrying different superscripts in the same row differs at P < 0.05. *The straws were frozen at 5 cm over the surface of liquid nitrogen for 15 minutes. #The straws were frozen at 8 cm over the surface of liquid nitrogen for 20 minutes.

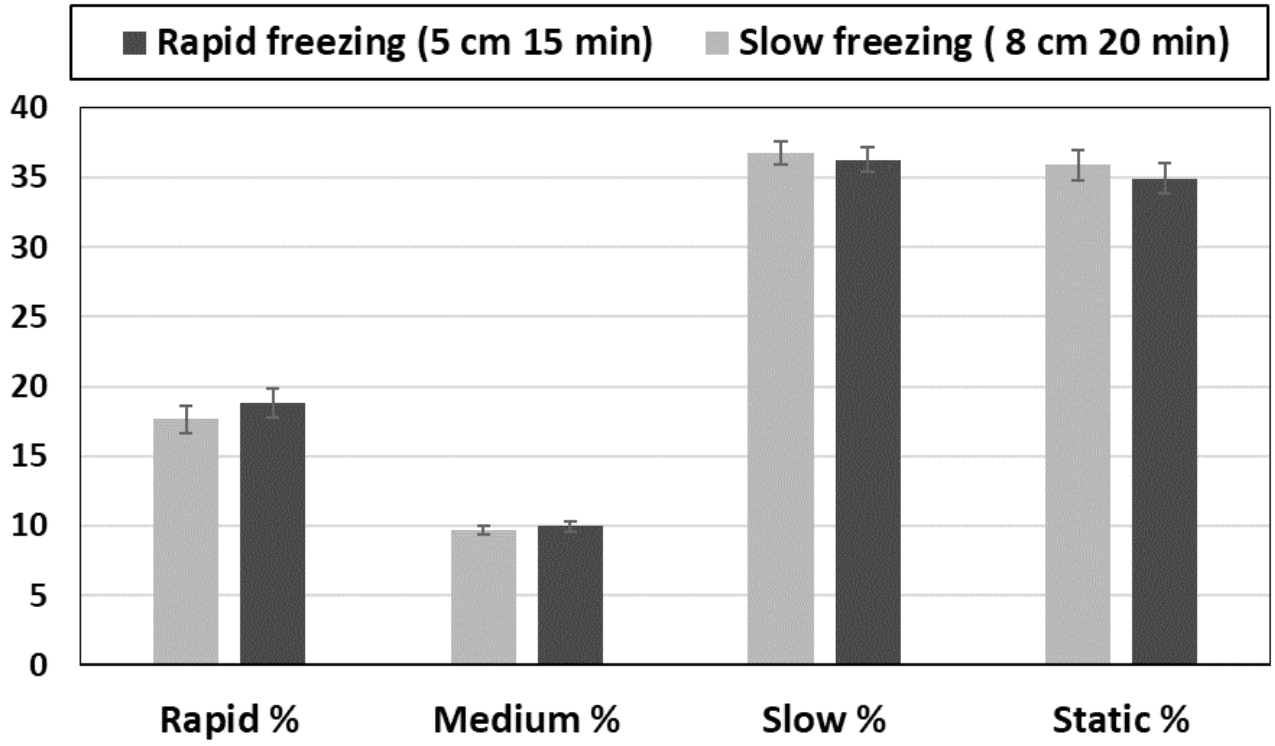


Figure 1: Effects of different freezing protocols on the percentages of the post-thawing velocity of Najdi ram sperm (mean ± standard error)

*The straws were frozen at 5 cm over the surface of liquid nitrogen for 15 minutes. #The straws were frozen at 8 cm over the surface of liquid nitrogen for 20 minutes. The sperms velocities were categorized to rapid (VCL >75 µm/s), medium (45 < VCL < 75 µm/s), slow (10 < VCL < 45 µm/s) or static (VCL < 10 µm/s).

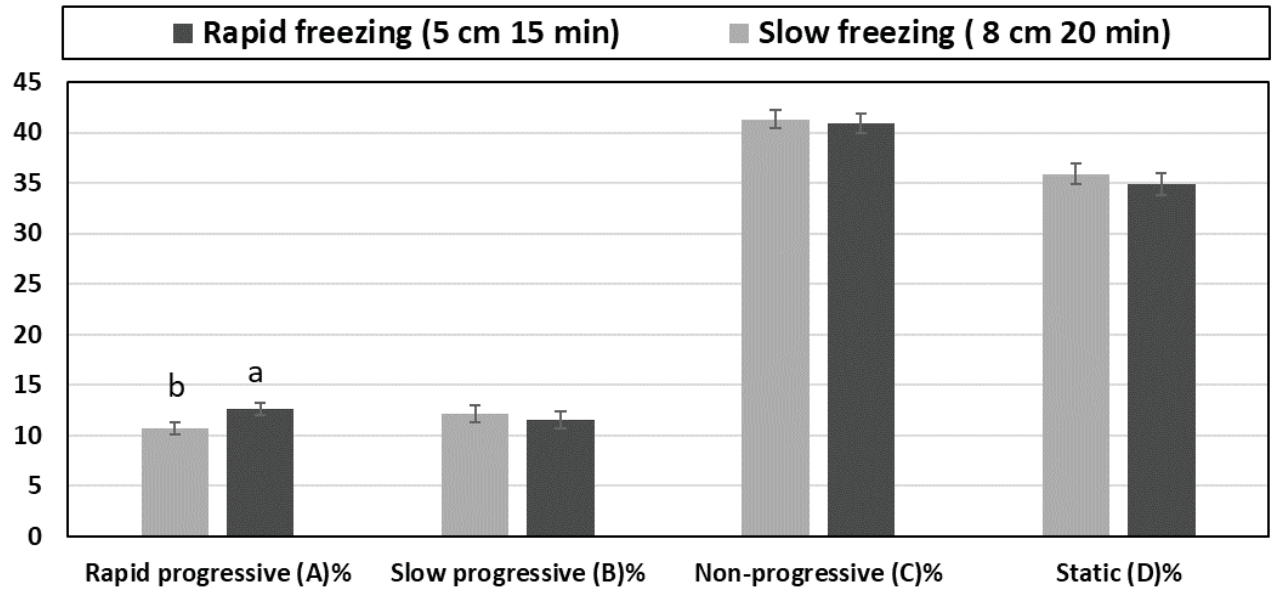


Figure 2: Effects of different freezing protocols on the percentages of the post-thawing motility grades (A, B, C, and D) of the Najdi ram sperms

a,b Means ± standard error carrying different superscripts within the same parameter differed at P<0.05. *The straws were frozen at 5 cm over the surface of liquid nitrogen for 15 minutes. #The straws were frozen at 8 cm over the surface of liquid nitrogen for 20 minutes. The motility results were categorized as rapid progressive (A), slow progres-sive (B), non-progressive (C) or static (D) according to WHO 4th edition.

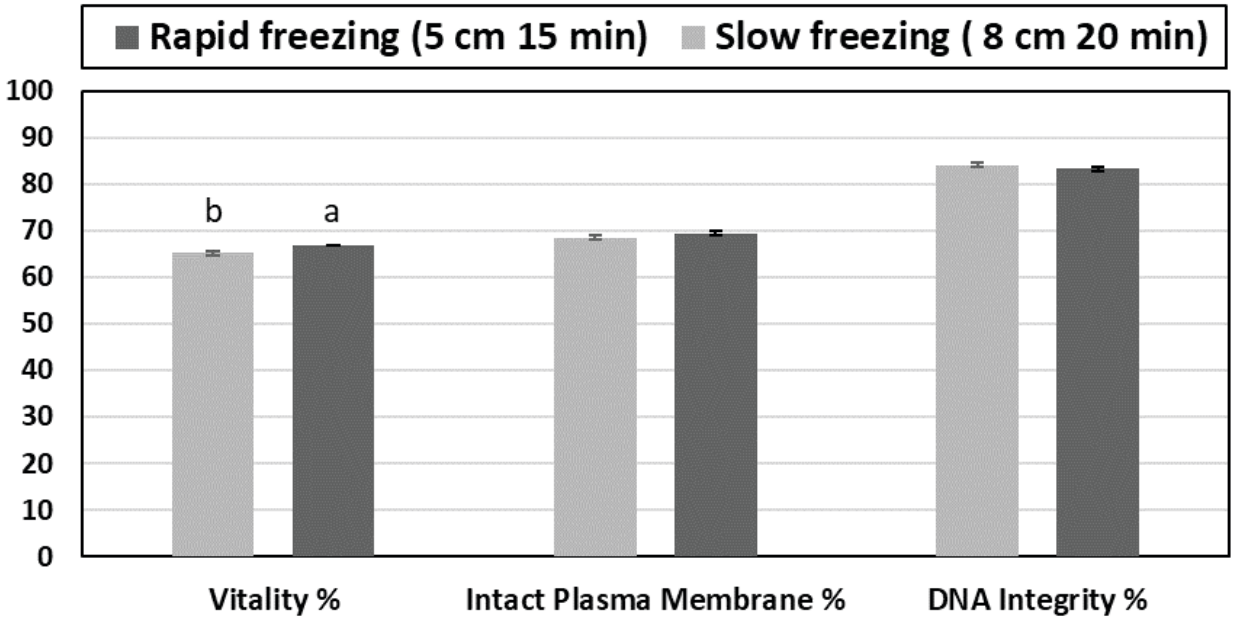


Figure 3: Effects of different freezing protocols on the percentages of post-thawing vitality, plasma membrane integrity and DNA integrity of Najdi ram sperms

a,b Means ± standard error carrying different superscripts within the same parameter differed at P<0.05. *The straws were frozen at 5 cm over the surface of liquid nitrogen for 15 minutes. #The straws were frozen at 8 cm over the surface of liquid nitrogen for 20 minutes.

Table 2: Effects of different freezing protocols on the percentage of sperm abnormalities after thawing and measurement of the morphology of the thawed Najdi ram spermatozoa

parameter	Freezing protocol	
	Rapid*	Slow#
Sperm Abnormalities (%)	16.80±0.37	16.09±0.37
Head length (µm)	10.79±0.44	10.91±0.44
Head width (µm)	5.12±0.12	4.94±0.12
Head area (µm²)	41.72±1.39	42.50±1.39
Head perimeter (µm)	28.72±0.90	29.36±0.90
Acrosome (%)	77.27±3.44	74.21±3.44
Elongation	0.34±0.02	0.37±0.02
Ellipticity	2.12±0.09	2.25±0.09
Regularity	1.04±0.02	1.00±0.02
Rugosity	0.66±0.02	0.64±0.02
Midpiece width (µm)	1.78±0.25	1.86±0.25
Midpiece area (µm²)	4.55±0.52	5.48±0.52
Distance (µm)	1.10±0.24	0.98±0.24
Angle (*)	20.45±5.55	20.52±5.55

[a,b] Means ± standard error carrying different superscript letters within the same row differed at $P < 0.05$. *The straws were frozen at 5 cm over the surface of liquid nitrogen for 15 minutes. #The straws were frozen at 8 cm over the surface of liquid nitrogen for 20 minutes.

with the explanation of previous research which reported that Rapid freezing leads to intracellular water crystallization, which might induce fewer cell damage than slow freezing, which produces severe dehydration (28).

In a manual freezing, the semen straws are placed on horizontal rack and frozen for 8–10 minutes in the vapor of liquid nitrogen at 4–6 cm above the level of liquid nitrogen (−75°C to −125°C). When the temperature drops below 5°C and nears to −10°C, the intracellular water freezes, putting sperms at risk of ice crystals formation.

Chemineau et al. (29) reported that 0.25 ml straws should be frozen 16 cm above liquid nitrogen for 2 minutes before being lowered to 4 cm for 3 minutes before being plunged into liquid nitrogen for storage. Pontbriand et al. (30) found that temperature variations of 6-24°C/min and 10-100°C/ min were acceptable, suggesting that ram sperm can tolerate a wide range of freezing rates. The temperature dropped at a controlled and programmed rate while utilizing an automatic freezing machine, from 4 to −5°C at 20°C/min, −5 to −110°C at 55°C/min, and −110 to −140°C at 35°C/min (31). Sperm cells are often frozen at a high rate (15–60°C/min), resulting in the best post-thawing results (31). Semen is slowly chilled at a rate of 0.1°C/min from water-bath temperature to 5° C and then frozen at a rate of 10–60° C/ min to temperatures as lower as −80°C before being stored in liquid nitrogen (32). Before plunging into liquid nitrogen, the final temperature should be reduced to at least −130° C, regardless of whether the freezing is done slowly or quickly, to stop all metabolic processes, including thermally driven chemical changes (33).

Post-thaw sperm motility, mitochondrial function, membrane integrity, and viability decreased after cryopreservation and thawing. Cryopreservation of ram sperm alters choline glycerophospholipids (34), (35). After cryopreservation, glycerophospholipids containing 22:6n-3 disappear (36). Cryopreservation impairs mitochondrial activity and damages mitochondria by removing two essential lipids (cardiolipin with 18:2n-6 and phosphatidylethanolamine with 20:4n-6) (36). After cryopreservation, sperm membrane lipids lose sterols, and sphingomyelin species with long-chain PUFA decrease (36). These lipid alterations influence most post-thawing parameters.

Conclusion

The two freezing protocols can be used to freeze ram semen. However, rapid freezing (5 cm for 15

minutes) was better than slow freezing (8 cm for 20 minutes) in ram semen cryopreservation. Beside saving time, rapid freezing protocol can improve the post-thawed ram sperm vitality, fast progressive motility, straight-line velocity, average pathway velocity and linearity.

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Original Research Article
Veterinary Medicine and The One Health Concept

THE EFFECTS OF DIETARY EUBIOTICS OR INTRAVENOUS AMINO ACID INFUSIONS ON NUTRIENT DIGESTIBILITY, RUMEN FERMENTATION, PERFORMANCE AND BLOOD PARAMETERS OF BUFFALO CALVES UNDER SUBTROPICAL CLIMATIC CONDITIONS

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Abstract: This study was conducted to compare the effects of dietary eubiotics or intravenous amino acid infusions (IVAAI) as two different growth promoters on nutrient digestibility, rumen fermentation, performance, and blood biochemical parameters of buffalo calves in subtropical climatic conditions. Thirty male buffalo calves (284.40 ± 18.45 kg) were randomly distributed into three groups and fed a basal diet (BD) of concentrate feed mixture and roughages. The first group was fed BD and considered as the control, the second group was fed the BD supplemented with eubiotics at 1.0 kg/ton of concentrate, whereas the third group was intravenously infused with amino acid (IVAAI) injection at a dose of 2.0 ml/100 kg body weight. Results showed that the total gain and the average daily gain were improved ($P < 0.05$) with dietary eubiotics. The digestibility of some nutrients was increased ($P < 0.05$) with dietary addition of eubiotics. In addition, eubiotics stabilize ($P < 0.05$) the rumen pH, which reduce the risk of subacute ruminal acidosis but increased ($P < 0.05$) ruminal NH3-N and total volatile fatty acids. The rectal temperature was decreased ($P < 0.05$) with eubiotics supplementation. In conclusion, the use of eubiotics induced superior positive effects on the digestibility of nutrients, rumen fermentation, rumen enzymes, rumen protein concentration, growth performance, feed conversion, blood parameters and ameliorated the harmful effects of thermal stress of buffalo calves in comparison with intravenous infusion of amino acids.

Key words: eubiotics; amino acids; buffalo calves; nutrient digestibility; rumen fermentation; growth performance

Introduction

Recently, there has been increasing worldwide legislation dedicated to limiting or banning the use of chemically synthesized antibiotics, which have been used for decades as growth promoters in animal production at sub-therapeutic doses under normal conditions; in order to prevent the emergence of drug resistance in human pathogens (1). This has accelerated the efforts of nutritionists to present natural, new, and safe antibiotic alternatives that are adequate for the animal production industry. Under stress conditions, banning or limiting the use of antibiotic growth promoters in animal feed would lead to highly reduced profits due to deteriorated intestinal barrier immunity and increased risk of pathogenic invasions (2).

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Dysfunction of the intestinal barrier causes intestinal epithelium failure and reduces the absorption of nutrients in animals. Various alternatives have been proposed, including probiotics, prebiotics, organic acids, exogenous enzymes, essential oils, amino acids, and vitamins to improve gut health and intestinal barriers (3, 4). Eubiosis means the correct balance of microflora in the intestinal tract, which is necessary for healthy gut performance. Supporting good animal gut health leads to reducing the need for antibiotics, ensuring effective utilization of natural resources, reducing the environmental pollution of farms, and improving farmers' incomes. eubiotic feed additives include five categories: organic acids, probiotics, prebiotics, phytogenics or essential oils, and gut health enzymes. All of these products positively contribute to microbiome modulation, leading to improved gut health. The combination of these different eubiotics would have a synergistic effect in improving animal performance (5).

Feeding of exogenous enzymes in the diet boosts the ruminal microbial enzyme activity, improves microbial adhesion to feed particles, and increases breakdown of dietary fiber (6). It has been demonstrated that amino acid infusion or ingestion is required to promote whole-body protein synthesis, minimize protein breakdown, and so elicit a positive net protein balance under heat-stressed conditions (7). Little work has gone into evaluating the feasibility of eubiotic combinations and amino acid supplementation in buffalos under heat-stress conditions. The use of combinations from these additives is hypothesized to show more pronounced benefits utilizing their cumulative and synergistic effects. Hence, the objective of this study was to evaluate the effects of dietary multicomponent eubiotics, or intravenous amino acids infusions (IVAAI) as two different growth promoters on nutrient digestibility, rumen fermentation, performance, and blood biochemical parameters of buffalo calves in subtropical climatic conditions.

Materials and methods

Animals, diets, and management

Thirty male buffalo calves that are clinically healthy, aged eleven to twelve months and weighing 284.40 ± 18.45 kg on average were used in this study. The animals were divided into three groups, each with ten animals. The experimental period was divided into two parts: a 15-day adaption period and a 110-day experimental period. The animals of each group were housed separately in pens, throughout the experiment, and were fed individually. The average ambient temperature inside the bunkers ranged between 36.0 to 45.0˚C and the relative humidity ranged between 60.5% to 65%. The temperature humidity index ranged from 75.33 to 83.01 on average. at 02:00 pm. The basal diet was prepared to meet the nutritional needs of growing calves according to NRC (8) recommendations. All the calves were fed a basal diet of 60% concentrate feed mixture (CFM) and 40% roughage (wheat straw (20 %) and Egyptian clover (20%)). The control group (1) received only the basal diet and no supplements. Group 2 were dietary supplemented with eubiotics at a rate of 1.0 kg/ton of concentrate mixture. The calves in group 3 were infused intravenously with amino acids (IVAAI) at a dose of 2.0 ml/100 kg body weight, through using jugular vein catheters with an automated peristaltic pump, and this dose was repeated biweekly. The chemical compositions of the experimental diet are presented in Table 1.

The multicomponent eubiotics consisted of: *Saccharomyces cerevisiae* ($20x10^{10}$ CFU), *Lactobacillus acidophilus* ($2x10^{9}$ CFU), *Lactobacillus plantarum* ($1.6x10^{9}$ CFU), *Lactobacillus casei* ($0.4x10^{8}$ CFU), total live bacteria ($2x10^{10}$ CFU), *Entrococcus faeciun, Bacillus licheniformis* ($6 x10^{9}$ CFU), ($4.0x10^{9}$ CFU), *Bacillus subtilis* ($6 x10^{9}$ CFU), lipase (2400 U), xylanase (1200 U), phytase (2400 U), cellulase (2400 U), amylase (20000 U), pectinase 400 U, β-gluconase (1000 U), protease (40000 U), fructo oligosaccharides (10 g), mannan oligosaccharides (10 g), calcium propionate (24 g), copper penta sulphate (10 g). The second growth promoter is an intravenous amino acid supplement, which contains 13 amino acids and vitamins. Each 100 ml of IVAAI contains Arginine 144 mg, Cysteine 320mg, Glycine 320mg, Glutamine 320mg, Histidine 132mg, Isoleucine 360mg, Leucine 428mg, Lysine 544mg, Methionine 320mg, Threonine 320mg, Tryptophan 86mg, Phenylalanine 500mg, Valine 360mg, Vitamin B1 400mg, Vitamin B2 17mg, Vitamin B6 34mg, Nicotinamide 800 mg.

Table 1: Chemical composition of the experimental diet (% on dry matter basis)

Items	CFM*	Egyptian clover	Wheat straw
Dry matter (DM)	89.40	18.43	91.2
Organic matter (OM)	94.70	93.78	92.7
Crude protein (CP)	15.22	18.78	3.55
Crude fiber (CF)	12.57	21.45	36.40
Ether extract (EE)	2.47	4.03	1.76
Nitrogen free extract (NFE)	69.74	55.74	58.29
Ash	5.30	6.22	7.30

*CFM, concentrate feed mixture. The concentrate feed mixture (CFM) consists of: 50% corn, 20 % wheat bran, 20% undecorticated cotton seed meal, 8 % soybean meal, 1% limestone, 0.5 % salt and 0.5% mineral -vitamin premix

To determine the animals' daily feed intake, the feed was daily offered to the animals, and the refusals were collected and weighed. The animals were weighed at the beginning and end of the experiment and biweekly throughout the experiment. By dividing the feed intake by the weight growth, the feed conversion ratio was obtained. Fresh water was available *ad libitum*. The calves were dewormed with an anthelminthic injection before the start of the trial and clinically examined to be sure that they are healthy.

Blood sampling and analysis

Every month, blood samples were collected from each calf's jugular vein 6 hours after the morning feeding. Then the blood samples were centrifuged at 3000 rpm for 15 minutes to separate the serum. The serum was collected and kept at 20°C until chemical analysis. Serum glucose, total protein, albumin, globulin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea, creatinine, and total cholesterol were tested colorimetrically using commercial kits and a spectrophotometer (Hitachi 911 automated analyzer) (Spinreact, Spain), and the analyses were performed according to the manufacturer's company.

Thermoregulatory responses

The number of flank movements per minute (breath/min) was used to calculate the respiratory rate (RR). Pulse rate (PR) was recorded using pulse oximeter apparatus (CMS60D- VET Handheld Veterinary Pulse Oximeter). A clinical thermometer was gently inserted into the rectum for one minute to determine rectal temperature (RT, °C). A portable infrared thermometer intended for temperature measurements was used to measure skin temperature (ST, °C), hair temperature (HT, °C), and ear temperature (ET, °C). All measurements were done at 2.00 pm every two weeks during the experimental period. The pulse rate and respiration rate were counted before measuring the body temperature. Air temperature and humidity were recorded during the experimental period using temperature / humidity thermometer at two pm. Temperature Humidity Index (THI) was estimated according to Mader et al. (9).

Digestibility trials

The nutrient digestibility of the experimental diets was determined at the end of the feeding trial using chromic oxide as an external marker in three digestibility trials. Each digestibility trial for each diet lasted for 14 days, with the first 7 days serving as an adaptation period, followed by 7 days of data collection. Each calf got exactly 10 g of powdered Cr2O3 on the first day of the preparatory period, which was manually mixed with the concentrate mixture. For a chemical analysis, daily feed samples were collected, mixed, dried, and ground through a 1 mm sieve screen. In addition, from day 8 to day 14, about 200 g of fresh feces were gathered twice daily by fecal grabbing and kept in a refrigerator.

The fecal samples from each animal were collected at the end of digestibility trial, dried at 60°C, and ground through a 1 mm mesh screen for chemical analysis. Using AOAC (10) methodologies, chemical analysis of feeds and excrement was

carried out. Goering and Van Soest (11) methods were utilized (ADL) to identify neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin. The nutrient composition of feces and feed from chromium was estimated using atomic absorption spectrophotometry (12). The nutrient digestibility was calculated using Maynard and Loosli (13) equations:

Digestibility of nutrient (%) = 100–(100 x % marker in feed / % marker in feces x % nutrient in feces / % nutrient in feed).

Rumen liquor parameters

Using a stomach tube, samples of rumen contents were obtained from each calf at the end of the digestibility trails, on the two successive days next to the collection period. Samples were taken immediately prior to feeding time, then three and six hours later, samples were filtered through four layers of cheesecloth to determine the pH using a digital pH meter (Beckman, Model 45, USA) and ammonia N content using the method of Conway (14). Furthermore, 0.8 mL of ruminal liquor filtrate was combined with 0.2 mL of a solution containing 250 g of metaphosphoric acid/L to halt microbial activity before being stored at −20 °C for volatile fatty acid (VFAs) measurement. The total volatile fatty acids were determined by the steam distillation method according to methodology of Warner (15).

Rumen enzyme activities

A subsample of 5 mL from the whole samples, collected at 3 h after the morning feeding of each calf, was preserved by the addition of a few drops of saturated mercuric chloride solution to inhibit microbial activity, and then refrigerated at -20 °C for rumen enzyme activities measurement. The enzyme activity in rumen fluid was determined using spectrophotometry (Unico, USA). The activities were determined following previously published protocols: cellulose and alpha-amylase were estimated according to Miller (16), lipase was determined according to Peled and Krenz (17), protease was measured according to Folin and Ciocalteu (18), and urease was measured according to Weatherburn (19). According to Lowry et al.(20) the quantities of extracellular protein in the crude enzyme were measured

Statistical analyses

The general linear model (G.L.M) of the SAS (21) program was used for statistical analysis. One-way ANOVA was used to assess the effect of treatments on feed intake, growth rate, feed conversion ratio, blood parameters, nitrogen retention, nutritional digestibility, feeding value, and rumen enzyme activities. Data on rumen liquid parameters were analyzed using SAS's procedure, which used time as a repeated measure and each animal as

Table 2: Effect of dietary eubiotic supplementation or intravenous infusion of AA on growth performance of buffalo calves

Items	Treatment			P-value
	Control	Eubiotics	IVAAI	
Initial weight (kg(	284.20 ± 19.51	284.60 ± 18.60	284.40 ±17.24	0.999
Final weight (kg(	376.80 ± 17.95	394.00 ± 17.71	383.00 ± 14.41	0.768
BW gain (kg)	92.60^{b} ± 3.54	109.40^{a} ± 2.52	98.60^{b} ± 3.68	0.011
Average daily gain (g)	841.82^{b} ± 38.22	994.54^{a}± 22.93	896.36^{b} ± 33.48	0.011
Feed intake (kg/d)				
DMI of concentrate	7.01 ± 0.21	7.22 ± 0.24	7.21± 0.22	0.756
DMI of roughages	3.22 ± 0.07	3.18 ± 0.04	3.13 ± 0.04	0.447
Total DM intake	10.22 ± 0.27	10.40 ± 0.25	10.33 ±0.25	0.886
Feed conversion ratio g DM/g gain	12.15^{a} ± 0.32	10.46^{b} ± 0.25	11.53^{a} ± 0.28	0.001

$^{a, b}$ *Means within the same row carrying different superscripts are significantly different at ((P<0.05).* IVAAI, intra venous amino acid infusions.

the experimental unit. Treatment, time, and the treatment x time interaction were all included in the model. The Duncan multiple range test (22) was used to evaluate the impact of treatments on parameters that were investigated. The results are shown as means and standard error (SE). Significant P values were defined as those less than 0.05 (P < 0.05).

Results

Growth performance

Calves fed the diet containing eubiotics gained more total body weight and daily weight gain than those fed IVAAI and control diets (Table 2). The feed intake of concentrate and roughages as well as the total DM intake were not significantly (P > 0.05) differed between groups. The feed conversion ratio was considerably (*P* < 0.05) improved in eubiotics group in comparison to the IVAAI and control groups (10.46 vs. 11.53 and 12.15 g DM/g gain, respectively).

Blood constituents

The results of blood metabolite analysis are shown in Table 3. The IVAAI increased (P < 0.05) the serum cholesterol concentration and decreased (P < 0.05) the urea-N and glucose concentrations as compared with other groups. However, there were no significant (P > 0.05) variations in other blood constituents among the treatments.

Table 3: Effect of dietary eubiotic supplementation or intravenous infusion of AA on blood serum constituents of growing buffalo calves.

Items	Treatment			P-value
	Control	IVAA		
Total protein, g/dl	7.56 ± 0.17	7.31 ± 0.21	7.34 ±0.15	0.581
Albumin (g/dl)	4.07 ± 0.17	3.86 ± 0.12	4.22 ± 0.11	0.207
Globulin (g/ dl)	3.41 ± 0.15	3.13 ± 0.10	3.04 ± 0.19	0.224
A/G ratio	1.22 ± 0.09	1.25± 0.05	1.46 ± 0.15	0.260
AST (U/l)	65.44 ± 1.37	65.91± 1.57	65.68 ± 1.67	0.830
ALT (U/l)	14.11 ± 0.48	14.22 ± 0.36	14.67± 0.44	0.635
Cholesterol, mg/dl	179.78^{b} ± 12.37	181.89^{b} ± 16.38	252.33^{a} ± 13.11	0.001
Urea-N (mg/dl)	11.67^{a} ± 0.51	11.70^{a} ± 0.73	9.30^{b} ±0.94	0.001
Creatinine, mg/dl	1.37 ± 0.07	1.39 ± 0.05	1.28 ± 0.04	0.388
Glucose, mg/dl	101.20^{a} ± 6.49	97.07^{a} ± 10.90	78.27^{b} ± 7.40	0.001

[a, b] Means within the same row carrying different superscripts are significantly different at ((P<0.05). IVAAI, intra venous amino acid infusions.

Table 4: Effect of dietary eubiotic supplementation or intravenous infusion of AA on thermoregulatory responses

	Treatment			P-value
Parameter	Control	Eubiotics	IVAAI	
RT (ºC)	39.06^{b} ± 0.08	38.82^{c} ± 0.10	39.37^{a} ± 0.06	0.021
ST (ºC)	37.22 ± 0.81	37.13 ± 0.56	37.43 ± 0.45	0.621
ET (ºC)	35.44± 0.87	35.38 ± 0.65	35.29 ± 0.87	0.835
RR (breath/min)	37.27 ± 0.45	37.77 ± 0.40	36.88 ± 0.40	0.354
PR (beat/min.)	65.56^{a} ± 1.45	64.22^{b} ± 1.50	64.12^{b} ± 1.51	0.002

[a,b,c] Means within the same row carrying different superscripts are significantly different at (P < 0.05). RT, Rectal temperature ; ST, Skin temperature; ET , Ear temperature ; RR, Respiration rate; PR, Pulse rate IVAAI, intra venous amino acid infusions.

Table 5: Effect of dietary eubiotic supplementation or intravenous infusion of AA on nutrients digestibility and nutritive value of experimental rations (%)

Item, %	Treatment			P-Value
	Control	Eubiotics	IVAAI	
Nutrient digestibility				
Dry matter, DM	63.66^{c} ± 0.04	65.97^{a} ± 0.41	65.03^{b} ± 0.21	0.002
Organic matter, OM	63.62^{b} ± 0.14	64.00^{ab} ± 0.28	64.88^{a} ± 0.31	0.034
Crude protein, CP	76.55 ± 0.56	77.52 ± 0.12	77.52 ± 0.40	0.226
Crude fiber, CF	55.75^{b} ± 0.30	64.79^{a} ± 0.98	57.45^{b} ± 0.42	0.001
Ether extract, EE	73.67^{b} ± 1.76	81.86^{a} ± 1.87	77.52^{ab} ± 0.64	0.018
Nitrogen free extract, NFE	62.50^{b} ± 0.78	64.26^{a} ± 0.20	64.56^{a}± 0.50	0.049
NDF	58.19^{c} ± 0.58	66.67^{a} ± 0.35	62.41^{b} ± 0.46	0.001
ADF	42.52^{c}± 1.58	49.26^{a} ± 0.45	46.14^{b} ± 0.87	0.001
Cellulose	37.29^{b} ± 1.76	43.43^{a} ± 0.69	40.46^{ab} ± 1.14	0.039
Hemicellulose	15.67 ± 1.62	17.41 ± 0.70	16.28 ± 1.11	0.609
ADL	5.23 ± 3.67	5.83 ± 0.24	5.68 ± 0.34	0.597
Nutritive value				
TDN	65.41 ± 0.32	65.73 ± 0.23	66.06 ± 0.25	0.255
SV	63.57^{b} ± 0.26	64.10^{b} ± 0.16	64.76^{a} ± 0.17	0.001
DCP	11.65^{b} ± 0.06	11.80^{a} ± 0.04	11.80^{a} ± 0.04	0.050

^{a,b} Means within the same row carrying different superscripts are significantly different at (($p<0.05$). NDF, Neutral detergent fiber; ADF, Acid detergent fiber; ADL, Acid detergent lignin; TDN, total digestible nutrient; SV, Starch value; DCP, digestible crude protein; IVAAI, intra venous amino acid infusions

Table 6: Effect of dietary eubiotic supplementation or intravenous infusion of AA on rumen parameters

Item	Treatment	Hours after feeding			Means of treatment	P- value	
		zero	3 hours	6 hours		Treat. effect	Treat. X time
pH	Control	6.53± 0.05	6.36^{B} ± 0.07	6.14 ± 0.02	6.36B ± 0.07	0.001	0.001
	Eubiotics	6.54 ± 0.04	6.87± 0.01	6.74 ± 0.03	6.72^{A} ± 0.05		
	IVAAI	6.56 ± 0.07	6.19 ± 0.1	6.29 ± 0.05	6.35^{B} ± 0.06		
Means of time		6.54 ± 0.03	6.48 ± 0.11	6.39± 0.09			
P- value (time)		0.46					
NH3-N, mg/100ml	Control	14.88 ± 0.07	23.23 ± 0.18	20.11 ± 0.57	19.41C± 0.68	0.001	0.055
	Eubiotics	15.08 ± 0.14	25.37 ± 0.0.23	19.09 ± 0.27	19.84^{B}± 0.84		
	IVAAI	15.26 ± 0.10	25.21 ± 0.21	20.18 ± 0.15	20.21^{A}± 0.80		
Means of time		15.07^{c}± 0.07	24.60^{a}± 0.22	19.79^{b}± 0.15			
P- value (time)		0.001					
TVFAs, meq/100ml	Control	8.78 ± 0.05	8.77 ± 0.12	8.77 ± 0.12	9.23C ± 0.14	0.001	0.376
	Eubiotics	9.40 ± 0.08	10.42 ± 0.10	9.49 ± 0.07	9.77A ± 0.10		
	IVAAI	8.98 ± 0.13	10.23 ± 0.18	9.06 ± 0.10	9.42B ± 0.13		
Means of time		9.05C ± 0.29	9.10^{b} ± 0.08	9.10b ± 0.08			
P- value (time)		0.001					

^{a,b,c} Means within the same row carrying different superscripts are significantly different at P (($p<0.05$). ^{A,B,C} Means within the same column (within each parameter) different superscripts differ significantly ($p<0.05$). IVAAI, intra venous amino acid infusions.

Table 7: Effect of dietary eubiotic supplementation or intravenous infusion of AA on ruminal enzymes activity

Items	Treatments			P-value
	Control	Eubiotics	IVAAI	
α-amylase activity (µg glucose/min/ml)	$4.73^{b} \pm 0.13$	$5.71^{a} \pm 0.08$	$4.88^{b} \pm 0.05$	0.001
Cellulase activity (µg glucose/min/ml)	$3.03^{b} \pm 0.08$	$4.46^{a} \pm 0.01$	$3.12^{b} \pm 0.08$	0.001
Lipase activity (µg p- nitrophenol/min/ml)	$4.73^{b} \pm 0.14$	$7.29^{a} \pm 0.22$	$5.22^{b} \pm 0.20$	0.001
Urase activity (µg NH_3/min/ml)	$34.47^{c} \pm 0.58$	$53.20^{a} \pm 2.83$	$42.04^{b} \pm 0.71$	0.001
Protease activity (µmol of tyrosine/min/ml)	$3.84^{b} \pm 0.24$	$4.68^{a} \pm 0.21$	$4.64^{a} \pm 0.21$	0.020
Rumen protein concentration (mg/ml)	$2.54^{b} \pm 0.09$	$3.11^{a} \pm 0.14$	$2.80^{ab} \pm 0.11$	0.006

^{a,b,c} Means within the same row carrying different superscripts are significantly different at (($p<0.05$). IVAAI, intra venous amino acid infusions

Thermoregulatory responses:

The rectal thermoregulatory responses are presented in Table 4. It was found that the rectal temperature of growing calves was increased ($P < 0.05$) with the infusion of IVAAI, and decreased ($P < 0.05$) with supplementation of dietary eubiotics in comparison with the control group but they were still within the normal range of body temperatures. The pulse rate was lower ($P < 0.05$) in all treatment groups than in the control. However, the skin temperature, ear temperature, and respiration rate were not significantly affected among the groups.

Nutrient digestibility and nutritive value

Dietary supplementation of eubiotics as a source of a microbial feed additive to the diets of growing calves increased ($P < 0.05$) the DM, CF, NDF, and ADF digestibility rates in comparison with IVAAI and control groups (Table 5). In addition, the digestibility of EE, NFE, and cellulose was significantly ($P < 0.05$) higher in the eubiotic group than in the control one. However, no significant ($P > 0.05$) differences were found between the eubiotic group and IVAAI group. Moreover, the intravenous (I/V) infusions of AA to growing calves improved ($P < 0.05$) the OM digestibility when compared to the control one. In addition, the feeding values including the starch value (SV) and digestible crude protein (DCP) were increased ($p < 0.05$) in treatment groups, when compared to the control group.

Rumen fermentation activities

Supplementation of eubiotics significantly increased ($P < 0.05$) the pH values in comparison to the control and IVAAI treated groups (Table 6). However, the concentrations of ruminal NH3-N and total short chain fatty acids (VFAs) were higher ($P < 0.05$) with the inclusion of eubiotics or I/V infusion of IVAAI to growing calves than the control one. The concentrations of total short-chain volatile fatty acids of calves that received dietary eubiotics were higher ($p < 0.05$) than in other groups. In terms of how sampling time affected rumen fluid parameters, the mean pH values were higher ($p < 0.05$) before feeding time and, then decreased at 3 and 6 hours after feeding. However, the concentrations of NH3-N and total VFAs decreased before to feeding, increased following feeding to reach their peak at 3 hours post feeding, and then began to decline once more at 6 hours post feeding. The interactions between the treatments and the time effect for ruminal pH and NH3-N concentration were significant ($P < 0.05$). The interaction between treatment and the time effects on total VFAs is not statistically significant ($P > 0.05$).

Rumen enzymatic activities

Regarding the effect of treatments on ruminal enzymatic activity, we found that ruminal α-amylase, cellulose, lipase and urase activities and rumen protein concentrations were significantly ($P < 0.05$) increased in eubiotic group when compared

to IVAAI and control groups (Table 7). Moreover, the protease activity was improved ($p < 0.05$) in both treatment groups when compared with the control one.

Discussion

Growth performance

In comparison to the control and IVAAI, intra venous amino acid infusions. groups, eubiotic supplementation in calves' diet enhanced the body weight and average daily gain by 18.14 and 10.95 %, respectively. This improvement might be due to increased nutrient digestibility in the eubiotic diet. This impact was found to be beneficial in the current study when calves were fed a fibrous diet (40% wheat straw and Egyptian clover) that was predicted to ferment and create a few amounts of lactobacili in the rumen. Furthermore, the product comprises two Bacillus bacteria strains (*B. subtilis* and *B. licheniformis*), both of which can suppress or create antimicrobials in the gastrointestinal tract and maintain gut health and improve the animal's performance (3, 23). In this context, the inclusion of exogenous enzymes in the diet of lambs enhanced the daily weight gain because of higher N intake and retention, as well as fiber digestion (24). The average daily gain of IVAAI treated growing calves was slightly higher than that of the control group. The numerical increase in body weight in AA supplemented group might be due to the better availability of infusing amino acids into the blood for metabolism and absorption by 100% and utilization sites by the various tissues in the body, resulting in better performance (25). The results reported by Kassube et al. (26), who suggested that infusing essential amino acids into cows exposed to the heat stress environment improved whole-body protein synthesis, confirm our findings. Similarly, supplementation of metabolizable amino acids to the finishing calves improved the performance and feed efficiency (27, 28). The addition of dietary eubiotics or IVAAI to calves had no impact on their intakes from concentrate, roughage, or total DM. This result confirms the finding of previous studies who stated that infused methionine, lysine, and branched-chain over needs (i.e. 135% of requirements) decreased dry matter intake and milk

Blood constituents

Except for cholesterol, urea nitrogen, and glucose, treatments did not affect the blood parameters examined in this study. The higher cholesterol concentration with infused amino acids in growing calves could be ascribed to decreased lipid metabolism in this group in comparison with the control group (30). The decreased blood urea nitrogen in the IVAA group may be due to lower amino acid deamination and improved AA absorption and utilization efficiency for tissue growth (31). The reduced serum glucose levels in the IVAA group could imply glucose elimination in peripheral tissues (e.g. muscle or adipose) (32). Similarly, infusions of methionine, lysine, and branched-chain AA decreased (P 0.01) plasma glucose levels, glutamate concentrations, plasma alanine, and aspartate compared to the control group (26). This suggests that glucose was absorbed in the small intestine and had an impact on the calves' growth.

Thermoregulatory responses

One of the strategies of supplementing eubiotics or infusing amino acids to calves in our study is to ameliorate the effects of thermal stress. The calves in this study were subjected to heat stress, and the addition of dietary microbial additives had only small impacts on rectal temperature and pulse rate. Supplementing heat-stressed dairy cows with a mixture of exogenous enzymes and yeast cultures decreased rectal temperature, suggesting a role in thermoregulatory processes (33, 34). Exogenous enzymes in eubiotics improve dry matter intake, nutrient digestibility, energy-use efficiency, water absorption, and the intestinal permeability and consequently could reduce the metabolic heat load coming rumen fermentation and reduce heat stress as clarified in previous study (35). Animals receiving feed additives/ supplements had lower rectal temperature (RT), respiratory rate (RR), and pulse rate (PR), because of a decrease in cortisol, indicating increased thermotolerance and performance (33, 34(. In addition, animals in the control group were unable to dissipate heat efficiently due to the high-temperature humidity index, resulting in increasing in rectal temperature. The increase in rectal temperature with an infusion of amino

acids could be related to protein and amino acid metabolism, which produces more heat as compared to fat and carbohydrate metabolism (36).

Nutrient digestibility

The majority of nutrients' digestibility rates were increased by the dietary addition of eubiotic to growing calves, especially crude fiber and its fractions, which may be related to its role in the establishment a healthy ruminal microflora and maintaining a ruminal pH to be more appropriate for ruminal digestion (37). The eubiotic contain multi-enzymes like cellulases, xylanase, and pectinase that have been found to increase fiber digestibility compared to components containing a single enzyme (38). Such additions enhanced the bacterial adhesion, stimulated the rumen microbiota, and interacted with other ruminal microorganisms. Eubiotic decreased the digesta viscosity and creates anaerobic conditions for cellulolytic bacteria, as well as provides vital nutrients for microbial activity and growth in the rumen (3, 37). Our findings are consistent with those of Sallam et al. (24), who found that adding eubiotics to sheep diets enhanced NDF and ADF digestibility by roughly 10% and 7.9%, respectively. The improvement in OM, NDF and ADF in IVAAI supplemented calves in comparison with the control group coincided with the results of many previous studies who found that supplementing protected proteins and amino acids to ruminant animals fed poor quality forages enhanced feed intake and nutrient digestibility (39, 40).

Rumen fermentation activities

The purpose of eubiotic supplementation in the diets of calves is based mainly on its positive effects on rumen fermentation. The components of eubiotics are bacteria, yeast, and exogenous enzymes are thought to have the potential to maintain ruminal pH and VFA, particularly lactate (3, 37). The rumen pH was increased when the eubiotics were added to the diet. This could be because probiotics stabilize rumen-dominant bacteria that consume more ruminal lactate, and consequently stabilize rumen pH at the range of (6.6 – 6.8) (41). The main benefits of probiotics for ruminants were improved ruminal digestion by increasing rumen pH (42), fiber digestion (43), and the production of microbial proteins (44). Probiotics decrease the concentration of rumen organic acids and may decrease the risk of SARA (subacute ruminal acidosis) (45). The higher ammonia nitrogen concentration in the rumen fluid of growing calves fed a diet containing eubiotics or intravenously infused with IVAAI may be attributed to the conversion of peptides and amino acids to ammonia by microbial activity. The energy needed for microbial protein synthesis is insufficient, and not all ammonia is converted into protein lead to elevation of rumen ammonia nitrogen (46). On the other side, the intravenous infusions of AA to growing calves improved the ruminal fermentation and production of VFA. Intravenous supplementation with amino acids could alter N recycling and thus potentially affect rumen microbial fermentation especially when the diet was deficient in RDP (47). These findings were agreed with those of Russell et al. (48) who found that adding free amino acids to the rumen ecosystem could be an important source of nitrogen and improves ruminal fermentation.

Rumen enzyme activities

The efficiency of the enzyme system found in the gastrointestinal tract determines how well animals digest and utilize nutrients present in feeds (49). Heat stress could affect the rumen microbial composition and metabolism (50). However, it was shown that adding microbial feed additives to the heat-stressed calves improved enzyme activity. This improvement in the enzyme activity might be attributed to an increase in the rumen's hydrolytic capability, owing to enhanced bacterial attachment, rumen microbial population stimulation, and synergistic actions with ruminal microorganism hydrolases (24, 51), since these enzymes are secreted by microbes in the GI tract (48). Eubiotics contain enzymes such as cellulases, xylanase, and pectinase, has been shown to improve microbial activity and rumen enzyme activity. These results could indicate a change in the colonizing bacteria's species profile because of the pre-feeding enzyme treatment of the feeds (6). Also, increases cellulolytic activity with enzyme treatment *in vivo* (52).

Conclusion

From the current study's findings, it could be concluded that both dietary supplementation of

eubiotics and intravenous amino acid infusions (IVAAI) improved the growth of buffalo calves through improving the digestibility rate of nutrients (particularly of cell wall constituents), rumen fermentation, and rumen enzyme activities. However, the addition of dietary eubiotics induced superior positive effects than IVAAI on all studied parameters, and ameliorated the harmful effects of thermal stress in growing buffalo calves.

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The authors report no potential conflicts of interest.

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Original Research Article
Veterinary Medicine and The One Health Concept

AN ALTERNATIVE ANTIVIRAL THERAPY OF NEWCASTLE DISEASE IN BROILER CHICKENS: A CLINICAL STUDY OF METHANOLIC *NEEM* LEAVES EXTRACT

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Abstract: Newcastle disease (ND) is an important viral disease that threatens the global poultry industry. The urgent need for a safe and effective antiviral alternative prompted us to evaluate neem methanolic leaf extract against Newcastle disease virus (NDv) in broilers. A total of Sixty Cobb chicks (day old) were allocated randomly into 4 equal groups; group A was a control negative, and group B was a control positive. Groups C and D received a methanolic neem leaf extract (MNLE) in drinking water for five days at 500 and 1000 µg/kg BW, respectively. At 25th days old, chicks in groups B, C and D were challenged via the oculonasal route with 0.2 ml of virulent NDv. The use of MNLE in groups C and D significantly reduced morbidity and mortality as well as the macroscopic and microscopic scoring lesions of all examined organs compared to the infected untreated group B. The high dose of MNLE (1000 µg/ kg BW; group D) was more efficient and significant in relieving the clinical and pathological abnormalities caused by ND challenge compared with the low dose (500 µg/ kg BW; group C).Moreover, positive effects of the herbal treatment on the experimentally ND-infected chickens were evidenced by reducing viral RNA concentrations in the oropharyngeal swabs at 3, 5, and 7 days post challenge and the infectivity titer of the virus isolated from the trachea, spleen, and cecal tonsils at 7 days post challenge. In conclusion, the study showed that MNLE had a positive and helpful role in the control of NDv infection and shedding. So, MNLE could be a source of inspiration for new alternative antivirals.

Key words: Newcastle disease; broilers, *Azadirachta Indica*; *Neem*; methanolic extract; antiviral

Introduction

Newcastle disease (ND) is a highly contagious viral disease of poultry affecting the respiratory, gastrointestinal, nervous, and reproductive systems. It leads to serious health consequences such as high morbidity and mortality, increased veterinary management costs, and long-term restrictions on international trade (1-2). Although ND was initially identified in 1926 as caused by avian paramyxovirus type 1, it continues to spread worldwide in both endemic and epidemic forms (3). There have been four defined panzootic ND, and the existence of a fifth panzootic was predicted to cause uncontrollable severe ND outbreaks by the current preventive strategies due to the rapid spread of new velogenic NDv VII sub-genotypes through Asia and the Middle East that threaten the poultry industry. Vaccination with live and inactivated NDv vaccines is the only commercial control measure, which does not always give complete protection to birds (4-5). As the world changes toward using safe herbs with historic medical properties, several screening

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studies and in vitro tests have been conducted to determine how well different herbs and their extracts fight viruses, especially NDv. Medicinal plants are antiviral alternatives, such as *Momordica balamina*, *Adansonia digitata*, *Artemisia annua*, *Azadirachta indica*, *Psidium guajava*, Moringa *oleifera* (6).

Azadirachta indica (Neem), a Meliaceae member, was used extensively in all Ayurvedic, Unani, and Chinese medicine to cure and prevent diseases because of being a rich source of antioxidants and other wide variety of bioactive compounds (7). This plant's leaves hold a variety of constituents, such as nimbin and its derivatives (nimbinene, 6-desacetyl-N-methylnimbinene, nimbandiol, nimbolide), ascorbic acid, and amino acids (as n-hexacosanol and 17-hydroxyazadiradione). Polyphenolic flavonoids, such as quercetin, and ß-sitosterol purified from neem leaves, have been shown by in vitro tests to have effective antiviral, antibacterial, and antifungal activities (8-9).

Antiviral activity of variable neem extracts has been previously documented against hepatitis C, human immunodeficiency virus, herpes, Dengue, Japanese encephalitis virus, coronaviruses, NDv, infectious bursal disease virus, and influenza viruses (10-16). Where several extractives of each of the neem parts as leaves, fruits, and bark, become known to have Invitro and Invivo antiviral activities against NDv and others in the culture of VERO cells and chicken embryos via virus inactivation and yield reduction besides interfering with their replication (7, 16). According to Helmy (16), the NDv replication in VERO cells and chicken embryos was fully inhibited with the methanol extract of neem leaves at concentrations of 3- 4 µg /ml and 4 µg /egg, respectively with the absence of cytotoxicity. The need for a specialized antiviral appears urgent with the frequent NDv epidemics facing poultry. This prompted us to apply a clinical evaluation of the methanolic neem leaves extract against Newcastle disease virus in broilers as an effective antiviral alternative from nature.

Material and methods

Preparation and analysis of methanolic Azadirachta Indica extract

Neem (*Azadirachta Indica*) leaves were collected from the faculty of Agriculture at Moshtohor, Benha University. Neem leaves dried under shade and ground efficiently to be a fine powder. Soak dried neem powder at room temperature for 24 hours in HPLC-grade methanol (80%) at a ratio of 1:5 and the mixture filtered. The obtained filtrate was concentrated at 45° C under a vacuum using a rotary evaporator. The obtained methanol neem leaves extract (MNLE) was emulsified in 20% dimethyl sulfoxide for the subsequent studies (17). Total phenolic and flavonoid contents were determined in the obtained extract that expressed as equivalents to gallic acid (GAE) and quercetin (QE), respectively. In addition, the antioxidant activities were assayed using ABTS and DPPH radical scavenging tests (18-19).

Challenge Virus

Virulent NDv isolate (NDV/CH/EG-Q/11/2018) belonged to class II (genotype VII) with accession No MN137991. The virus was kindly provided in the form of infectious allantoic fluid from our colleague Asmaa Desouky, Department of Avian and Rabbit Diseases, Faculty of Veterinary Medicine, Benha University. Later, virus was propagated ($10^{8.5}$ EID_{50}/0.1ml) in the laboratory of Newcastle Department, Veterinary Serum and Vaccine Research according to OIE, (2).

Experimental design

The experiment was conducted at the Laboratory Animal Research Center, Faculty of Veterinary Medicine, Benha University. All procedures used in this experiment were approved by the Institutional Animal Care and Use Committee of Benha University and followed the guidelines of the National Institute of Health in Egypt (Ethical No. BUFVTM 07-11-21).

A total of sixty Cobb chicks (day old) were obtained from a local hatchery in Egypt and allocated randomly into 4 equal groups of 15 birds as follows; group A was as control negative without any treatment, and group B was as control positive challenged untreated group. Groups C and D were challenged and received the prepared neem leaves extract in the drinking water for five days starting from the day of infection at a dose of 500 and 1000 µg/kg BW, respectively. At 25^{th} days old, chicks in groups B, C and D were challenged via the oculonasal route with 0.2 mL of virulent NDv class II (genotype VII) with titer

of 10^6 EID_{50}/chick (20). All chicks were observed during the trial and examined for clinical signs, and postmortem lesions. Morbidity and mortality were recorded daily.

Parameters evaluating the efficacy of the methanolic neem leaves extract

Clinicopathological examination and lesion scoring

At 7 days post challenge (dpc), clinical and postmortem examination was applied on three humanely slaughtered birds from each group. The trachea, lung proventriculus, intestine, cecal tonsils, spleen, and kidneys were examined macroscopically and scored from 0 to 3 according to the lesion severity (21). Part of the trachea, proventriculus, cecal tonsils, and spleen were collected for the histopathological examination. These tissue samples were fixed in 10% formalin, then dehydrated in the grades of alcohol concentrations, cleared in xylene, and embedded in molten wax. Sections of 5 µm thick were made and finally stained by H and E. The stained slides were examined microscopically, and lesion scoring was applied (21).

Viral shedding

At 3, 5, and 7 dpc, five oropharyngeal swabs were collected from each group to estimate the virus shedding using reverse transcription-Real time polymerase chain reaction (RRT-qPCR) as follows; Total RNA was extracted from 500 µl of the prepared oropharyngeal swabs using the RNeasy® Mini Kit (Qiagen). RT-qPCR experiment was performed in a Bio-Rad real-time thermal cycler CFX96™ using primers for Matrix gene of NDv (Forward; 5′-CCT GAG GAG AGG ATT TGC TA-3′ & reverse; 5′-AGT GAT GTG CTC GGA CCT TC-3′; and probe (5′-[FAM] TTC TCT AGC AGT GGG ACA TGC [TAMRA]-3′ that were designed by Wise et al., (22). TOPreal™ One-step RT-qPCR kit (enzynomics) was used in the amplification. All procedures were conducted according to the manufacturer's instructions. For primer set, the RT step was 30 min at 50°C, followed by 15 min at 95°C. The cycling conditions for the APMV-1 matrix primers consisted of 40 cycles of 10 s of denaturation at 94°C, 30 s of annealing at 58°C, and extension at 72°C for 10 s. Samples were always run-in triplicate to test for reproducibility. Challenge NDv strain was used for positive control and for the determination of the limit of detection for the assay. RNA concentration was quantified by the cycle threshold (Ct) method and interpreted versus the standard curve to NDv EID_{50} (22).

Infectivity titer of the challenged virus in SPF eggs embryos

Three samples of trachea, spleen and cecal tonsils from each group were collected at 7 dpc and prepared to estimate the infectivity titer of the challenged virus in SPF eggs embryos as follows; Each clarified homogenate sample was diluted in sterile phosphate buffer saline (PBS) twelve fold serial dilution (1 part of sample to 9 part of PBS), and each dilution was injected in five SPF embryonated chicken eggs via the allantoic cavities at a dose of 0.2 ml per egg. The negative control eggs were injected with physiological saline. All the eggs were incubated at 37°C. All the embryos that died on the first day were discarded. The infectivity titer was calculated using the Reed and Muench (23) method and expressed in $\log_{10}$ EID_{50}/ml.

Statistical analysis

Data were analyzed by one way ANOVA, and by multiple repeated measures for viral shedding using SPSS 16, (2007) to determine the differences between individual treatments and corresponding controls. Results were represented as means ± standard error.

Results

Total phenolic and flavonoid contents and antioxidant activities of neem leaves

The extraction yield of neem leaves was 11.93 g/100 g on a dry weight basis. Total phenolic and flavonoid contents in each gram of MNLE were estimated as 82.1 mg GAE and 105.30 mg QE, respectively. Moreover, ABTS and DPPH radical scavenging activities of MNLE were detected as 61.25 and 63.18 %, respectively.

Effect of methanolic neem leaves extract on clinical and pathological outcomes after NDv challenge

The challenge of 25-day-old broilers by NDv genotype VII resulted in depression, ruffled feathers, conjunctivitis, and nasal discharge beginning at the 3rd dpc in all experimentally infected groups (B, C, and D). At the 5th dpc, birds in the challenged groups showed swollen heads, sneezing, rales, and greenish diarrhea that involved 100 % of the challenged birds in the positive control group (B), and the mortalities started early in this group to reach 60 %. Postmortem examination of dead birds revealed congestion of musculature and trachea with marked hemorrhages on the proventriculus, small intestine, cecal tonsils, and rectum. On day 7 post-challenge, birds in positive control group B reported significant pathological changes with the highest lesion scores, including congestion in trachea and lungs; edematous and hemorrhagic proventriculus; hemorrhagic ulcers along the intestine and cecal tonsils; mottled spleen and inflamed kidneys (Figure 1; Table 1). On the other side, all birds in group A (negative control) appeared clinically healthy throughout the experiment.

Using MNLE at 500 and 1000 µg/ kg BW for five successive days to control the ND infection in groups C and D, morbidity and mortality rates lowered to74 & 53.3% and 26.67 & 20 %, respectively. As shown in Table (1) and Figure (1), significant decreases in total lesion scoring were reported for all organs examined from groups C and D treated with MNLE when compared with those of the positive control group (B). The high dose of MNLE (1000 µg/ kg BW; group D) was more efficient and significant in relieving the clinical and pathological abnormalities caused by ND challenge when compared with the other dose given to birds in group C (500 µg/ kg BW).

Table 1: Effect of methanolic neem leaf extract on the clinical and pathological outcomes the broilers challenged with Newcastle disease virus

Items			Experimental groups			
			Group A (-ve)	Group B (+ve)	Group C (500 µg/ kg BW)	Group D (1000 µg/ kg BW)
Morbidity rate			0	100 %	74 %	53.3%
Clinical findings*	**Ruffled feathers and de-pression.**		0	15/15	11/15	8 /15
	Conjunctivitis and nasal discharges		0	15/15	10/15	9 /15
	Swollen head.		0	7/15	3/15	1/15
	Greenish diarrhea		-	++	+	+
Mortality rate			0	60 %	26.67%	20%
Le-sion scoring ⌘	**Macroscopic**	**Trachea**	0^{d}	3^{a}	2.33 ± 0.33^{ab}	1.67 ± 0.33^{c}
		Lung	0^{d}	2.67 ± 0.33^{a}	1.67 ± 0.33^{b}	1^{c}
		Proventriculus	0^{d}	3^{a}	2^{b}	1.33 ± 0.33^{c}
		Intestine	0^{d}	3^{a}	2^{b}	1 ± 0.58^{c}
		Cecal tonsils	0^{d}	3^{a}	1.67 ± 0.33^{c}	0.67 ± 0.33^{c}
		Spleen	0^{c}	2.33 ± 0.33^{a}	1^{b}	0.33 ± 0.33^{bc}
		Kidney	0^{c}	2.67 ± 0.33^{a}	1^{b}	0^{c}
	Micro-scopic	**Trachea**	0^{c}	4.33 ± 0.33^{a}	2^{b}	1.67 ± 0.33^{b}
		Proventriculus	0^{c}	3.67 ± 0.33^{a}	2.33 ± 0.33^{b}	2.33 ± 0.33^{b}
		Cecal tonsils	0^{c}	3.33 ± 0.33^{a}	2^{b}	1.33 ± 0.33^{b}
		Spleen	0^{d}	3.67 ± 0.33^{a}	2.67 ± 0.33^{b}	1.67 ± 0.33^{c}

Clinical signs were recorded as ruffled feathers, depression, conjunctivitis, nasal discharges, swollen head and green-ish diarrhea (No of affected chicks/ total No). ⌘ Following slaughtering of selected birds; macroscopic and micro-scopic lesions as congestion, hemorrhages and so on were scored in the harvested organs (trachea, lungs, proventric-ulus, cecal tonsils, intestine, spleen, and kidney). Severity scores were absent (0); Mild (1); Moderate (2); Severe (3). Lesion scoring was expressed as mean ± standard error.

Effect of methanolic neem leaves extract on the histopathological changes in broiler chicks after challenge with NDv

Histopathological changes of the control and NDv-challenged chickens were analyzed on day 7 post challenge, as shown in Table (1) and Figure (2). No histological alterations were detected in all examined organs from control negative group A. Tracheal sections (T) of control positive group B showed hemorrhage and obvious epithelial desquamation in the tracheal lumen, in addition to note of focal deciliated areas. Significant differences in the histological tracheal alterations score were evident between the treated groups C and D and the infected untreated group B. Similarly, treated group C and D proventricular (P) sections showed slight edema, congestion, and mild lymphocytic infiltration which significantly improved histological score when compared with group B (positive control sections) that showed severe mononuclear cell infiltration with severe hemorrhages and necrosis, loss of the lining epithelium, fusion, and shortening of the plicae.

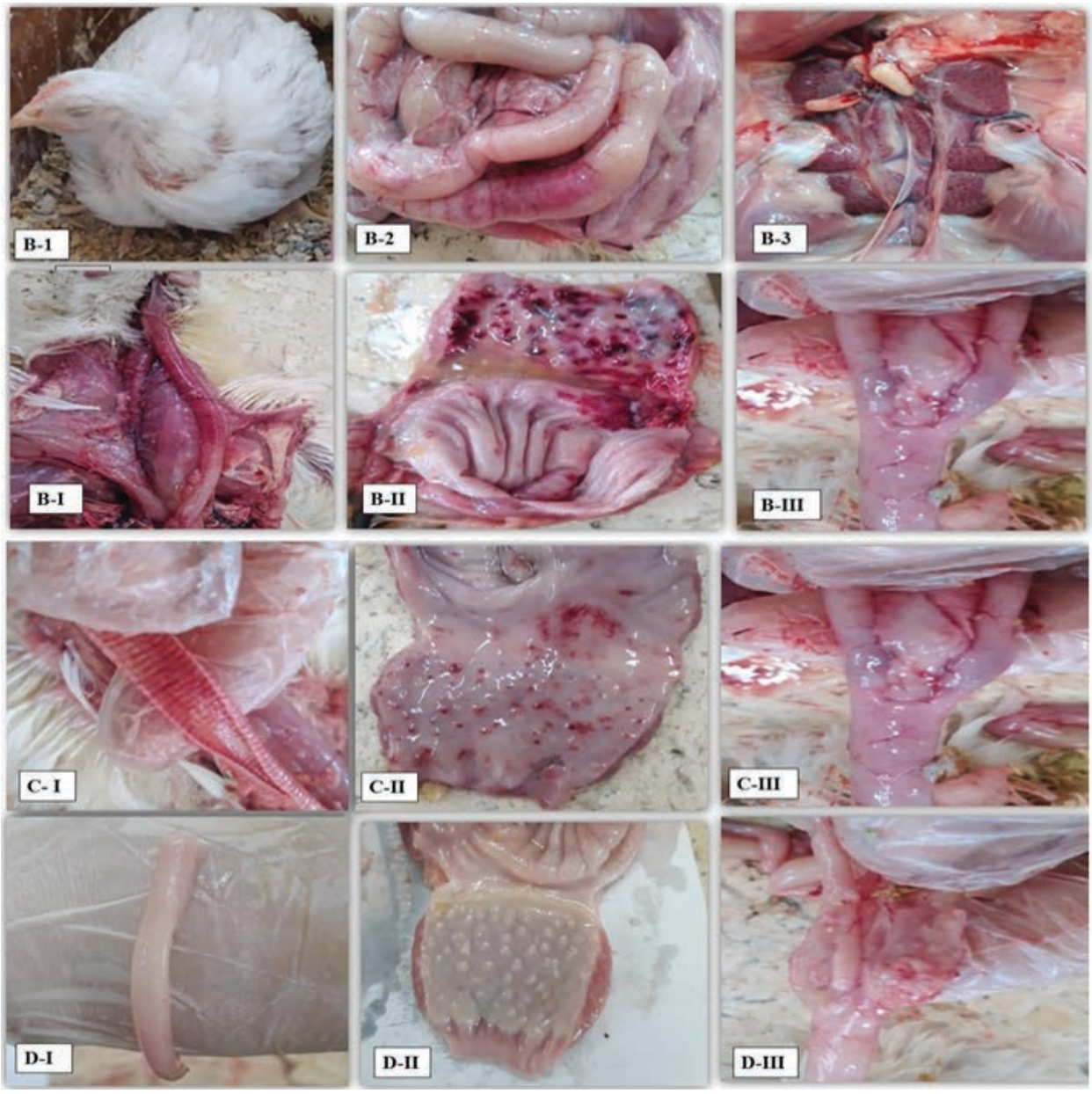


Figure 1: Clinicopathological picture of positive control group showing morbid bird with ruffled feathers, conjunc-tivitis, and nasal discharge (B-1); hemorrhagic ulcers along the intestine of dead bird (B-2) with inflamed kidneys (B-3). Moreover, several pathological lesions with varied scores were observed in the examined trachea (I), proventriculus (II), and cecal tonsils (III) of the experimental groups (B, C, and D) on day 7 post challenge with NDv

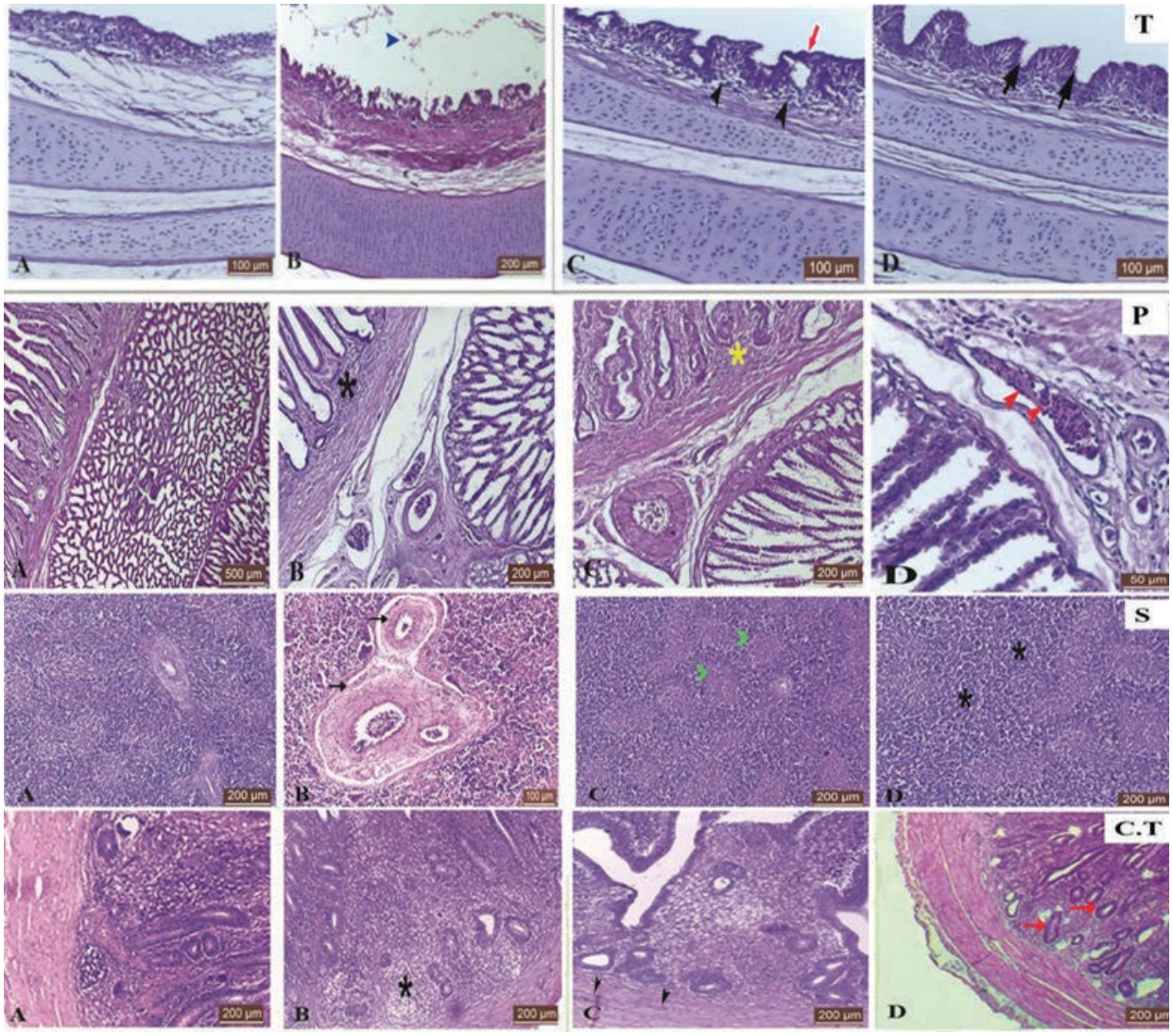


Figure 2: Histological H and E stained sections of the trachea (T), spleen (S), proventriculus (P), and cecal tonsils (C.T) from the experimental groups (A, B, C, and D) of the broilers on day 7 post challenge with NDv challenge; bar in photos indicates lens magnification. Tracheal sections (T) from control negative (A) showing apparent nor-mal histological structure; control positive group (B) showing hemorrhage, deciliation and epithelial desquama-tion (blue arrowhead); (C) showing hyperemia and lymphocytic infiltration (black arrowhead) with focal areas of decil-iation (red arrow) from group C, and (D) showing edema with no evidence of deciliation (black arrow) from group D. In proventriculus sections (P) were (A) control negative group showing apparent normal histo-logical structure, (B) group B showing mononuclear cell infiltration (black star), (C) group C showing mild lymphocytic infiltration (yellow star), (D) group D showing lymphocytic infiltration and increased accumulated gland secretions (red ar-rowhead). Spleen sections (S) for control negative (A) showing apparent normal histo-logical structure, (B) group B showing thickening of blood vessels wall (black arrow), (C) group C showing mild to moderate lymphocytic degeneration (green arrowhead), (D) of group D showing proliferated lymphoid follicles (black star). Sections of cecal tonsil (C.T); negative control (A) showing normal histology. (B) Group B showing mild lymphoid depletion, (C) group C showing mild transmural invasion of inflammatory cells with hemorrhages, and (D) group D showing few active follicles

Moreover, examination of the cecal tonsil (C.T) sections in the positive control group (B) showed severe transmural inflammatory cell invasion with increased bleeding, while C.T sections in treated groups C and D showed slight lymphatic depletion and mild intramural invasion of lymphocytes resulting in a significant decrease in their scores compared to the positive control group B. Significance decrease in histopathological lesion scoring with efficient proliferation in lymphoid follicles were also observed in the spleen sections (S) of the treated groups (C and D) with comparison to the control positive group B that showed severe multifocal lymphoid depletion and diffuse necrosis.

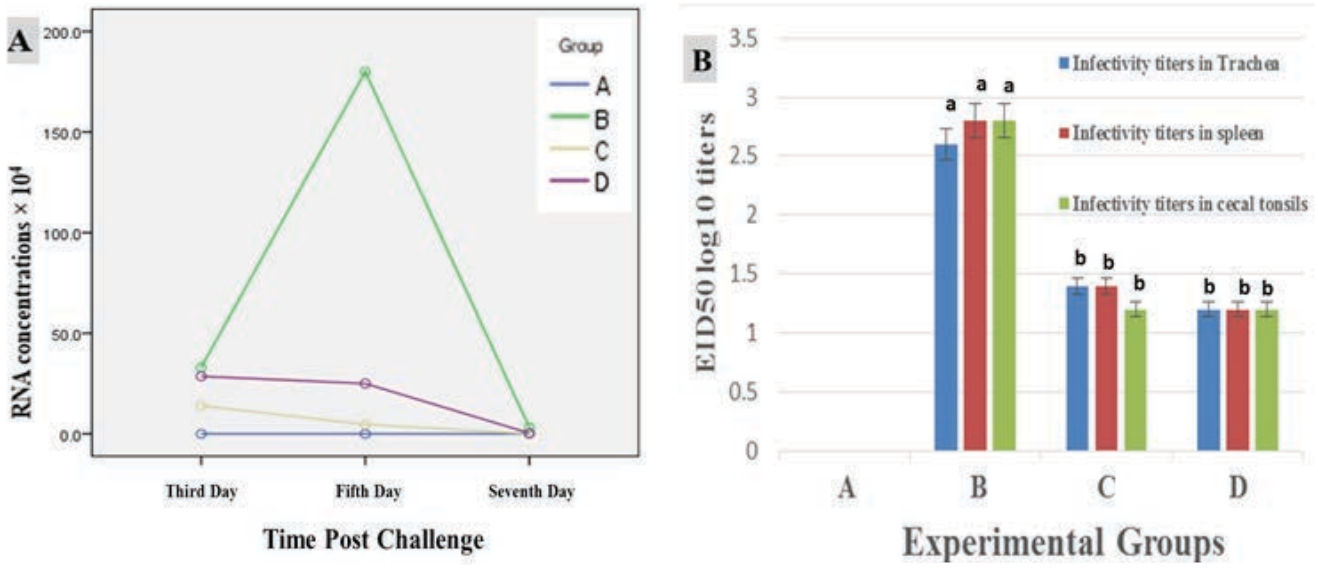


Figure 3: Shedding and infectivity titration of Newcastle disease virus in different groups of broilers. (A) Mean viral nucleic acid concentration value of real time RT-PCR and equivalent EID50/mL of NDv genotype VII are detected in oropharyngeal swabs on days 3rd, 5th, and 7th post challenge and (B) Infectivity titers of reisolat-ed NDv genotype VII from the trachea, spleen, and cecal tonsil in specific pathogen free chicken embryos on day 7th post challenge. Small letter (a, b) showed significant difference between the groups.

Effect of methanolic neem leaf extract on viral shedding and infectivity titer after challenge with NDv

The virus was shed early at the 3rd dpc from the oropharyngeal swabs of the control positive group B with mean RNA concentrations of approximately $3.3x10^5$ EID_{50}/ml (Figure 3). Virus shedding continued on days 5 and 7 dpc with mean RNA concentrations of approximately $1.8\ x10^6$ and $3.1x10^4$ EID_{50}/ml, respectively. Moreover, the challenged ND was reisolated in ECE with infectivity titers of $10^{2.6}$, $10^{2.8}$, and $10^{2.8}$from the trachea, spleen, and cecal tonsils of the control positive group B at 7dpc, respectively. Worthily, the highest RNA concentrations and infectivity titers were reported in the positive control group (B) at all three interval periods. A significant decrease in the viral shedding and infectivity titer after challenge with NDv genotype VII was recorded in birds treated with the MNLE at two different doses in groups (C & D) compared with those of the control positive group (B). Interestingly, no significant difference was reported between the treated groups (C and D) and the negative control group (A) in the viral shedding at the 7th dpc. The challenged NDv and its RNA expressions were not detected in all examined samples from the negative control birds (group A).

Discussion

Newcastle disease is a transmissible and notifiable poultry disease in the OIE list A. It has the potential for severe and rapid spread, irrespective of national borders, adversely affecting global poultry production (24). Despite immunization and medication programs against ND, it causes yearly 40–60% of poultry losses (25). Most references to NDv in poultry are chicks, which are most affected. However, the severity of clinical signs does not vary only according to the inherent virulence of the virus but also according to some host-related factors such as age, route of infection, immune status, and concomitant environmental stress (4, 26). The infected untreated birds of the control positive group in the current study early showed the clinical signs of ND at 2 dpc as appetite loss, depression, greenish diarrhea, respiratory signs, and nervous signs, in addition to the morbidity and mortality rates in this group were more significant than control negative and other treated groups.

Moreover, we saw several notable pathological changes in different organs for NDv infection, such as congestion of musculature and trachea with marked hemorrhages on the proventriculus, small intestine, cecal tonsils, and rectum during necropsy. The histopathological examination

of the trachea, proventriculus, cecal tonsil, and spleen showed congestion, hemorrhages, and lymphocytic infiltrations. Similar observations of several organs were recorded previously in the field, and experimental ND infection (21, 26-29) was attributed to specific tropisms and the extensive dissemination of the velogenic ND strains to multiple body systems after initial replication (4).

Herbal extracts have been used medicinally since ancient times due to their antiviral potential and manageable side effects. Several neem extracts are known for their powerful therapeutic effect, which may be an excellent option for treating viral infections and protecting herds. For that, this work studied the impact of the methanolic neem leaves extract on the control of ND in broilers at doses of 500 and 1000 µg/kg BW (groups C and D). MNLE doses showed a delay in the onset of the disease. They significantly decreased mortalities, clinical signs, and pathological findings in the examined organs caused by ND challenge compared with the control positive group (B). This result was consistent with the findings of Tolba (29), who reported the protective efficacy of *Moringa Oleifera* extract in chickens against NDv infection without immunization. These findings were supported by previously report on the invitro ability of neem leaves to suppress several viruses as hepatitis C, human immunodeficiency virus, herpes, Dengue, Japanese encephalitis virus, coronaviruses, NDv, infectious bursal disease virus and influenza viruses (10-16).

Consequently, the improved survival and tissue health in the infected birds highlight the efficient and protective action of MNLE. This came supported with the previous finding of Helmy (16) about anti-NDv activity of MNLE in Vero cells and chicken embryo. Moreover, the amerioliative action of MNLE may be attributed to ability of different the neem tree parts in the inhibition of inflammatory factors such as nitric oxide and tumor necrosis factor (17), and improving the expression of the immune mediators such as interleukins, interferon, and cytokines in mucosal surfaces (30-32), high nutritional value of neem leaves in the broiler chicken that used as feed components (33) due to its high protein content and an abundance of minerals and vitamins such as calcium, phosphorus, magnesium, iron, vitamin C, and beta-carotene (8-9).

MNLE alleviated not only the disease picture of ND but also virus shedding at 3, 5, and 7 dpc from oropharyngeal swabs of the infected chicks and viral isolation at 7 dpc from the trachea, spleen and cecal tonsils, which were significantly reduced due to the ability of phytochemicals in neem leaves to stop essential steps in the life cycle of the ND virus, like getting into cells and making copies of themselves (7, 34-35). This is correlated with our findings from the high amount of total phenolic and flavonoids in MNLE with high potential scavenging and antioxidant activities. These came in agreement with previous reports about the effectiveness against NDv of the *Azadirachta indica* extracts and other herbal extracts such as *Synadenium glaucescens*, *Artemisia annul L.,* and *Moringa Oleifera* (29, 36-37). Similarly, Helmy (16) found the methanol extract of neem leaves at 3- 4 µg /ml and 4 µg /egg completely inhibiting the NDv replication in VERO cells and chicken embryos, respectively, with the absence of cytotoxicity. The presence of phytochemicals in herbal as phenolic, anthroquinones, flavonoids, and tannins can protect the body tissues and improve survival rates, in addition, to being responsible for its antiviral activity and potential for treating a wide range of diseases (6, 38-40). In conclusion, the pathological changes caused by a Newcastle disease infection could be mitigated by MNLE, which would also reduce NDv shedding and infectivity. This plant is used in traditional medicine and may provide a model for developing novel antivirals.

Conclusion

In the current study, the neem leaf had a high extraction yield and quantity of total phenolic and flavonoids, as well as potential scavenging and antioxidant capabilities. Consequently, the methanolic neem leaf extract was used to control the ND infection at a dose of 500 and 1000 µg/kg BW which resulted in a significant reduction in morbidity and mortality rates, as well as the macroscopic and microscopic lesion scoring of all examined organs, when compared to the infected untreated group. Moreover, Positive benefits of herbal treatment for experimentally ND-infected chicken were evidenced by a reduction of viral RNA concentrations in oropharyngeal swabs at 3, 5, and 7 dpc and the infectivity titer of the isolated virus from the trachea, spleen, and cecal

tonsils at 7 dpc. MNLE could counteract tissue damage generated by Newcastle disease virus infection and reduce its shedding and infectivity. Traditional cures like this plant could be a source of inspiring alternative new antivirals.

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All authors contributed to conceptualization, methodology, investigation, resources, and editing supervision. Writing original draft and Statistical analysis – Sawsan Sami Elbasuni. All authors have read and agreed to the published version of the manuscript.

The authors declare no conflict of interest

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Original Research Article
Veterinary Medicine and The One Health Concept

ANTI-FIBROTIC AND ANTIOXIDANT AMELIORATIVE EFFECTS OF NARINGENIN AGAINST THIOACETAMIDE INDUCED LIVER FIBROSIS

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Abstract: Liver fibrosis still life-threatening problem and searching out ameliorative products motives many scientists, so this work evaluated the anti-fibrotic and antioxidant role of naringenin (NAR) against thioacetamide (TAA) induced liver fibrosis. Fifty adult male albino rats randomly divided into 5 groups (10 each); the first kept as control; the second treated I/P by 200 mg/kg TAA twice a week for 8 weeks; the third was gavaged daily with 50 mg /kg/ b.wt of NAR for 8 weeks; the fourth was co-treated by TAA and NAR while the fifth was treated with TAA for 8 w then gavaged daily by NAR for 1 month. TAA administration significantly increases the hepatic cell enzymes (ALT, AST, ALP and GGT) in the serum referring to hepatic cell destruction with an increase in hepatic MDA with a reduction in GSH concentrations, antioxidant enzyme activities as well as down regulation of their expression levels. NAR administration either with or after TAA ameliorates this effect suggesting its antioxidant ability. In the fibrotic pathway, TAA treatment up-regulates the expression levels of fibrogenic biomarkers (TGF-β, collagen 1α and fibronectin) genes while NAR down-regulates these genes suggesting its anti-fibrotic ability. Histopathological analysis confirms the biochemical results. In conclusion, NAR ameliorates the deleterious effect of TAA through its antioxidant and anti-fibrotic abilities.

Key words: naringenin; thioacetamide; liver fibrosis; fibrogenic markers

Introduction

Liver is the main metabolic regulatory and detoxifying organ in the body (1). Liver diseases including hepatitis and liver cancers representing a global health problems, threatening lives with great loses in health and money (2).

Fibrosis of the hepatic cells is a common pathological process leading to hepatic cell failure, cirrhosis with a high risk for developing hepatocarcinoma (HCC). Liver fibrosis is characterized primarily by increasing the deposition of extracellular matrix proteins such as collagen in hepatic cells leading to hepatic architecture distortion with nodular formation, alteration of blood flow, portal hypertension, HCC, and ultimately liver failure (3, 4). The major fibrotic biomarkers are collagen, fibronectin, nidogen, laminin and transforming growth factor beta-1 (TGF-β1) (5).

Hepatic cell fibrosis is commonly associated with the most of chronic hepatic diseases and pathologically characterized by inflammation with parenchymal damage. The common causes of liver fibrosis are viral hepatitis especially hepatitis C, alcoholic abuse, metabolic diseases such as Wilson's disease and hemochromatosis, obesity, biliary diseases, parasitic diseases such as Schistosomiasis and chronic drug, toxin and chemical exposure (6). Till now no satisfactory

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anti-fibrotic substances can be used for treatment or prevention of liver fibrosis despite a great progress in the mechanisms and treatment of liver fibrosis.

Experimentally, TAA is considered a classical hepatotoxic reagent used for induction of hepatic fibrosis and cirrhosis in rats. TAA can results in liver diseases and centrilobular damage in acute applications (7,8) while in chronic applications results in liver cirrhosis (9, 10) through the induction of membrane damage, oxidative stress and accumulation of lipid droplets in the hepatocyte cytoplasm to enhance inflammation and liver injury in rodents (11).

Medicinal plants have always been a part of human culture, and up to 80% of the world population relies on this system for some aspects of primary health care especially as antioxidant protecting the body from oxidative stress and reactive oxygen species (12). The natural compounds have been studied extensively and are relevant to many illnesses including liver diseases as they play an important role in the prevention of fibrosis and thereafter in cirrhosis (13). Recently, the interest in natural antioxidants has expanded extensively as a result of their valuable impacts of avoidance and hazard decrease (14). It is well known that consumption of antioxidants is related to a decreased danger of a few sicknesses, for example, cardiovascular, cancer and liver diseases (15).

NAR, 4', 5, 7-trihydroxy flavanone is a bioflavonoid present in tomato, citrus fruits (oranges, lemons, grapefruit, tangerines), cherries, grapefruit and coca (16, 17). Some reports has been demonstrated the NAR use for a prevention of experimentally induced acute liver damage by alcohol, carbon tetrachloride (CCl4), heavy metals or lipopolysaccharide (18,19). Also, there is some other repots associated NAR with anticancer properties and anti-fibrotic properties of NAR. (20, 21). NAR was reported to have the ability to prevent deposition of collagen in rat's haptic cells treated by diethyl-nitrosamine (DENA) but with no clear explained mechanism (22). Du et al., reported the anti-fibrotic effect of NAR in lung tissue and connected it with down-regulation of TGF-β activity. However, according to our knowledge, there is no studies on the anti-fibrotic effect of NAR against liver fibrosis (23). Therefore, the main purpose of our interest was directed to investigate the possible protective effects of NAR and its mechanism of protection against TAA induced liver fibrosis.

Materials and methods

Chemicals and reagents

TAA and NAR were purchased from Sigma-Aldrich Chemical Co., (St. Louis, MO, USA), while all other chemicals used in this experiment were purchased from analytical grade Co., India.

Study design

Fifty male adult albino rats, with 6 months age and average weighting at the beginning of the experiment (120±15) gm were purchased from the breeding unit of laboratory animal farm (Helwan, Egypt) and housed in a standard cages in groups of 5 rats per cage in animal House of the faculty of Vet. Med. Zagazig University, Egypt under controlled conditions (temperature 23±1°C, a 12:12 light/dark cycle), and relative humidity (50–60%) and were given a standard diet and water ad libitum.

Animals were kept for two weeks under control conditions for acclimatization before the beginning of the experiment.

Rats were randomly divided into 5 groups, 10 rats each; the first group kept as a control group and didn't receive any treatment all over the experiment; the second group treated I/P with 200 mg/kg b.wt. TAA twice a weeks for 8 weeks according to Bruck, et al., (24); the third group was gavaged daily with NAR at dose of 50 mg /kg/ b.wt according to Mershiba et al., (21); the fourth group was co-treated by TAA and NAR by the previous mechanisms for 8 weeks as a preventive group; the fifth group was treated with TAA for 8 w then gavaged daily by NAR for 1 month as a treated group.

Ethical Approval

The experiment was applied under the instructions of National Institutes of Health Guide for the Care and Use of Laboratory animals (NIH Publications No. 8023, revised1978) that approved by the faculty of Vet. Med. Zagazig University.

Sampling

At the end of the experimental period, the rats were subjected to overnight fasting then decapitated, blood samples were collected and centrifuged at 3.000 rpm for 10 min. for serum separation for determination of serum ALT, AST, ALP and GGT. The serum was stored at -20°C until used.

Hepatic tissues were collected rapidly after decapitation, washed in normal saline and divided into 3 parts for biochemical, molecular and hitopathological examinations. The first part accurately weighed and homogenized in chilled potassium chloride (1.17%) using tissue homogenizer (Potter–Elvehjem) for determination of antioxidant status. The second part was taken, weighted and kept in liquid nitrogen to follow up the changes of gene expression of TGF-β1, Collagen 1α, Fibronectin, CAT, SOD and GPx. The third part was taken and kept in 10 % formalin for histopathological examination.

Biochemical investigations

Serum ALT (EC 2.6.1.2), AST (EC 2.6.1.1), ALP (EC 3.1.3.1) and GGT (EC 2.3.2.2) were determined using the commercial kits according to the methods described by Breuer (25) for ALT and AST and Moss et al. (26) for ALP and GGT. Malonyldialdehyde (MDA) was determined as a marker for lipid peroxidation according to the method described by Buege and Aust, (27). GSH (reduced glutathione) was also determined according to the method described by Ellman (28). Glutathion peroxidase (GPx) (EC 1.11.1.9), Glutathione reductase (GRD) (EC 1.8.1.7), superoxide dismutase (SOD) (EC 1.15.1.1), and Catalase (CAT) (EC 1.11.1.6), were also determined according to the methods described by Hussein et al., (16). All pervious parameters were measured by Shimadzu spectrophotometer (UV 120-02).

Molecular investigation

Hepatic total RNA of all groups was isolated using RNeasy Mini kit (Qiagen) (Cat. No. 74104) and following the manufacture instructions. Checking the quantity and purity of isolated RNAs performed OD260/OD280 using ND-1000 Spectrophotometer NanoDrop®. RNA samples which have a purity of 1.8 or more only were used for the synthesis of cDNA using RevertAidTM H Minus (Fermentas, USA). One µl of cDNA with 1 µl of each primer (10 pmol/µl), 9.5 µl of RNase-free water and 12.5 µl of SYBR Green Master Mix to reach a total volume of 25 µl was used for Semi-quantitative RT-PCR using a Rotor-Gene Q cycler (Qiagen) using a BioRad® SYBR® Green PCR Kit. The primer sequences and thermal cycler profile for TGF-β1, Collagen 1α, Fibronectin, CAT, SOD, GPx and β-actin were listed in table 1. The relative fold changes (2-ΔΔCt) were calculated in accordance to the method of Livak and Schmittgen (29) by determining the $2^{-\Delta\Delta Ct}$.

Table 1: Oligonucleotide primer sequences and real-time PCR conditions

Gene name	Primer sequence	Annealing temp./ number of cycles	Accession number
TGF-β1	F 5′- GGGCTACCATGCCAACTTCTG -3′ R 5′-GAGGGCAAGGACCTTGCTGTA-3′	60 °C / 30 cycles	NM_021578.2
Collagen 1 α	F 5’-GACATGTTCAGCTTTGTGGACCTC-3′ R 5′-GGGACCCTTAGGCCATTGTGTA-3′	59 °C/ 60 s (40 cycles)	NM_053304.1
Fibronectin	F 5′-TGGCTGCCTTCAACTTCTC-3′ R 5′-AGTCCTTTAGGGCGGTCAAT-3′	58 °C/ 60 s (40 cycles)	NM_019143.2
CAT	F 5′-GTCCGATTCTCCACAGTCGC-3′ R 5′-CGCTGAACAAGAAAGTAACCTG-3′	58 °C/ 60 s (30 cycles)	S50336.1
SOD	F AAGCATGGCGATGAAGG-3′-5′ R GAGACTCAGACCACATAGGGA-3′-5′	55 °C/ 60 s (25 cycles)	NM_017050.1
GPx	F 5′-CACAGTCCACCGTGTATGCC-3′ R 5′-AAGTTGGGCTCGAACCCACC-3′	55 °C- 60 s (28 cycles)	Z21917.1
β-actin	F 5′-ACCACAGCTGAGAGGGAAATCG-3′ R 5′-AGAGGTCTTTACGGATGTCAACG-3′	59 °C/ 60 s (30 cycles)	BC063166.1

Statistical analysis

The obtained results were analyzed using SPSS version 22 Software and expressed as the mean ± standard error (SE) using one-way ANOVA for data analysis and followed by Post hoc Duncan's tests for comparison of groups using p-value < 0.05 to show a significant statistical difference.

Histopathological examination

Hepatic tissues were collected, washed in normal saline and fixed in 10% buffered neutral formalin solution, dehydrated in gradual ethanol (70-100%), cleared in xylene, and embedded in paraffin. Five-micron thick paraffin sections were prepared and then routinely stained with hematoxylin and eosin (HE) dyes (30) and then examined microscopically.

Results

Biochemical investigation

The effects of TAA treatment and/ or NAR on the serum and hepatic tissue parameters were reported in table 2. TAA treatment resulted in hepatic cell dysfunction which indicated by a significant increase in the serum hepatic function indicative enzymes (ALT, AST, ALP and GGT); whereas NAR administration for a healthy rats didn't show any significant change in the hepatic enzymes serum levels. Co-treatments of NAR and TAA minimized the deleterious effects of TAA treatment by decreasing the serum levels of hepatic enzymes. Treatment with NAR after TAA treatment resulted in reduction of the hepatic enzymes serum levels but in a lesser extent than performed in the fourth protective group (table 2). TAA treatment resulted also in a significant increase in the hepatic lipid peroxidation biomarker MDA with a reduction in the hepatic levels of GSH and other antioxidant enzymes, GPx, GR, SOD and CAT. NAR treatment either with TAA treatment or after TAA treatment success to improve the hepatic antioxidant status by decreasing MDA and increasing antioxidant substance levels and enzymes activities (Table 2). The degree of improvement was higher when NAR administrated with TAA as a co-treated substance.

Molecular investigation

The results showed in figure 1 represented that, TAA treatment increases the expression levels TGF-β, collagen 1 α and fibronectin genes with a reduction in the expression levels of antioxidant enzymes genes (CAT, SOD and GPx). NAR administration alone didn't significantly affects the expression levels of the above mentioned

Table 2: Effect of TAA and/ or Naringenin (NAR) on some serum and hepatic tissue parameters.

	Control	TAA group	NAR group	TAA&NAR co-treated group	TAA then NAR treated group
Serum ALT (U/L)	34.32±3.62^{d}	96.36±4.56^{a}	32.2±2.25^{d}	52.85±3.15^{c}	69.7±3.8^{b}
Serum AST (U/L)	62.25±4.46^{d}	128.75±4.62^{a}	58.43±2.68^{d}	73.92± 5.82^{c}	95.45±5.85^{b}
Serum ALP (U/L)	88.42±3.52^{d}	156.2±4.5^{a}	86.53±3.65^{d}	96.75±6.22^{c}	117.32±6.65^{b}
Serum GGT (U/L)	22.46±1.6^{d}	67.45±3.54^{a}	21±2.72^{d}	37.22±3.62^{c}	51.56±3.36^{b}
Hepatic MDA (µmol/L)	9.2± 0.76^{d}	24.6±2.55^{a}	7.14±0.54^{e}	14.35±1.14^{c}	20.66±1.53^{b}
Hepatic GSH (mg/gm tissue)	24.52±1.55^{b}	10.63±1.68^{e}	27.27±2.25^{a}	18.14±2.1^{c}	14.4±1.69^{d}
Hepatic GPx (µmol NADPH /mg protein)	62.4±5.2^{a}	25.06±2.42^{d}	61.4±2.6^{a}	48.54±2.67^{b}	35.94±3.01^{c}
Hepatic GR (U/ gm tissue)	22.32± 1.68^{a}	9.85±0.88^{d}	23.28±1.16^{a}	17.25±1.23^{bc}	13.28±1.02^{c}
Hepatic SOD (eu/mg protein)	44.35± 2.07^{a}	23.52±2.12^{d}	42.83±4.33^{a}	36.95±1.15^{b}	29.84±1.76^{c}
Hepatic CAT (µmol H_2O_2 decomposed/ gm tissue	88.9±3.51^{a}	45.3±2.65^{d}	90.4±2.44^{a}	77.7±2.1^{b}	62.3±2.85^{c}

Means within the same columns and bearing different superscripts are significantly different at (P< 0.05).

genes whereas it improved the deleterious effects resulted due to TAA administration when it administrated either with or after its treatment. The co-treated effect of NAR was higher and better that the treatment after induction of liver cell fibrosis.

Histopathological findings:

The control group showed normal hepatic parenchyma including central vein, hepatic cords, and sinusoids. (Figs. A). Hepatic tissue treated with TAA showed multi-portal fibrous strands extended to interlobular septa with focal interstitial inflammatory cells with collagen fibrous deposits (Figs. B1). With high power Focal interstitial inflammatory cells aggregations were also seen beside Hepaticellular necrosis replaced by round cells infiltrations with disorganized hepatic parenchyma by collagen fibrous deposits (Fig B2). The liver sections from rats received only NAR revealed normal hepatic parenchyma without any collagen fibers deposits. (Figs. C). The liver sections from rats received with TAA with NAR as a preventive to liver fibrosis didn't show any fibrosis and appeared nearly normal hepatocytes, with mild centro-lobular ballooning degeneration of a few cells as seen (Figs. D). The liver sections from rats received TAA then treated by NAR showed congested blood vessels with moderate portal fibrosis and diffuse ballooning degeneration (Fig. E).

Discussion

Fibrosis of hepatic cells representing a dangerous medical problem due to its morbidity and mortalities as it is the main leader to cirrhosis, liver failure and hepatocellular carcinoma (31). Experimentally, TAA is ideally used as a model for liver fibrosis for testing antifibrotic effect of different drugs or natural products in rodents (32). Decreasing the risk of liver fibrosis or preventing it is a great hope of many scientists. Many trials about substance that decreasing the risk of hepatic cell fibrosis by discovering compounds either natural or synthetic was performed by many researchers (33). The direction towards natural antioxidants to minimize the deleterious effects was started many years ago (34). In our research we used TAA (the potent toxin and carcinogens) for induction of hepatic cell fibrosis as well as NAR as a potent antioxidant flavonoid to study its potential antifibrotic effects.

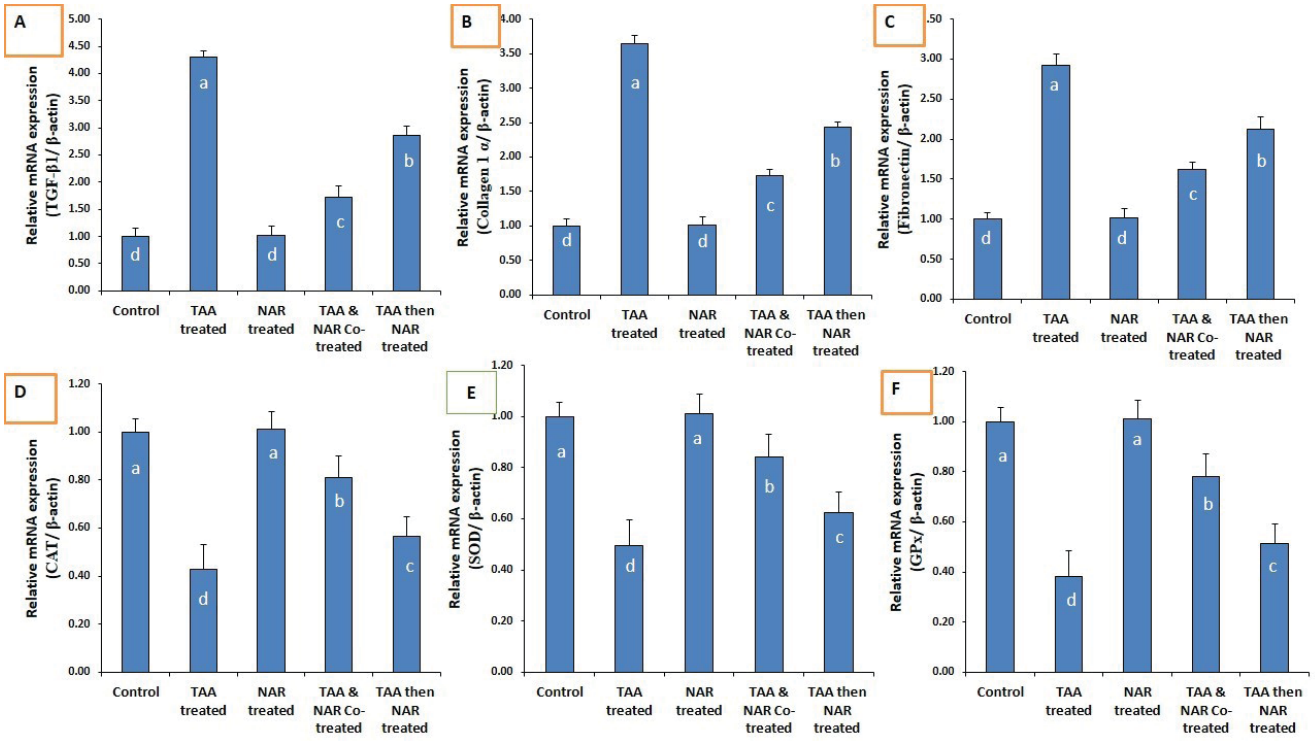


Figure 1: Effect of TAA and/or NAR administration on mRNA expression levels of TGF-β1, collagen 1α, Fibronectin, CAT, SOD and GPx genes in hepatic tissues Different letters (a, b, and c) by bars indicate significant differences ($p < 0.05$).

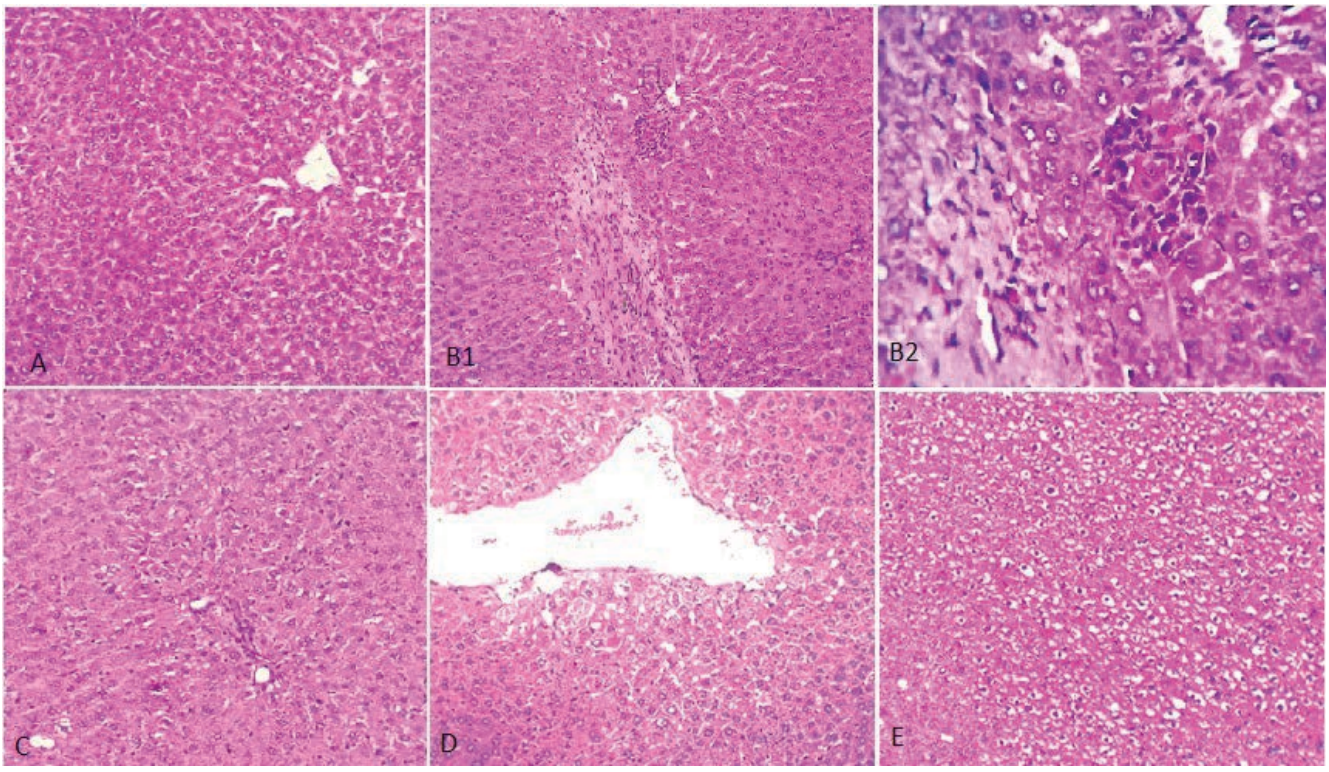


Figure 2: Hisopathological examination of hepatic tissue of rat by H&E stain at scale bar 100µm

A) Control group that showed normal hepatic parenchyma without any fibrosis or any lesions. B1) TAA treated group and showed fibrous strands extended to interlobular septa with thickening and fibrosis (star) beside inflamma-tory cells aggregation (arrow). With high power (Scale bare 50µm), hepatic cells showed interstitial necrosis with round cell infiltrations beside disor-ganized hepatic parenchyma and the cells were replaced by collagen fibrous de-posits (B2). C) The group treated with naringinin only, the hepatic cells were normal without any lesions, the same as control group. D) The preventive group treated with TAA & naringenin at the same time, hepatic cells showed ap-parently normal hepatic parenchyma with mild centro-lobular ballooning degeneration of a few cells. E) The nar-inginin treated group after induction of liver fibrosis by TAA, the hepatic cells showed diffuse ballooning degenera-tion with congested blood vessels.

The results obtained in our experiment after TAA treatment confirmed its ability to induced hepatic cell fibrosis as it results firstly in hepatic cell dysfunction showed in serum increase of hepatic enzymes ALT, AST, ALP and GGT with induction of hepatic cell lipid peroxidation observed in increase of MDA levels (table 2). At the same time it destroyed the antioxidant system by decreasing GSH levels and decreasing the mRNA expression levels of GPx, CAT and SOD with decreasing their enzyme activities. This creates a defect in free radical/ antioxidant status in the cell.

The pathogenesis produced by TAA for induction of hepatic cell fibrosis based mainly on induction of inflammation in hepatic cell through up-regulating the inflammatory cytokines (35-38) which also confirmed by our results represented in figure 1 that showed the up-regulatory effect of TAA on inflammatory and fibrotic markers gene expression (TGF-β, collagen 1α and fibronectin). For more confirmation, histopathological examination of hepatic cells was performed showing that, fibrotic strands from interlobular septa with collagen fibrous deposits in low power, after zooming in the collagen fibrous deposits were very clear (figure 2).

NAR, the natural flavonoid was reported for its antioxidant power especially against hepatic illness (39). Its ability to improve hepatic status were examined by its administration as a protective agent with TAA treatment and as a treatment agent after induction of hepatic fibrosis by TAA. The obtained results showed that, NAR alone didn't affect the fibrotic or antioxidant status in normal non treated rats which suggests its safety as natural safe product; after its administration with or after TAA treatment it improves the hepatic cell function firstly by decreasing the serum hepatic enzymes levels (ALT, AST, ALP and GGT) together with improving the antioxidant status through decreasing the hepatic lipid peroxidation

marker MDA and increasing the GSH levels with increasing both antioxidant enzymes activities and gene expression. The ability of NAR to improve hepatic function may be due to its ability to prevent membrane damage due to its antioxidant properties (41). NAR has the ability to prevent lipid peroxidation via its hydroxyl groups that facilitates its adherence to polar groups of lipid bilayer as well as interacting with hydrophobic tail of phospholipids by its nonpolar nucleus reducing the deleterious effects of free radicals on membranes (42). On the other hand, TAA treatment decreased GSH levels; however, NAR preserved normal hepatic GSH levels; this may be due to its up-regulation effect on the expression of glutathione reductase, which catalyzes the reduction of oxidized glutathione to the reduced form (43) and GPx that detoxifies H_2O_2 utilizing two molecules of GSH (44). As well as its effect on their activities.

The new here its role in down regulation of expression levels of inflammatory and fibrotic biomarkers (TGF-β, collagen 1α and fibronectin) that plays a significant role in minimizing the deleterious effect of TAA treatment. Liu et al., (44) previously also examined NAR for its effect on TGF-1 for first time suggesting its role in decreasing the expression levels of TGF-1 gene via disruption of TGF-”1-Smad3 signaling pathway. Down regulation of NAR to fibrotic markers plays the main role in its antifibrotic effect against the fibrosis induced by TAA in this experiment.

Hitopathological examination of hepatic cells supports the obtained biochemical results and confirmed the role of NAR in improvement of hepatic cell status by decreasing the collagen deposition caused by TAA treatment (figure 2).

From all of the above mention we can concluded that, NAR flavonoid may have hepatic ameliorative effect against TAA treatment, and can improve the hepatic cell function through its antioxidant and anti-fibrotic effect.

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The authors declare no conflict of interest.

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Original Research Article
Veterinary Medicine and The One Health Concept

PREVALENCE OF MULTIDRUG RESISTANT SHIGA TOXIN PRODUCING *E. coli* IN THE MILK OF CATTLE, BUFFALOES, AND CAMEL

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Abstract: Milk is regarded as an important source of essential amino acids, vitamins, and trace elements. Milk from camel is consumed in many Arabian countries as a traditional Medicine. However, few reports investigated the microbiological quality of raw camel milk in a comparison with cattle and buffalo's milk. This study was conducted to investigate the bacteriological quality of raw milk collected from cattle, buffaloes, and camel. Besides, Isolation and identification of different *E. coli* serotypes were carried out. Moreover, molecular confirmation of the recovered *E. coli* isolates via amplification of *16S rRNA* and detection of shiga toxin coding genes (*stx1*, and *stx2*) were done using PCR. Additionally, the antibiogram of the recovered *E. coli* isolates was screened using the disk diffusion method. The obtained results indicated that milk samples collected from camels had the lowest microbial counts compared with that of cattle and buffaloes. *E. coli* was isolated from the collected samples of cattle, buffaloes, and camel at 50%, 20%, and 10%, respectively. Various *E. coli* serotypes were identified in the present study with the ability of three and four *E. coli* isolates recovered from cattle milk samples to harbor *stx1*, and *stx2*, respectively. All recovered *E. coli* isolates showed multidrug resistance profiling. Therefore, pasteurization of milk prior to use, and adoption of strict hygienic measures are highly recommended to avoid the risk of human exposure to shiga toxin producing *E. coli*.

Key words: shiga toxin; *E. coli*; milk; camel; drug resistance

Introduction

Milk is regarded as a major source for the nutrients needed to maintain the human health. Milk proteins such as casein and whey proteins have been proven to be crucial for a number of human metabolic, physiological, and other nutritional processes. Additional benefits of milk proteins include their ability to function as antioxidants, boost the immune system, and guard against gastrointestinal infections. Even while other animal and plant proteins include potential bioactive peptides, milk proteins are now the primary source of many physiologically active peptides (1, 2). Milk is a rich source of trace elements such as calcium and magnesium. Milk sources in Arab countries involve cattle, buffalo, and camel. The latter is particularly regarded as an ethnic food with many therapeutic functions and used in the traditional medicine (3). However, milk is implicated in the transmission of several foodborne pathogens such as *E. coli*, *Salmonella spp.*, *Listeria spp.*, and others (4, 5).

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Microbial contamination of milk is controlled by the hygienic practices adopted during the milking process, processing, and distribution. The poor microbiological quality of a food product is a direct indicator for the hygienic procedures followed during production of such a product (6).*Escherichia coli (E. coli)* is a typical resident of both human and animal digestive systems. Its detection in animal products therefore refers to fecal contamination. *E. coli* is moreover one of the foodborne pathogens associated with the emergence of various human sickness outbreaks (7). Shiga toxin producing *E. coli* (STEC) is the cause of numerous hospitalizations of people all over the world (8). Several non O157 *E. coli* pathotypes are classified as STEC like O26, O45, O103, O111, O121, and O145 (9). One major task of the food safety and zoonoses sector is the continuous monitoring and surveillance of the foodborne pathogens like *E. coli* in various food subjects.

Microbial drug resistance is a challenging problem that poses serious risks to the public's health. In livestock production, antimicrobials are frequently used to prevent and control bacterial infections. However, the continuous and the uncontrolled usage of antimicrobials may result in the emergence of organisms with multidrug resistance properties (10, 11).

Taken the previous notes, this study was conducted to investigate the prevalence of *E. coli* in the milk of cattle, buffalo, and camel retailed in Egypt. Furthermore, detection of shiga toxin coding genes (*stx1*, and *stx2*) in the recovered *E. coli* isolates was done using PCR. Moreover, the antibiogram of the serodiagnosed-*E. coli* pathotypes was screened using the disk diffusion method.

Material and methods

Collection of Samples

Sixty random milk samples were collected from cattle, buffaloes, and camel (20/each). The collected milk samples (100 mL/each sample) were purchased from local stores and dairy farms in Sharkia governorate, Egypt. Samples were apparently normal with no alterations in their sensory characteristics. Samples were transferred cooled without delay to the laboratory for bacterial isolation and identification.

Bacteriological examinations

Milk samples were processed for bacteriological analysis in accordance with the APHA-recommended technique (12). Briefly, 25 mL of each sample were mixed with 225 ml of sterile buffered peptone water 0.1% to create a dilution of 10^{-1}. Further tenfold decimal serial dilution were created by aseptically transferring one ml from the prepared dilution (10^{-1}) to another sterile tube containing 9 ml of sterile 0.1% buffered peptone water.

Total bacterial Count (TBC)

Using plate count agar, TBC was estimated according to the method of APHA (12). Cultured plates were incubated for 24 h at 35 ± 2 °C, all colonies including pinpoint size were recorded.

TBC/g = average No. of colonies × reciprocal of dilution

Counted colonies expressed as log 10 cfu/g.

Determination of most probable number (MPN) of Coliforms

The three-tube approach was employed to calculate the MPN of coliforms (12). Three test tubes containing MacConkey broth with inverted Durham's tubes were inoculated with one mL of each generated dilution. The test tubes were then incubated at 37°C for 24-48 hours. Positive tubes that produced gas and acid were recorded. The recommended tables were used to determine MPN of coliforms that were most likely to exist.

Determination of MPN of E. coli

In tubes containing 7 ml of *E. coli* (EC) broth (Himedia, Mumbai), loopfuls from positive tubes demonstrating acid and gas productions on MPN of coliforms assays were inoculated. The tubes were then incubated at 44.5°C for 24-48 hours (12). Tubes with acid and gas production were recorded as positive. According to the suggested tables, the MPN of *E. coli* was calculated.

Isolation of Escherichia coli

Using the APHA procedure, *E. coli* was isolated using Eosin Methylene Blue (EMB) agar (12). A loopful of EC broth from each positive tube was

spread onto EMB agar. The inoculated plates underwent a 24-hour incubation period at 37°C. *E. coli* colonies typically had a dark purple core and were metallic green in appearance. Purified suspected colonies were sub-cultured onto nutrient slope agar and incubated for further examinations. Staining and biochemical assays were used to identify the isolates. Using specific *E. coli* antisera sets, the recovered *E. coli* isolates were subjected to serological identification (Difco, Detroit, USA) (13).

Bacterial DNA preparation and detection of 16S rRNA- E. coli specific gene and shiga toxin producing genes in the identified isolates

Each isolate of *E. coli* from the glycerol stock underwent DNA extraction using the previously described procedure (8). A PCR analysis was used to confirm isolation of E. coli via amplification of E. coli specific *16S rRNA*, and identify genes encoding the shiga toxins (*stx1*, and *stx2*). Table 1 displayed the amplified product sizes and primer sequences. A Thermal Cycler was used to carry out the amplification (Eppendorf, Hamburg, Germany). PCR assays were performed utilizing Dhanashree and Mallya's technique (14). A denaturing step at 95°C for three minutes was followed by 35 cycles of 95°C for 20 seconds, 58°C for 40 seconds, and 72°C for 90 seconds as the amplification conditions. The last cycle lasted 5 min at 72°C. The reference strains *E. coli* O157:H7 Sakai and *E. coli* K12DH5α were used as positive and negative strains, respectively. On a 2% agarose gel electrophoresis (Applichem, Germany, GmbH) in a 1x TBE buffer stained with ethidium bromide, amplified DNA products were visualized.

Antibiotic resistance of the recovered E. coli

The disc diffusion method was used to assess the antibiogram of the recovered *E. coli* isolates. Nutrient agar and the used antimicrobial discs were acquired from Oxoid in Hampshire, United Kingdom. We used the Clinical and Laboratory Standards Institute's (CLSI) experimental guidelines (16). Additionally, using the method outlined by Singh et al. (17), the Multiple Antibiotic Resistance (MAR) index for each tested *E. coli* isolate was calculated as follows.:

MAR index = No. of resistance / Total No. of tested antibiotics

Isolates classified as intermediate were considered sensitive for MAR index

The tested antimicrobial discs (Oxoid Limited, Hampshire, UK) were ampicillin (10 µg) (AM), cephalothin (30 µg) (CET), chloramphenicol (30 µg) (C), ciprofloxacin (5 µg) (CIP), enrofloxacin (5 µg) (ENR), erythromycin (15 µg) (E), gentamicin (10 µg) (GEN), kanamycin (30 µg) (K), nalidixic acid (30 µg) (NA), neomycin (30 µg) (N), oxacillin (1 µg) (OX), oxytetracycline (30 µg) (OXY), penicillin (10 IU) (P), and trimethoprim/sulfamethoxazole (25 µg) (SXT).

Statistical analysis:

All values are expressed as means ± SD. Statistical significance was evaluated using the Tukey–Kramer HSD test. $P < 0.05$ was used to indicate statistical significance using JMP statistical package, SAS Institute Inc., Cary, NC.

Table 1: Oligonucleotides' sequences used in the present study

Primer	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
16S rRNA (F)	5′ CTTTCAGCGGGGAGGAAGG ′3	390	(15)
16S rRNA (R)	5′ TCAACCTCCAAGTCGACATCGT ′3		
stx1 (F)	5′ ACACTGGATGATCTCAGTGG ′3	614	(14)
stx1 (R)	5′ CTGAATCCCCCTCCATTATG ′3		
stx2 (F)	5′ CCATGACAACGGACAGCAGTT ′3	779	
stx2 (R)	5′ CCTGTCAACTGAGCAGCACTTTG ′3		

Results

All examined milk samples from all animal species had normal organoleptical characteristics at sensory evaluation (Data are not shown). Bacteriological examination of the examined milk samples at the present study revealed that the average values of TBC were 4.84 ± 0.28, 3.69 ± 0.15, and 2.69 ± 0.21-log cfu/g in the examined cattle, buffaloes, and camel milk samples, respectively (Fig. 1A). The calculated mean values of the Most probable number of coliforms (MPN) were 3.20 ± 0.15, 2.59 ± 0.24 and 1.71 ± 0.22-log MPN/g in the same examined samples, respectively (Fig. 1B).

MPN of *E. coli* was also evaluated using EC broth. The calculated values of MPN of *E. coli* in the examined samples were 2.92 ± 0.15, 2.13 ± 0.11, and 1.17 ± 0.08-log MPN/g, respectively (Fig. 2A). The prevalence rates (%) of *E. coli* in the examined samples were 50% in cow's milk samples, 20% in buffalo's milk samples, and 10% in the examined camel's milk samples, respectively (Fig. 2B). Serotyping of the recovered *E. coli* revealed six serotypes, namely *E. coli* O2:H6 at 31.25%, *E. coli* O26:H11 at 25%, *E. coli* O55:H7 at 18.75%, *E. coli* O78:H- at 12.5%, *E. coli* O86:H11 at 6.25%, and *E. coli* O127:H6 at 6.25% (Fig. 3). All recovered *E. coli* isolates had *16S rRNA* as detected by PCR. Besides, detection of shiga toxin coding genes (*stx1*, and *stx2*) among the recovered *E. coli* isolates demonstrated that *stx1* could be detected in three *E. coli* serotypes (*E. coli* O2:H6, *E. coli* O26:H11, and *E. coli* O55:H7) recovered from cow's milk. While *stx2* could be detected in four *E. coli* serotypes (*E. coli* O2:H6, *E. coli* O26:H11, *E. coli* O55:H7, and *E. coli* O78:H) recovered from cow's milk (Table 2).

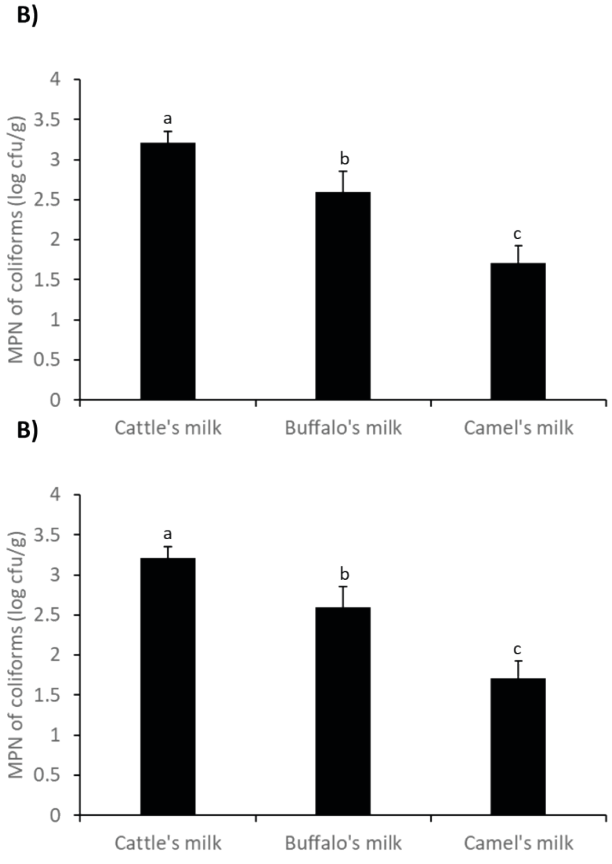


Figure 1: A) Total bacterial count B) MPN of coliforms. Values represent means ± SD in the examined milk samples of cattle, buffaloes, and camel. Columns with different letter are significantly different at $p < 0.05$

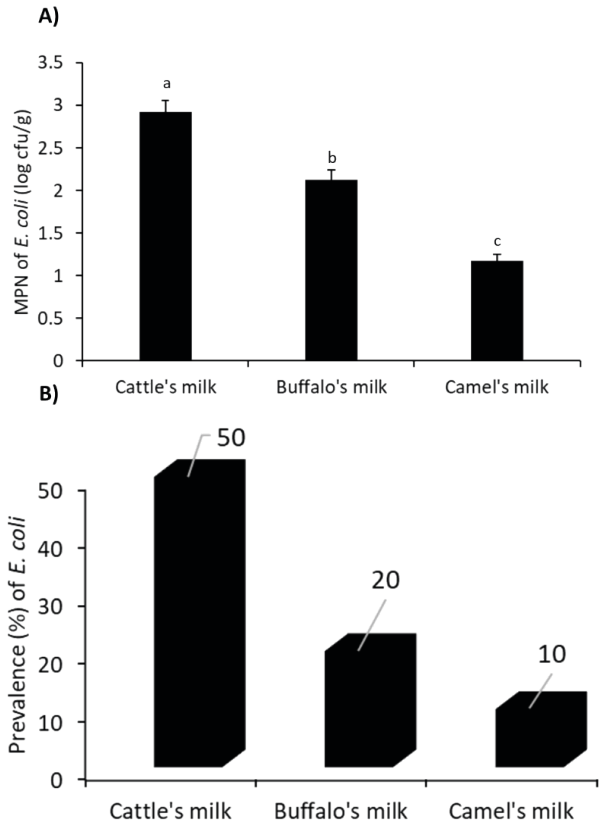


Figure 2: A) MPN of *E. coli*, B) prevalence (%) of *E. coli* isolation (n = 20/each). Values of MPN of E. coli represent means ± SD in the examined milk samples of cattle, buffaloes, and camel. Columns with different letter are significantly different at $p < 0.05$

Table 1: Antimicrobial resistance profiling and detection of *16S r*RNA, *stx1*, and *stx2* genes in the recovered *E. coli* isolates from milk samples collected from cattle, buffaloes, and camel

Isolate number	Serotype	Origin	*16S rRNA*	*stx1*	*stx2*	antimicrobial resistance profiling	MAR index
1	*E. coli O2: H6*	Cow's milk	+	+	+	AM, CN, C, CP, En, E, G, K, NA, N, OX, T, P, SXT	1
2	*E. coli O2: H6*	Cow's milk	+	-	+	AM, CN, C, CP, En, E, G, K, NA, N, OX, T	0.857
3	*E. coli O2: H6*	Cow's milk	+	-	-	AM, CN, C, CP, En, E, G, K, NA, N	0.714
4	*E. coli O2: H6*	Buffalo's milk	+	-	-	AM, CN, C, CP, En, E, G, K, NA	0.642
5	*E. coli O2: H6*	Camel's milk	+	-	-	AM, CN, C, CP, En, E	0.428
6	*E. coli O26: H11*	Cow's milk	+	+	+	AM, CN, C, CP, En, E, G, K, P, K, NA	0.785
7	*E. coli O26: H11*	Cow's milk	+	-	-	AM, CN, C, CP, En, E, G, P	0.571
8	*E. coli O26: H11*	Buffalo's milk	+	-	-	AM, CN, C, CP, En, E	0.428
9	*E. coli O26: H11*	Camel's milk	+	-	-	AM, G, K, NA, T	0.357
10	*E. coli O55: H7*	Cow's milk	+	+	+	AM, CN, C, CP, En, E, G, K, NA, N, OX, T	0.857
11	*E. coli O55: H7*	Cow's milk	+	-	-	AM, CN, C, CP, En, E, G, K, NA, N	0.714
12	*E. coli O55: H7*	Buffalo's milk	+	-	-	AM, CN, C, CP, En, E, G, K, NA	0.643
13	*E. coli O78: H-*	Cow's milk	+	-	+	AM, CN, C, CP, En, E, G, K, NA, N, P	0.785
14	*E. coli O78: H-*	Buffalo's milk	+	-	-	AM, CN, C, CP, En, E, K, NA	0.571
15	*E. coli O86: H11*	Cow's milk	+	-	-	AM, CN, C, CP, En, E, G, K, NA, N, OX, P	0.857
16	*E. coli O127: H6*	Cow's milk	+	-	-	AM, CN, C, CP, En, E, G, K, NA, N, OX	0.786
						Average MAR index	0.688

Table 2: Antimicrobial resistance rates among the recovered E. coli serotypes

	O2		O26		O55		O78		O86		O127	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
AM	5	100	4	100	3	100	2	100	1	100	1	100
CN	5	100	3	75	3	100	2	100	1	100	1	100
C	5	100	3	75	3	100	2	100	1	100	1	100
CP	5	100	3	75	3	100	2	100	1	100	1	100
En	5	100	3	75	3	100	2	100	1	100	1	100
E	5	100	3	75	3	100	2	100	1	100	1	100
G	4	80	3	75	3	100	1	50	1	100	1	100
K	4	80	2	50	3	100	2	100	1	100	1	100
NA	4	80	2	50	3	100	2	100	1	100	1	100
N	3	60	2	50	2	66.66	1	50	1	100	1	100
OX	2	40	2	50	1	33.33	0	0	1	100	1	100
T	2	40	1	25	1	33.33	0	0	0	0	0	0
P	1	20	2	50	0	0	1	50	1	100	0	0
SXT	1	20	0	0	0	0	0	0	0	0	0	0

No. Number of isolates, %: Percentage of isolates, AM: Ampicillin; CN: Cephalothin; C: Chloramphenicol; CP: Ciprofloxacin; En: Enrofloxacin; E: Erythromycin; G: Gentamicin; K: Kanamycin; NA: nalidixic acid; N: Neomycin; OX: Oxacillin; T: Oxytetracycline; P: Penicillin; SXT: trimethoprim/sulfamethoxazole

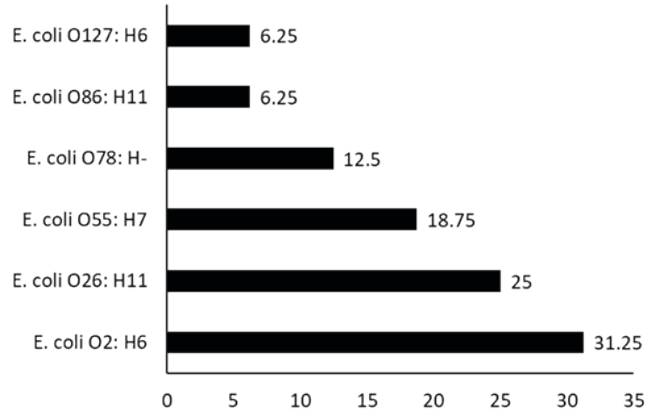


Figure 3: Prevalence (%) of different *E. coli* serotypes recovered in the present study

Tables 2 and 3 show the drug resistance profiling of the recovered *E. coli* pathotypes in the current investigation. The resistance profile of *E. coli O2: H6* was 100% to AM, CN, C, CP, En, and E; 80% to G, KA, and NA; 60% to N, and less than 50% to the other tested antimicrobials. 78.94% to polymyxin B; 73.68% nalidixic acid and less than 50% to other tested antimicrobials. *E. coli O26: H11* had 100% resistance to AM; 75% to CN, C, CP, En, E, and G; While 50% or less to the other tested antimicrobials. *E. coli O55: H7* had 100% resistance to AM, CN, C, CP, En, E, G, K, and NA, 66.66% to N; While less than 50% to the other tested antimicrobials. *E. coli O78: H-* had 100% resistance to AM, CN, C, CP, En, E, G, NA, and N; While 50% or less to the other tested antimicrobials. The recovered isolate of *E. coli O86: H11* had 100% resistance to all tested antimicrobials, but sensitive to T, and SXT. Similarly, the recovered isolate of *E. coli O127: H6* had 100% resistance to all tested antimicrobials, but sensitive to T, P, and SXT. The recovered isolates had an average MAR index of 0.688.

Discussion

Milk is regarded as an essential nutrient for all ages due to its high content of protein, fat and minerals. Camel milk in particular has been used for centuries as a food and as a traditional medication for many diseases such as diabetes, allergy, and asthma (18). However, milk can be easily contaminated during the production cycle starting from milking, processing, packaging, and distribution. Few reports investigated the bacteriological status of camel milk in comparison with the milk of other species. In the present study, the bacteriological status of the milk collected from three animal species, cattle, buffaloes, and camel, was evaluated. Cattle milk had significantly ($p < 0.05$) the highest bacterial counts in terms of TBC, MPN of coliforms, and MPN of *E. coli*, followed by samples collected from buffaloes. While camel had the lowest counts of such parameters. Similarly, *E. coli* was isolated from the milk samples of cattle, buffaloes, and camel at 50%, 20%, and 10%, respectively. In agreement with the recorded results of the present study, camel milk was found contaminated at higher levels in Samara-Logia Town of Afar National Regional State, Northeast Ethiopia, compared to the recorded values in the present study, as TBC, and total coliforms counts (log 1o cfu/mL) were 6.37, and 4.87, respectively. *E. coli* was also isolated at higher rate 24.6% in the same study (19). *E. coli* was also isolated from raw milk marketed at Taif region (Western Saudi Arabia) (20). Raw milk samples collected from Peninsular, Malaysia had higher TBC counts compared with the present study. Besides, *E. coli O157:H7* was isolated at 33.5%, while not detected in the current investigation (21). Raw cow's milk collected from Shahrekord, Iran was found contaminated with coliforms and *E. coli* at higher rates at 79%, and 69%, respectively. In Egypt, raw milk marketed in Dakahlia governorate was found highly contaminated as TBC and total coliforms counts were recorded at 5.46×10^7 and 8.42×10^6, respectively (23). The obtained results of the present study revealed identification of several *E. coli* pathotypes. Similarly, *E. coli O26:H11, E. coli O55:H7, E. coli O78:H-, E. coli O111:H4, and E. coli O127:H6* were recovered and identified from dairy products retailed in Egypt (15). Shiga toxin coding genes are also detected in three *E. coli* isolates for *stx1*, and 4 isolates for *stx2*. Likely, shiga toxin related genes were also detected in E. coli isolated from raw milk in Taif, Saudi Arabia (20). Consumption of raw milk was associated with the onset of several outbreaks and food poisoning cases worldwide. For instances, an outbreak of *E. coli O157: H7* associated with raw milk consumption was reported in the Pacific Northwest, United States during 2005 (24). Moreover, raw milk consumption was linked to foodborne infection cases with shiga toxin producing *E. coli* in several European countries (25).

Development of drug-resistant pathogens, which is viewed as a serious health threat, is mostly

caused by the uncontrolled use of antibiotics in animal production (10, 11). The recovered *E. coli* pathotypes in the current investigation were characterized by a high prevalence of multidrug resistance. Studies from Brazil, Egypt, and Romania all reported isolation of multidrug resistant *E. coli* in raw milk (26, 27, 28). Therefore, it is strongly advised that antimicrobials be used responsibly in the livestock production and in dairy farms.

In conclusion, the current investigation identified and isolated multidrug-resistant and shiga toxin-producing *E. coli* from milk sold in Zagazig, Egypt, from cows, buffaloes, and camels. Therefore, when handling milk, extra hygiene precautions should be used. Additionally, it is strongly advised against consuming any raw milk, even from camel milk. Furthermore, it is strongly recommended to do ongoing research on the occurrence of STEC in milk.

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Conflict of interest: None

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Original Research Article
Veterinary Medicine and The One Health Concept

GENOTYPIC CHARACTERIZATION OF SOME PATHOGENIC FUNGI ISOLATED FROM POULTRY AND THEIR SURROUNDINGS

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Abstract: Regarding high morbidity, mortality, and production losses, fungi infections have their importance among infectious illnesses and seem to be one of the main challenges facing poultry producers. This study aims to identify the genotypic characteristics of some fungi isolated from poultry. To reach this end, in El-Gharbia Governorate, Egypt, a total of 210 birds with a history of respiratory distress were randomly selected from a variety of private farms and hatcheries. The birds were sacrificed; tissue pieces were collected. In addition, a total of 87 samples of the poultry surroundings including 40 samples of poultry ration, 14 bedding materials, 4 air samples, and 29 water samples were collected. Using traditional fungal isolation, four fungal species were recovered, namely, *Aspergillus niger, Aspergillus flavus, Cladosporium perangustum,* and *Penicillium chrysogenum*. PCR was performed by fungus-specific universal primer pairs (ITS1 and ITS4) to identify and describe the genotype of isolated fungi. All examined isolates' ITS1-5.8SrDNA regions could be amplified. A purified PCR product was sequenced according to the Emerald Amp GT PCR master mix. This was initially performed to establish sequence identity to GenBank accession numbers. The rRNA gene for 5.8 sRNA divides the two ITS sections, which are situated between the 18S and 28S rRNA genes. ITS-1 gene sequence of the isolated *Cladosporium perangustum* (GeneBank accession number was OM 407392). The Sequence of the ITS-1 of isolated *Penicillium chrysogenum* (GeneBank accession numbers for studied nucleotide sequences were OM407401; OM407402; OM403685, and OM403686). For the examined nucleotide sequences, the GeneBank accession number for the ITS-1 internal transcribed spacer region of single *Aspergillus niger* was OM407391. GeneBank accession numbers for the isolated *Aspergillus flavus* ITS-1 sequence examined nucleotide sequences were OM403676, OM403677, and OM403678. In conclusion, genotypic characterization confirmed the phenotypic traditional fungal identification in the present study. Aspergillus species are the major fungi associated with birds in Egypt farms. The predominantly identified species were *Aspergillus flavus* and *Penicillium chrysogenum.*

Key words: phenotypic; genotypic; molecular; fungi; poultry

Introduction

Poultry is one of the fastest-growing elements of Egypt's agricultural industry, providing income to both skilled and unskilled laborers. It also provides an extra source of income as well as an affordable and accessible supply of protein for several poor households (1). However, lack of knowledge and skills, insufficient finance at all levels, and marketing are all challenges in chicken production (2). Chickens are susceptible to a variety of severe infections that reduce productivity and affect welfare, resulting in significant mortality in some situations. Regarding high morbidity, mortality, and production losses, fungi have their importance among infectious illnesses and appear to be a significant barrier for chicken producers (3). Many fungal infections affect poultry production, including Aspergillosis, Candidiasis, Dactylariosis, Favus, Mucormycosis, Histoplasmosis, and Cryptococcosis. Aspergillosis and Candidiasis are the most common fungal illnesses, although

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Cryptococcosis and Histoplasmosis are also zoonotic (4).

Fungi cause disease in two ways: i) damaging the host's bodily tissues and infiltrating them, and ii) creating mycotoxin during harvesting and storage of crops, which results in illness, immunosuppression, and decreased production potential when ingested (5).

Infections with fungi are frequently linked to morbidity and mortality in birds. Even when the best conditions of culture, harvest, storage, and handling were used, fungi and their toxins are inherent pollutants of the environment, particularly foods. The introduction of outside air containing conidia, which may readily fulfill the nutritional requirements of many fungal species, including Aspergillus species, is another possible source of contamination (6). The majority of fungal diseases in poultry are manifested as high morbidity, mortality, and production on a sporadic basis, but they can also be manifested as an outbreak (5).

The fungal infection primarily affects poultry's respiratory and neurological systems, causing the host to experience pathological changes including inflammation, lesions, and illness that ultimately result in death (3). Risk factors that enhance the possibility of acquiring a fungus and making the situation worse include stress, immunosuppressive sickness, insufficient vitamin D, poor hygiene, chronic use of drugs that impede natural bacterial flora, and malnutrition (7).

The most common fungal illnesses in poultry are aspergillosis and candidiasis (8). Because it causes economic losses in the poultry business, aspergillosis has arisen as a major poultry health concern for poultry producers and human health regulators. Aspergillosis is an infectious, non-contagious disease affecting human beings, mammals, and primarily wild or domestic birds. The most harmful fungus that affects poultry, *Aspergillus fumigatus*, causes aspergillosis, a disease of lung with hematogenous dissemination that causes necrosis and granulomatous cavities (9). Due to the lower spore size compared to other Aspergillus species, Aspergillus fumigatus infection in poultry is more prevalent. *A. flavus, A. terreus, A. glaucus, A. nidulans, and A. niger* are other Aspergillus species that can harm birds (10).

Traditional microscopic and cultural features are still often utilized to identify fungi. Conidial and mycelial color, colony reverse color, colony diameter, soluble pigments, and exudate production are used to make macro-morphological identification. Microscopically, seriation, vesicle size and form, conidia, and stipe morphology all play major roles in fungal identification **(**11). However, these approaches are time-consuming, involve extensive technological skill, and are occasionally ineffective due to the unusual characteristics of isolates. Hence, it is now possible to identify fungi more quickly and accurately thanks to molecular methods (12).

PCR allows the rapid detection of fungal species directly from clinical specimens with good specificity, DNA barcoding with dual loci (ITS) offer optimal accuracy and next generation sequencing technologies offer highly discriminatory analysis of genetic diversity including outbreak investigation, and for drug resistance characterization. Advances in molecular technologies will further enhance routine fungal diagnostics (13).

The aim of this study is to identify the genotypic characteristics of some fungi isolated from poultry and their surroundings.

Material and methods

Sample collection

In El-Gharbia Governorate, Egypt, a total of 210 birds with a history of respiratory distress were randomly selected from a variety of private farms and hatcheries (180 broiler chicks at age of 1:10 days, 5 Baladi breeds at age of 30:45 days, and 25 Saso chickens at age of 30 days). When compared to other farms between November 2020 and July 2021, these farms displayed a distinct mortality rate. The birds' tissues (lung, air sac, liver, crop, and trachea) were taken from each bird.

In addition, a total of 87 samples of the surroundings of the poultry including (40 samples of poultry ration, 14 bedding materials, 4 air samples and 29 water samples) were collected.

Identification and mycological examination according to previous method (14)

The prepared samples were cultured on to Sabouraud dextrose agar plates with a sterile bent glass rod. The cultured plates were incubated at 25°C and examined daily for five days. Mold colonies were picked up and sub-cultured on slope

agar for further identification. The mold culture was purified by subculturing on Sabouraud agar plates, incubated at 25°C for 3-5 days and examined for macro and micromorphological characters.

Genotypic and molecular characterization

The genotypic characterization was performed at Animal Health Research Institute, Eldokki, Cairo, Egypt.

Extraction of DNA

For identification and genotypic characteristics of the isolated fungi, PCR was carried out using the QIAamp DNeasy Plant Mini kit Catalogue No. 69104, the internal transcribed spacer1 (ITS 1 Oligonucleotide primer sequence**:** TCCGTAGGT-GAACCTGCGG), and (ITS 4 Oligonucleotide primer sequence: TCC TCC GCT TAT TGA TAT GC) according to a previous method (15).

This was performed according to QIAamp DNeasy Plant Mini kit instructions.

Making a traditional PCR Master Mix

The Emerald Amp GT PCR master mix (Takara) Code No. RR310A kit was used to sequence a purified PCR product. This was initially carried by mixing Emerald Amp GT PCR master mix (2x premix) 12.5 *µl*, PCR grade water 5.5 *µl*, Forward primer (20 pmol) 1 *µl* , Reverse primer (20 pmo1) 1*µl* and Template DNA 5 *µl* to verify the sequence's connection to GenBank accessions. **Table (1)** displays the temperature and timing parameters for the two primers during PCR. 100 bp gene ruler DNA ladder from Fermentas (cat. no. SM0243) was used during electrophoresis. During electrophoresis grade agarose (1.5 g) (16) was used after the addition of 0.5 g/ml ethidium bromide. The positive control, the negative control, and 20 µl of each PCR product sample were placed onto the gel. A gel documentation system was utilized to take pictures of the gel, and computer software was used to analyze the results.

Phylogenetic research

The sequences (17) in MEGA (Molecular Evolutionary Genetics Analysis is asoftware enables comparative analysis of molecular sequences in phylogenetics) were compared using the CLUSTAL W multiple sequence alignment technique, version 1.83 of the MegAlign module of Lasergene DNAStar software Pairwise, and phylogenetic analysis was performed using maximum likelihood, neighbor joining, and maximum parsimony (18).

Results

Incidence of molds in poultry samples

From 210 birds with 1050 tissue pieces, 306 fungal spp. were isolated and identified. The highest incidence was in lungs among the broiler (25%) and Baladi (0.9%) birds followed by the air sac (16.44%) then trachea (14.37). In Saso birds, the highest incidence was in air sacs (6.2%) followed by the lungs (5.5%) then the trachea (4.4%).

Mycological examination of 87 surroundings samples revealed the isolation of 19 strains mold spp**.,** the mycotic incidence was the highest in ration (50%) followed by the bedding (28.5%)**.**

The most prevalent fungi isolated are *Aspergillus spp.* especially *Aspergillus flavus,* and *Peniciillium spp.*

Using traditional fungal isolation, four fungal species were recovered, namely, *A. niger, A. flavus, Cladosporium perangustum,* and *Penicillium chrysogenum.* The recovered isolates were further identified, and their genotyping characteristics were determined using PCR and a universal primer unique to fungi. The rRNA gene for 5.8 sRNA divides the two ITS sections, which are situated between the 18S and 28S rRNA genes (Fig. 1-4).

Molecular identification of the isolated molds

Confirmatory identification, phylogenetic analysis, and multiple alignments:

The sequence of the ITS-1 of isolated *Cladosporium perangustum* (ES2: isolated from ration) GenBank accession number for the studied nucleotide sequence was OM 407392. More than 99 percent of the sequences found for the ITS-1 region matched the relevant GenBank sequences (accession no. MT645915; KT600413; KT600414; HM148147 and HM148141) (Fig. 5).

Table 1: Cycling conditions of the different primers during conventional PCR

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
ITS	95˚C 5 min.	94˚C 30 sec.	56˚C 40 sec.	72˚C 50 sec.	35	72˚C 10 min.

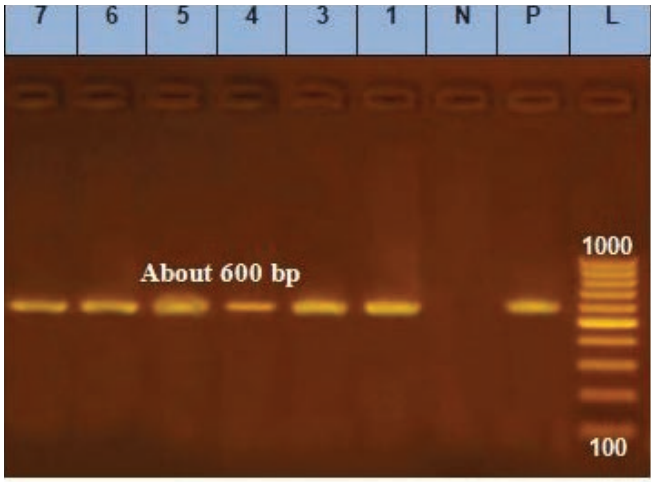


Figure 1: PCR findings for six fungal isolates. Amplification of the 600 bp fragment of ITS1 gene from tested isolates (Lanes 1-7) showed a positive amplicon migration with the molecular size of about 600 bp using the molecular DNA size marker.

L: 100 bp ladder. P: control positive.
N: control negative.

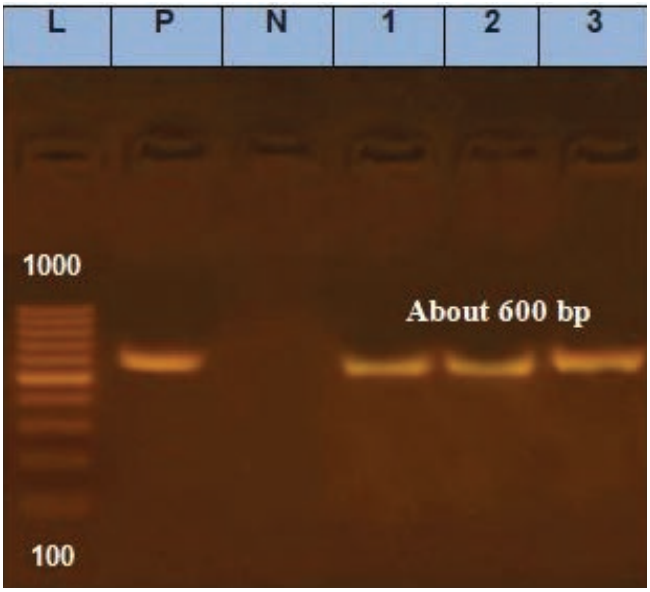


Figure 2: PCR findings for three fungal isolates. Amplification of the 600 bp fragment of ITS1 gene from test-ed isolates (Lanes 1_ 3) showed a positive amplicon migration with the molecular size of about 600 bp using the molecular DNA size marker. L: 100 bp ladder. P: control positive. N: control negative.

The sequence of the ITS-1 of the isolated *Penicillium chrysogenum* (ES6: isolated from bedding materials, ES7: isolated from lung tissue and ES1: isolated from ration) GenBank accession numbers for the studied nucleotide sequences were OM407401; OM407402; OM403685, and OM403686. Over 99 % of the matched GenBank sequences and the ITS-1 region sequences were comparable (accession no. KP27816).

GenBank accession numbers for the examined nucleotide sequences were OM407391 for the isolated *Aspergillus niger* (ES9: isolated from bedding materials and air samples) isolate's internal transcribed spacer-1 region (ITS-1). The sequences obtained for the ITS-1 region obtained were 100% identical to the corresponding GenBank (accession no. MG 228419; MG228418; MG228417; MG228416; MH855726, and MH855928). For the examined nucleotide sequences, the GenBank accession codes for the ITS-1 of isolated *Aspergillus flavus* (ES4: isolated from lung tissue, ES5: isolated from air sac and ES8: isolated from trachea) were OM403676, OM403677, and OM403678. The sequences obtained for the ITS-1 region were 100% identical to the corresponding GenBank (accession no. MT292809; CP051089; MT462229; MT629885; MN095128, and MH864265).

Discussion

The present study was conducted aiming to the genotypic characterization of some fungi isolated from broiler, Baladi, and Saso chicken from different farms in Egypt.

The most prevalent fungal species isolated was Aspergillus species especially *Aspergillus flavus* and *Penicillium spp.* These findings were in line with a previous report (19), where *Aspergillus spp.*, and *Penicillium spp.*, were the most prevalent fungal species detected in poultry.

Fungal diseases are a severe threat to human and animal health around the world. Infections with fungi are frequently linked to morbidity and mortality in birds. Even when the best conditions of culture, harvest, storage, and handling were



Figure 3: ITS-1 and ITS-2 Nucleotide sequence alignment of *A. flavus; A. niger; P. chrysogenum and C. perangustum* and other intra- and interspecies fungal strains

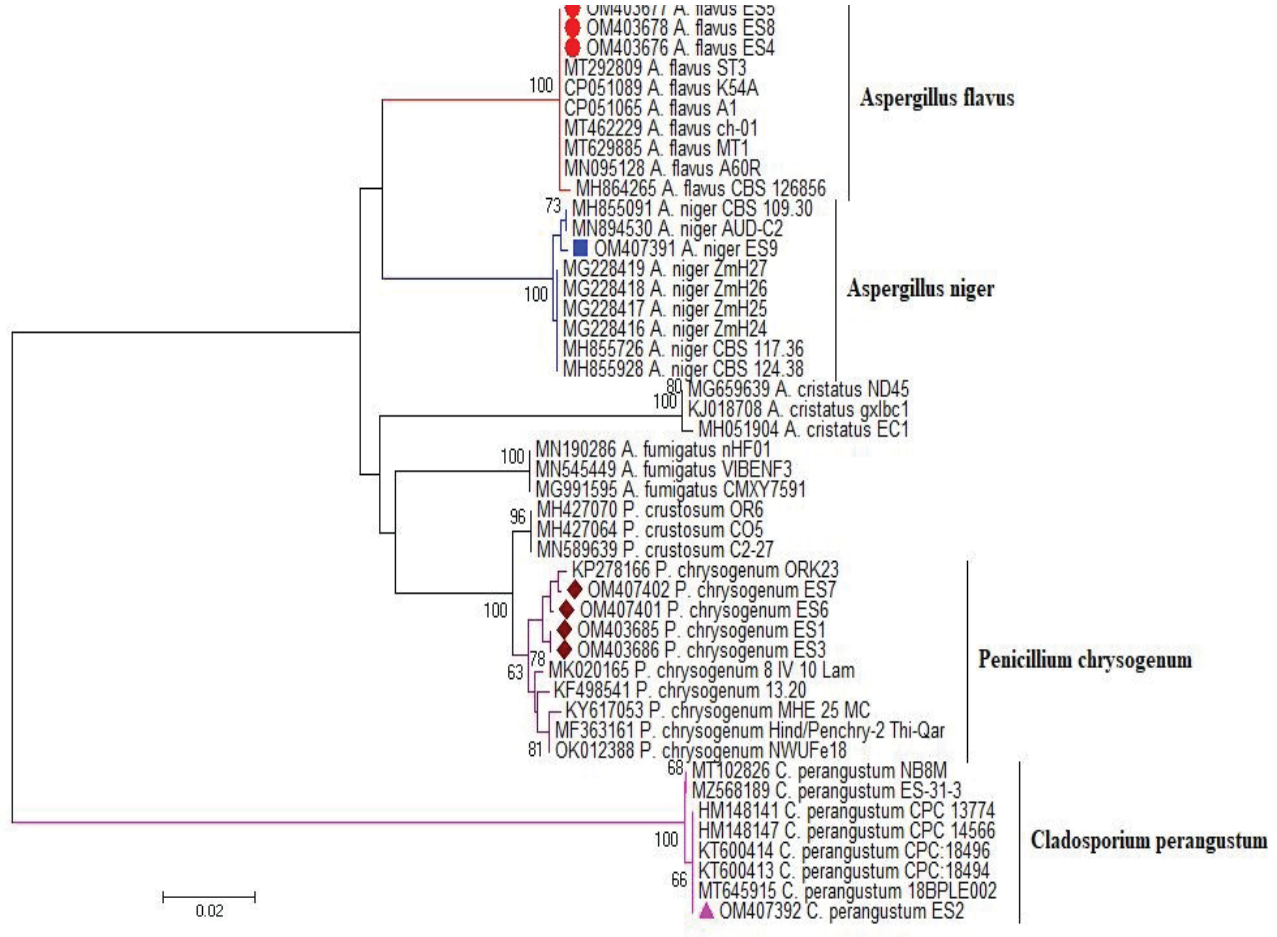


Figure 4: Phylogenetic analysis of strains (ES4, ES5, ES8, ES9, ES1, ES6, ES7, ES3, and ES2) with other intra- and interspecies strains. The tree is showing that the strains are very close to the other stains. Other strains are showing lesser or greater distance, filled squares indicate our isolated strains

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		
1		99.8	99.8	99.8	99.8	85.8	85.8	85.6	64.7	64.9	64.7	85.3	85.8	85.4	86.0	85.8	85.3	76.7	76.9	84.3	84.3	1	MH864265 A. flavus CBS 126856
2	0.2		100.0	100.0	100.0	86.0	86.0	85.8	64.9	65.1	64.9	85.4	86.0	85.6	86.2	86.0	85.4	76.9	77.1	84.5	84.5	2	MT629885 A. flavus MT1
3	0.2	0.0		100.0	100.0	86.0	86.0	85.8	64.9	65.1	64.9	85.4	86.0	85.6	86.2	86.0	85.4	76.9	77.1	84.5	84.5	3	OM403676 A. flavus ES4
4	0.2	0.0	0.0		100.0	86.0	86.0	85.8	64.9	65.1	64.9	85.4	86.0	85.6	86.2	86.0	85.4	76.9	77.1	84.5	84.5	4	OM403677 A. flavus ES5
5	0.2	0.0	0.0	0.0		86.0	86.0	85.8	64.9	65.1	64.9	85.4	86.0	85.6	86.2	86.0	85.4	76.9	77.1	84.5	84.5	5	OM403678 A. flavus ES8
6	11.7	11.4	11.4	11.4	11.4		100.0	99.8	64.0	64.2	64.0	85.6	86.8	85.6	86.0	86.2	85.8	76.5	76.9	88.1	88.1	6	MG228419 A. niger ZmH27
7	11.7	11.4	11.4	11.4	11.4	0.0		99.8	64.0	64.2	64.0	85.6	86.8	85.6	86.0	86.2	85.8	76.5	76.9	88.1	88.1	7	MH855726 A. niger CBS 117.36
8	11.9	11.7	11.7	11.7	11.7	0.2	0.2		63.8	64.0	63.8	85.4	86.6	85.4	85.8	86.0	85.6	76.3	76.7	87.9	87.9	8	OM407391 A. niger ES9
9	37.1	36.7	36.7	36.7	36.7	38.8	38.8	39.2		99.8	100.0	65.3	66.0	65.3	65.7	65.9	65.1	61.4	61.2	64.0	64.0	9	HM148141 C. perangustum CPC 13774
10	37.1	36.7	36.7	36.7	36.7	38.8	38.8	39.2	0.0		99.8	65.5	66.2	65.5	65.9	66.0	65.3	61.6	61.4	64.2	64.2	10	KT600413 C. perangustum CPC:18494
11	37.1	36.7	36.7	36.7	36.7	38.8	38.8	39.2	0.0	0.0		65.3	66.0	65.3	65.7	65.9	65.1	61.4	61.2	64.0	64.0	11	OM407392 C. peranguhstum ES2
12	13.0	12.7	12.7	12.7	12.7	13.4	13.4	13.6	37.5	37.5	37.5		97.4	99.4	99.3	99.4	99.3	77.8	78.2	87.3	87.3	12	KP278166 P. chrysogenum ORK23
13	12.5	12.3	12.3	12.3	12.3	12.6	12.6	12.9	36.5	36.5	36.5	2.2		97.9	98.1	97.9	98.1	79.3	79.7	88.4	88.4	13	KF498541 P. chrysogenum 13.20
14	12.7	12.5	12.5	12.5	12.5	13.4	13.4	13.6	37.5	37.5	37.5	0.6	1.6		99.4	99.3	99.4	78.2	78.5	87.7	87.7	14	OM403685 P. chrysogenum ES1
15	12.2	12.0	12.0	12.0	12.0	13.1	13.1	13.3	37.0	37.0	37.0	0.6	1.6	0.4		99.8	98.9	78.5	78.9	88.1	88.1	15	OM407401 P. chrysogenum ES6
16	12.5	12.2	12.2	12.2	12.2	12.9	12.9	13.1	36.7	36.7	36.7	0.4	1.8	0.6	0.2		98.7	78.4	78.7	87.9	87.9	16	OM407402 P. chrysogenum ES7
17	13.0	12.7	12.7	12.7	12.7	13.1	13.1	13.4	37.9	37.9	37.9	0.8	1.4	0.6	1.0	1.2		78.0	78.4	87.9	87.9	17	OM403686 P. chrysogenum ES3
18	15.9	15.6	15.6	15.6	15.6	15.5	15.5	15.8	37.6	37.6	37.6	14.3	12.5	13.8	13.5	13.7	14.1		99.6	77.8	77.8	18	MH051904 A. cristatus EC1
19	15.8	15.6	15.6	15.6	15.6	15.2	15.2	15.5	38.3	38.3	38.3	14.0	12.2	13.5	13.2	13.4	13.8	0.2		78.2	78.2	19	MG659639 A. cristatus ND45
20	12.3	12.0	12.0	12.0	12.0	9.3	9.3	9.6	37.7	37.7	37.7	9.0	7.9	8.6	8.3	8.6	8.3	13.7	13.4		100.0	20	MN190286 A. fumigatus nHF01
21	12.3	12.0	12.0	12.0	12.0	9.3	9.3	9.6	37.7	37.7	37.7	9.0	7.9	8.6	8.3	8.6	8.3	13.7	13.4	0.0		21	MN545449 A. fumigatus VIBENF3
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		

Figure 5: Sequence distance for confirming the percentage of identity between the isolated strains and other related stains on GenBank and all are identical by more than 99% or 100%

used, fungi and their toxins are inherent pollutants of the environment, particularly foods. Other contamination might include improper bedding management, poor quality feedstuff, or admission of outside air containing conidia that can easily meet the nutrient requirements of many fungal species, including *Aspergillus spp.,* (6).

Rapid and accurate separation of pathogenic *Aspergillus spp.* has become critical for selecting effective antifungal medication. Furthermore, species identification is essential for epidemiological and management purposes, such are the correct assessment of incidence rates, observation of the advent of novel species, and invasive aspergillosis outbreaks (20). Additionally, molecular characterization of *Aspergillus spp.* by PCR and sequencing of PCR products were very important)21). The goal of the current study was to genotypically characterize certain fungi that were isolated from broiler, Baladi, and Saso chicken as well as their surroundings, including their diet, bedding, air, and water samples, from various farms in Egypt.

In the present study, the molecular identification and genotyping characters of the isolated fungi were performed using PCR-based identification techniques. The identification systems for the isolated pathogens were based on ITS 1 and ITS 2 regions with the use of 18S or 28S rDNA as target DNA. This agreed to previous reports)7, 22(.

The fungi's sequenced ITS regions supported the morphological investigations' conclusions. In this investigation, the PCR products of the examined samples were identified as *Cladosporium perangustum, Penicillium chrysogenum, A. niger,* and *A. flavus*. The sequences obtained for the ITS-1 region of the isolated *Cladosporium perangustum*, and the isolated *Penicillium chrysogenum* were 99% identical to the corresponding GenBank records, while for the isolated *A. niger*, and *A. flavus* were 100% identical to the corresponding GenBank records.

It is concluded that *Aspergillus spp.* is associated with poultry birds in Egypt farms. The genotypic characterization of fungi from poultry and the surroundings showed that the predominantly identified species were *A. flavus* and *Penicillium chrysogenum*.

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Original Research Article
Veterinary Medicine and The One Health Concept

A COMBINED APPROACH OF MULTIPLE CORRESPONDENCE ANALYSIS AND HIERARCHICAL CLUSTER ANALYSIS FOR PROFILING RELATIONSHIPS AMONG CATEGORICAL RISK PREDICTORS: A BLUETONGUE CASE STUDY

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Abstract: Bluetongue (BT) is a non-contagious virus in the Reoviridae family that infects both wild and domestic animals. It causes economic losses and reduces infected animals' production and reproduction. This study aims to show the utility of MCA and HCCA in identifying relationships between categorical variables used as risk factors for Bluetongue disease. Six categorical variables (age, sex, season, species, locality, and BT serotyping) of 233 apparently healthy animals were screened for studying BT. Based on visualized information of MCA and HCA among variable categories, disease profiles were identified. The first two MCA dimensions retained up to 27% of the total inertia contained in the data. The positive BT results, summer, and old animals categories were loaded in the first dimension, while negative cases, Al-mounfia and winter categories were related to the second dimension. HCA identified three clusters. Cluster 1 was characterized by frequent and largely exclusive seronegative BT animals 91.67 % of animals in the cluster were seronegative, negative BTV category is the most important and related to cluster 1 with positive v-test=8.75. Cluster 3 can named a cluster of seropositive BT, up to 88% of cases were seropositive. We can conclude that seropositive BT is associated with summer and old age categories, whereas seronegative BT is associated with young age and winter categories, and thus MCA and HCA provide convenient and easy-to-interpret analytical tools for assessing categorical data relationships.

Key words: categorical data; multiple correspondence; inertia; factominR; hierarchical cluster

Introduction

Bluetongue virus (BTV) is a non-contagious vector-borne infection of both domestic and wild animals that belongs to the genus Orbivirus and the family Reoviridae. Bluetongue virus infection is spread to susceptible animals such as sheep, goats, and deer by mosquitoes, ticks, and species of biting midges (Culicoides) (1). Reduction in weight, low milk yield, wool breakage, and other economic losses are caused by this disease (2), and also can cause a high mortality rate (70%) in highly susceptible sheep (3).

Data in epidemiological studies almost collected in vast volumes using surveys and the response pattern involves many categories which may be either binary, ordinal, or nominal. Researchers are frequently interested in investigating the links between such sets of category variables. Consider doing independent chi-square tests for each pair of variables, or, in the case of binary or ordinal data, viewing a correlation matrix of the bivariate associations. However, for a high number of categorical variables, this paired technique would become tedious and the findings would be

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difficult to describe. More considerably, such a technique would only indicate the existence of a relationship but not which response categories are associated (4).

Multiple correspondence analysis (MCA) is one of a family of descriptive approaches and an extension of correspondence analysis that allows studying the pattern of relationships between numerous categorical dependent variables. CA's multivariate extension is used to analyze tables with three or more variables. Furthermore, MCA can be thought of as an expansion of principal component analysis for categorical variables, revealing patterns in large data sets. MCA aids in the distinct description of patterns of relationships using geometrical methods by situating each variable/unit of analysis as a point in a low-dimensional space. MCA can be used to map variables as well as individuals, allowing the creation of complex visual maps whose structure can be analyzed. Furthermore, this technique has the ability to connect both variable-centered and case-centered approaches (5).

The MCA technique, as opposed to the orthogonalization technique that underpins PCA, employs a distance measure. MCA converts the relationship between discrete variable categories into coordinates in a multidimensional space. It assigns scale values to the discrete variable categories and maximizes the variance of those scores to determine the correlations between the variables and the proximity of subjects. Points pointing in the same direction as the origin are strongly linked. The mean is represented by points near the origin, whereas points farther from the origin depart from the mean (6).

Clustering is a type of unsupervised learning job that discovers underlying structures in unlabeled data. Those are divided into homogeneous groups or clusters, with intracluster items having high resemblance while being quite distinct to objects in other clusters. Over the years, many clustering approaches have been suggested and implemented. Clustering methods are classified into two types: hierarchical clustering and partitional clustering. While hierarchical clustering uses agglomerative or divisive algorithms to build a hierarchy of partitions (i.e., a dendrogram) across the dataset, partitional clustering usually assumes a fixed number of clusters and strives to maximise homogeneity within the clusters (7).

Hierarchical cluster algorithms portray data as a tree of nodes, with each node representing a possible data categorization. Hierarchical algorithms can be used to cluster categorical data in two different manners: an agglomerative (bottom-up) and divisive (top-down). The latter, on the other hand, is less common. The agglomerative algorithm's core idea is to use a similarity metric to gradually assign objects to tree nodes. The fundamental drawback of hierarchical clustering is its poor pace. Another issue is that the clusters may merge, causing these methods to cause information distortion (8).

The chi-square test or logistic regression is frequently used in epidemiologic studies to assess the relationship of qualitative risk factors. MCA is not as widely used as these methods, despite its importance and convenience in representing relationship patterns in data. The purpose of this study is to use MCA and HCCA to describe and assess the relationship between bluetongue risk factors in five Egyptian governments and bluetongue seropositivity in small ruminants. Using the MCA graphical plot and HCCA clusters, it is simple to identify, if possible, any hidden patterns of animals based on these risk factors.

Material and methods

Source of data

The data were obtained from a previous epidemiologic study on 233 apparently healthy animals: 125 sheep (42 males and 83 females) and 108 goats (47 males and 61 females). The animals were screened for bluetongue seropositivity using competitive ELISA from April 2018 to March 2019. The description of the data is shown in table 1.

Methodology and model

There are K variables, each with a level of Jk, and the total number of Jk is equal to J. There are also as many observations as I. X represents the $I \times J$ matrix. When a correspondence analysis is performed on the indicator matrix, two groups of factor scores are produced: one for rows and one for columns. In general, these factor scores are adjusted so that the variance equals the appropriate eigenvalues.

Table 1: Epidemiologic qualitative variables for studying bluetongue

variables	Categories
1. Species	Sheep or goat
2. Age	Young (6- < 18 months), moderate (18- < 36 months), and old (≥ 36 months)
3. Sex	male and female
4. locality	Al-Sharkia, Al-Monfia, Al-Menia, Al-Giza, and Al-Suis
5. Season	winter, spring, summer, and autumn
6. Bluetongue	Positive or negative serotyping.

The probability matrix $Z = N\text{-}1X$ is calculated as the initial stage in the investigation. Where N is the total value of the arrangement's matrix, r is the vector of the total row Z (i.e., r = Z1, where 1 is the vector of 1), and c is the vector of the whole column. Dc = diag {c} and Dr = diag {r}. The factor score is calculated by decomposing a single number in the following equation:

$$D_r^{-\frac{1}{2}}(Z - rc^T) \qquad D_c^{-\frac{1}{2}} = P\Delta Q^T$$

(Δ is a diagonal matrix of single values, and Λ=Δ2 is a matrix of eigenvalues). The row and column factor scores (respectively) are obtained by means of the following equation:

$$F = D_r^{-\frac{1}{2}} P\Delta \qquad G = D_c^{-\frac{1}{2}} Q\Delta$$

Distance squared (x2) of rows and columns can be denoted in the following equation:

$$d_r = \text{diag}\{FF^T\} \quad d_c = \text{diag}\{GG^T\} \quad (9).$$

Hypothesis and assumptions:

MCA is assumption-free, and when working with categorical data, it can represent both linear and non-linear connections equally well. These are significant advantages over more traditional methodologies, which may require linear correlations between variables and a prior hypothesis is about likely variable interactions (10).

Validity of MCA:

The validity of MCA was assessed according to Rodriguez-Sabate *et al.* (10) by:

1. Inertia: Which depicts data dispersion around their center of gravity G (or centroid) and is used as a measure of information. The term inertia is employed by analogy with the notion of "moment of inertia" in practical mathematics, which stands for the integral of mass times the squared distance between the centroids. Where;

$$\Delta^2 = \sum_i p_i + d_i^2$$

p_i is the marginal relativefrequency (or mass) of row i, and is the chi-square distance between the row i's profile and the average row profile, All aforementioned equations are analogous for column profiles (11). $d_i = \sqrt{\sum_j \left(\frac{(p_{ij}-p_{+j})^2}{p_{+j}}\right)}$

2. Total inertia which represents the inertia of all the dimensions analyzed, and ranged from 0 which denotes no information to 1 which denotes all available information.
3. Contribution of each variable in extracted dimensions, and ranged from 0 to 1.
4. The quality of a variable (cosine2) Cosine2 is a value represents the squared cosine value of the angle created by the point with the specified dimension. The closer the number is to one, the better the representation of the variable in the computed dimensions.
5. Eigenvalues Indicate the relative weight of each dimension in relation to total inertia (it is normalized to 1 which represents all the information of all the variables in all the dimensions). The first dimension always had the largest eigenvalue, which decreased steadily through the remaining dimensions. This variable (together with cumulative inertia) is typically used to determine the number of dimensions to include in the MCA. As a result, dimensions with eigenvalues less than 0.05 are commonly ignored.

Cluster analysis

Clustering is defined by two functions: the distance function and the linkage function. The distance function calculates the distances between the points, while the linkage function calculates the distance between clusters. Clustering outcomes frequently differ depending on the functions used. The distance between two points is defined as:

$$\varphi_{j,k} = \|p_j - p_k\| = \sqrt{(x_j - x_k)^2 + (y_i - y_k)^2}$$

Which is the Euclidean distance. The clusters depend on the choice of a clustering distance $d \geq 0$. Then, if two points satisfy $\varphi_{j,k} \leq d$, they are in the same cluster. Next, let A and B be two clusters containing points a_α and b_β, then the distance between two clusters is defined by:

$$\varphi(A, B) = \min_{\alpha,\beta} \varphi(a_\alpha, b_\beta)$$

which is known as single-linkage merge criterion (12, 13).

Ward's linkage is most commonly used with the Euclidean squared distance measure (14). At each step, Ward's linkage combines the two clusters, C_m and C_k, into one cluster C_l, that minimizes the total within-cluster squared error. The within-cluster squared error $S(C_l)^2$ of the lth cluster is defined as:

$$S(C_l)^2 = \sum_{i \in C_l} \sum_{p=1}^{P} (x_{ip} - \frac{1}{|C_l|} \sum_{j \in C_l} x_{jp})^2$$

where $|C_l|$ is the number of observations in cluster C_l, P is the number of variables measured for each observation, x_{ip} and x_{jp} denote the pth variable corresponding to observations i and j.

The total within-cluster squared error is the sum of within-cluster squared error over all K clusters (14, 15):

$$\sum_{l=1}^{K} S(C_l)^2$$

Software used

All analyses were conducted by SPSS version 25 (Armonk, NY: IBM Corp) and RStudio (16) using R (17).

Results

The results show that ten dimensions were extracted and described 100% of information of data. The first two dimensions 1 and 2 are sufficient to retain 27% of the total inertia (variation) contained in the data as shown in fig. 1. In table 2. The dimension's eigenvalue that is a measure

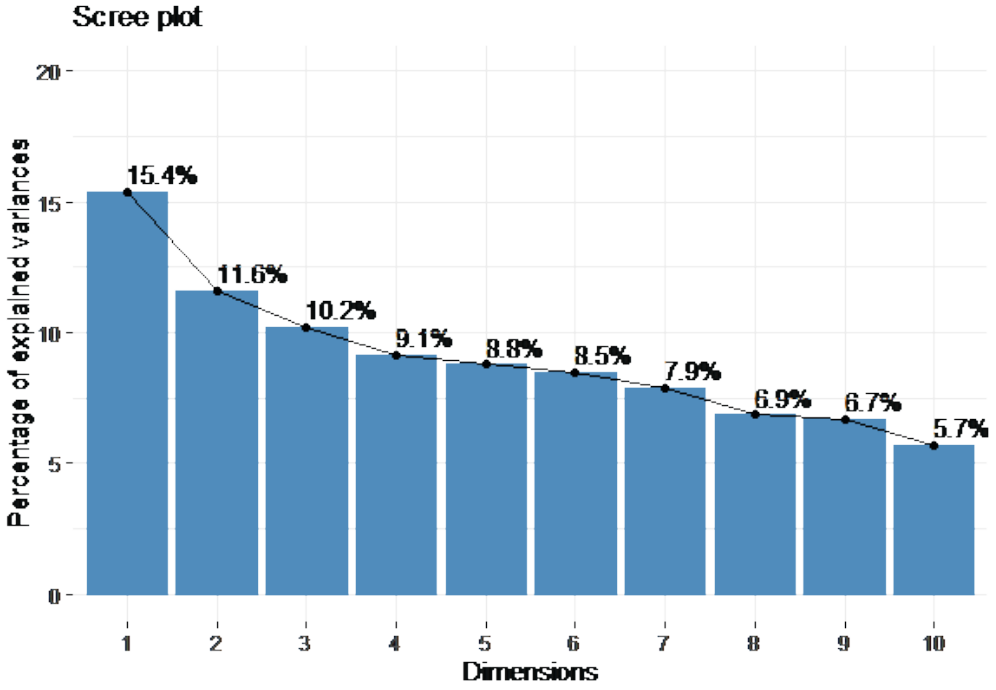


Figure 1: Scree plot for the main 10 extracted dimensions

Table 2: Discrimination and contributions of variables for the 1st and 2d dimensions

	Dimension 1			Dimension 2		
	Discrimination (R^2)	**contribution**	**Cos2**	**Discrimination (R^2)**	**contribution**	**Cos2**
Species	0.152	8.24%	0.3	0.131	9.43%	0.26
Sex	0.163	8.83%	0.32	0.092	6.63%	0.18
Age	**0.344**	18.65%	0.52	0.117	8.41%	0.17
Locality	**0.350**	18.94%	**0.53**	**0.408**	29.26%	0.45
season	**0.341**	18.45%	**0.47**	**0.474**	34.04%	0.63
BTV result	**0.496**	**26.87%**	1	0.170	12.22%	0.34
eigenvalues	0.31			0.23		
percentage of variance	15.39%			11.61%		

Cos2 denotes the squared cosine; Discrimination measures quantify the variance in each indicator.

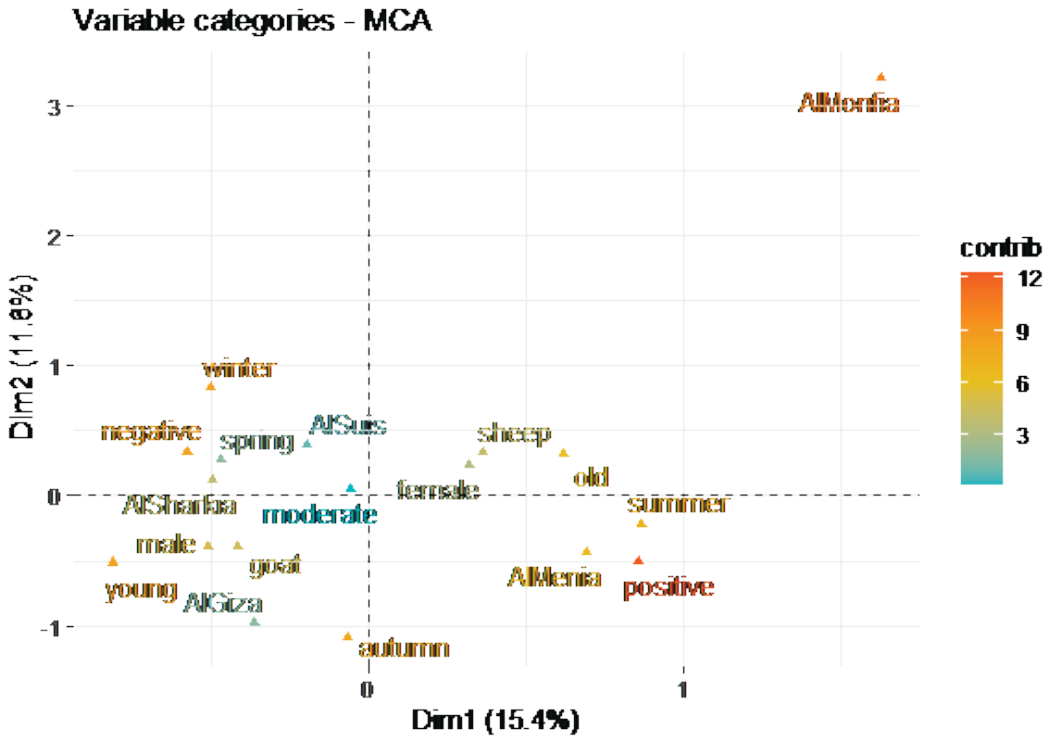


Figure 2: variable contributions on dimensions. Variable categories with low contribution values will be colored in "white", variable categories with mid contribution values will be colored in "blue", variable categories with high contribution values will be colored in "red"

of the amount of variance for which it accounts. Dimension 1 accounts for 0.31 of variance with percentage of 15.39% and dimension 2 for 0.23 of variance with 11.61%.

Table (2) and figure (2) help to find the variables that are most closely related to each dimension. The squared correlations between variables and the dimensions are used as coordinates. BTV result, locality, age, and season represent the indicators that most discriminating and making the largest contribution to dimension one. While locality and season are the most discerning indicators and contribute the most to dimension two. The squared cosine (cos2), which gauges the degree of association between different variable categories and a particular axis, measures discrimination (dis.), contribution (ctr.), and representation quality table (2). The most contributing and discriminating variables for the first dimension are BTV result (dis. = 0.496, ctr. = 26.87%), locality (dis. = 0.35, ctr. =18.94%), age (dis. = 0.344, ctr. =18.65%) and season (dis. = 0.341,

ctr. =18.45%). The cos2 of variable categories that can be extracted as in table (2). If a variable category is well represented by two dimensions, the sum of the cos2 is closed to one. The result BTV variable is the variable with highest cos2 = 1, then locality=0.53, age= 0.52, and sex=0.47. For the second dimension the most contributing and discriminating variables are season (dis. = 0.474, ctr. = 34.04%) and locality (dis. = 0.408, ctr. = 29.26%).

Table (3) lists all of the BTV-contributing factors in this study in descending order of significance, considering the coefficient of determination (R2) and the P value of the overall test (F-test). The R^2 value ranges from 0 to 1, with 0 being no relationship and 1 being a very strong relationship between the qualitative variable and the MCA dimension. The presence of sex categories (male and females) are somewhat far from positive and negative categories of bluetongue results, which is also confirmed by the significance of variables.

Whereas no other correlation was found as shown in table (4). Only correlations above 0.30 were considered to have meaningful practical significance. Table (5) shows the contribution of variables' categories for each of the first two dimensions. For dimension 1; the highest contributions and cos2 with positive coordinate (coord.) were for positive BTV (ctr. =16.03, cos2. =0.5, coord. = 0.86), summer season (ctr. = 11.98, cos2. =0.31, coord.= 0.86), old (ctr. = 8.73, cos2. =0.26, coord.= 0.62), and Al-Minea (ctr. = 8.55, cos2. =0.24, coord.= 0.69). When looking at the second dimension; the highest contributions and cos2 with positive coordinate were for Al- Monufia (ctr. = 19.06, cos2. =0.27, coord.= 3.21), winter

Table 3: Significance of test results for key BTV contributing factors in the top 2 dimensions

	R^2	P-value		R^2	P-value
Dim.1			**Dim.2**		
Result	0.496	< 0.00001	**season**	0.47	< 0.00001
Age	0.344	< 0.00001	**Locality**	0.41	< 0.00001
Season	0.341	< 0.00001	**Result**	0.17	< 0.00001
locality	0.3499	< 0.00001	**Species**	0.13	< 0.00001
sex	0.16	< 0.00001	**Age**	0.12	< 0.00001
species	0.15	< 0.00001	**sex**	0.09	< 0.00001

In dimension 1, age correlated (transformed variables) significantly with species (r = 0.26, r < 0.001), locality (r = 0.207, r < 0.001), and season (r = 0.142, r < 0.05); sex correlated with locality (r = 0.222, r < 0.001), locality correlated with season (r = 0.201, r < 0.01) dissimilar correlations were found for dimension 2, just age correlated with species (r = 0.154, r < 0.05) and sex with season (r = 0.13, r < 0.05).

Table 4: variables relationship on dimensions1&2

	Species	Sex	Age	Locality	Season
Dim.1					
Species	1				
Sex	0.102^{NS}	1			
Age	0.26**	0.101^{NS}	1		
Locality	0.099^{NS}	0.222**	0.207**	1	
season	0.045^{NS}	0.125^{NS}	0.142*	0.201** (0.002)	1
Result					
Dim.2					
Species	1				
Sex	-0.102^{NS}	1			
Age	0.154*	0.036^{NS}	1		
Locality	0.096^{NS}	0.057^{NS}	0.051^{NS}	1	
season	-0.013^{NS}	0.13*	0.012^{NS}	0.126^{NS}	1
Result					

NS: non-significant correlation P > 0.05; * significant correlation P<0.05; ** highly significant correlation P < 0.01

Table 5: Contributions of variables' categories for each dimension of top 2 dimensions

	contributions	Cos2	coordinates	contributions	Cos2	coordinates
	Dim.1			Dim.2		
Species						
Goat	4.42	0.15	**-0.42**	**5.06**	**0.13**	**-0.39**
Sheep	3.82	0.15	**0.36**	**4.37**	**0.13**	**0.34**
Sex						
Female	3.37	**0.16**	**0.32**	**2.53**	0.09	**0.24**
male	5.46	**0.16**	**-0.51**	**4.10**	0.09	**-0.39**
Age						
Young	10.22	**0.26**	**-0.82**	**5.37**	**0.1**	-0.51
Moderate	0.06	**0.00**	**-0.06**	**0.06**	**0.00**	0.05
old	8.37	**0.26**	**0.62**	**2.98**	**0.07**	0.32
Locality						
Al-Giza	0.37	**0.01**	**-0.37**	**3.57**	**0.05**	-0.98
Al-Minea	8.55	**0.24**	**0.69**	**4.54**	**0.09**	-0.44
Al-Monfia	3.70	**0.07**	**1.63**	**19.06**	**0.27**	3.21
Al-Sharqia	6.01	**0.20**	**-0.5**	**0.49**	**0.01**	0.12
Al-Suiez	0.31	**0.01**	**-0.2**	**1.60**	**0.03**	0.39
Season						
Winter	3.89	**0.1**	**-0.5**	**13.96**	**0.27**	0.83
Spring	2.53	**0.06**	**-0.47**	**1.16**	**0.02**	0.28
Summer	11.98	**0.31**	**0.86**	**0.99**	**0.02**	-0.22
autumn	0.05	**0.00**	**-0.07**	**17.93**	**0.32**	-1.09
BTV result						
Positive	16.03	**0.5**	0.86	**7.92**	**0.17**	**-0.5**
negative	10.84	**0.5**	-0.58	**4.93**	**0.17**	**0.34**

Cos2: is the quality or squared correlations of each dimension

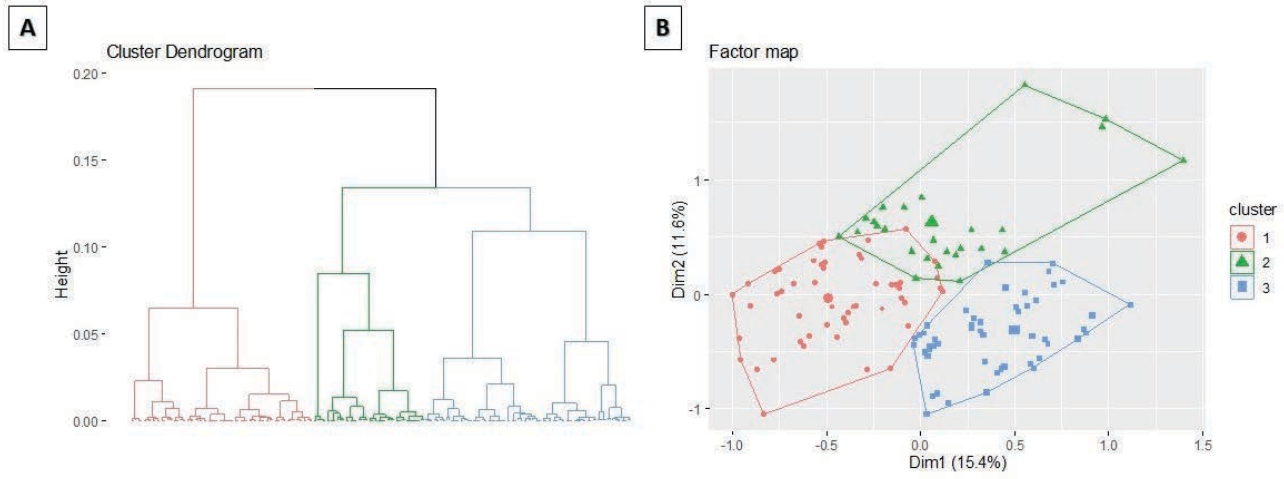


Figure 3: (A): Dendrogram of correspondence scores revealing the three clusters. The hierarchical tree suggests a clus-tering into three clusters. Figure (B): the three clusters on factor map

(ctr. = 13.96, cos2. =0.27, coord.= 0.83), negative BTV results (ctr. = 4.93, cos2. =0.17, coord.= 0.34), and sheep (ctr. = 4.37, cos2. =0.13, coord.= 0.34).

Data from all MCA dimensions were utilized in the HCCA to determine the animals' profiles and clusters. The results show three clusters figure 3. "BTV result" and "season" are the variables that best describe the division into three clusters. Only the variables' categories whose p-value is less than 0.02 are used. Only the categories whose p-value is less than 0.02 are used. Cluster 1 (negative BTV young aged animals): This group was characterized by frequent and largely exclusive seronegative BTV animals 91.67% of animals in the cluster are negative, negative BTV category is the most important and related to cluster 1 with positive v-test=8.75. 71.88% of animals in this cluster

are from Al-Sharkia governorate and 52% are young age. Cluster 2: 75% of cases recorded in winter (v-test= 7.64), up to 40% in Al-Suis, and 62% are old age. Cluster 3 (seropositive BTV): Out of all animals in this cluster 87.64% are positive BTV, 62.92% of cases are recorded from Al-Minea governorate, 55% and up to 35% are in summer and autumn respectively. 73% are females, and 52% are old.

Discussion

The study investigated the relationship among six qualitative variables involving 18 categories using MCA and HCA. MCA is appealing because it provides a bi plot depiction of both variables and variables' categories, which is not available in many qualitative data relationship analytical tools. HCA based on data of MCA revealed that data is well separated by season and BTV result into three clusters. The seropositive BT category was related to summer, and Al-Minea categories this finding is in contrast with Malek and Abou EL-wafa (18) who recorded more seropositive of BTV in non-hot months compared to hot months. Old age, female, and sheep categories are associated and all are grouped and associated with positive BTV category. These findings are consistent with (19) who reported that seroprevalence increases with age, probably a reflection of increased duration of exposure. In a previous study (20) they found that the seroprevalence rates were increased with increase of age in sheep and goats. However, in a seroprevalence study of Mozaffari and Khalili (21) in the south-east of Iran the results showed that seroprevalence rates were decreased with the increasing of age in sheep. The left side of plot mainly obtain negative BTV results, which associated with winter and spring. The governorates that showed more negative BTV were Al-Suiez, Al-Sharqia, and Al-Giza, which is in contrast with (22) who reported the two governorates with the highest prevalence with Beni-seuif governorates. The categories that show a degree of association to negative BTV are male, goat, and young age categories. The bottom-left quadrant shows the association between male, young, goat, and Al-Giza which appears more related to negative results compared to positive results. The presence of sex categories (male and females) are somewhat far from positive and negative categories of bluetongue results, which is also confirmed by the significance of variables in table (7). Sex demonstrated lower significance and correlation to the dimensions. The result is in line with (20) who applied chi-square and found non-significant effect of sex to seropositivity of BTV. However, Malek and Abou EL-wafa (18) reported that sex is significantly related to seropositivity and BTV is more recorded in females.

Conclusion

In large-scale qualitative data sets with more than two variables or survey-collected epidemiologic data, multiple correspondence analysis is a useful technique for identifying relationships. Additionally, it is interpretable for assessing relationship patterns and free of assumptions. By grouping data points together based on their homogeneity, HCA can be used for categorical data after MCA to display data patterns and reveal the underlying structure of BT data (positive or negative serotyping) in relation to other variables and/or categories.

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Original Research Article
Veterinary Medicine and The One Health Concept

SQUAB AND QUAIL MEATS: MICROBIAL STATUS AND PREVALENCE OF MULTIDRUG-RESISTANT SHIGA TOXIN-PRODUCING *E. coli*

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Abstract: The consumption of exotic meats, such as squab and quail meats, is common in many parts of the world. However, little is known about the safety of these foods. This study aimed to evaluate the microbial characteristics of squab and quail meats, with a particular interest in the prevalence of Shiga toxin-producing *Escherichia coli* (STEC). Meat samples were examined for total bacterial counts, total mold counts, most probable number of coliforms, total *E. coli* counts, and the prevalence of STEC. The presence of virulence genes *(stx1, stx2, and eae)* in STEC isolates was also investigated. Results from microbial analyses revealed poor hygienic status of squab and quail meats. *E. coli* was isolated from 16% and 10% of squab and quail meats, respectively. Six *E. coli* serotypes were recovered, including *O26, O78, O111, O114, O119,* and *O127*, and STEC genes were detected in all these isolates. Squab liver had the highest *E. coli* prevalence rates, followed by gizzard, heart, spleen, and breast muscles. The prevalence of *E. coli* in quail meat samples was similar across all tissues. STEC serotypes showed notable multidrug resistance profiles. We then used ascorbic and rosmarinic acids to increase the safety of breast muscle. Treatment of breast muscles with these acids significantly improved their microbial safety. These findings highlight the potential role of squab and quail meats as a vehicle for STEC transmission to humans, and the beneficial effect of treatment with ascorbic and rosmarinic acids on enhancing the safety of exotic meats.

Key words: food safety; shiga toxin; *E. coli*; squab; quails; meat

Introduction

The consumption of pigeon squab and quail meats is common in many countries, such as Egypt, Italy, and China. meat is characterized by its higher degree of tenderness and juiciness compared with other poultry meats. Squab meat is similar to that of the duck in terms of color and fatty skin with higher vitamin, minerals and protein content (1, 2). Similarly, quail meat is rich in biodigestible proteins, vitamins, and minerals, and its composition is similar to broiler meat. (3).

There are many sources in which microbial contamination may occur in poultry meat, including the bird itself (e.g., feathers and excreta), the operator (e.g., hands, hair, and clothes), the use of contaminated raw materials, washing water, collecting containers, and equipment (4). Contamination of poultry meat with foodborne pathogens is a particularly a major public health concern. Human infection with multidrug-resistant pathogens may result in severe health problems (5, 6).

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Shiga toxin-producing *Escherichia coli* (STEC) is a major foodborne pathogen responsible for significant losses worldwide. Diarrheagenic *E. coli* mainly causes enteric infections. Currently, diarrheagenic *E. coli* are categorized based on their dominant virulence attributes into enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EAEC), diffusively adherent (DAEC) and enterohemorrhagic (EHEC)/ Shiga toxin-producing *E. coli* (STEC) (6, 7). The latter is classified into two broad categories: O157 STEC and non-O157 STEC. *E. coli* O157 is responsible for about 90% of human cases, while the remaining cases are associated with other serotypes such as *O26, O45, O103, O111, O121* and *O145* (8, 9).

STEC serotypes can be transmitted to humans mainly through contaminated food, including contaminated poultry meat. However, the potential role of squab and quail meats as a vehicle for STEC transmission to humans remains unknown.

Rosmarinic acid (RMA) is one of the phytochemicals which represents the major component of rosemary, a plant that is cultivated worldwide and used as a flavoring agent in many dishes. Rosmarinic acid possesses significant antioxidant and antimicrobial properties (10). Likely, ascorbic acid (ASA) also has positive effects in wound healing, as an antioxidant, and with significant antimicrobial activities as reported against *Campylobacter spp.*, *Listeria monocytogenes, Staphylococcus aureus*, and *Mycobacterium tuberculosis* (11). Therefore, the first objective of this study was to evaluate the microbial status and the prevalence of STEC in squab and quail meats and giblets. The second objective was to characterize STEC isolates and to assess the antimicrobial effect of treating breast muscle with rosmarinic (RMA) and ascorbic (ASA) acids.

Materials and methods

Sample collection and preparation

Two hundred samples of squab and quails (100 each) were collected from farmed birds that had been slaughtered and distributed in local markets in Sharkia Governorate, Egypt.

Samples were prepared according to APHA (12). Briefly, 90 ml of 0.1% sterile buffered peptone water (LAB104, LAB M, UK) were used to homogenize 10 g of each sample using a sterile meat homogenizer (M-p3-302, mechanic, Precyzina, Poland) for 1-2 minutes at 2000 rpm. Such homogenate represents the dilution of 10^{-1} and further ten-fold decimal serial dilutions were prepared.

Determination of total bacterial count (TBC)

The total number of bacteria was calculated according to the APHA protocol. Aliquots (1 mL) of each dilution were transferred to a sterile Petri dish plate. For each plate, add 12–15 mL of plate count agar (Difco, Detroit, Michigan, USA) cooled to 45 ± 1 °C, mix well, and then allow to harden before incubating inverted for 48 hours at 35 ± 2 °C. Record all colonies including pinpoint size colony forming units as TBC in plates with 25-250 colonies per dish.

TBC/g = average No. of colonies × reciprocal of the dilution

Counted colonies expressed as log 10 cfu/g.

Determination of total mold count (TMC):

Total mold counts were determined by the pour plate technique using Sabouraud's dextrose agar media (Oxoid, UK) supplemented with chloramphenicol 100 mg/L. Plates were incubated in the dark at 25 °C for 5-7 days. and the plates were checked daily for mold growth. Estimation of total mold count was obtained by direct counting of the inoculated plates (12).

TMC/g = average No. of colonies × reciprocal of the dilution

Determination of the most probable number (MPN) of Coliform:

Three tubes most probable number (MPN) method was used (12). Briefly, Aliquots (1 mL) were inoculated into three separate test tubes containing MacConkey broth on inverted Durham's tubes. Tubes were then incubated at 37 °C for 24-48 h. Tubes with visual acid and gas production were considered positive and MPN of coliforms was calculated according to the recommended tables. To determine the MPN of *E. coli*, positive tubes were inoculated into tubes containing 7 ml of *E. coli* (EC) broth (Himedia, Mumbai) using a 10 µL loop (12). After inoculation at 44.5 °C for 24-48 hrs, positive tubes (showing acid and gas

production) were used to calculate the MPN of *E. coli* according to the recommended tables.

Isolation of Escherichia coli

Eosin Methylene blue (EMB) agar was used for the isolation of *E. coli* according to APHA protocol (12). From each positive tube of EC broth, a loopful of culture was streaked onto EMB agar. The inoculated plates were incubated at 37 °C for 24 h. Typical colonies of *E. coli* (metallic greenish colonies with dark purple center) were purified and sub-cultured onto a nutrient agar slopes and incubated for further confirmation by Gram's staining and biochemical tests (12).Biochemically identified isolated of *E. coli* were then subjected to serological examination using antisera sets specific for *E. coli* (Difco, USA) as previously described (13).

DNA extraction and gene detection

Enriched *E. coli* isolates in nutrient broth were centrifuged at 1500 rpm for 10 min. The pellet was lysed with TE buffer, then frozen for 10 min, and incubated at 98°C for 10 min. The concentration of the released DNA was measured using Nanodrop (ND-1000, Nanodrop Technologies, USA). Tested *E. coli* were examined by PCR for harboring Shiga toxin-coding genes, including *stx1, stx2,* and *eae*. The previously published protocol (14) was followed during all PCR procedures and the used primers were stated in Table 1.

Antimicrobial sensitivity testing,

Antimicrobial sensitivity testing was carried out according to the guidelines of Clinical and Laboratory Standards Institute (CLSI) (15). The tested antimicrobials were trimethoprim/ sulfamethoxazole (SXT), ampicillin (AM), enrofloxacin (En), cephalothin (CN), ciprofloxacin (CP), chloramphenicol (C), erythromycin (E), kanamycin (K), gentamicin (G), nalidixic acid (NA), oxacillin (OX), oxytetracycline (T), penicillin (P), and neomycin (N). Multiple antibiotic resistance index was calculated according to the following equation Singh *et al.* (16):

MAR index= Number of resistance (Isolates classified as intermediate were calculated as sensitive for MAR index) antibiotics/ Total number of antibiotics tested.

Improvement of the microbial status of the quail's breast muscle

The effect of RMA and ASA acids in reducing the microbial load of quail's breast muscle was investigated (11). Quail's breast samples (n = 20) were first examined for their microbial quality. Samples were then grouped into groups (n = 4). Samples were immersed in 1% RMA (Group 1), 2% RMA (Group 2), 1% ASA (group 3), 2% ASA (Group 4), or a combination of the two acids (Group 5; 2% each) for 30 min. The microbiological examination was then conducted as described above.

Statistical analysis

All values are expressed as means ± SE, and measurements were carried out in duplicates. Microbial counts were converted into base logarithms, 10 of colony-forming units (cfu) per g (log 10 cfu/g). Statistical analysis was performed using One-way analysis of variance (ANOVA), followed by the Tukey–Kramer HSD post-hock test. Significance was considered when $P < 0.05$.

Results

The obtained results of the present study revealed higher microbial loads in quails than those in squab samples. TBC counts in quails were (log 10 cfu/g): gizzard 6.5 ± 0.19 and 6.2 ± 0.55; liver 6.1 ± 0.24, and 5.4 ± 0.23; heart 5.5 ± 0.18, and 4.2 ± 0.18; spleen 5.2 ± 0.32, and 4.5 ± 0.20; breast muscle 3.2 ± 0.25, and 2.8 ± 0.14 of quail, and squab respectively.

The average TMC in the quail's samples was 5.5 ± 0.22, 5.2 ± 0.18, 4.8 ± 0.18, 4.2 ± 0.16, and 2.8 ± 0.16 in the examined liver, gizzard, heart, spleen, and breast muscle, respectively. These counts were 4.3 ± 0.18, 4.4 ± 0.25, 3.7 ± 0.28, 3.5 ± 0.22, and 2.5 ± 0.33 in the same samples of squab. MPN count of coliforms (log 10 cfu/g) were 5.2 ± 0.18, 4.2 ± 0.20, 3.5 ± 0.18, 3.2 ± 0.15, and 2.8 ± 0.18 in the examined gizzard, liver, heart, spleen, and breast muscle of quails, respectively. These values were 4.5 ± 0.11, 3.6 ± 0.14, 3.2 ± 0.33, 3.0 ± 0.22, and 2.5 ± 0.13 in the same samples of squab. MPN counts of *E. coli* (log 10 cfu/g) were 4.2 ± 0.18, 3.6 ± 0.21, 3.2 ± 0.18, 3.1 ± 0.20, and 2.4 ± 0.33 in the examined

gizzard, liver, heart, spleen, and breast muscle of quails, respectively, and 2.8 ± 0.33, 3.2 ± 0.15, 2.8 ± 0.11, 2.6 ± 0.10, and 2.1 ± 0.22 in the same samples of squab (Fig. 1).

E. coli was recovered from 16% and 10% of squab and quail samples, respectively. Six *E. coli* serotypes were isolated from the samples, including *E. coli O127: H6, O114: H32, O78: H–, O26: H11, O119: H6* and *O111: H–*. *E. coli* O26 was the most prevalent serotype in squab samples (50%), followed by *O127, O78*, and *O119*. In quail samples, *E. coli O127* was the most prevalent serotype (40%), followed by *E. coli O26* (20%), *O78* (20%), *O111*(10%) and *O114* (10%) (Fig. 2).

In squab samples, livers had the highest prevalence rate of *E. coli* (37.5%), followed by gizzard (25%), heart (12.5%), spleen (12.5%), and muscle (12.5%). By contrast, the recovery of *E. coli* in quail samples was similar across all tissue samples (20%) (Fig. 3).

The detection of Shiga-toxin genes varied among STEC isolates. In addition, all isolated *E. coli*

Table 1: PCR primers used in the present study

Shiga toxin coding genes	Primer sequence (5'-3')	Size (bp)	Gene Bank (Acc. No)	Tm°
stx1	F-ACACTGGATGATCTCAGTGG R-CTGAATCCCCCTCCATTATG	614	MF039301	58
stx2	F-CCATGACAACGGACAGCAGTT R-CCTGTCAACTGAGCAGCACTTTG	779	MF039302	58
eae	F-GTGGCGAATACTGGCGAGACT R-CCCCATTCTTTTTCACCGTCG	890	AJ875041	58

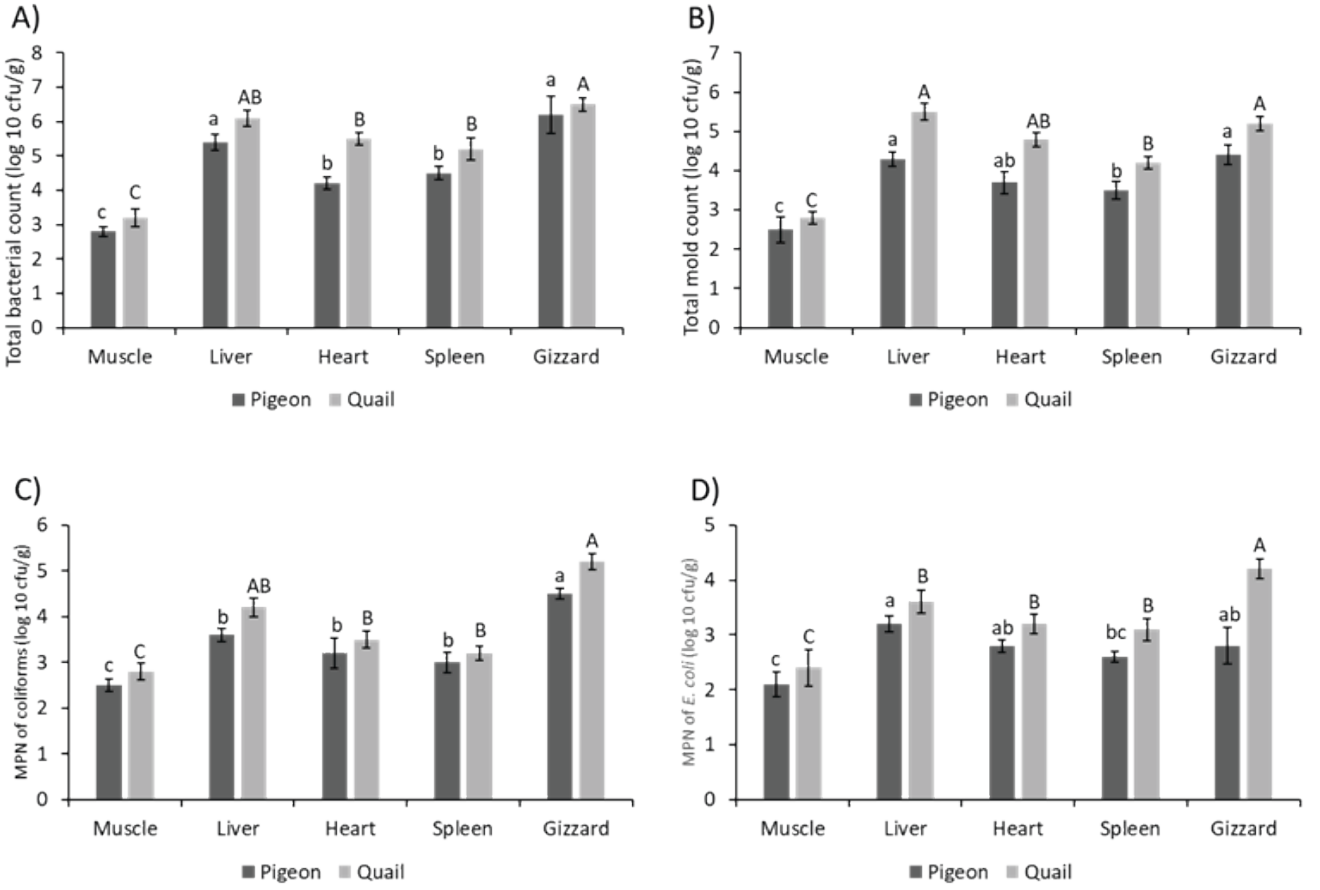


Figure 1: Indicators of the microbial quality of the examined squab and quail samples

A) Total bacterial count (TBC); B) Total mold count (TMC); C) Most probable number (MPN) of coliforms; D) Most probable number (MPN) of *E. coli*. Data represent means ± SD where n = 20 samples for each tissue. Columns carrying different letter a, b, c in case of squab, or A, B, C in case of quail are significantly different at $P < 0.05$. Microbial counts were changed into its log 10 cfu/g.

serotypes had notable multidrug resistance profiles. For example, the MAR index of *O26* ranged between 0.428 and 1, whereas that of *O127* was between 0.642 and 1 . The lowest MAR index was observed in *O114* at 0.357 (Table 2).

The antimicrobial resistance of the recovered *E. coli O26* isolates showed pan-resistance (100%) to AM, CN, CP, En, E, and P, 90% resistance to C, 80% to G, 70% to K, 60% to NA, 50% to N, 40% to OX, 30% to T, and 205 to STX. *O78* isolates showed 100% resistance to AM, G, K, NA, T, and P, 66.66% resistance to CN, C, CP, En, E, N, OX, and 33.33% resistance to SXT. *O127* isolates showed100% resistance to AM, CN, C, CP, En, K, NA, N, and P, 90% resistance to E, 80% resistance to G, 70% resistance to OX, 40% resistance to T, and 20% resistance to SXT. Isolates of *O111* were 100% resistant to AM, CN, CP, En, E, and P, but were sensitive to the other tested antimicrobials. The *O114* isolates showed 100% resistance to AM, CP, En, E, and P, while sensitive to the other tested antimicrobials. Isolates of *O119* were 100% resistant to AM, CP, En, K, NA, N, and P, but were sensitive to the other tested antimicrobials (Table 3).

Treatment of breast muscle with a combination of ASA and RMA at a concentration of 2% was the most effective in reducing TBC counts by 62.5%, TMC by 71.43%, MPN of coliforms by 82.14%, and MPN of *E. coli* by 91.67% (Table 4).

Discussion

Exotic meats, such as those of squab and quails, are becoming popular as an alternative source of meat in many countries around the world. In this study, the hygienic status of squab and quail meats and giblets was investigated. Total bacterial and coliform counts are regarded as acceptable indicators for hygienic measures and fecal contamination of foods (18). In Egypt, the upper allowable limit of TBC in poultry meat is 5 log 10 cfu/g and of coliforms is 2 log 10 cfu/g but must be free from mold spores (17). Results showed higher counts for the tested hygienic indicators, including TBC, TMC, MPN of coliforms, and MPN of *E. coli*. These high levels reflect the poor hygienic measures adopted during the preparation and processing of such meat sources. Similarly, Javadi and Safarmashaei (19) found the bacterial contamination of broiler meat sold in Iran had average values of ~ 5 log cfu/g for TBC and ~ 4 log cfu/g for coliforms, respectively. However, higher values (6.6 to 7.1 log 10 cfu/g for TBC) were found in poultry slaughtered in traditional shops in Morocco Amara *et al.* (20). It should be noted that, in Egypt, squab and quails are slaughtered and processed in conventional poultry slaughterhouses located near chicken and other birds. In many cases, the scalding tanks and water used in the wash process are the same. Therefore, there is a high risk of microbial cross-contamination between different birds processed at the same slaughterhouse (21).

STEC is a major foodborne pathogen that causes fatal complications, especially in vulnerable populations such as young children and the elderly. Between October 2002 and February 2003, a cluster of *E. coli O157:H7* hemorrhagic colitis was discovered in Canada.

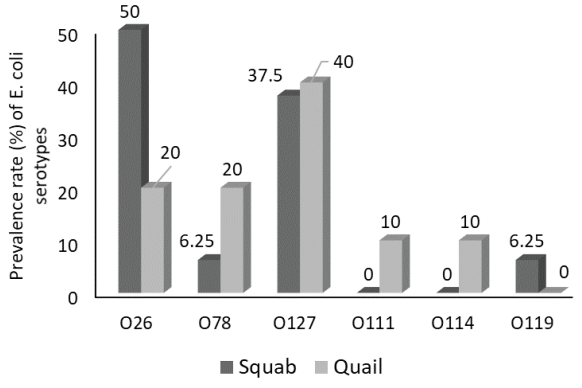


Figure 2: Prevalence (%) of different *E. coli* serotypes isolated from the examined squab and quail samples (n = 100/each) based on serodiagnosis

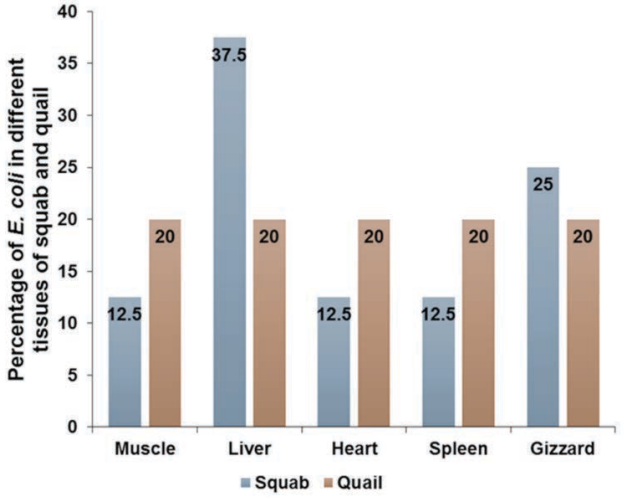


Figure 3: Prevalence (%) of *E. coli* in the examined tissue samples of squab and quails

Table 2: Characterization of the recovered *E. coli* serotypes in the present study

Species	Isolate number	Sero-type	Origin	*Shiga-toxin coding genes*			Antimicrobial resistance profiling	MAR index
				stx1	*stx2*	*eae*		
Squab (Prevalence rate = 16%)	1	O26	liver	+	+	+	AM, CN, C, CP, En, E, G, K, NA, N, OX, T, P, SXT	1
	2	O26	gizzard	-	-	-	AM, CN, C, CP, En, E, G, K, NA, N, OX, T, P	0.928
	3	O26	gizzard	-	-	-	AM, CN, C, CP, En, E, G, K, NA, N, OX, P	0.857
	4	O26	heart	+	-	+	AM, CN, C, CP, En, E, G, K, NA, N, P	0.785
	5	O26	spleen	+	-	+	AM, CN, C, CP, En, E, G, K, NA, P	0.714
	6	O26	muscle	-	-	-	AM, CN, C, CP, En, E, G, K, P	0.642
	7	O26	muscle	+	-	-	AM, CN, C, CP, En, E, G, P	0.571
	8	O26	liver	+	-	-	AM, CN, C, CP, En, E, P	0.5
	9	O78	liver	+	+	-	AM, G, K, NA, T, P	0.428
	10	O127	liver	+	+	+	AM, CN, C, CP, En, E, G, K, NA, N, OX, T, P, SXT	1
	11	O127	gizzard	-	-	-	AM, CN, C, CP, En, E, G, K, NA, N, OX, T, P	0.928
	12	O127	gizzard	+	+	-	AM, CN, C, CP, En, E, G, K, NA, N, OX, P	0.857
	13	O127	heart	-	-	-	AM, CN, C, CP, En, E, G, K, NA, N, P	0.785
	14	O127	spleen	-	-	-	AM, CN, C, CP, En, E, K, NA, N, OX, P	0.785
	15	O127	liver	+	+	+	AM, CN, C, CP, En, K, NA, N, P	0.642
	16	O119	liver	+	+	-	AM, CP, En, K, NA, N, P	0.5
Quail (Prevalence rate = 10%)	17	O26	muscle	-	-	-	AM, CN, CP, En, E, P	0.428
	18	O26	liver	+	-	+	AM, CN, C, CP, En, E, G, K, NA, N, OX, T, P	0.928
	19	O78	muscle	+	-	-	AM, CN, C, CP, En, E, G, K, NA, N, OX, T, P, SXT	1
	20	O78	heart	-	-	-	AM, CN, C, CP, En, E, G, K, NA, N, OX, T, P	0.928
	21	O127	heart	-	+	-	AM, CN, C, CP, En, E, G, K, NA, N, OX, T, P, SXT	1
	22	O127	gizzard	+	+	+	AM, CN, C, CP, En, E, G, K, NA, N, OX, T, P	0.928
	23	O127	gizzard	-	-	+	AM, CN, C, CP, En, E, G, K, NA, N, OX, P	0.857
	24	O127	spleen	-	-	-	AM, CN, C, CP, En, E, G, K, NA, N, P	0.785
	25	O111	spleen	-	-	-	AM, CN, CP, En, E, P	0.428
	26	O114	liver	+	+	-	AM, CP, En, E, P	0.357

Table 3: Antimicrobial resistance rates among the recovered *E. coli* serotypes in the present study

	O26		O78		O127		O111		O114		O119	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
AM	10	100	3	100	10	100	1	100	1	100	1	100
CN	10	100	2	66.66	10	100	1	100	0	0	0	0
C	9	90	2	66.66	10	100	0	0	0	0	0	0
CP	10	100	2	66.66	10	100	1	100	1	100	1	100
En	10	100	2	66.66	10	100	1	100	1	100	1	100
E	10	100	2	66.66	9	90	1	100	1	100	0	0
G	8	80	3	100	8	80	0	0	0	0	0	0
K	7	70	3	100	10	100	0	0	0	0	1	100
NA	6	60	3	100	10	100	0	0	0	0	1	100
N	5	50	2	66.66	10	100	0	0	0	0	1	100
OX	4	40	2	66.66	7	70	0	0	0	0	0	0
T	3	30	3	100	4	40	0	0	0	0	0	0
P	10	100	3	100	10	100	1	100	1	100	1	100
SXT	2	20	1	33.33	2	20	0	0	0	0	0	0

(No.) indicates the number of isolates in this serotype, (%) indicates the percent of the positive isolates to the total, number. AM: ampicillin, CN: cephalothin, C: chloramphenicol, CP: ciprofloxacin, En: enrofloxacin, E: erythromycin, G: gentamicin, K: kanamycin, NA: nalidixic acid, OX: oxacillin, T: oxytetracycline, P: penicillin, N: neomycin, SXT; trimethoprim/sulfamethoxazole

Table 4: Reduction rates of microbial counts of the quail's breast muscle by the use of RMA, ASA, or their mixture

	Control	RMA 1%	RMA2%	ASA 1%	ASA 2%	RMA + ASA
TBC	0	12.50	43.75	21.88	53.13	62.50
TMC	0	14.28	42.86	35.71	57.14	71.43
MPN coliforms	0	21.43	57.14	28.57	71.43	82.14
MPN *E. coli*	0	16.67	66.67	37.50	79.17	91.67

This outbreak was caused by the ingestion of unpasteurized Gouda cheese (22). Furthermore, *E. coli O104:H4* was responsible for an outbreak in Germany in May 2011 that infected over 3000 people and resulted in 50 deaths (23). In addition, 19 people in six states in the United States were infected with shiga toxin-producing *E. coli O121* (24). In the present study, *E. coli* was isolated from 16% and 10% of squab and quail meat samples, respectively. These results support a previous study showing the presence of *E. coli* in quail carcasses at different percentages (25). Similarly, Darwish et al. (2015) were able to isolate *E. coli* from 16% of duck meat and giblets. *E. coli* was also isolated from feral pigeon feces in India (26). In the present study, the STEC serotypes *O127, O114, O78, O26, O119*, and *O111* were identified. The serotypes *O26, O86, O114, O119*, and *O127* are among the human EPEC (27). Likely, *E. coli O26* was detected in rabbit carcasses and calves from Switzerland and Brazil, respectively (28, 29). The presence of these pathogenic STEC serotypes in squab and quail meats is concerning and warrants attention.

The high levels of *E. coli* in the liver and gizzard may be attributed to the potential migration of *E. coli* from the intestinal tract to the liver and gizzard. Unhygienic practices during slaughtering, de-feathering, and processing of slaughtered squab and quail may also have contributed to these contamination levels. Similar to these findings, *E. coli* was isolated from the liver, heart, and gizzards of processed poultry and from retail poultry in Trinidad (30, 31).

STEC strains are characterized by harboring a group of virulence genes such as *stx1, stx2*, and *eae. Stx1* and *stx2* are the principal toxin-coding genes in STEC, whereas *the eae* gene facilitates the adhesion of some STECS to the intestinal cells. Therefore, it is a critical determinant for the pathogenicity of *stx1* and *stx2* (32). Interestingly, *stx1*, *stx 2,* and *eae* genes were detected in the identified *E. coli* serotypes at variable rates. Likely, *stx1* and *stx2* genes were previously detected in STEC isolated from meat samples in Argentina (33), India (34) and meat served at hospitals in Egypt (35). Infection with STEC may result in serious complications such as hemolytic uremic syndrome, hemorrhagic colitis, and bloody diarrhea (36).

Antimicrobials are commonly used in poultry farms for disease treatment and prevention, as well as growth promoters. However, the abuse of antimicrobials have contributed to the development of multidrug-resistant pathogens (37). In this study, all STEC serotypes were resistant to multiple drugs. The obtained results go in agreement with those reported in several previous studies (4, 35, 38).

RMA is the active ingredient found in rosemary, whereas ASA is widely distributed in fruits, tomatoes, green leaves, and broccoli (39). Verghese *et al.* (40) found ASA to be effective against *E. coli* and *Klebsiella pneumonae* in broth. Similarly, ASA had antimicrobial effects against *Salmonella* in a cheese model (41). Both ASA and RMA were found effective against the growth of *Listeria monocytogenes* in soft cheese (11). Therefore, we used these acids to reduce microbial contamination of breast muscle. When combined at 2%, these acids were highly effective in reducing the microbial load in meat samples. The reduction rates for TBC, TMC, MPN of coliforms, and MPN of *E. coli* were 62.50%, 71.43%, 82.14%, and 91.67%. Similarly, the antimicrobial activities of ASA and RMA might be attributed to their prooxidation action against ROS generation in microbial cells leading to leakage of protein and sugar and subsequently microbial cell death Shivaprasad *et al.* (42).

Conclusions

These findings demonstrated unsatisfactory hygienic conditions during the preparation of squab and quail meats. In addition, meat and giblets from squab and quails should be

considered as a vehicle for multidrug-resistant STEC. ASA and RMA are promising antimicrobial candidates that can be used during the processing of poultry meat.

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This study was conducted according to the guidelines of Zagazig University, Egypt, and no living animals were used in this study.

All authors approved to participate in this research work and in the manuscript. All authors approved this manuscript to be published. The authors declare that they have no conflict of interest.

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Original Research Article
Veterinary Medicine and The One Health Concept

PREVALENCE, ANTIMICROBIAL RESISTANCE PROFILES OF CLINICAL AND SUBCLIN-ICAL MASTITIS IN LACTATING COW WITH ASSESSMENT OF TREATMENT TRIAL

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Abstract: This study looked at the prevalence of bovine mastitis in a dairy farm in Egypt's Ismalia Governorate, as well as the phenotypic and genotypic characteristics of the causative bacteria and their antimicrobial susceptibility. Also, a treatment trial with a combination of Cefalexin and Kanamycin was evaluated. The total prevalence of mastitis was 31.82% (119/374) at cow level and 17.01% (247/1452) at quarter level. 261 isolates were detected. As major microorganisms, 74.33% are Staphylococcus spp. and 25.67% are Streptococcus spp. The isolates were tested against 15 antimicrobial agents, with Gentamycin (CN), Ciprofloxacin (CIP), Cefalexin (CL), and Kanamycin (K) having the lowest percentage of resistant bacteria. Molecular characterization of isolated pathogens and antimicrobial resistance genes was performed by PCR on 15 isolates. *blaTEM-1*was the most frequently detected gene, followed by *aadA1, dfrA1, cmlA, sul1,* and *tetA*. 110 infected udder quarters were enrolled for 21 days to evaluate the treatment with Terrexine LC intramammary suspension 10g (Cefalexin ph. Eur 200mg, Kanamycin monosulfateph.Eur 100,000 I.U.) on six occasions at 12-hour intervals and Gentamycin intramuscular injection (1 cm/20 kg BW for 3-5 days) in cows with systemic reactions. A highly significant reduction was recorded for the log10 SSC, log10 TBC, and the level of LDH in milk after treatment compared to their level before treatment (P-value 0.0001***). The milking season, severity of mastitis, or type of microorganism isolated prior to treatment have no effect on the recovery rate (P-value 0.05). In conclusion, bacterial isolates in the present study revealed multidrug resistance to the majority of commonly used antimicrobial agents, so antibiotic usage must be restricted. PCR is a helpful technique for the detection of resistant bacteria. Treatment of bovine mastitis with a combination of antibiotics significantly improves the bacteriological cure, SSC, TBC, and the level of LDH in milk.

Key words: bovine mastitis; drug resistance; resistance gens; treatment; Cafalexin; Kanamycin

Introduction

Bovine mastitis is a devastating and great challenge to dairy production, disturbing animal health and welfare in addition to causing considerable economic losses and a public health hazard. It lowers milk yield and quality, raises treatment and labour costs, a causes milk to be held after treatment, resulting in premature culling or death of the infected cow (1, 2).

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Mastitis is caused by a wide range of pathogens that vary in their virulence, abilities to stimulate an immune response, and structures of their cell walls, resulting in differing susceptibilities to antimicrobials, so accurate determination of the cause is important to prescribe the appropriate treatment (3).

Mastitis pathogens are classified into 1-Contagious bacteria (*S. aurus, S. agalactia*, and *mycoplasma*) that spread from an infected quarter during milking.*2.* Environmental bacteria (*S. uberis*, S. dysagalctia, E. coli*, and other coliforms*) that are commonly present in cows' environments. (4).

The diagnosis of clinical mastitis depends on udder examination and changes in the physical and chemical properties of milk, as well as an increase in somatic cell count, while the diagnosis of subclinical mastitis is more difficult and depends on indirect techniques such as the California mastitis test, an ,electrical conductivity test, and the detection of body enzymes released due to tissue damage, such as the lactate dehydrogenase enzyme. (5,6)

The lactate dehydrogenase enzyme is present in the cytoplasm of all animal cells. During udder infection, the activity of this enzyme increases as a result of udder tissue damage, making it a responsive indicator for early detection of mastitis and a reliable outcome to evaluate treatment success (6, 7).

Microbiological examination of milk is considered to be the standard method for diagnosing bovine mastitis, despite its limitations, such as the time needed for culturing and the costs (8).

PCR is a unique molecular technique that was developed in order to provide higher sensitivity and specificity while minimising costs. (9)

Mastitis is the most common disease that leads to the use of antimicrobials on dairy farms. Imprudent use of antimicrobials can lead to a rise in antimicrobial resistance, and there is a risk of the subsequent spread of resistant genes to other microbial populations. (10)

Combining two or more antibiotics may reduce the emergence and spread of resistant pathogens by increasing bactericidal activity against specific pathogens due to a synergistic effect, or it may allow antimicrobial action against more pathogens that are not affected by a single antibiotic (11, 12).Also, this combination may extend the period between dose administrations or decrease the number of administrations required, or both, so it can achieve similar or enhanced bactericidal activity using a lower total amount of antibiotics (13).

Our study aimed to investigate the prevalence of bovine mastitis in dairy cows, study antimicrobial resistance, and assess the recovery rate through intramammary infusions of cephalexin and kanamycin.

Material and methods

The protocol for this study was reviewed and approved by Zagazig University's Ethical Committee on Animal Care and Use Experimentation (ZU-IACUC Committee), with approval number ZU-IACUC/2 /F/165/2021.

Study design

This research was carried out in a dairy farm in Ismailia Governorate, Egypt, between 2020 and 2021.The farm was purposefully selected based on the high population of cows (374 black and white Holstein lactating cows) and the diversity of cattle age and lactation stage.

The farm was raised using an open system, with machinery milking three times per day.The udder was washed with water before and after milking teat dipping. The soil was muddy and scraped every six months.The farm was screened with the California mastitis test (CMT) every 2 months. Dry cows were treated with long-acting udder infusions. Prior to our study, mastitis was treated in the farm without the regular application of antimicrobial sensitivity to the causative pathogen.

Clinical examination

Cows were generally examined for systemic illness and for clinical mastitis with a special physical examination of each udder, teat, and secreted milk.

Sample collection

Aseptic milk samples were collected from each quarter separately. Udder was carefully cleaned and disinfected with 70% alcohol. After discarding the early jets of milk, 10 ml of milk from each quarter was collected in a screw-capped bottle. The samples were then labelled and transported to Zagazig University,Faculty of Veterinary Medicine, infectious diseases laboratory for examination.Each milk sample was physically checked for any abnormalities in colour, consistency, and the presence of blood, clots, and flak.

California mastitis test

Apparently healthy udder quarters with apparently normal milk were tested using the California mastitis test(CMT) to detect the subclinical mastitis. The test procedures were

performed according to the prior study (14), and the reading of the test was scored as 0, trace, 1, 2, and 3. A milk sample was counted as positive if it had a reading of 1-3.

Isolation and identification of the causative agent

Ten microliters of each milk sample were streaked onto blood agar (Oxoid, Hampshire, UK; CM0271 with 5% sheep blood) and incubated at 37 C for 24-48 h. The colony morphology and type of hemolysis were used to identify the growth on blood agar at first.Aloopful of pure culture was smeared and stainedwith Gram´s stain to differentiate the shape of bacteria. Separate colonies from blood agar were subcultured onto mannitol salt agar (Oxoid Ltd., Hampshire, UK) for primary identification of the genus Staphylococcus. And to Edward Media (Oxoid, Hampshire, UK) for the identification of the genus Streptococcus spp. The colonies were morphologically and biochemically identified (15).

Antimicrobial Susceptibility Testing (16)

The sensitivity of the isolated strains was determined using the disc diffusion technique against 15 antimicrobial agents commonly used in Egypt for the treatment of mastitis and of public health importance (Gentamicin CN (10 g/ml), Streptomycin S (10 g/ml), Kanamycin K (30 g/ml), Ciprofloxacin CIP (5 g/ml), Vancomycin VA (30 g/ml), Cefalexin CL (30 g/ml) Amoxicillin+ Clavulanic acid AMC(30 µg/ml), Ceftriaxone CRO(30 µg/ml), Penicillin G P(10 µg/ml), Doxycycline DO(30 µg/ml), Chloramphenicol C(30 µg/ml), Erythromycin E(15 µg/ml), Oxacillin OX(1µg/ml), Sulfamethoxazole + Trimethoprim SXT (25 µg/ml)and Clindamycin DA(2 µg/ml)).

A few colonies of similar morphology were mixed in a tube with 5 ml of sterile 0.85% physiological saline, and the turbidity was adjusted to match a 0.5 McFarland standard tube (1.5 x 10^8 CFU) and streaked onto Mueller-Hinton agar plates. The antimicrobial discs were applied and incubated at 37 C for 24–48 h. The diameter of the zone of growth inhibition was measured to determine the degree of sensitivity.It was interpreted according to the national committee for the Clinical Laboratory Standards Institute (17). Isolates were considered multidrug-resistant (MDR) if they were resistant to at least three different classes of antimicrobial agents (18).

Determination of the minimum inhibitory concentration (MIC)

MIC values of antibiotics were determined by the microdilution method following the recommendations of Papich(19).

Molecular Identification of Isolates and Resistance Genes

Extraction of DNA

DNA was extracted from the from biochemically identified bacterial isolate according to QIAamp DNA mini kit instructions (Catalogue no.51304). QIAGEN protease, 200 µl of the sample, 200 µl buffer were mixed in a 1.5 ml microcentrifuge tube. The tube was incubated at 56˚C for 10 min. 200 µl ethanol (96%) were added. The mixture was carefully applied to the QIAamp mini spin column (in a 2ml collecting tube). 500 ml buffer AW1, 500 ml buffer AW2 and 100 µl buffer AE were added separately.

Preparation of the PCR Master Mix

According to Emerald Amp GT PCR mastermix (Takara) Code No.RR310Akit:PCR reactions were of 25 µlvolum (Emerald Amp GT PCR mastermix (2x premix) 12.5*µl*, PCR grade water 4.5*µl,* Forward primer (20 pmol), Reverse primer (20 pmol) 1 *µl*for each andTemplate DNA 6 *µl).* Eight pairs of oligonucleotide primers were used in conventional PCR assays for amplification of *16S RNA* gene of genus *staphylococcus*, *tuf*gene of genus *Streptococcus spp*and six antimicrobial resistance genes (Table 1).

Agarose gel electrophoreses

20 µl of each PCR product samples, negative control and positive control were loaded to the gel. The power supply was 1-5 volts/cm of the tank length. The run was stopped after about 30 min and the gel was transferred to UV cabinet.

The gel was photographed by a gel documentation system and the data was analyzed through computer software.

Treatment

One hundred and ten udder quarters with intramammary infection (16 showed sub clinical mastitis and 94 with clinical mastitis) form total 247 infected quarters (146 with sub clinical mastitis and 101 with clinical mastitis) were enrolled in this study for 21 days after treatment with Terrexine LC intramammary suspension 10g (Cefalexin ph. Eur 200mg, Kanamycin monosulfateph.Eur 100,000 I.U.; Univet Limited) on six occasions at 12 h intervals. A gentamycin intramuscular injection was administered to 32 cows (1 cm/20 kg BW for 3-5 days) and showed a systemic reaction (Table 2).

Each quarter was sampled at 0, 7, 15,21days after initiation of treatment to evaluate the bacteriological cure, level of somatic cell count(SCC),total bacterial count(TBC) and lactate dehydrogenase (LDH) activity.

Bacteriological cure

Bacteriological cure was assessed by sample taken from affected quarter on(7,15and21)days after treatment were free of the bacterial species isolates in the pre-treatment.

Somatic cell count and total bacterial count

Somatic cells as well as total bacterial count were measured at quarter level. Milk samples were collected from each quarter just before treatment and on 7, 15, and 21 days aftertreatment. These parameters were enumerated in raw cow's milk by The FOSS BacSomatic™ analyser. The samples were tested within 2h after collection. Udder quarters enrolled in treatment were with SCC ≥ 500,000 cells/ml pre-treatment.

Lactate dehydrogenase activity

LDH activity was measured using the Health Mate™ milk LDH mastitis test DFI-car. It is a dipstick semi-quantitative colorimetric test to determine elevated levels of LDH in milk as early indicator of mastitis. The test strip is dipped in 5mL of fresh cow milk for 2minutes then read the change in its colour.

Analysis of the data

The analysis of the datawas carried out in the R environment for statistical computing and visualization (28), which is an open-source dialect of the S statistical computinglanguage, and were expressed the mean ± standard deviation (SD) or percentage (%). For normality investigation, the Shapiro–Wilks test was used for numerical data, and the Chi-square test ($\chi 2$) was used for categorical data. The means of samples with normal distribution and of sufficient size were compared by Student's t test and one-way ANOVA. A p value < 0.05 was considered significant.

Table 1: Oligonuclotide primers for molecular identification of *Staphylococcus spp.*, *Streptococcus spp.* and antimicrobial re-sistance genes)

Target gene	Primers sequences	Amplified segment (bp)	Reference
16S RNA	AACTCTGTTATTAGGGAAGAACA CCACCTTCCTCCGGTTTGTCACC	756	**(20)**
tuf	CCACCTTCCTCCGGTTTGTCACC TGG GTT GAT TG AACC TGG TTT A	110	**(21)**
blaTEM-1	AGGAAGAGTATGATTCAACA CTCGTCGTTTGGTATGGC	535	**(22)**
aadA1	TATCAGAGGTAGTTGGCGTCAT GTTCCATAGCGTTAAGGTTTCATT	484	**(23)**
dfrA1	TGGTAGCTATATCGAAGAATGGAGT TATGTTAGAGGCGAAGTCTTGGGTA	425	**(24)**
TetA	'GGCGGTCTTCTTCATCATGC 'CGGCAGGCAGAGCAAGTAGA	502	**(25)**
Sul 1	CC GATATTGCTGAGGCGG CCAACGCCGACTTCAGCT	435	**(26)**
cmlA	CCGCCACGGTGTTGTTGTTATC CACCTTGCCTGCCCATCATTAG	698	**(27)**

Table 2: 110 udder quarters enrolled for treatment with Cefalexin and Kanamycin monosulfate

No. of treated quarters	Clinical	Subclinical
	94	16
Treatment	- Terrexine LC intramammary suspension 10g (Cefalexin ph. Eur 200mg, Kanamycin monosulfateph.Eur 100,000 I.U.; UnivetLimited,UK) on six occasions at 12 h intervals. - Gentamycin intramuscular injection was administrated to 32 cows (1cm/20kg Bw for 3-5 days) showed systemic reaction.	Cases of subclinical mastitis were isolated and milked six times daily for one month, then retested with CMT and the positive cases were treated with an antibiotic.

Results

Prevalence of mastitis on the farm before treatment

At cow level:

A total of 374 lactating cows were tested, and 119 were found to have clinical or subclinical mastitis, for a total prevalence of 31.82%.

At the quarter-udder level:

44 udder quarters (2.9%) were completely dried out of a total of 1496 examined.From the remaining 1452 examined quarters, 101 (6.96%) revealed different degrees of inflammation (asymmetry, redness, hotness, and pain during palpation). Also, changes in the secreted milk from these quarters varied from slight deformity in milk quantity and milk colour to clot formation, bloody milk, and the appearance of yellow fluid or pus secreted from the affected quarters. 146 quarters (10.06%) were diagnosed as subclinical mastitis using the California mastitis test, with the total prevalence of mastitis at 17.01% (247/1452) (Table 3).

Microbiological results

261 bacterial isolates were yielded from a total of 247 milk samples. The isolates were characterised by phenotypic characteristics and biochemical reactions as two major microbial species. 194 (74.33%) *Staphylococcus spp.* and 67 (25.67%) Streptococcus *spp.* isolates (Table 4).

Antimicrobial sensitivity

All of the bacteria isolated were multidrug resistant**.**The highest proportion of antimicrobial-resistant bacteria was recorded for AMC, followed by E, S, C, and P, and the lowest for K, CIP, CL, and OX. No resistance was recorded for VA.

Regarding the Staphylococcus spp. isolates, the highest percentage of resistance was recorded for AMC (60.82%), and the lowest resistance rates were recorded for K, CIP, and CL (3.61% for each); no resistance was recorded for VA. For Streptococcus spp. isolates, the highest resistance rate was recorded for AMC (100%) and the lowest rate was recorded for CL (11.94%). No resistance was recorded for CN, K, CIP, OX, CRO, and VA (Table 5).

Table 3: Prevalence of mastitisat quarter level

		Clinical					Subclinical					Total (1452*)
At quarter level	N	Q1	Q2	Q3	Q4	Total	Q1	Q2	Q3	Q4	Total	247
		23	28	17	33	101	24	49	44	29	146	
	P(%)	1.58	1.93	1.17	2.27	6.96	1.65	3.37	3.03	1.99	10.06	17.01

Q: quarter; N : number; p: percentage ; *Other quarters (N= 44) were either dried or blind& excluded from the total examined number.

Table 4: Prevalence of isolated bacteria

Isolates	*Staphylococcus spp*			*Streptococcus spp*			
No. of isolates	CNS	*S. aurus*	Total	*S. uberis*	*S. agalactia*	*S. dysagalacti*	Total
	111	83	194	32	23	12	67
Prevalence	42.53%	31.80%	74.33%	12.26%	8.81%	4.60%	25.67%

Table 5: Antimicrobial resistance of *Staphylococcus spp.* and *Streptococcus spp.* isolated from lactating cows with mastitis

Antimicrobial Disc (Concentration **µg/ml**)	Microrganism	Resistance No (%)	Total (%)
CN (10)	Staph Strept	21 (10.8) 0 (zero)	21(8.05)
S (10)	Staph Strept	76 (39.18) 48 (63.16)	124 (47.51)
K (30)	Staph Strept	7(3.61) 0 (zero)	7 (2.68)
CIP (5)	Staph Strept	7(3.61) 0 (zero)	7 (2.68)
VA(30)	Staph Strept	0(zero) 0 (zero)	0 (0.00)
CL(30)	Staph Strept	7(3.61) 8 (11.94)	15 (5.75)
AMC(30)	Staph Strept	118(60.82) 67 (100)	185 (70.88)
CRO (30)	Staph Strept	35 (18.04) 0 (zero)	35 (13.41)
P (10)	Staph Strept	55 (28.35) 42 (62.69)	97 (37.16)
DO(30)	Staph Strept	49 (25.26) 34 (50.75)	83(31.80)
C (30)	Staph Strept	83 (42.78) 34 (50.75)	117 (44.83)
E (15)	Staph Strept	83 (42.78) 50 (74.63)	133(50.96)
OX (1)	Staph Strept	21(10.82) 0 (zero)	21 (8.05)
SXT (25)	Staph Strept	62 (31.96) 34 (50.75)	96(36.78)
DA (2)	Staph Strept	49 (25.26) 17 (25.37)	66 (25.29)

PCR and resistance gens

Eight *Staphylococcus spp.* and seven *Streptococcus* isolates, with phenotypic resistance to a large number of antimicrobial agents, were confirmed by PCR in addition to the detection of six resistance genes. β-lactamase gene (*blaTEM-1)* was the most detected resistance gene (86.67%), followed by aminoglycoside adenylyl-transferase gene (*aadA1)*, dihydrofolate reductase gene (*dfrA1),* chloramphenicol resistance gene *(cmlA),*Sulfonamide resistance gene *(sul1),* and finally tetracycline resistance gene (*tetA)*, with a percentage of 60.00%, 60.00%, 47.67%, 40.00%, and 26.67%, respectively (Table 6).

Treatment evaluation

Clinical and Bacterial cure

By the 21st day after treatment, 92.55 % of clinical cases (87/94) were cured where the appearance of milk and mammary glands returned to normal**.** In addition, the bacteriological cure was achieved in 88.18% of enrolled quarters(97/110).

Milk yield

No significant change in the level of milk production per cow after treatment (18.39± 4.75) comparing its level before treatment (17.87± 4.51; P-value 0.76) (Figure 1).

Table 6: Antimicrobialresistantprofileof 15 isolates(8 *Staphylococcus spp* and 7 *Streptococcus spp*)

NO	M.O	PHENOTYPE	RESISTANTGENES
1	*S.aurus*	CN, S, CL, AMC, CRO, P, DO, C, E, DA, TE, AX, CX	*cmlA, aadA1, bla*$_{TEM-1}$*, dfrA1, sul1, tetA*
2	*S.aurus*	S, AMC, C, DO, E, OX, SXT, DA, TE, CX	*aadA1, bla*$_{TEM-1}$*, sul1*
3	*S.aurus*	CN, S, AMC, CRO, P, DO, C, E, OX, SXT, DA, TE, AX, CX	*aadA1, bla*$_{TEM-1}$*, tetA*
4	CNS	S, AMC, CRO, P, DO, C, DA, TE	*cmlA, aadA1, bla*$_{TEM-1}$*, tetA*
5	CNS	CN, S, CIP, AMC, CRO, P, DO, C, E, OX, SXT, DA, IP, M, AX	*cmlA, aadA1, bla*$_{TEM-1}$*, dfrA1*
6	CNS	CN, S, K, AMC, CRO, C, SXT, CX	*cmlA, aadA1, bla*$_{TEM-1}$*, dfrA1*
7	CNS	CN, S, AMC, CRO, C, SXT, CX	*aadA1, dfrA1, sul1*
8	CNS	S, AMC, CRO, P, DO, E, CX	*cmlA, aadA1, bla*$_{TEM-1}$*, sul1*
9	Strept	S, AMC, P, E, SXT	*dfrA1, sul1, aadA1, bla*$_{TEM-1}$
10	strept	S, AMC, E, SXT	*dfrA1, sul1,*
11	strept	S, AMC, P, DO, C, E, DA	*cmlA, dfrA1, bla*$_{TEM-1}$
12	Strept	S, AMC, P, DO, C, SXT	*cmlA, bla*$_{TEM-1}$
13	Strept	S, AMC, C, E, SXT	*bla*$_{TEM-1}$
14	Strept	S, AMC, CRO, P, C, E	*bla*$_{TEM-1}$
15	Strept	S, AMC, CRO, P, C, E	*bla*$_{TEM-1}$

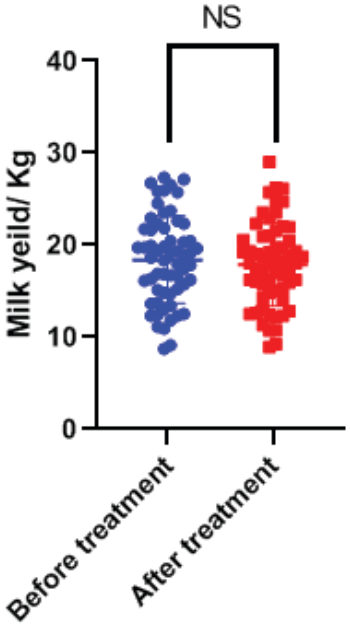


Figure 1: Change in milk yield at cow level before and after treatment with combination of Kanamycin and Ce-falexin

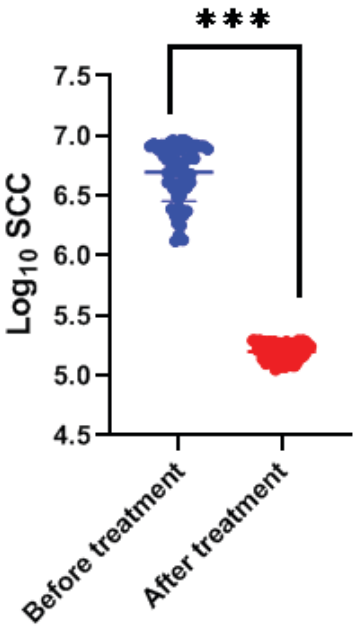


Figure 2: Change in $\log_{10}$ SCC at quarter level before and after treatment with combination of Kanamycin and Ce-falexin

SCC

Quarter-level SCC gradually declined in milk samples during the posttreatment follow-up. A high significance was recorded for the log10 SSC in milk at 21stday after treatment (5.20± 0.07) compared the level before treatment (6.69 ±0.24 P-value <0.0001***) (Figure 2).

Total bacterial count

The TBC was increased before treatment to 7.40 ±0.21 which was then significantly decreased to 3.67 ±0.16 after treatment (Figure 3).

Milk samples revealed significant increase LDH Activity as high as 497.33 ±14.36 U/L before treatment that restored its normal level after treatment(Figure 4).

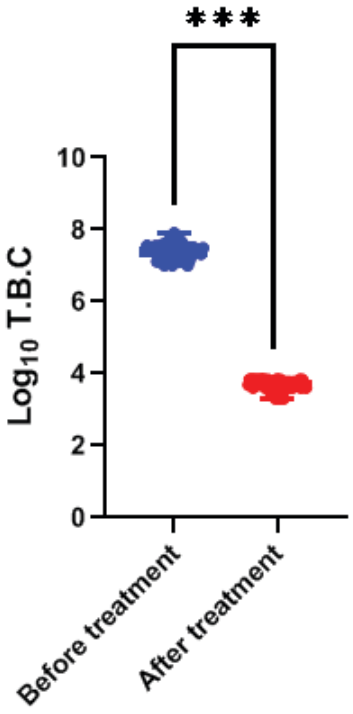


Figure 3: Change in log10 TBC at quarter level before and after treatment with combination of Kanamycin and Ce-falexin

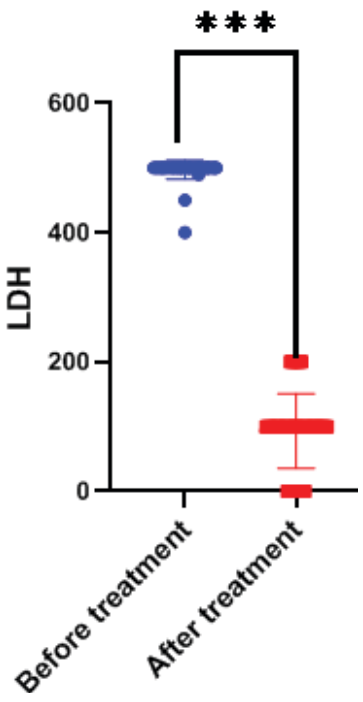


Figure 4: Change in LDH activity at quarter level before and after treatment with combination of Kanamycin and Cefalexin

Lactat dehydrogenase activity

Table 7: The development of the 4 outcomes (milk yield, Log_{10} SCC, Log_{10} TBC and LDH)in relation to day of treatment

parameters	Before treatment	After treatment			P-value (Anova test)	Significance
	0 day	7days	15 days	21 days		
Milk yield	17.87± 4.51	17.50± 4.52	17.49± 4.77	18.39± 4.75	0.76	Not significance
Log_{10} TBC	7.40 ±0.21	5.13± 0.18	4.34 ±0.20	3.67 ±0.16	<0.0001***	Highly significance
Log_{10} SCC	6.69 ±0.24	5.51 ±0.09	5.35 ±0.13	5.20± 0.07	<0.0001***	Highly significance
LDH	497.33 ±14.36	93.33 ±57.83	10.00 ±30.25	-	<0.0001***	Highly significance

Discussion

Bovine mastitis is the most common production disease in dairy herds worldwide, not just in developed countries.It is responsible for several production effects (29).

The obtained results on 374 lactating cows revealed that the prevalence of mastitis was 31.82% (119/374) at the cow level and 17.01% (247/1452) at the udder quarter level, out of which 6.96% (101/1452) was clinical mastitis and 10.06% (146/1452) was subclinical mastitis. This prevalence of bovine mastitis was similar to the results obtained in other previous studies in Egypt (30), A higher prevalence of 52.1% and 49.9% for subclinical mastitis was recorded by Algammal et al. (31) in cattle in the same governorate. Mbindyo et al. (32) in Kenya reported a higher percentage for total mastitis (80%; 316/395) at cow level and (51.6%; 815/1580) at quarter level.

In the present study, CNS was the most prevalent pathogen (42.53%), followed by *S. aurus* (31.8%), *S.uberis*(12.26%), *S. agalactia (8.81%),* and *S. dysagalactia (4.59%).* This is in line with previous findings (33-34) that CNS were the dominant isolated bacteria from clinical mastitis

in cattle. Also, CNS was recorded as the major pathogen implicated in udders with subclinical mastitis in different countries (35–36).

Previously, CNS was proven to cause mild subclinical mastitis, so it was considered a minor mastitis pathogen compared with *Staphylococcus spp*aureus, streptococci, and coliforms (37).

Recently they associated with many economic losses due to significant increases in SCC and reductions in milk yield and quality (38, 39); moreover, it can cause intraalveolar fibrosis of the udder tissue and loss of secretory function of this tissue.

S. aurus had a prevalence of 31.80% in the current study.Nearly similar percentages were recorded by Mousa et al. (40) and Moustafa et al. (41) but a lower percentage of *S. aureus* (5.6%) was reported by El-Ashker et al. (42) and a higher percentage of 45.6% and 46.5% was recorded by Abdel-Tawab et al. (43) and El-Faramawy et al. (44) from clinical mastitis cases. The infection with *S. aurus* is transmitted from animal to animal through milker's hands, contaminated equipment, and a bad hygienic environment (45).

In this study, the prevalence of streptococcal mastitis was 25.67% (*S.uberis*(12.26%), *S. agalactia* (8.81%), and *S. dysagalactia* (4.59%). This related to the results recorded by Duse et al. (46) for *S.uberis* (11.4%), but they reported different prevalences for *S. agalactia (1.2*%) and *S. dysgalactia*(15.8%). A lower prevalence of streptococcal mastitis (5.0%) was found by Hasan et al. (47). Abd El-Aziz et al. (48) reported a higher percentage of *S.uberis* (20.59%) in lactating cows.

S.dysgalactiae detected in mastitis milk were 14 times higher than those detected in non-mastitis milk, suggesting that this species is a major pathogen in mastitis cases (49).

Successful treatment of bovine mastitis depends upon the selection of an appropriate antibiotic, so frequent surveillance of mastitis pathogens and updating information about their antibiotic resistance are very important. Here, we tested the isolated strains against 15 antimicrobials that have veterinary and public health importance. A high rate of resistance was observed. All of the isolates were drug resistant.This disagrees with Duse et al. (46), who reported low antibiotic resistance to many tested compounds among gramme-positive agents isolated from bovine mastitis.

A phenotypically high proportion of resistant isolates was found for amoxicillin + clavulanic acid, chloramphenicol, erythromycin, sulfa + trimethoprim, and penicillin. This partially agrees with Mbindyo et al. (32) in Kenya, who recorded the highest resistance for ampicillin followed by erythromycin in *Staphylococcus spp*. Abd El-Aziz et al. (48) in Egypt observed 100% resistance to cefalexin in *S.uberis* isolated from clinical bovine mastitis. In our study, cefalexin showed a low resistance rate in both *Staphylococcus spp.* and *Streptococcus spp.* isolates (3.61% and 11.94%, respectively). This difference in the antimicrobial resistance pattern reflects the need for regular monitoring of the antibiotic sensitivity of isolated pathogens and the strategic use of antibiotics.

Low proportions of resistant staphylococcal isolates were recorded for kanamycin and gentamicin, and no streptococcal resistant isolates were found for them. This strongly agrees with the results of Hasan et al. (47). This result indicated that optimum doses of kanamycin, cefalexin, gentamycin, and ciprofloxacin may be the drugs of choice to treat the most cases of bovine mastitis.

The molecular characterization of different resistance genes is an important measure to investigate the emergence and spreading of resistance. This study investigated six resistance genes in 15 isolates (8 *Staphylococcus spp. and 7Streptococcus spp.)* using PCR. (*blaTEM-1)* was the most detected resistance gene.It is the β-lactamase gene that mediates resistance to β-lactam antibiotics; it hydrolyzes the β-lactam ring and inactivates the antibiotic (50).

Molecular analysis of resistance genes provides a rapid screening method for investigating and controlling drug resistance.

In our study, treatment of bovine mastitis with a combination of antibiotics recovered significant improvements in the measured parameters (bacteriological cure, SSC, TBC, and the level of LDH), in addition to improvements in cow, udder, and milk states after treatment.

In our study, clinically and bacteriologically cured quarters were 92.55 percent and 88.18 percent, respectively.Clinical cure is the main goal on farms, but it is not a reliable outcome to assess antimicrobial therapy effectiveness as inflammations of the mammary gland and physical changes in milk are self-limiting and

usually return to their normal appearance within 7 days (51).

Bacterial cure in present study is near to that found by McDougall (52). Using acombination of lincomycin plus neomycin.

Cefalexin is a first generation cephalosporin. It inhibits the synthesis of bacterial cell wall with a time-dependent bactericidal activity, but kanamycin is an aminoglycoside that inhibit of bacterial protein synthesis. Its bactericidal activity is proportional to its concentration.This difference in the mechanisms of action allows a marked difference in the kinetics of killing (12).

Kanamycine's bactericidal activity is greatly reduced by the presence of milk, as is that of other aminoglycosides (53), but synergism observed between cefalexin and kanamycine overcomes this reduction (13).

Maneke et al. (54) proved a faster and greater bacterial killing rate for the combination of cefalexin and kanamycin at lower antibiotic concentrations than those observed with either drug alone.

In this study, post-treatment milk yield per cow did not obviously differ from that recorded pretreatment. The same result was revealed by Sérieys et al. (55), and Fuenzalida and Ruegg (56). Assessment of milk yield requires a prolonged follow-up period (57); this may be the reason why no significant difference in milk yield was recorded in the present study or other previous studies with similar results.

Our results proved a gradual decrease in SCC at the quarter level to near the normal value during the follow-up period after treatment. This is in line with that mentioned by Ruegg(3), who recommended that assessment of SCC responses be performed at the quarter level rather than the composite milk samples and should continue for at least 21 days post treatment, but a gradual (rather than immediate) decline should be expected and etiology influences the rate at which SCC returns to normal.

LDH is a sensitive mastitis indicator in cattle. The enzyme is primarily released by damaged udder epithelial cells, invading microorganisms, and leukocytes, which disintegrate as a response to inflammation by the animal's immune system (58)So the more severe the inflammatory response, the higher the level of this enzyme. LDH is a reliable, highly sensitive marker for early detection of udder infections and assessment of mastitis therapy (6).

Early detection of mastitis is of paramount importance in controlling the disease. The Health MateTM milk LDH mastitis test is a rapid, easily applied test. It does not require complicated steps, instruments, or the skimming of milk samples, making it an ideal mastitis screening test.

Conclusion

Regular updating of information about mastitis-causing pathogens and their antibiotic susceptibility is a very important measure. Molecular analysis of antibiotic resistance genes is a helpful screening method for investigating and controlling drug resistance. Treatment of bovine mastitis with a combination of antibiotics significantly improves the bacteriological cure, SSC, TBC, and the level of LDH in milk.

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Original Research Article
Veterinary Medicine and The One Health Concept

MORPHOLOGY OF THE DROMEDARY CAMEL STOMACH WITH REFERENCE TO PHYSIOLOGICAL ADAPTATION

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Abstract: Dromedary camels (*Camelus dromedarius*) are adapted to their desert habitat where they are able to survive and reproduce despite very high temperatures, little vegetation, and limited water availability. The mechanism of thermoregulation in camels is highly efficient, which makes them maintain the appropriate body temperature to carry out their physiological activities. Compared to other mammals, camels are environmentally tolerant as they can be bred for milk and meat production in areas with scant natural resources depending on their unique physiological, anatomical and behavioral characteristics. The camel's digestive system has unique morphological features that make it highly adapted to its natural environment. Although the stomach of camels is divided into compartments, as in ruminants, they are referred to as pseudo-ruminants because they do not have the clearly divided four-chambered stomach found in true ruminants. However, their stomach is larger and more efficient in dealing with dry, tough, and fibrous food. Therefore, the current study aimed to review the gross anatomical and histological peculiarities and characteristic features of the dromedary camel stomach with respect to their physiological importance. The morphological characterization of the dromedary camel stomach might elucidate the functions of its different compartments. Thus, this review could add to our understanding of the physiology of the digestive system in dromedary camels.

Key words: dromedary camel stomach; morphology; desert habitat

Introduction

The worldwide population of camels is about 18 million, and about 16.5 million of them are dromedary or one-humped camels (1). Dromedary camels (*Camelus dromedarius*) are reared mainly in North and East Africa as well as in the eastern and western parts of Central Asia. They are economically important to low-income families in many countries of Africa and Asia as they are raised for the production of meat, milk, wool, and hides, as well as for transportation and as draft animals (2, 3).

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Dromedary camels are uniquely adapted to the harsh environment of arid and sub-arid regions, which are characterized by shortage of vegetation and water, high ambient temperatures, and other harsh conditions (4, 5). The ability of dromedaries to withstand adverse conditions is attributed to their morphological features, physiological adaption mechanisms, and behavioral attitudes (6). It has been reported that dromedary camels are able to cope with dehydration due to their efficient urinary (4) and digestive systems (7). Camels are able to withstand high ambient temperature, hunger, and thirst for long durations (6, 8). This ability has been attributed to their adaptive homeostatic mechanisms (8) and their high potential for converting the scanty

resources of the desert environment into milk, wool, and meat (9, 10, 11). Thus, camels are considered as a good source of food in areas where the performance of other animals is adversely affected (2).

The digestive system of dromedary camel is highly adaptive to its natural habitat. The lips are long and thick with the upper one being split, which assists in selection and prehension of food when grazing on thorny desert bushes and shrubs (12). The esophagus is long (13) and protected by keratinized stratified squamous epithelium and mucous glands (13, 14). The long intestines in camels results in an increased surface area that enables better nutrient and water absorption (12, 15). The large size of the camel liver facilitates better digestion and blood detoxification than the livers of other species (12). However, the stomach is believed to play the most important role in the adaptation mechanism of the dromedary camel digestive system. The stomach is large and has a great capacity for digesting cellulose, specific and differentiated motility, very active microflora, and significant food mixing ability (11). Additionally, the camel stomach plays an important role in the maintenance of water balance during dehydration and rehydration (16).

This study reviews the gross anatomy, light microscopy, and electron microscopy of the dromedary camel stomach in relation to its physiological activity. The review might be helpful in understanding the digestive physiology of dromedary camels and their ability to withstand desert conditions.

Gross anatomy of camel stomach

The anatomical structure of the camel stomach is shown in Figures 1, 2 and 3.

Camels belong to the order Artiodactyls (even-toed ungulates), sub-order Tylopoda (pad-footed), and family Camelidae (17, 18, 19). Camelids and ruminants are taxonomically and behaviorally different (18, 19). Species of the two sub-orders independently underwent parallel evolution to develop a compound stomach with large cranial part (forestomach). The specific design of the stomach in Tylopoda and Ruminantia together with its related function and physiological actions confirm their non-homologous parallel evolution (20). In this respect Camelids are called modified- or pseudo-ruminants as they have segmented stomach like that found in true ruminants, but their stomach is segmented into three compartments: compartment 1 (C1), compartment 2 (C2) and compartment 3 (C3) rather than four (rumen, reticulum, omasum and abomasum) in true/typical ruminants (21, 22, 23; 24; 25). The stomach of adult camels extends from the diaphragm to the pelvic inlet, occupying the major part of the abdominal cavity (26). The categorization of the different parts of camel stomach seems to be controversial (25). According to many studies the stomach is divided into three compartments C1, C2 and C3 in dromedary camels (21, 22, 23, 25, 27), *Lama glama* (12, 28) and alpaca (20, 29). On the other hand, some other authors recorded four compartments (C1, C2, C3, and C4) in dromedary camel stomach (30, 31). Furthermore, the stomach in Bactrian camels has been considered as a single cavity with multiple different cardiac glands (24). It seems that most of the recent studies believe in the three compartment categorization of camel stomach depending on its morphological, histochemical and functional characteristics. Thus, the current review adopts the three-compartment segmentation of camel stomach i.e. compartment 1 (C1), compartment 2 (C2) and compartment 3 (C3).

C1:

This is the first and largest compartment which comprises 83 % of the total stomach volume (32) and is considered as the analogous to the rumen in true ruminants (29, 31, 32). C1 is round in shape and located on the left part of the abdominal cavity, extending from the diaphragm at the level of the seventh rib to the caudal border at the level of the twelfth rib (31). External transverse groove and internal transverse muscular ridge divide C1 into a cranial portion (cranial sac) and a caudal portion (caudal sac) (29, 31, 32, 33). The opening separating C1 from C2 and that separating C2 from C2 are small in dromedary and alpaca camels compared to true ruminants (29). However, the relatively small C2 is not entirely separated from C1 in camelids (27). The interior of C1 contains two glandular sacs: the cranioventral glandular sac and the caudodorsal glandular sac (22, 26, 27, 29, 31, 34, 35, 36, 37). The cranioventral and caudodorsal glandular sacs are denoted by a crescent-shaped

pillar internally that is corresponding to the transverse groove. Each sac contains glandular chambers formed by four walls (pillars); the caudodorsal sac is larger, irregular, and more sacculated, as compared to cranioventral sac (31). Recently, two sacculated regions in C1 and a third comb-like one have been reported in C2 (20). It has been stated that there are two regions in C1 of dromedary camel: non-glandular region and glandular regions; the non-glandular region is large and relatively smooth and constitutes 53.2% of the entire gastric mucosa (25). A ventricular muscular groove, like that found in true ruminants connecting C1 and C3, is also present in Llama and dromedary camels; during the neonatal period, fluid ingesta bypass C1 and C2 via this groove to enter C3 (29, 36).

C2:

C2 is the second and smallest gastric compartment constituting 6% of the total gastric volume (32). It is situated on the right part of abdominal cavity in dromedary camels (27, 30, 31, 36) and guanaco and llama (28). C2 is described as pear- (26) or kidney- (25) shaped in dromedary camels. Wang *et al.* (2000) described it as elliptically concave that continues cranially with the pro-ventriculus and the caudal glandular sac (38). Lechner-Doll *et al.* (1995) showed that in dromedary camels C2 is not completely separated from C1 (27). In *Llama and Guanaco*, C2 empties into C3 through a short and thick muscular tube that controls the rate of material movement into C3 (28).

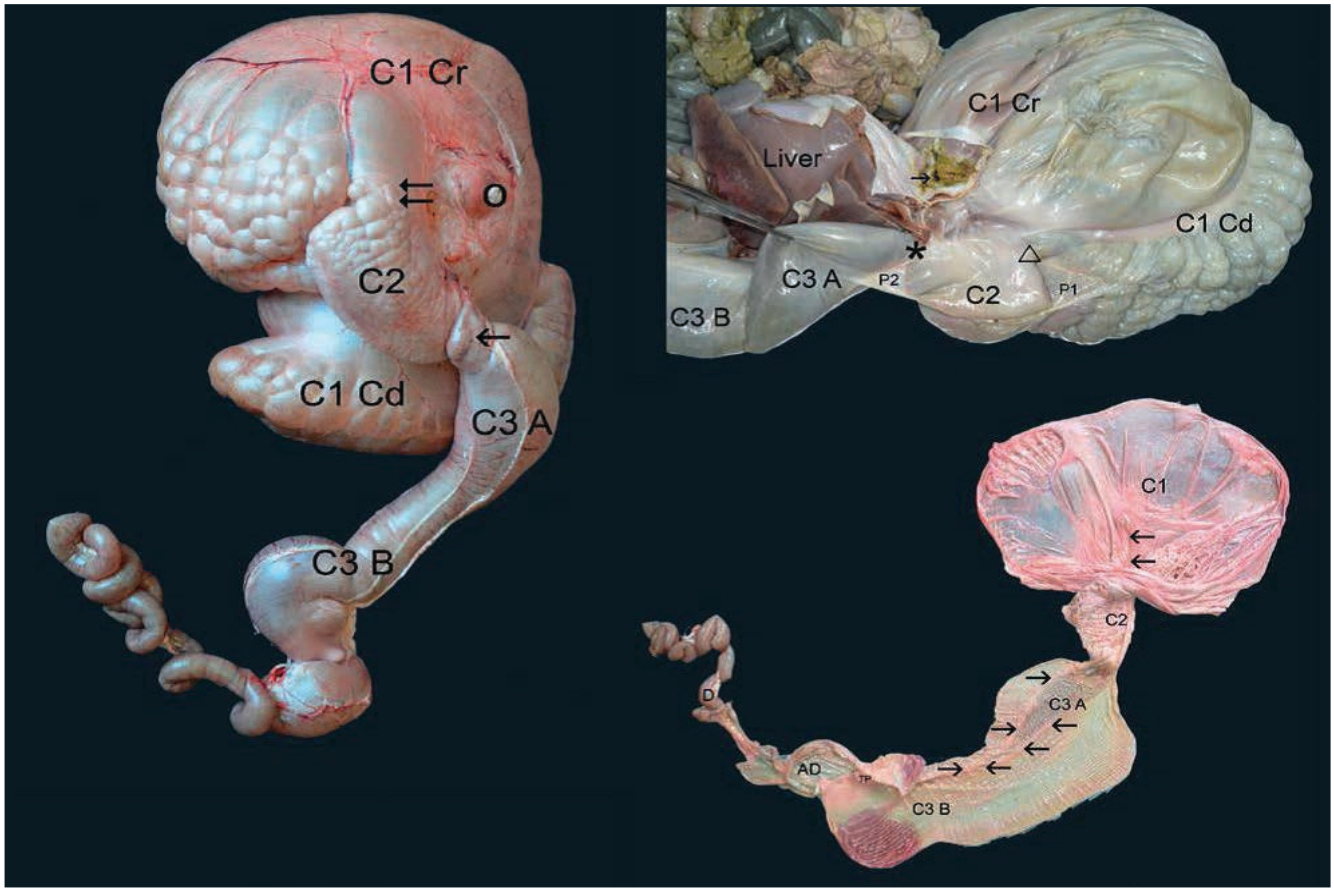


Figure 1: (From Pérez et al., 2016 after getting permission):

A. Dromedary camel stomach. C1 Cr: Cranial part of first gastric compartment; C1 Cd: Caudal part of first gastric compartment; C2: Second gastric compartment C2; C3A and C3B: Proximal and distal parts of third gastric com-partment C3; O: Ostium car-diacum; Double arrow: Position of the orifice between C1 and C2; Arrow: Position of the orifice between C2 and C3A. B. Alpaca gastrointestinal tract showing external position of orifices of C2 and peritoneal folds that delimited C2. C1 Cr: Cranial part of first gastric compartment; C1Cd: Caudal part of first gastric compartment; C2: Second gastric compartment C2; C3A and C3B: Proximal and distal parts of third gastric com-partment C3; Triangle: Position of the orifice between C1 and C2; * Position of the orifice between C2 and C3A; P1: Peritoneal fold between C1caudal and C2; P2: Peritoneal fold between C2 and C3A. C. Internal view of the drome-dary stomach after dorsal incision. C1: First gastric compartment; C2: Second gastric compartment; C3A and C3B: Proximal and distal parts of third gastric compartment C3; TP: Torus pyloricus; AD: Ampulla duodeni; D: Duode-num; Arrows; Gastric groove. Scale bar = 10 cm

The internal surface of C2 shows several longitudinal and transverse muscular bands which form large and small chambers (31). The interior of C2 is similar to C1, except for the numerous bands and folds that form smaller and deeper chambers (26, 30). The entire gastric mucosa, except for the gastric groove, is found to be in the form of interconnected folds similar to, but smaller, than those found in glandular sacs (25).

C3:

C3 is described as a long tube located on the right part of the abdominal cavity under C2 (27, 29, 34, 31, 36). In dromedary camel and Alpaca it is composed of two parts: proximal and distal (29). The proximal part is the initial dilated part and the distal part is elongated and connects to the duodenum.

Internally, C3 is entirely lined by glandular and its mucosa is in the form of about 50 longitudinal folds that decrease craniocaudally and increase in the middle (27, 31, 34, 36). Like the abomasum in true ruminants, C3 in camels contains two regions: the fundic region and the pyloric region (22, 26, 31, 36, 39). The fundic region consists of thick longitudinal folds and the pyloric region is formed of thin longitudinal folds (30). However,

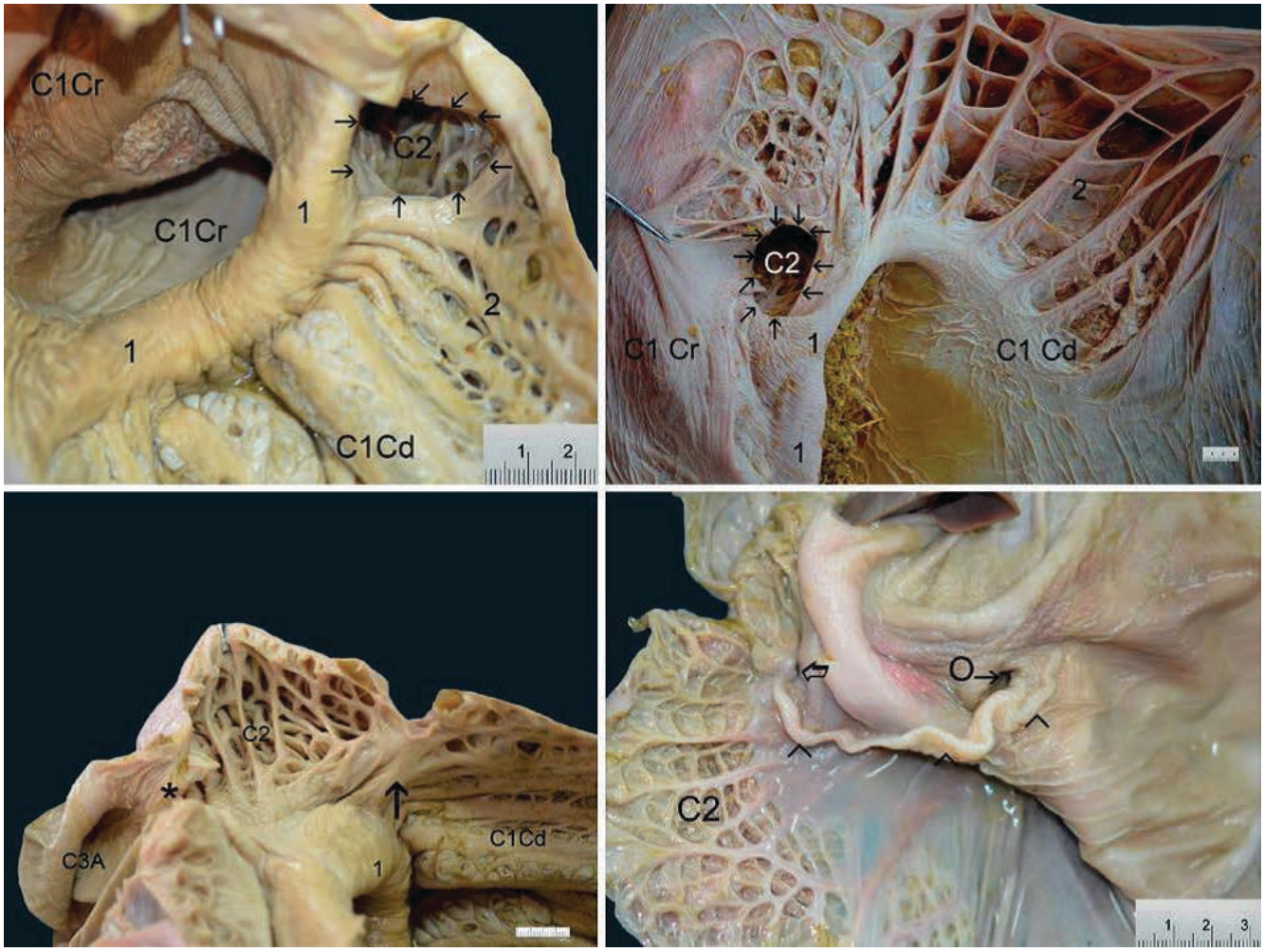


Figure 2: (From Pérez et al., 2016 after getting permission):

A. Internal view of cranial part of C1 and C2 of alpaca stomach. C1Cr: Cranial part of first gastric compartment; C1 Cd: Caudal part of first gastric compartment; C2: Second gastric compartment C2; Arrows: Orifice between C1 and C2; 1: Transverse pillar of C1; 2: Glandular sac area of C1 Cd. B. Internal view of cranial part of C1 and C2 of dromedary camel stomach. C1Cd: Caudal part of first gastric compartment; C2: Second gastric compartment C2; Arrows: Orifice between C1 and C2; 1: Transverse pillar of C1; 2: Glandular sac area of C1Cd. C. Internal view of cranial part of C1 ventral, C2 and C3 A of alpaca stomach after dorsal incision of C1 dorsal and the dorsal walls of orifices between C1 to C2 and C2 to C3. C1 Cd: Caudal part of first gastric compartment; C2: Second gastric compartment C2; C3 A: First part of the third gastric compartment; Arrow: Opened orifice between C1 and C2; Asterisk: Opened orifice between C2 and C3 A; 1: Transverse pillar of C1. D. Internal view of cranial part of C1 and C2 of alpaca stomach.O: Ostium cardiacum; C2: Second gastric compartment C2; Arrow: Orifice between C2 and C3A; Arrowheads: Sulcus ventriculi in the inner side of C2. Scale bars in cm.

three regions have been reported in the interior of C3 in dromedary camel: cranial region with slender tortuous folds, middle region with tight folds and distal region with characteristic thick wavy folds like those found in true ruminant's abomasum (25).

Histology of camel stomach

The different compartments of the dromedary camel stomach consist of four tunics: tunica mucosa, tunica submucosa, tunica muscularis, and tunica serosa (30, 31). All compartments in dromedary stomach are glandular except C1 which exhibits both glandular and non-glandular mucosae (Fig. 4A, B) (40). The latter authors stated that the glandular mucosa in C1 is only found in the regions of glandular sacs, whereas the non-glandular mucosa is present elsewhere. Similar findings have also been reported in other camelids (27, 28, 33, 41). However, eight histological regions are found in dromedary camel stomach (25). The first region, which constitutes about 53.2% of the stomach, is non-glandular and occupies the body of the first compartment. The other seven regions have glandular mucosae. Whereas keratinized stratified squamous epithelium lines the mucosa of the entire true ruminant's forestomach (rumen, omasum, abomasum), it is only restricted to the dorsal parts of Cl and C2 in camelids (27, 41, 42). The latter authors mentioned that the ventral part of C1, C2 and the entire internal surface of

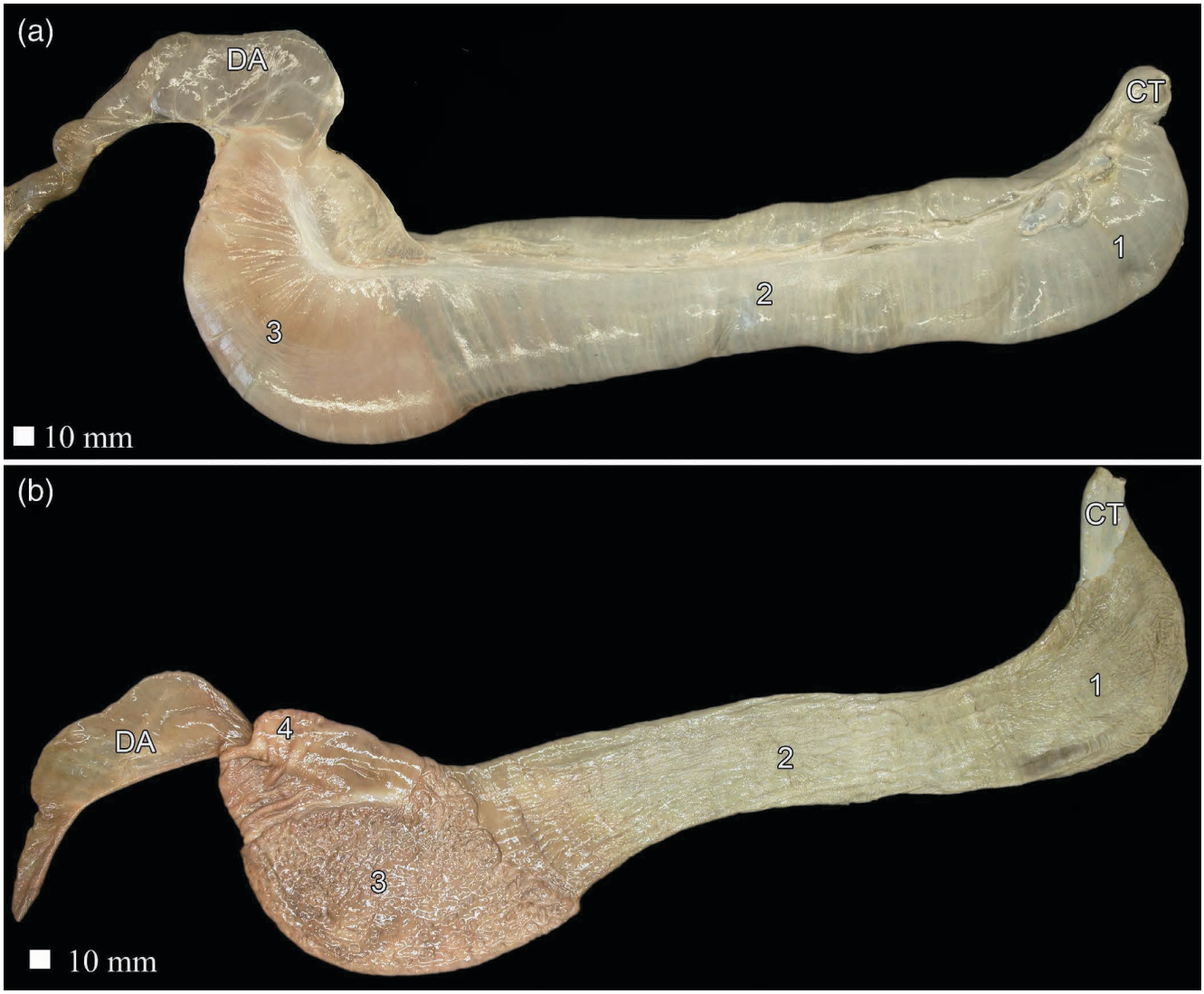


Figure 3: (From Vater et al., 2021 after getting pretermission)

External (a) and internal (b) morphology of C3. CT, Connection tube; DA, duodenal ampulla. 1: Initial bend of C3; 2: straight part of C3; 3: caudal loop of C3; 4: torus pyloricus

C3 are lined by simple columnar epithelium with deep tubular glands underneath. They conclude that this region is similar to the cardiac region of true ruminant's abomasum. The mucosal lamina propria is in the form of loose connective tissue which contains gastric glands in glandular regions (30, 31, 43). While muscularis mucosa is absent in the non-glandular region, it constitutes a thin layer of circular smooth muscle fibres in the glandular region (30, 31). However, the muscularis mucosa is totally absent in glandular sacs of dromedary camels (35). The tunica submucosa of dromedary camel stomach is formed of connective tissue with some blood vessels as well as nerve and smooth muscle fibres; the tunica muscularis which follows the submucosa is in the form of smooth muscle fibers arranged as an inner circular layer and an outer longitudinal layer and it is followed by serosa which is a thin layer of connective tissue covered by mesothelium (30, 31).

In C1 of the camelids stomach, including dromedaries, the epithelium of the non-sacculated region is keratinzed stratified squamous, whereas it is simple columnar in the glandular sac area (27, 30, 31). However, it has been reported that the glandular sacs in dromedary camel have only keratinized stratified squamous epithelium (35). The lamina propria of C1 consists of loose connective tissue with simple branched glands (30, 31, 33, 42). These glands have also been described as deep tubular in shape (41- 43).

In C2, whereas the walls and floors of the glandular saccules are lined by simple columnar epithelium, longitudinal bands, which are non-glandular, are lined by keratinized stratified squamous epithelium (30, 31,). Both stratified squamous and simple columnar epithelia in C2 have also been reported in other camelids (27). On the other hand, the stratified squamous epithelium of C2 is found to be non-keratinized (30). Moreover, the entire mucous membrane of C2 is reported to be lined by simple columnar lining (43). The muscularis mucosa in C2, which is in the form of circular smooth muscle layer separating the lamina propria from the submucosa, is only found in the glandular region, (30, 31). In C2 the muscularis mucosa of the bands consists of a longitudinal layer of smooth muscle fibers, whereas it is in the form of a smooth muscle bundle in the upper regions of the primary folds (30).

The mucosa of C3 in camels is highly folded and entirely glandular; it is lined by simple columnar epithelium followed by a lamina propria which

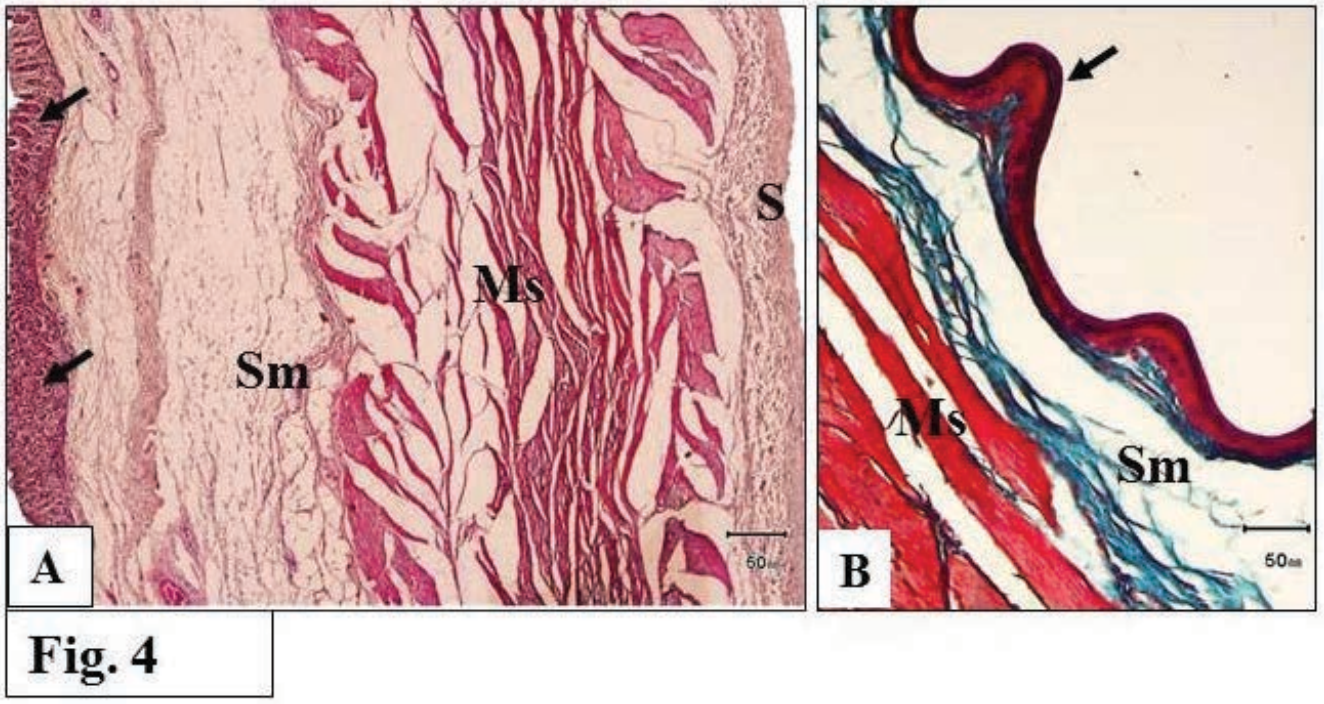


Fig. 4

Figure 4: (From Abuagla, 2014, PhD thesis)

A: Glandular area of C1 showing simple columnar epithelium and mucosal gastric glands (Arrows); submucosa (Sm), tunica muscularis (Ms) and serosa (S). H&E stain. X4. **B:** Non-glandular area of C1 showing keratinized stratified squamous epithelium (Arrows), submucosa (Sm) and tunica muscularis (Ms). Massons' trichrome stain. X10.

contains gastric glands (20, 27, 33). Similar findings have also been noted in ruminant abomasum (27, 43). Large gastric glands are observed in dromedary camel C3 which are continuous with numerous gastric pits (39). The greater curvature of tunica mucosa of the terminal dilated part of C3 bears very thick folds which divide the mucosa into nearly regular region with gastric areas; this region contains the proper gastric glands (fundic glands) which are somewhat different from those in the true ruminants (22). However, the terminal distended part of C3 considered as a separate fourth compartment, named C4 (30, 31). While in one study C4 is divided into three histological regions: cardiac, fundic, and pyloric regions (31), other two studies divided it into two regions: fundic and pyloric regions, considering the cardiac region as a narrow band between the fundic region and C3 (30, 34). The mucosa in the cardiac region of C4 is folded and the epithelial lining is simple and columnar, whereas the lamina propria contains simple branched tubular glands with mucus secreting cells (30, 31, 34). The lamina propria is surrounded by a thin muscularis mucosa of circular smooth muscle fibers (30, 31, 34). Osman (1999) stated that the smooth muscle fibers of muscularis mucosa do not reach the folds or laminae of C3 as it does in ruminants (30). The gastric glands of C4 in dromedary camel are lined by three types of cells: mucous cells, parietal cells, and chief cells (31). These cell types have previously been reported in the same species (30, 34). The mucous cells are tall and found in the glandular neck region. The chief cells are basophilic and mainly basally located. The parietal cells are numerous compared to the chief cells. They are rounded and acidophilic and located in the basal and parietal regions (30, 31). The gastric glands in the basal part mainly contain mucus-secreting cells with some parietal cells (21, 30, 31). However, the pyloric glands have been observed with mucous cell types that with spherical or flat nuclei occupying the glandular basal part (30).

Ultra-structurally, the dromedary saccular area is folded and studded with numerous glands of various sizes and shapes including cup-shaped, cap-shaped and flower-shaped which are lined by folded cells (Fig. 5A, B) (40). Also in Llama guanaco, transmission electron microscopy reveals glandular sac epithelial cells with tiny microvilli (42). In dromedary camels the columnar cells lining the glandular sacs are closely packed together and they present basal rounded or oval nuclei and rich

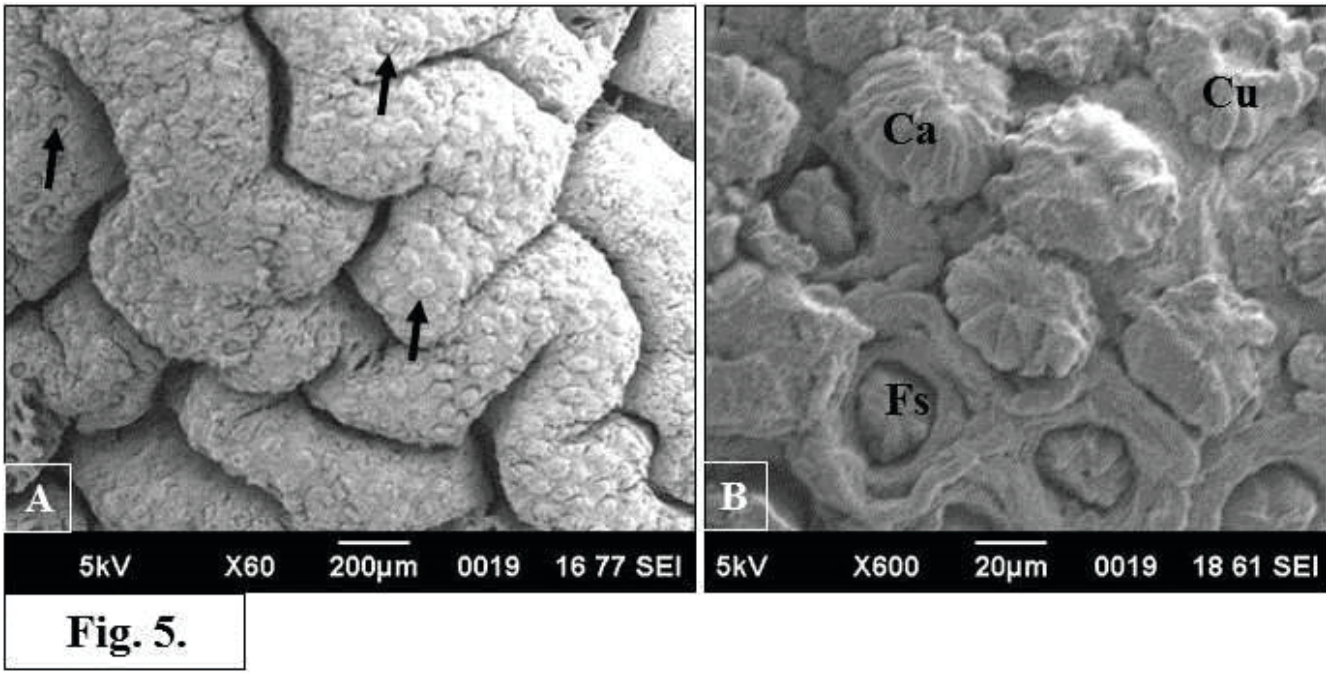


Figure 5: (From Abuagla, 2014, PhD thesis)

A: Scanning electron microphotograph of glandular area of C1 showing folded mucosa with different gastric glands (Arrows). X60. B: Scanning electron microphotograph of glandular area of C1 with cup-shaped (Cu), cap-shaped (Ca) and flower-shaped (Fs) glands. X600.

in mitochondria, rough endoplasmic reticulum, Golgi apparatus, numerous secretory granules and several dense bodies, especially in their apical cytoplasm (Fig. 6A,B) (40). It has also been stated that the plasma membrane of abomasal glands shows prominent enfolding in some epithelial cells of dromedary (31) and Llama (41, 42) camels. The mitochondria are numerous and mainly occupying the supra-nuclear cytoplasm of many cells in the epithelium of cranioventral and caudodorsal sacs in dromedary camels (31). The mitochondria are numerous and mainly occupying the supra-nuclear cytoplasm of many cells in the epithelium of cranioventral and caudodorsal sacs in dromedary camels (31). In llama, the mitochondria are slender or elongated in shape with a dense matrix filling wide parts of the upper cytoplasm (41). Additionally, the dromedary camel (31) and *Llama guanaco* (41) have well-developed Golgi apparatuses as well as numerous secretory granules and dense bodies in the apical cytoplasm and glandular cells of the cranioventral and caudodorsal sacs. It has also been noted that in Bactrian camels the glandular cells in the sac area have low- and high-density granules in the supra-nuclear cytoplasmic region (24).

Adaptation of camel stomach

Physiologically, camel stomach is similar to true ruminants in several aspects, including microbial fermentation, re-gurgitation, re-chewing, and re-swallowing of ingested food (18, 19). It has been reported that slow circular movements between the cranio-ventral and caudo-dorsal sacs of rumen facilitates microbial activity (27). The large size of the dromedary C1 and C2 and true ruminant's rumino-reticulum results in increased storage capacity and efficient digestion of forage rich in crude fiber (12). Gastric differentiation of camels enables using C1 and C2 as a fermentation vat because it offers complex media for anaerobic bacteria (44). Moreover, the large forestomach in grazers and browsers like dromedary camels could be linked to the fact that they require more retention time for the digesta (45). It has been mentioned that the relatively small orifice between C2 and C3 can only allow for passage of a certain size of food particles which ensures adequate food fermentation and absorption in forestomach in camels and cattle (45 46). However, two important morphological differences between the forestomach in camels and true ruminants

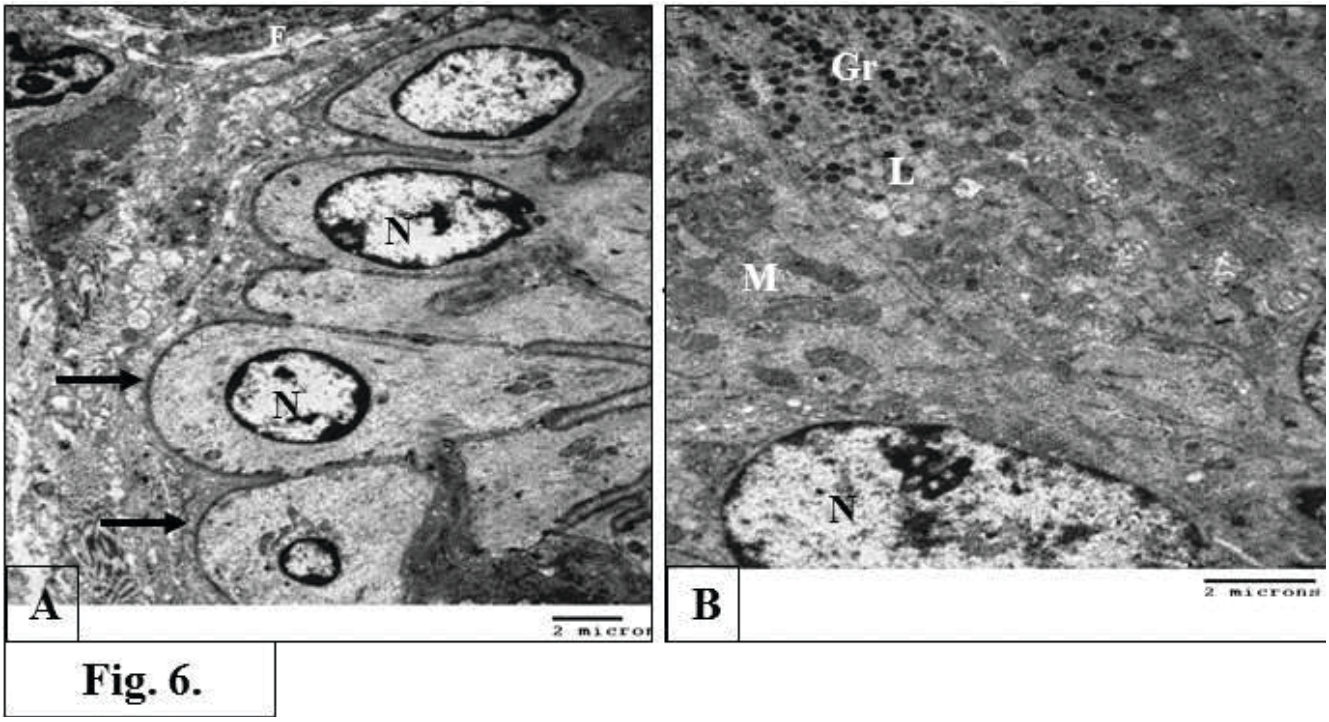


Figure 6: (From Abuagla, 2014, PhD thesis)

A: Transmission electron microphotograph of glandular area of C1 showing closely packed columnar or pyramidal epithelial cells (Arrows) with oval nuclei (N). X3600. B: Transmission electron microphotograph of glandular area of C1 showing oval nuclei (N), supra-nuclear mitochondria (M), lipid droplets (L) and secretory granules (Gr). X5800.

have been stated (29). Firstly, whereas in true ruminants the reticulum lies along the major axis of the rumen (cranial to it), the major axis of the C1 is not aligned with C2 in camelids. Secondly, in true ruminants, the opening between the rumen and reticulum is relatively larger as compared with the opening between C1 and C2. Thus, the forestomach of camels contains a bottleneck between C1 and C2 which might explain why they cannot process high amount of food compared to taxonomic ruminants. It has been mentioned that the glandular sacs in camels allows for increased gastric surface area which increases the absorption rate of volatile fatty acids (VFAs) 3 - 4 times that of other ruminants (47). Moreover, the glandular sacs might perform rapid absorption of water and solutes (41). The glandular sacs could also be considered as a means of protecting camels from dehydration in hot environmental conditions (12). It is reported that water-deprived sheep lose much more rumen water than camelids (46) which is related to the anatomical differences between camels and true ruminants. This could also be related to the extremely slower water turnover in camels (48). The later author concluded that water dynamics in the alimentary canal allows camels to survive and produce during dry periods (48). Similarly, the fatty acid absorption in the llama stomach is faster than in true ruminants (49, 50). The absorption of soluble particles and water in camel stomach occurs mainly in C3 (51). In this respect, the contents of C3 in the camel stomach are dry, which strongly suggests significant water absorption (52). The glandular microscopic structure of the camel gastric compartments is responsible for their high digestibility coefficient (32). The keratinized stratified epithelium of the upper digestive tract, including non-glandular parts of C1 and C2, enables camel to deal with dry and thorny forage in desert (53). The large gastric glands in C3 fundic region indicates that the camel stomach is more prepared for glandular digestion than fermentative digestion as compared to true ruminants (25). C3 parietal cells arrangement and chief cells behavior can preserve muco-substance during fastening or diminishing of the food (22). The columnar cell lining and prominent enfolding in the plasma membrane of the glandular sacs could be considered as an indication of the absorptive function in the surface and glandular epithelial cells. Despite the prominent differences in morphology between camels and true ruminants, both show similar dependence on salivary glands for provide the mucus, alkali, and fluid needed for the fermenting food in the forestomach (34). This indicates the dominance of the absorptive function of the camel stomach over its secretory function. It has also been shown that the mucus found in the surface epithelium of the camel stomach might mainly be a protective function (54). While the latter authors claim that that the function of the glandular endocrine cells in camel stomach is unknown, they are considered important in water-electrolyte balance during dehydration (55). It has been noted that the ruminal endocrine and secretory activity in camels could account for nitrogen retention (54). The latter authors claimed that nitrogen decline in both urine and feces together with sodium renal loss enable camels to maintain a relatively stable extracellular volume (54). It has also been stated that the flow of water urea-nitrogen in the same direction accounts for the lower water content in the feces and urine of camels compared to that of sheep (56). Thus, the camel has a far more efficient mechanism of nitrogen conservation than other ruminant animals (57). It has been reported that concerning the recycling of urea, 12 days of dehydration in camels are equal to 2 days of dehydration in sheep (56). Ultrastructure review shows that the gastric secretory activity in camels could be indicated by the presence of many mitochondria, well-developed Golgi bodies, and numerous secretory granules and dense bodies in the supra-nuclear cytoplasm of glandular epithelial cells (31).

Conclusion

The data reviewed in this work shows morphological and physiological adaptations of the dromedary camel stomach. These adaptive mechanisms of camel stomach reflect its capability to deal with limitations of food and water in harsh habitat compared to other mammalian species. Moreover, this review could be considered as a contribution toward a better understanding of the unique digestive system of the dromedary camel.

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Original Research Article
Veterinary Medicine and The One Health Concept

EVALUATION OF ANTHELMINTIC, HEMATOLOGICAL AND SERUM BIOCHEMICAL EFFECTS OF HERBAL DEWORMER ON THE CATTLE

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Abstract: Cattle play an important role in the food chain as a major milk and meat producer. Helminths affect the health and production of livestock which need to be controlled efficiently and economically. The high usage of anthelmintic drugs has increased anthelmintic resistance to all available classes resulting in failed treatment options and economic losses. Herbs, which may be better options for animal and food safety, are among the best candidates to control these parasites. For this purpose, a research experiment was conducted. Twelve Sahiwal heifers were used for the evaluation of the anthelmintic activity of herbal dewormers composed of various parts of local herbs. Animals were divided into 4 groups A, B, C and D, all the groups, except group D were given herbal dewormer powder. In an experiment of 30 days, doses were given on a weekly basis. Fecal samples for egg counts, blood and serum samples were obtained fortnightly and subjected to statistical analysis. Results revealed that the animals receiving herbal dewormer @ 1400 mg/Kg had the best results and egg counts and hematological values were significantly ($P<0.05$) better than the control animals. The results of the study suggested that the herbal dewormer was efficient in reducing the worm counts as well as beneficial for the hematological profile of the cattle.

Key words: cattle; helminths; EPG; ECR; anthelmintic; herbal

Introduction

Infectious agents i.e., viruses, bacteria, fungi, protozoa, and helminths, etc. are the major causes of death in life on the earth (1, 2). Among the infectious agents, parasites are the major causes of mortalities and morbidities in animals. Helminths are among the dangerous parasites which affect a wide range of animals and pose a threat to their productive and reproductive efficiencies (3). They are among the major threats to food security because of their great impact on the milk and meat production of animals (4). Among the food animals, cattle have vital importance because of their huge share in milk and meat production globally (5). Cattle are greatly affected by helminths, which reduce their milk and meat production and may even cause mortalities (6). The surety of food security through efficient cattle milk and meat production needs proper control of these parasites (2, 7). Among helminths, nemathelminths and platyhelminths are two phyla that contain the parasites of the helminths. *Trichostrongylus* from nemathelminths and *Paramphistomum cervi* from phylum platyhelminths are among the major species found in cattle (8). Multiple researchers have reported about these parasites are found frequently in cattle (9).

Routine control of these parasites is dependent on the chemical anthelmintics which are being used practically (10, 11). Progressively, helminths

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are being resistant to these drugs which makes it impossible to use these drugs in near future (12). Multiple drug resistance has been reported in various infectious agents including bacteria, protozoa, and helminths (13, 14). Additionally, these drugs are harmful to milk and meat consumers due to their toxic residual metabolites in these products. These issues are issues of great concern for scientists, so they are trying to find alternatives (15). Vaccines, organic acids, and many other alternatives have been suggested by scientists (14, 15). Researchers are focusing to develop commercial products for proper control of these drugs (16, 17).

Plants have been among the most prominent options for their use because of their safe mechanism and ease of availability (18, 19). Multiple plants have been tested to control helminths, but the use of a single plant may not be sufficient to control these varieties of parasites (20, 21). This phenomenon needs a proper herbal product made up of various plants belonging to different families having diverse active compounds for their safe and effective use (19). For this purpose, a plant-based herbal dewormer was formulated to find out the cumulative effect of various plants simultaneously. In this research effect of herbal dewormer was evaluated against total helminths, *Trichostrongylus* spp. and *Paramphistomum* spp. along with evaluation of the effects of these herbs on blood and chemical procedures.

Material and methods

Plant material:

Various parts of the plants were obtained from commercial resources and verified at the Department of Botany, University of Agriculture, Faisalabad, Pakistan. They were ground to make a powder for use in the cattle. Detailed composition of the plants used for the formulation of herbal dewormer is given in the Table (1).

Animals

The research was conducted on the cattle from cattle section, Livestock Production and Research Institute, Bahadurnagar, Okara, Pakistan. Adult female heifers of the Sahiwal breed (18-24 months old) which were not pregnant, were selected and screened for egg counts. 12 cattle were selected for the research.

Table 1: Chemical composition of herbal dewormer

Sr. No	Family name	Scientific name	composition (%)
1	*Apiaceae*	*Foeniculumvulgare*	18
2			
3		*Trachyspermumammi*	18
4		*Cuminumcyminum*	3
5	*Fabaceae*	*Glycine soja*	10
6		*Sansevieriatrifasciata*	10
7			
8		*Casia fistula*	4
9	*Combretaceae*	*Combretum indicum*	4
10		*Terminalia chebula*	4
11	*Linaceae*	*Linumusitatissimum*	4
12	*Nitrariaceae*	*Peganumharmala*	4
13	*Asclepiadaceae*	*Leptadenia Reticulata*	3
14	*Theaceae*	*Camellia sinensis*	4
15	*Brassicaceae*	*Lepidium sativum*	3
16	*Cucurbitaceae*	*Citrulluscolocynthis*	3
17	*Rosaceae*	*Rosa sericea*	4
18	*Lamiaceae*	*Mentha spicata*	2
19	*Gentianaceae*	*Swertia* L.	2

Experimental design

Selected animals were grouped equally distributed into four groups A, B, C and D. Groups A, B and C were administered the herbal formulation at a rate of 1000 mg/Kg, 1200mg/Kg and 1400 mg/Kg orally in the powder form. Group D was maintained as negative control. Trial continued for 30 days. The animals were given the dose at a week interval and the fecal samples were collected fortnightly. Weight gains, blood and serum samples were also collected fortnightly.

Eggs per gram of feces and percent reduction in egg count

Standard, sterile methods of collection of fecal samples were observed and collected samples were transported to the university of Agriculture, Faisalabad. MAFF (22) method of egg counting was used, and the following equation was used for the determination of egg counts.

Weight Gains

The weights of the animals under study were taken initially and at the end of the trial. Data obtained was subjected for estimation of weight gain, average daily weight gains and percent weight gains (23).

Hematology and serum biochemistry

Blood and serum samples were collected on day 0, 15 and day 30. Standard procedures were adopted to evaluate effects on blood parameters i.e., Red blood cells (RBCs) white blood cells (WBCs), Packed Cell Volume (PCV) and hemoglobin concentration. Spectrophotometric kits were used to analyze the serum parameters of liver and renal efficiency (3).

Statistical analysis

Egg per gram, serum and blood values were subjected to the analysis of variance and means were compared through the Tukey test using Minitab® 26.0. Percent egg count reduction and graphs were formed using Excel® 365 (24).

Results

Eggs per gram of feces and percent reduction in egg count

Significant (P<0.05) reduction in EPG was observed in the treated groups when compared to the control, over the period of time. A dose-dependent response was observed in the treated groups (Table 2). Group C had the lowest EPG among all the groups and the highest percent reduction was also observed in all the groups. Group C had the highest percent Reduction of eggs i.e., 80.97% for total helminths 83.8% for *Trichostrogylus* and 81.43% for *Paramphistomum* (Table 3).

Hematology and serum biochemistry

Group C animals showed significantly (P<0.05) higher RBC, PCV and Hb values at the end of the trial. Lower WBC counts were observed in the treated groups and group C had significantly

Table 2: Helminths, *Trichosrtongylus*, and *Paramphistomum* egg counts per gram of feces in Sahiwal cattle on days 0, 15, and 30 of trial

	Eggs per gram								
	Total helminths			Trichostrongylus			Parsmphistomum		
	Day 0	Day 15	Day 30	Day 0	Day 15	Day 30	Day 0	Day 15	Day 30
A	2300 ± 217.94^{a}	1716.66 ± 125.83^{b}	1116.66 ± 125.83^{b}	783.33 ± 104.08^{a}	650 ± 50^{a}	400 ± 50^{b}	533.33 ± 76.37^{a}	400 ± 50^{ab}	300 ± 50^{ab}
B	2316.66 ± 125.83^{a}	1500 ± 50^{b}	916.66 ± 152.75^{b}	800 ± 132.28^{a}	550 ± 50^{a}	333.33 ± 76.37^{bc}	516.66 ± 125.83^{a}	316.66 ± 28.86^{ab}	166.66 ± 28.86^{b}
C	2266.66 ± 175.59^{a}	1100 ± 50^{c}	433.33 ± 76.37^{c}	816.66 ± 104.08^{a}	333.33 ± 28.86^{b}	133.33 ± 28.86^{c}	516.66 ± 104.08^{a}	233.33 ± 28.86^{b}	100 ± 50^{b}
D	2283.33 ± 301.38^{a}	2316.66 ± 225.46^{a}	2333.33 ± 251.66^{a}	716.66 ± 125.83^{a}	716.66 ± 104.08^{a}	733.33 ± 125.83^{a}	500 ± 180.27^{a}	483.33 ± 175.59^{a}	516.66 ± 175.59^{a}

Values with similar letters within a column are statistically nonsignificant (p>0.05)

lower white blood cells over the passage of time (Table 4). There was a non-specific trend in the serum biochemical values except serum protein and albumins among the groups with the passage of time (Figure 1-9). Serum albumins and proteins were significantly (P<0.05) higher in group C (Figure 5, 6).

Weight Gains

Weight gain, average daily weight gain and percent weight gain of all the groups was evaluated and it was estimated that the group C had the best weight gains among all the groups. All the treated groups had the dose dependent response across the concentrations (Table 5).

Table 3: Percent reduction in egg counts on day 0, 15 and 30 of the trial

	Fecal Egg count reduction (%)								
	Total helminths			Trichostrongylus			Parsmphistomum		
	Day 0-15	Day 15-30	Day 0-30	Day 0-15	Day 15-30	Day 0-30	Day 0-15	Day 15-30	Day 0-30
A	25.21 ± 3.71	34.95 ± 5.42	51.48 ± 1.79	16.61 ± 4.88	38.61 ± 2.97	48.88 ± 1.92	24.83 ± 2.52	23.74 ± 17.94	42.67 ± 13.64
B	35.09 ± 4.88	38.62 ± 12.13	60.49 ± 5.81	29.25 ± 18.7	38.53 ± 17.19	58.63 ± 2.99	37.05 ± 10.88	47.61 ± 4.12	67.24 ± 4.12
C	51.33 ± 3.22	60.61 ± 6.56	80.97 ± 2.05	59.02 ± 1.99	60.31 ± 5.49	83.8 ± 1.72	54.29 ± 4.17	58.33 ± 17.55	81.43 ± 6.25
D	-1.76 ± 3.48	-0.65 ± 1.13	-2.4 ± 2.5	-0.42 ± 6.52	-2.08 ± 3.6	-2.38 ± 4.12	3.43 ± 13.09	-8.11 ± 8.34	-3.7 ± 6.41

Table 4: Hematological parameters evaluated in the cattle on day 0, 15 and 30 of the experiment

	Red Blood Cells (10^6/uL)			Hemoglobin(g/deciliter)			PCV (%)			White Blood cells ($x10^3$/uL)		
	DAY 0	DAY 15	DAY 30	DAY 0	DAY 15	DAY 30	DAY 0	DAY 15	DAY 30	DAY 0	DAY 15	DAY 30
A	3.2 ± 0.09a	3.36 ± 0.05b	3.56 ± 0.11b	8.54 ± 0.09a	9.4 ± 0.16b	9.82 ± 0.12c	23.8 ± 0.6a	25.63 ± 0.86bc	27.6 ± 1.95c	5.23 ± 0.08a	5.03 ± 0.16ab	4.77 ± 0.04ab
B	3.26 ± 0.2a	3.43 ± 0.15ab	4.2 ± 0.1a	8.44 ± 0.22a	9.63 ± 0.13b	10.33 ± 0.15b	25.7 ± 0.87a	27.28 ± 1.47b	33.5 ± 2.83b	5.41 ± 0.4a	4.98 ± 0.06b	4.82 ± 0.05b
C	3.46 ± 0.15a	3.73 ± 0.15a	4.46 ± 0.05a	560.75 ± 478.48a	10.33 ± 0.15a	10.96 ± 0.2a	24.2 ± 2.33a	32.5 ± 0.95a	39.53 ± 0.95a	5.55 ± 0.12a	4.41 ± 0.05c	4.22 ± 0.08c
D	3.2 ± 0.09a	3.33 ± 0.05b	3.33 ± 0.15b	8.47 ± 0.21a	8.39 ± 0.18c	8.36 ± 0d	24.46 ± 1.95a	24.23 ± 1.02c	24.4 ± 0.81c	5.39 ± 0.19a	5.46 ± 0.2a	5.35 ± 0.2a

Values with similar letters within a column are statistically nonsignificant (p>0.05)

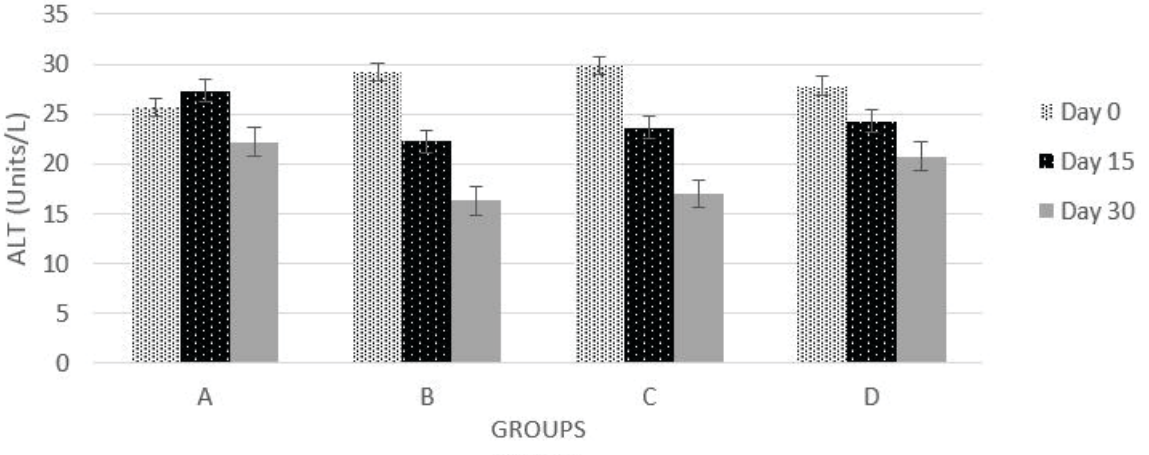


Figure 1: Alanine aminotransferase (ALT) values of Sahiwal cattle on days 0, 15 and 30 of trial

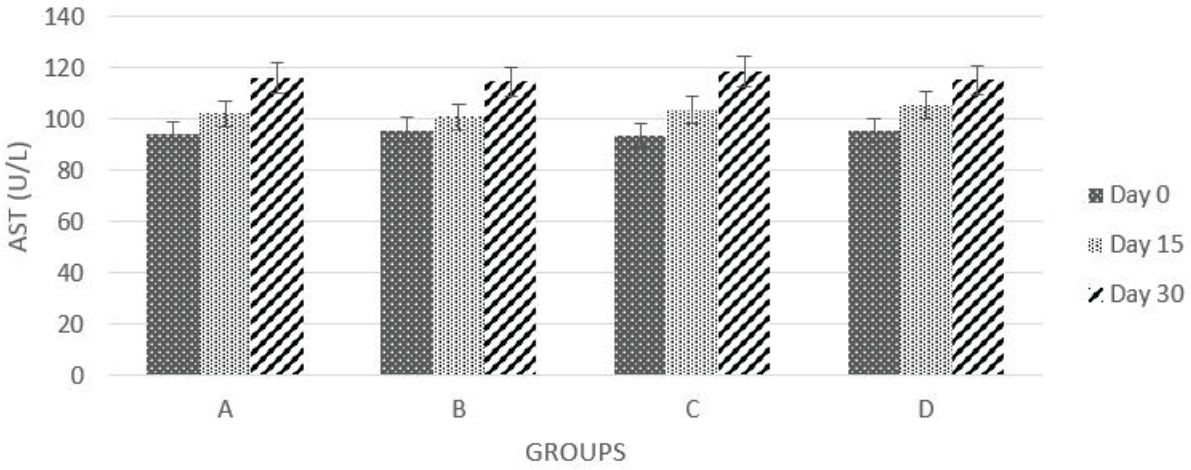


Figure 2: Aspartate Transferase (AST) values of Sahiwal cattle on days 0, 15 and 30 of trial

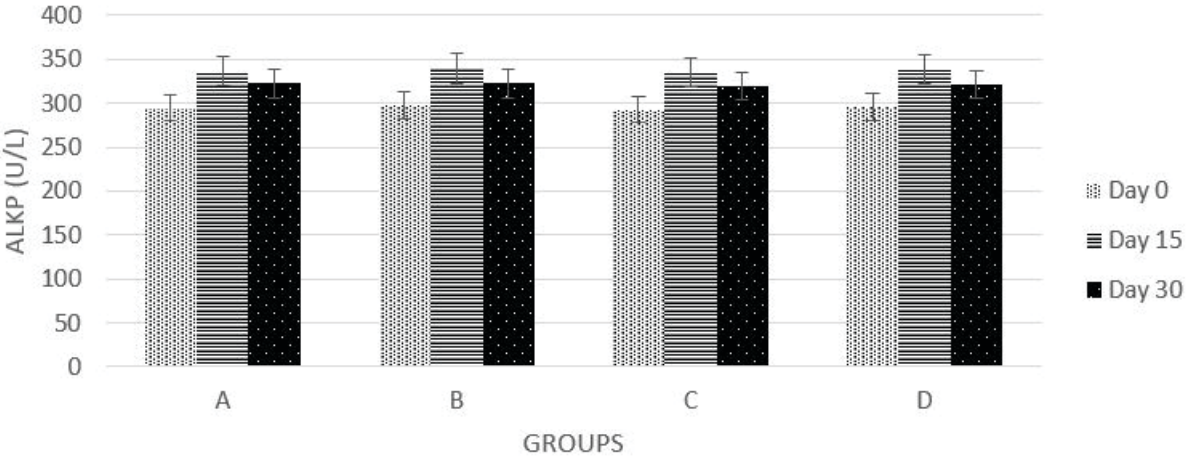


Figure 3: Alkaline Phosphatase (ALKP) values of Sahiwal cattle on days 0, 15 and 30 of trial

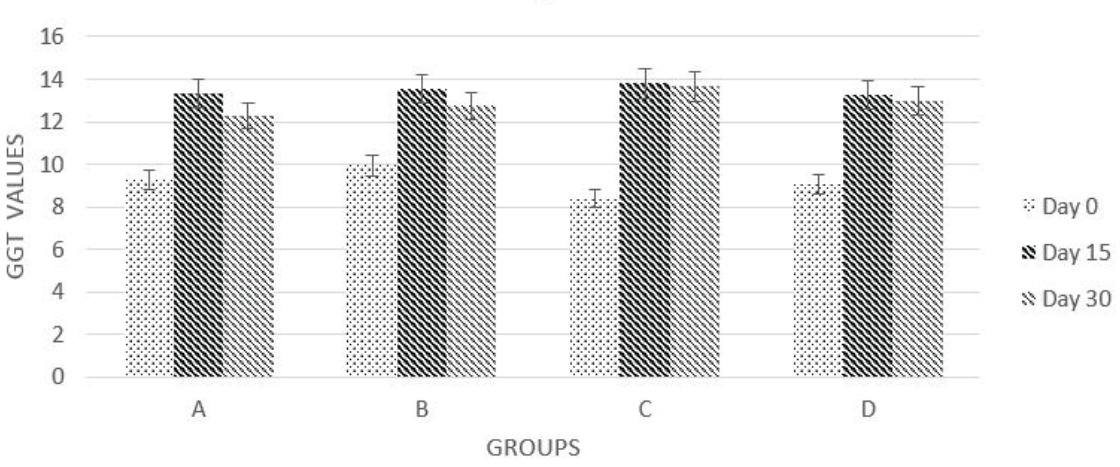


Figure 4: Gamma Glutamyl Transferase (GGT) values of Sahiwal cattle on days 0, 15 and 30 of trial

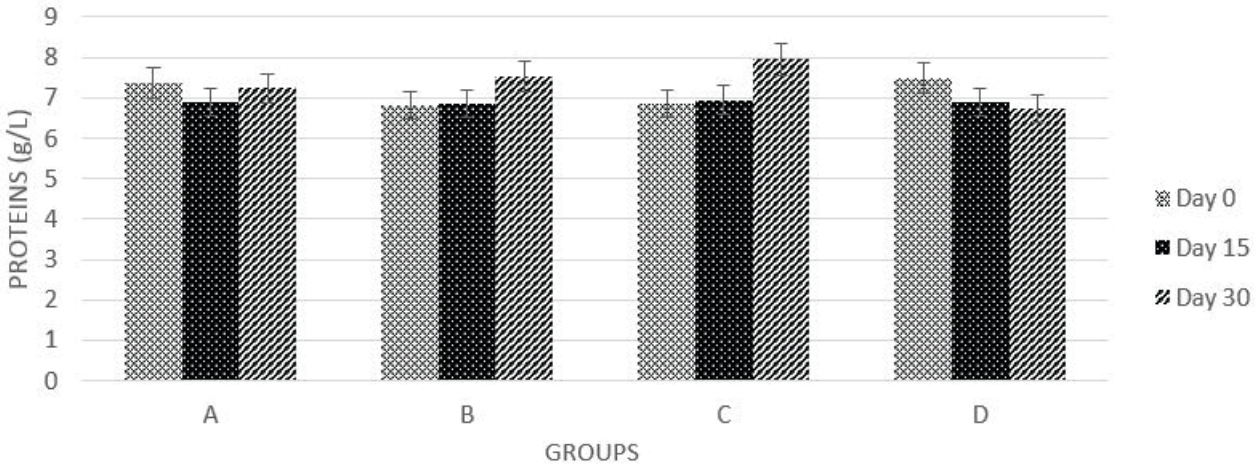


Figure 5: Total serum Protein values of Sahiwal cattle on days 0, 15 and 30 of trial

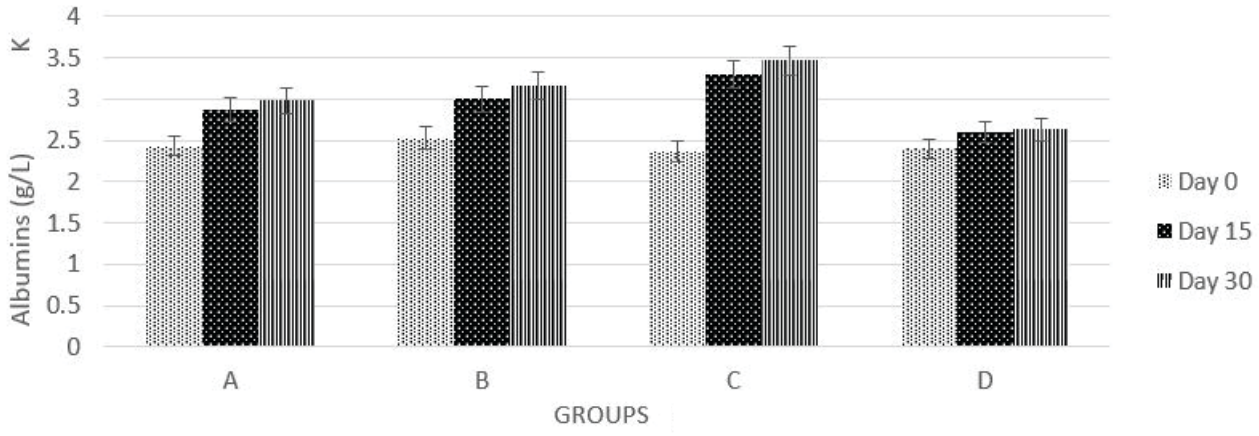


Figure 6: Serum Albumin values of Sahiwal cattle on days 0, 15 and 30 of trial

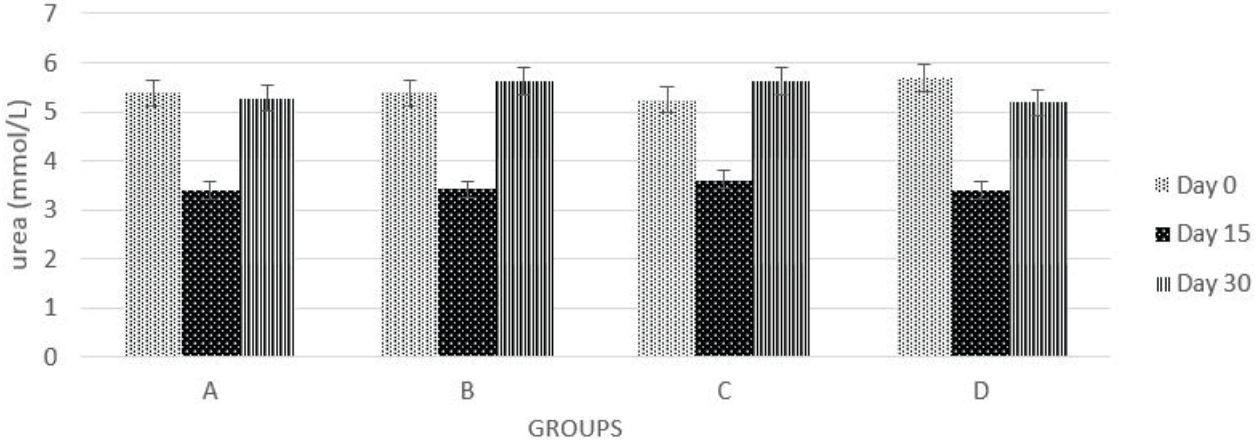


Figure 7: serum Urea values of Sahiwal cattle on days 0, 15 and 30 of trial

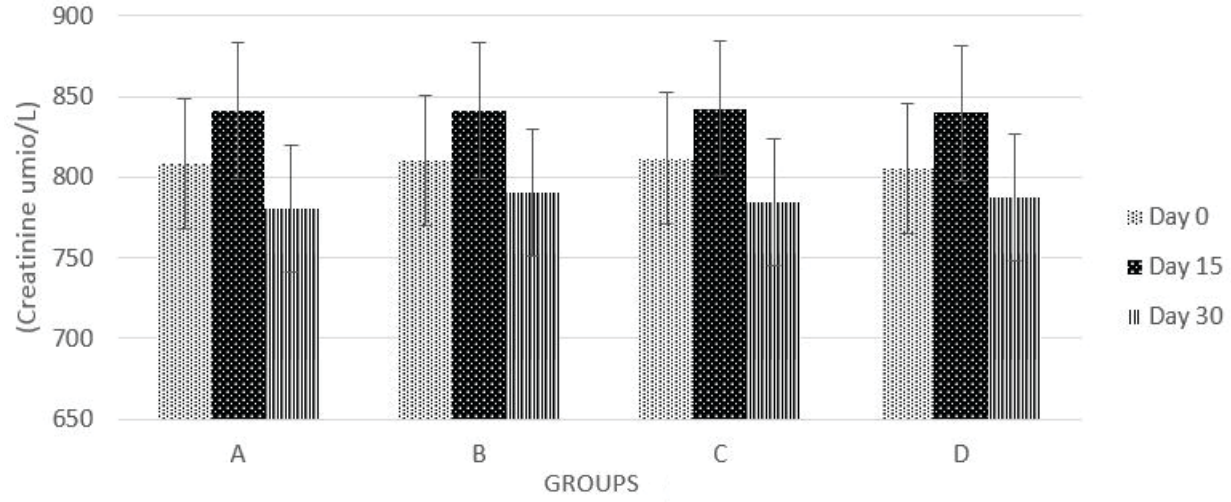


Figure 8: Creatinine values of Sahiwal cattle on days 0, 15 and 30 of trial

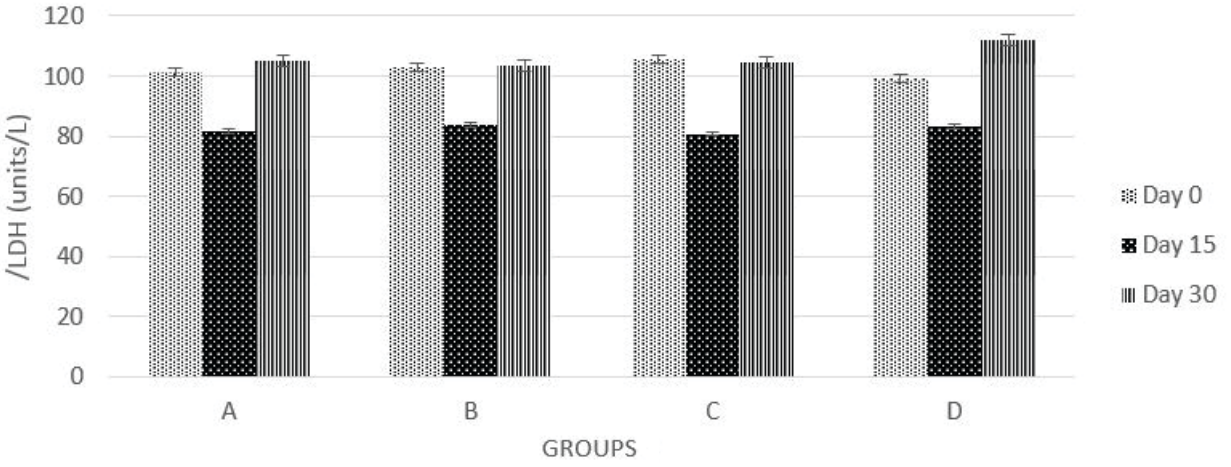


Figure 9: Lactate Dehydrogenase (LDH) values of Sahiwal cattle on days 0, 15 and 30 of trial

Table 5: Weight gain, Average daily weight Gain and Percent weight gain of Sahiwal cows during experiment

	Initial weights (Kg)	Final weights (Kg)	Weight Gain (Kg)	Average daily weight gain (grams)	Percent Weight Gain (%)
A	223.66 ± 1.6	238.66 ± 5.5	15 ± 3.9	500 ± 130.17	6.26 ± 1.47
B	217.83 ± 2.51	238.33 ± 3.05	20.5 ± 3	683.33 ± 100	8.59 ± 1.19
C	225 ± 2.29	249.16 ± 2.02	24.16 ± 0.28	805.55 ± 9.62	9.7 ± 0.19
D	215.66 ± 2.75	226.83 ± 3.17	11.16 ± 1.44	372.22 ± 48.11	4.92 ± 0.6

Discussion

In this research, Sahiwal heifers were used for evaluation of the anthelmintic activity of the herbals belonging to various families of plants the herbal dewormer showed a great reduction in the fecal egg counts. The herbal dewormer showed excellent efficiencies in reducing the egg counts and they reduced the egg count of both *Trichostrongylus* and *Paramphistomum* spp. efficiently. Hematological profile was also efficiently improved by the herbal dewormer. Herbal dewormer showed 80.97% reduction in total helminth egg counts, 83.8% reduction in nemathelminths (*Trichostrongylus*), and

81.43% reduction in platyhelminths (*Paramphistomum*) eggs.

Many researchers have conducted anthelmintic trials of the herbals and reported that the herbals were efficient in reducing the egg counts and reducing the helminths in the ruminants (3, 25-28). Similar results have been reported by Abbas et al. (3) who conducted a trial to evaluate the anthelmintic effects of same herbal dewormer in the goat species**.** They concluded that the herbal dewormer was efficient in reducing the egg counts reduced the egg counts efficiently. It improved hematological profile and had a positive effect on the egg counts. Results of our study were in line with Kaitay et al. (28). They proved that the extract of pomegranate peel was efficient in controlling the parasite members of phylum nemathelminths in the goat, cattle and sheep efficiently. These studies suggest that the plants have efficient role in controlling the gastrointestinal helminths. Our dewormer was beneficial for reducing total helminths including nemathelminths (*Trichostrongylus*) and platyhelminths (*Paramphistomum*). These activities have been reported by other researchers which represent that the plants have ability to reduce the nemathelminths as well as platyhelminths (29).

Anthelmintic activities of the plants are mainly attributed because of the presence of active compounds in the plants. These herbals contain various bioactive compounds including phenolics, flavonoids, thymols and terpenoids etc. (30). These compounds are in varying concentrations and have altering concentrations in different plants. These compounds can control the various types of helminths because of diverse mode of actions of their constituents (30). Many phytochemicals i.e., thymols usually inhibit the reproductive efficiency of the helminths and restrict oocyst shedding (31). While some phytochemicals like nicotinic compounds cause paralysis of the worms leading to their inability to attach to the body hence direct removal from the body (32, 33). There were multiple plants that contained a variety of compounds belonging to these groups. Many researchers have reported that the plants in combination show the higher effects than the single plant because there is increased diversity of bioactive compounds, and their mode of actions (34).

In current research the herbal dewormer improved the hematological profile of the cattle. Increased red blood cells, PCV and Hemoglobin contents can directly be correlated with the reduced number of worms (35). Parasites feed upon blood and have drastic effects on circulatory blood cells leading to anemia and lead to low circulatory blood cells, lowered hemoglobin and decreased packed cell volume (36). Our herbal dewormer was efficient in reducing the worms hence causing less blood and serum metabolites loss. Reduction in WBCs is observed when the infectious agents are reduced, leading to lower circulatory immune cells which was shown in current research (37). Likewise, the improvement in serum proteins and albumins was observed in the Sahiwal cattle being given herbal dewormer. Parasites cause serum proteins loss when they are higher in number (38, 39), our herbal dewormer reduced worms efficiently, so there was significant ($P<0.05$) increase in the serum proteins and albumin counts in the treated animals. Improved weight gains observed in this research can be attributed to reduction in worm burden which improves nutrient availability and feed uptake of the animals (3, 40).

There are some limitations in this research as many researchers (41) have reported improvement in ALT, AST and creatinine values, but in present research no significant effect was observed. All the serum values including ALT, AST, ALKP, urea, LDH and creatinine were in normal ranges, that may be a reason that the animals remained in normal physiological states (42). Along with it these differences can be affected by multiple other parameters which were not considered in this research i.e., feed intake, type and amount of disease, other diseases which were not caused by the helminths.

Conclusion

The results of this research depict that the food animals are always at a threat of helminths and their safe and economical control can be achieved by the herbal mixtures. Herbal mixtures are safe, effective, and economical alternatives for control of parasites of livestock. multiple other species should be included in the research, so that the herbal formulations for all the species may be formulated. Long term studies of these herbals should be conducted to estimate any hazardous effect on prolonged status.

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Original Research Article
Veterinary Medicine and The One Health Concept

ANTIOXIDANT ACTIVITY AND INHIBITORY EFFECT OF 2,4,4'-TRIHYDROXYCHALCONE ON DIGESTIVE ENZYMES RELATED TO OBESITY

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Abstract: The aim of this study was to investigate the antioxidant activity and inhibitory effect of 2,4,4'-trihydroxychalcone on digestive enzymes related to obesity, including sucrase, α-amylase, and lipase. The in vitro antioxidant activities of three concentrations of 2,4,4'-trihydroxychalcone (100, 300, and 500 µg/ml) were determined using 2,2-diphenyl-1-picrylhydrazyl radical scavenging, reducing power, and ferrous ion chelating assays. Moreover, in vitro inhibition of lipase, α-amylase, and sucrase enzyme activities by 2,4,4'-trihydroxychalcone was determined using a specific assay for each enzyme. 2,4,4'-Trihydroxychalcone has been shown to have antioxidant properties and inhibits sucrase, α-amylase and lipase activities. These findings suggest that 2,4,4'-trihydroxychalcone demonstrated an antioxidant activity and can effectively inhibit the key enzymes related to obesity.

Key words: 2,4,4'-trihydroxychalcone; DPPH radical scavenging activity; reducing power; ferrous ion chelating activity; sucrase; α-amylase; lipase

Introduction

Obesity is a global epidemic associated with significant morbidity and mortality in adults (1). Worldwide, 40% of women and 39% of men aged ≥18 years are overweight (2). In Saudi Arabia, the prevalence rates of overweight and obesity are increasing, with an overall prevalence of 35% (3). As obesity causes the development of metabolic disorders such as diabetes mellitus, hypertension, cardiovascular diseases, and inflammation-related pathologies, reducing the incidence of overweight and obesity is of considerable importance to public health (4).

A positive energy balance resulting from a chronic disparity between the intake of energy and its expenditure leads to weight gain and eventually obesity (5). Between 45% and 65% of the total caloric intake is accounted for by carbohydrates in their various forms, making them the most important energy source (6). The major dietary carbohydrates are of plant origin and can be found in grains, tubers, and legumes (7). The digestion of these starchy foods begins in the mouth by the action of salivary α-amylase, which hydrolyses the α-1,4 bonds in starch. The products of this process are shorter polysaccharides and maltose (8). The

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digestion processes of shorter polysaccharides and maltose continue in the small intestine by the action of pancreatic α-amylase and α-glucosidases, producing glucose (8,9). Moreover, sucrase (isomaltase) catalyses the sucrose hydrolysis to yield glucose and fructose (8). In addition to carbohydrates, fats represent approximately 35% of the daily energy intake where dietary triacyl glycerides are the predominant fat in foods and are hydrolysed by enzymes called lipases (6). Pancreatic lipase removes fatty acids at carbon 1 and 3, producing a mixture of 2-monoacylglycerol and free fatty acids, thereby facilitating their uptake (8).

To reduce the energy intake through gastrointestinal mechanisms without altering any central mechanism, reduction of dietary carbohydrate and fat absorption from the intestine through the inhibition of their digestive enzymes appear to be effective in reducing obesity (10). Although α-amylase inhibitors such as acarbose and pancreatic lipase inhibitors such as orlistat are safe drugs for weight loss, they have unpleasant side effects. Therefore, the interest in studying the inhibition of digestive enzymes by plant derived phytochemicals has recently increased (10). Among the different classes of phytochemicals, phenolic compounds have been increasingly recognised to be the most active inhibitors of α-amylase, sucrase and lipase enzymes (10).

Chalcones are a class of flavonoids that belongs to phenolic compounds (11). They are naturally occurring bioactive compounds and known to have several medicinal and pharmaceutical applications (12). Over the last recent years, few studies started investigating the inhibitory effect of chalcones on enzymes but this area still largely unexplored (13-15). 2,4,4'-Trihydroxychalcone (isoliquiritigenin) is one of the chalcone family that is found in liquorice (*Glycyrrhiza uralensis*), shallots, and soybeans (16,17). This chalcone has been reported to exhibit anti-inflammatory and anti-tumour properties (18). However, the antioxidant activity and inhibitory effect of this compound on digestive enzymes related to obesity are still largely unexplored and might be an excellent target for the development of safe and effective anti-obesity drug. Therefore, the aim of this study was to investigate the antioxidant activity and inhibitory effect of three concentrations of 2,4,4'-trihydroxychalcone (100, 300, and 500 μg/ml) on digestive enzymes related to obesity, including sucrase, α-amylase, and lipase.

Materials and methods

Chemicals

2,4,4'-trihydroxychalcone (cat.no.961-29-5), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (cat. no.1898-66-4), quercetin (cat.no.117-39-5), Vitamin C (cat.no.50-81-7), Sucrase (≥300 U/mg solid) (cat.no.9001-57-4), α-amylase (≥1000 U/mg) (cat.no.9000-90-2), Porcine pancreatic lipase (PPL, type II ≥100 U/mg) (cat.no.9001-62-1), orlistat (cat.no.96829-58-2) and Acarbose (cat.no.56180-94-0) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid (TCA) (cat.no.76-03-9), potassium ferricyanide (cat.no.13746-66-2), ferric chloride (cat. no.7705-08-0), Ferrozine (cat.no.63451-29-6), Ethylenediaminetetraacetic acid (EDTA) (cat. no.60-00-4), ferrous chloride (cat.no.7758-94-3), sucrose (cat.no.57-50-1), 3,5-dinitrosalicylic acid (cat.no.609-99-4), sodium phosphate dibasic (cat. no.7558-79-4), Sodium phosphate monobasic (cat. no.10049-21-5), sodium chloride (cat.no.7647-14-5), potassium sodium tartrate tetrahydrate (cat. no.6381-59-5), sodium hydroxide (cat.no.1310-73-2), p-Nitrophenyl α-D-Glucopyranoside (cat.no.3767-28-0), Sodium Carbonate (cat. no.497-19-8), Tween 80 (cat.no.9005-65-6) were purchased from E. Merck (Darmstadt, Germany). 2,4-Dinitrophenyl butyrate (cat.no.24273-19-6) was purchased from cymitquimica. All chemicals and reagents used in all experiments were of analytical grade or purer.

Measurement of the antioxidant activity of 2,4,4'-trihydroxychalcone

DPPH Radical-scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was used to determine the radical scavenging activity of 2,4,4'-trihydroxychalcone (19). To achieve this, 0.15 mM of the DPPH radical was prepared in methanol, and 1 ml of the solution was mixed with 1 ml of different concentrations of the 2, 4, 4'-trihydroxychalcone solution (100, 300, and 500 μg/ml). The reaction mixture was left at

room temperature for 15 min. The absorbance of the mixture was recorded at 517 nm. The negative control was prepared in a similar way but without 2,4,4'-trihydroxychalcone. Different concentrations of vitamin C and quercetin (100, 300, and 500 µg/ml) were used as positive controls. The experiment was repeated four times for two independent days, and the average absorbance was calculated. The percentage of the DPPH scavenging activity was then calculated using the following equation:

% DPPH radical scavenging activity = $[(A_0 - A_1)/A_0] \times 100\%$,

where A_0 is the absorbance of the negative control and A_1 is the absorbance of the 2,4,4'-trihydroxychalcone or positive controls.

Reducing power assay

The reducing power of 2,4,4'-trihydroxychalcone was determined using Oyaizu's method (20). At different concentrations, 2.5 ml of 2,4,4'-trihydroxychalcone (100, 300, and 500 µg/ml) was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide (1.0%), and the mixture was incubated in a water bath at 50°C for 20 min. Trichloroacetic acid (2.5 ml, 10%) was added to the mixture and then centrifuged for 10 min at 3000 rpm. The supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 0.1%), and the absorbance was then measured at 700 nm. The negative control was prepared in a similar way but without 2,4,4'-trihydroxychalcone. Different concentrations of vitamin C (100, 300, and 500 µg/ml) were used as positive controls. The experiment was repeated four times for two independent days, and the average absorbance was calculated. The absorbance values were then used to indicate the reducing power of the antioxidants.

Ferrous ions-chelating activity

The chelating activity of ferrous ions by 2,4,4'-trihydroxychalcone was determined using the method of Dinis et al. (21). Different concentrations of 2,4,4'-trihydroxychalcone (100, 300, and 500 µg/ml) at 0.4 ml were added to a solution of ferrous chloride (0.2 ml, 2.0 mM). The reaction was started by adding ferrozine (0.4 ml, 5 mM), and the total volume was adjusted to 4 ml with methanol. The mixture was then shacked and incubated for 10 min at 37°C. The absorbance of the mixture was recorded at 562 nm. The negative control was prepared in a similar way but without 2,4,4'-trihydroxychalcone. Different concentrations of ethylenediaminetetraacetic acid (EDTA; 100, 300, and 500 µg/ml) were used as positive controls. The experiment was repeated four times for two independent days, and the average absorbance was calculated. The percentage inhibition was then calculated using the following equation:

% Chelating activity of ferrous ions = $[(A_0 - A_1)/A_0] \times 100\%$,

where A_0 is the absorbance of the negative control and A_1 is the absorbance of 2,4,4'-trihydroxychalcone or the positive controls.

Measurement of digestive enzymes activity

Sucrase inhibition assay

The effect of 2,4,4'-trihydroxychalcone on sucrase activity was examined using Honda and Hara's method (22). Different concentrations of 2,4,4'-trihydroxychalcone (100, 300, and 500 µg/ml) at 50 µl were mixed with a sucrase solution (50 µl, 4.8 U/µl) and incubated for 10 min at 37°C. The enzyme reaction was started by adding the sucrose solution (100 µl, 60 mM). After 30 min, the reaction was stopped by adding a 3,5-dinitrosalicylic acid reagent (200 µl), and the mixture was incubated in a boiling water bath for 5 min. After the addition of distilled water (2.0 ml), the absorbance of the mixture was read at 540 nm using an ultraviolet (UV)-visible spectrophotometer. The negative control was prepared in a similar way but without 2,4,4'-trihydroxychalcone. The experiment was repeated four times for two independent days, and the average absorbance was calculated. The enzyme activity inhibition was calculated as follows:

Inhibition (%) = $[(A_{control} - A_{sample})/A_{control}] \times 100\%$,

where $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance of 2,4,4'-trihydroxychalcone.

α-Amylase inhibition assay

The α-amylase inhibition activity of 2,4,4'-trihydroxychalcone was estimated using the method of Oboh et al. (23) with some modifications. The α-amylase enzyme (40 µl, 5 U/ml) was mixed with a sodium phosphate buffer (0.36 ml,

0.02 M, pH 6.9 with 0.006 M NaCl) and 0.2-ml 2,4,4'-trihydroxychalcone (100, 300, and 500 µg/ml), and incubated for 20 min at 37°C. The starch solution in the sodium phosphate buffer (300 µl, 1%) was then added, and the mixture was re-incubated for 20 min. The reaction was stopped by adding a 3,5-dinitresalicylic acid reagent (0.2 ml). The mixture was then mixed well and kept in a boiling water bath for 5 min. After the addition of distilled water (6 ml), the absorbance of the mixture was read at 540 nm using a UV-visible spectrophotometer. The negative control was prepared in a similar way but without 2,4,4'-trihydroxychalcone. Different concentrations of acarbose (100, 300, and 500 µg/ml) were used as positive controls. The experiment was repeated four times for two independent days, and the average absorbance was calculated. The enzyme activity inhibition was calculated as follows:

Inhibition (%) = $[1 - (A_{sample}/A_{control})] \times 100$,

where $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance of 2,4,4'-trihydroxychalcone or the positive controls.

Lipase inhibition assay

The lipase inhibition activity of 2,4,4'-trihydroxychalcone was estimated using the method of Won et al. (24) with some modifications. A reaction mixture containing porcine pancreatic lipase (0.1 ml, 200 units/ml in 0.1 M potassium phosphate buffer, pH 6.8, 1% tween 80) and 0.1 ml of 2,4,4'-trihydroxychalcone (100, 300, and 500 µg/ml) was prepared. After adding 0.5 ml of 25 mM 2,4-dinitrophenyl butyrate as a substrate, the reaction was started. Then, the reaction mixture was kept at 37°C during the assay. After 1 minute of incubation, 2,4-dinitrophenol was released by the lipase and measured at 360 nm using a UV-visible spectrophotometer. The negative control was prepared in a similar way but without 2,4,4'-trihydroxychalcone. Different concentrations of orlistat (100, 300, and 500 µg/ml) were used as positive controls. The experiment was repeated four times for two independent days, and the average absorbance was calculated. The enzyme activity inhibition was calculated as follows:

Inhibition (%) = $[1 - (A_{sample}/A_{control})] \times 100$,

where $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance of 2,4,4'-trihydroxychalcone or the positive controls.

Statistical analysis

To assess the differences in antioxidant activity and inhibitory effect between the three different concentrations of 2,4,4'-trihydroxychalcone on α-amylase and lipase enzymes, two-way analysis of variance (ANOVA) was performed followed by the Tukey test. One-way ANOVA followed by the Bonferroni test was used to test the differences of the three different concentrations of 2,4,4'-trihydroxychalcone in terms of their inhibitory effects on the sucrase enzyme. All independent analyses were replicated in quadruplicates, for which the results were expressed as mean ± SEM. All statistical analysis was performed using the statistical software GraphPad Prism 8. The statistical significance levels were based on $p < 0.05$.

Results

The antioxidant activity of 2,4,4'-trihydroxychalcone

DPPH radical scavenging activity

Figure 1 shows the DPPH radical scavenging activity of the three different concentrations of vitamin C, quercetin, and 2,4,4'-trihydroxychalcone. At a concentration of 100 µg/ml, vitamin C showed the highest scavenging effect (97.2%), followed by quercetin (95.7%) and then 2,4,4'-trihydroxychalcone (57.4%). At a concentration of 300 µg/ml, vitamin C showed the highest scavenging effect (97.6%), followed by quercetin (95.9%) and then 2,4,4'-trihydroxychalcone (61.88%). The same trend was found when the concentration was increased to 500 µg/ml where vitamin C still had the highest scavenging effect, followed by quercetin and then 2,4,4'-trihydroxychalcone (97.88%, 96.3%, and 74.7%, respectively).

In addition, the results showed the relationship between the concentration of the three compounds and their DPPH scavenging activity (Figure 1). For vitamin C and quercetin, the results showed that their scavenging activity did not changed with the increment in the concentrations. In contrast, 2,4,4'-trihydroxychalcone showed a significant gradual increase from 57.4% at 100 µg/ml to 74.7% at 500 µg/ml.

Reducing power assay

Figure 2 shows the reducing power of vitamin C and 2,4,4'-trihydroxychalcone where increasing absorbance indicates an increase in reducing power. At each concentration (100, 300, and 500 μg/ml), vitamin C showed higher reducing power than 2,4,4'-trihydroxychalcone. In addition, the results of vitamin c showed that the significant difference in reducing power occurred only at concentrations of 100 μg/ml and 500 μg/ml. On the other hand, the results of 2,4,4'-trihydroxychalcone showed a direct proportional increase with the concentration.

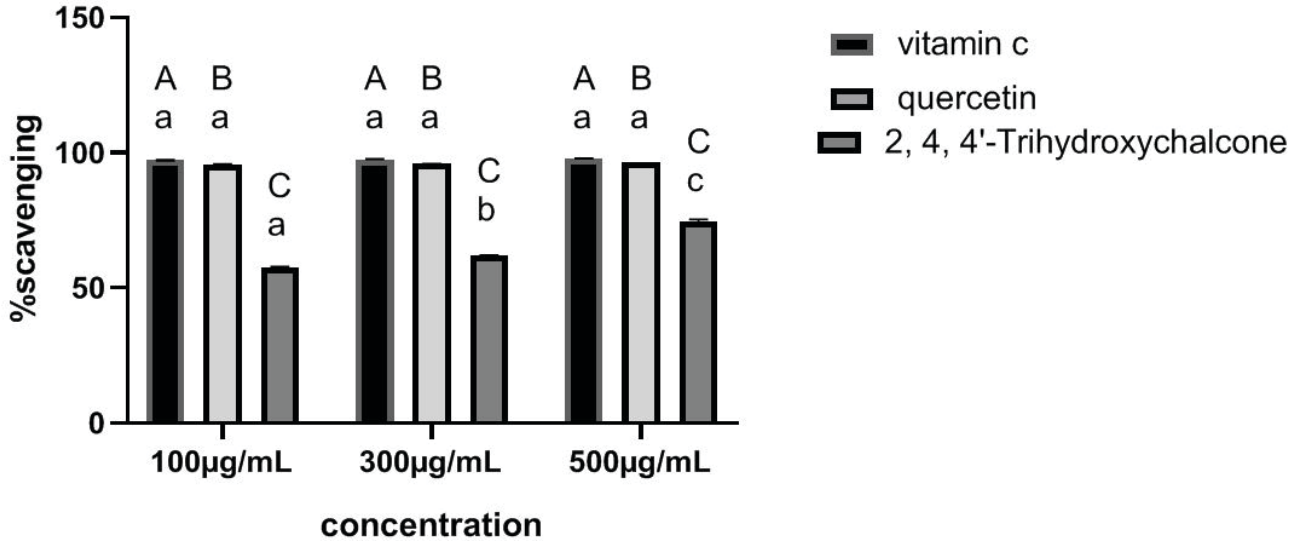


Figure 1: DPPH radical scavenging activities of vitamin C, quercetin, and 2,4,4'-trihydroxychalcone

Different upper-case letters denote significant differences ($p < 0.05$) between the different types of compounds at the same concentration. Different lower-case letters denote significant differences ($p < 0.05$) between the different concentrations of the same compound. Values are presented as mean ± SEM. Error bars shows the SEM. DPPH: 2,2-diphenyl-1-picrylhydrazyl, SEM: standard error of the mean.

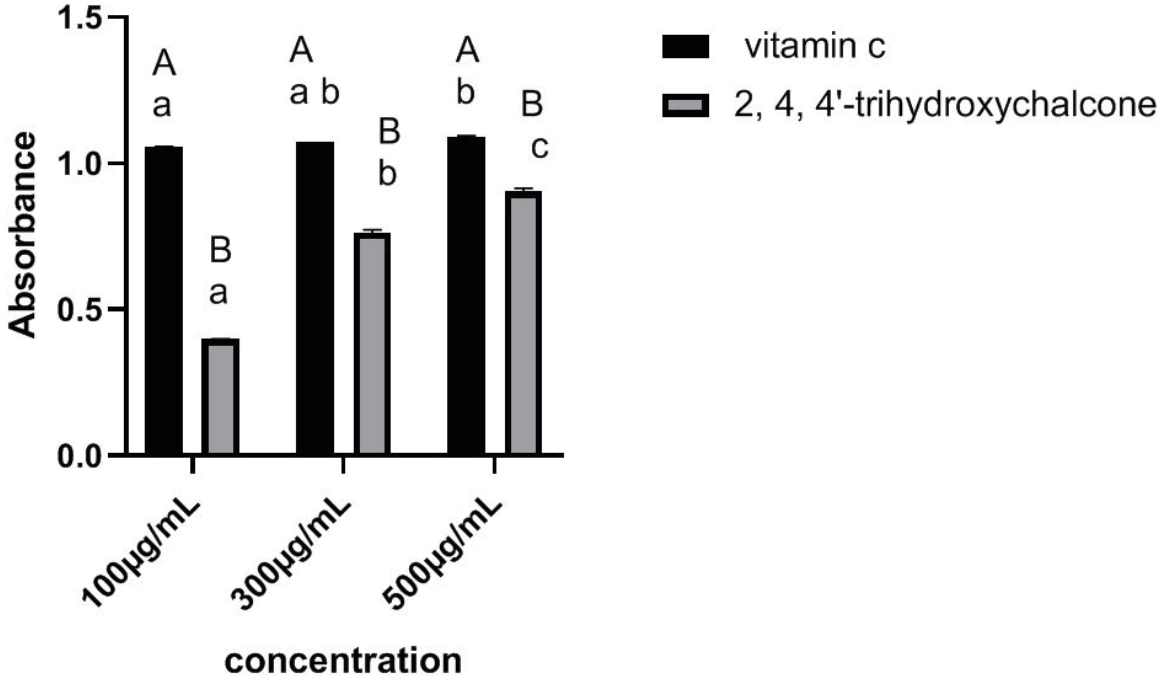


Figure 2: Reducing power of vitamin C and 2,4,4'-trihydroxychalcone

Different upper-case letters denote significant differences ($p < 0.05$) between the different types of compounds at the same concentration. Different lower-case letters denote significant differences ($p < 0.05$) between the different concentrations of the same compound. Values are presented as mean ± SEM. Error bars shows the SEM. SEM: standard error of the mean.

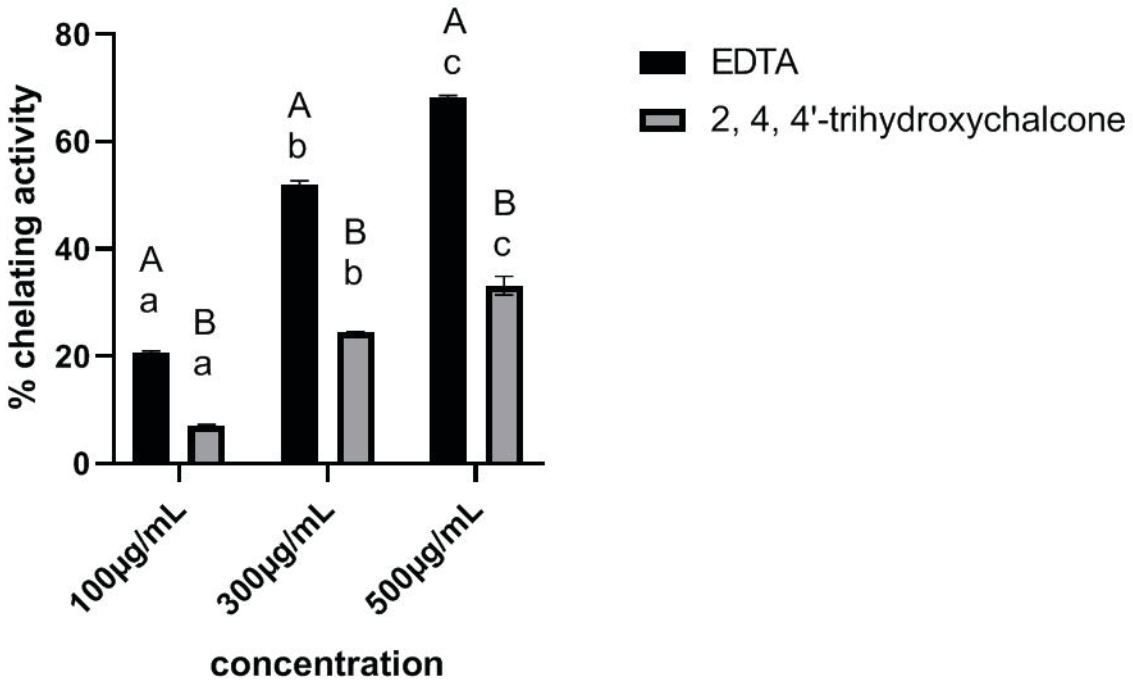


Figure 3: The Ferrous ions-chelating activity of EDTA and 2,4,4'-trihydroxychalcone

Different upper-case letters denote significant differences ($p < 0.05$) between the different types of compounds at the same concentration. Different lower-case letters denote significant differences ($p < 0.05$) between the different concentrations of the same compound. Values are presented as mean ± SEM. Error bars shows the SEM. EDTA: ethylenediaminetetraacetic acid, SEM: standard error of the mean.

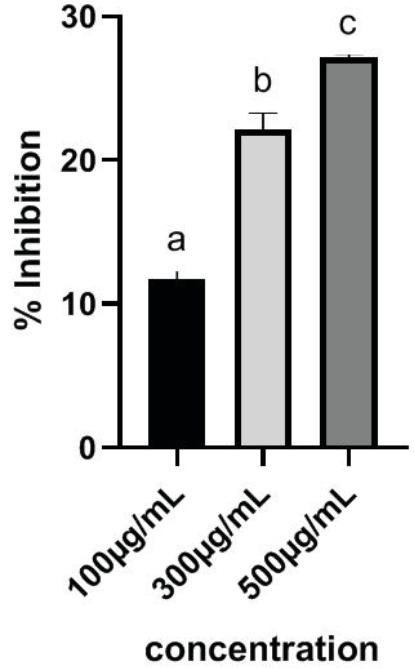


Figure 4: Sucrase inhibition activity by 2,4,4'-trihydroxychalcone.

Different lower-case letters denote significant differ-ences ($p < 0.05$) between the different concentrations. Values are presented as mean ± SEM. Error bars show the SEM. SEM: standard error of the mean.

Ferrous ions-chelating activity

The chelating activities of the ferrous ions of EDTA and 2,4,4'-trihydroxychalcone (100, 300, and 500 µg/ml) are shown in Figure 3. Results of 100 µg/ml showed that EDTA has a higher chelating activity (20.58%) than 2,4,4'-trihydroxychalcone (7.125%). A concentration of 300 µg/ml also showed that EDTA has higher chelating activity (52.04%) than 2,4,4'-trihydroxychalcone (24.42%). Similarly, the same trend was observed with 500 µg/ml where the chelating activities of EDTA and 2,4,4'-trihydroxychalcone were 68.22% and 33.20%, respectively.

In addition, the results showed a proportional relationship between the compound's concentration and the chelating activity. The EDTA results showed that the chelating activity was increased from 20.58% at 100 µg/ml to 68.22% at 500 µg/ml. Likewise, the results for 2,4,4'-trihydroxychalcone showed that the chelating activity was increased from 7.125% at 100 µg/ml to 33.20% at 500 µg/ml.

Digestive enzymes activity

Sucrase inhibition

The sucrase inhibition activities of the different concentrations of 2,4,4'-trihydroxychalcone (100, 300, and 500 µg/ml) are shown in Figure 4. The inhibitory activity was found to be proportional to the increment in the concentration where the results showed that 100, 300, and 500 µg/ml of 2,4,4'-trihydroxychalcone lead to 11.73%, 22.17%, and 27.12% sucrase inhibition, respectively α-Amylase inhibition.

The inhibitory effects of α-amylase at different concentrations of acarbose and 2,4,4'-trihydroxychalcone (100, 300, and 500 µg/ml) are shown in Figure 5. 2,4,4'-Trihydroxychalcone showed a higher inhibitory effect (23.87%) than acarbose (7.12%) at 100 µg/ml. In contrast, when the concentrations of acarbose and 2,4,4'-trihydroxychalcone were increased to 300 and 500 µg/ml, the two compounds exhibited similar inhibitory effects.

Moreover, the results showed a proportional correlation between 2,4,4'-trihydroxychalcone and acarbose in terms of concentration and inhibitory activity. The results for acarbose showed an increase in inhibitory effect from 7.12% at 100 µg/ml to 37.55% at 500 µg/ml. Similarly, 2,4,4'-trihydroxychalcone showed an increase in inhibitory effect from 23.87% at 100 µg/ml to 37.96% at 500 µg/ml.

Lipase inhibition

The results, as shown in Figure 6, indicate that the different concentrations of orlistat and 2,4,4'-trihydroxychalcone (100, 300, and 500 µg/ml) inhibit lipase activity. Orlistat showed a significantly higher inhibitory activity (63.58%) than 2,4,4'-trihydroxychalcone (57.71%) at 100 µg/ml.

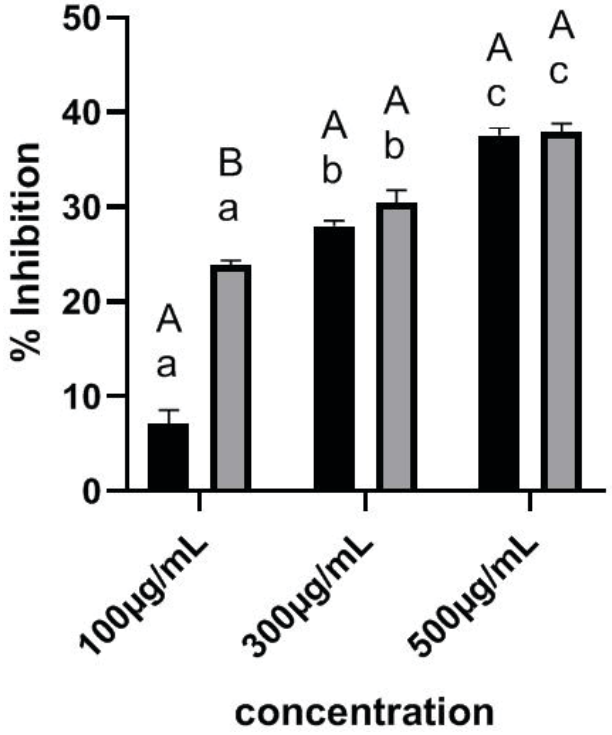


Figure 5: α-Amylase inhibition activities of acarbose and 2,4,4'-trihydroxychalcone

Different upper-case letters denote significant differ-ences ($p < 0.05$) between the different compounds at the same concentration. Different lower-case letters denote significant differences ($p < 0.05$) between the different concentrations of the same compound. Values are pre-sented as mean ± SEM. Error bars show the SEM. SEM: standard error of the mean.

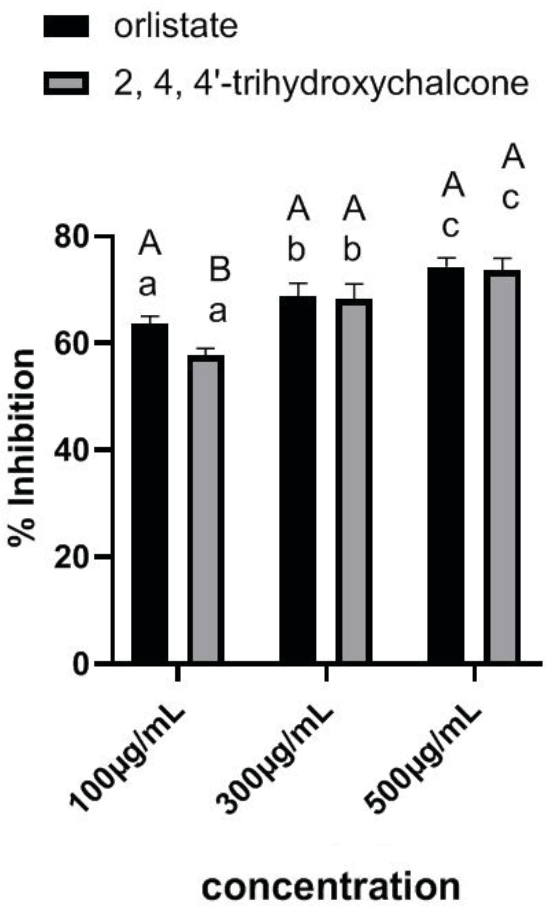


Figure 6: Lipase inhibition activities of orlistat and 2,4,4'-trihydroxychalcone

Different upper-case letters denote significant differences ($p < 0.05$) between the different compounds at the same concentration. Different lower-case letters denote significant differences ($p < 0.05$) between the different concentra-tions of the same compound. Values are presented as mean ± SEM. Error bars show the SEM. SEM: standard error of the mean.

By contrast, no significant differences were found in lipase inhibition between orlistat and 2,4,4'-trihydroxychalcone at higher concentrations (300 and 500 µg/ml).

In addition, the concentrations and inhibitory activities of 2,4,4'-trihydroxychalcone and orlistat were found to be proportvionally associated. The inhibitory effect of orlistat was increased from 63.58% at 100 µg/ml to 74.21% at 500 µg/ml. Similarly, the inhibitory effect of 2,4,4'-trihydroxychalcone was increased from 57.71% at 100 µg/ml to 73.64% at 500 µg/ml.

Discussion

The present study investigated the antioxidant activity and inhibitory effect of 2,4,4'-trihydroxychalcone on obesity-related digestive enzymes.

The antioxidant activity of 2,4,4'-trihydroxychalcone

DPPH Radical scavenging activity

The results of the present study showed that 2,4,4'-trihydroxychalcone has a DPPH radical scavenging activity. This finding is in line with previous studies that investigated the DPPH scavenging activity of several extracts containing flavonoids (25-29). The DPPH radical is a commercially oxidising radical that is reduced by antioxidants. In this assay, the violet colour of DPPH changes into a pale-yellow colour because of the acceptance of DPPH to the hydrogen atoms donated by antioxidants [30]. This explains the high dense yellow colour presented in this study which indicates the high scavenging activity of 2,4,4'-trihydroxychalcone.

The results also showed a proportional relationship between the 2,4,4'-trihydroxychalcone concentration and the DPPH scavenging activity. These results agree with those of previous studies that showed that increasing concentrations of extracts containing flavonoids increased the DPPH scavenging activity (30-35).

Reducing power assay

In this study, a reduced power activity was observed for 2,4,4'-trihydroxychalcone. Finding in the present study is consistent with findings of previous studies that investigated the reducing power of several plant flavonoids (25,29,36). The presence of 2,4,4'-trihydroxychalcone in the assay resulted in the reduction of Fe^{3+} to Fe^{2+} via the electron donation of the compound. The yellow colour of the test solutions changed to various shades of light green to dark blue, indicating the amount of Fe^{2+} complex formed (37).

The reducing power of 2,4,4'-trihydroxychalcone was gradually increased with the increase in its concentration. Several previous studies showed similar results where the reducing power was increased by increasing the concentration of extracts containing flavonoids (32-35).

Ferrous ions-chelating activity

The activity of 2,4,4'-trihydroxychalcone in chelating ferrous ions was confirmed in this study. Similarly, findings of previous studies revealed the chelating activity of ferrous ions by different plant extracts containing flavonoids (35,38-40). In this test, Ferrozine is capable of forming red complexes with Fe^{2+} [38]. In the presence of 2,4,4'-trihydroxychalcone, the formation of these red complexes was decreased. This could be explained by the inference of 2,4,4'-trihydroxychalcone with the formation of the ferrous and ferrozine complex, which suggests that it has a chelating activity and captures ferrous ions before ferrozine binding (41,42).

Moreover, the chelating of ferrous ions by 2,4,4'-trihydroxychalcone was gradually increased with the increase in its concentration. This is supported by previous studies which reported that the chelating activities of extracts containing flavonoids on ferrous ions were increased when the concentrations of the extracts increased (35,40).

Digestive enzyme activity

Sucrase inhibition

In this assay, sucrase hydrolysed sucrose to glucose and fructose. Then, these reducing sugars reduced 3,5-dinitrosalicylic acid (DNS) to 3-amino-5-nitrosalicylic acid (a reddish-brown compound) (43,44). In the presence of 2,4,4'-trihydroxychalcone, the formation of this reddish-brown compound was reduced, indicating the inhibition of sucrase enzyme. To our knowledge, this is the first study to investigate the effect of a

chalcone compound on sucrase activity. However, our results are in accordance with the findings of a previous study which stated that flavonoids extracted from tamarix gallica inhibited sucrase activity up to 30% (45).

The results also showed a direct correlation between the 2,4,4'-trihydroxychalcone concentration and the inhibition activity. A similar pattern of results was observed in previous studies (46,47).

α-Amylase inhibition

The DNS colorimetric method was used to measure amylase activity by using starch as the substrate. Under alkaline conditions, the colour of the solution was changed from yellow to reddish brown when the reducing sugar (glucose) reduced 3,5-dinitrosalicylic acid to 3-amino-5nitrosalicylic acid (48-49). In the presence of 2,4,4'-trihydroxychalcone, the formation of the reddish-brown compound was reduced, indicating that 2,4,4'-trihydroxychalcone binds to α-amylase enzyme and reduces its activity up to 40%. This result is in line with those of a previous study which reported that different flavonoids extracted from tartary buckwheat inhibited α-amylase activity up to 45% (50). Similar inhibition % were also reported with synthetic chalcone compounds (51).

When comparing the effect of 2,4,4'-trihydroxychalcone with that of an acarbose drug, 2,4,4'-trihydroxychalcone demonstrated a greater inhibitory effect than acarbose at 100 µg/ml and similar inhibitory effects when their concentrations were increased to 300 and 500 µg/ml. The use of acarbose was associated with several gastrointestinal symptoms, including flatulence, abdominal distension, and diarrhoea (52). Accordingly, 2,4,4′-trihydroxychalcone could be considered a good source of natural compound in slowing down the breakdown and absorption of carbohydrates.

In addition, the results of the present study showed that the inhibition activity of α-amylase was directly proportional to the concentration of 2,4,4'-trihydroxychalcone. A similar result was previously reported by different studies which found an increase in the inhibitory effect of flavonoids on α-amylase with the increase in its concentration (50,53-55).

Lipase inhibition

In the lipase activity assay, 2,4-dinitrophenyl butyrate was used as the substrate. Upon hydrolysis, this substrate liberates the strongly absorbing 2,4-dinitrophenolate anion (DNP), which can be measured spectrophotometrically (56). In the presence of 2,4,4'-trihydroxychalcone, the formation of DNP was reduced, indicating that 2,4,4'-trihydroxychalcone binds to lipase enzyme and reduced its activity up to 70%. Studies that investigated lipase inhibition using several flavonoid-containing extracts from cinnamon, cardamom, gloves, Rosa damascene and white and blue poppy seeds or using other chalcone compounds extracted from licorice have reported similar % inhibition (57, 55, 58, 59).

In the intestine, lipase inhibitors block the action of this enzyme, and as a result, the conversion of dietary triglyceride to monoglyceride and fatty acid is reduced. Therefore, dietary fat is less likely to be absorbed (60). Orlistat is the only over-the-counter weight-loss drug approved by the Food and Drug Administration and European Medicines Agency that inhibits gastric and pancreatic lipases in the lumen of the gastrointestinal tract and therefore decreases the absorption of dietary fats (61). At long-term clinical trials in adults, orlistat reduced dietary fat absorption by approximately 30% and weight gain by approximately 5% (62). Orlistat has several side effects, including gastrointestinal problems (fatty stool and faecal incontinence), interfering with nutrients and drugs, and liver damage (61). When comparing the effect of 2,4,4'-trihydroxychalcone with that of orlistat drug, orlistat demonstrated a greater inhibitory effect than 2,4,4'-trihydroxychalcone at 100 µg/ml, but the two showed similar inhibitory effects when their concentrations were increased to 300 and 500 µg/ml. Thus, 2,4,4′-trihydroxychalcone could be a promising natural alternative to orlistat.

Along with its increased concentration, 2,4,4'-trihydroxychalcone exhibited increased lipase inhibitory activity. This result agrees with previous results that showed that extracts containing flavonoids had an increased inhibitory effect as their concentrations increased (55,58).

Limitations and future research

This study has limitations, as α-amylase and lipase were derived from porcine pancreas, not human pancreas. Another limitation is that sucrase was derived from baker's yeast rather than from human pancreas. Future studies should examine the mode by which 2,4,4'-trihydroxychalcone inhibits enzymes and investigate the effects of 2,4,4'-trihydroxychalcone on digestive enzymes in animals and humans.

Conclusion

The findings stated that 2,4,4'-trihydroxychalcone demonstrated an antioxidant activity and can effectively inhibit the key enzymes related to obesity.

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Original Research Article
Veterinary Medicine and The One Health Concept

PHENOTYPIC CHARACTERIZATION OF SOME PATHOGENIC FUNGI ISOLATED FROM POULTRY AND THEIR SURROUNDINGS IN EL–GHARBIA GOVERNORATE, EGYPT

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Abstract: Poultry production is affected by several fungal diseases. Such fungal infection occurs in poultry farms via using a moldy litter, or ingestion of contaminated drinking water or moldy ration. In this study, a total of 210 birds with a history of respiratory distress of different breeds were collected randomly from sporadic different private farms and hatcheries in El–Gharbia Governorate, Egypt. The birds were sacrificed, then a total of 1050 tissue specimens from lung, air sacs, liver, crop and trachea were collected. In addition, 40 samples of poultry ration, 14 bedding materials, 4 air samples and 29 water samples were also collected. The collected samples were cultured on Sabouraud's agar plates. Macromorphological and micromorphological fungal examinations were performed for phenotypic characterization. Histopathological examinations were also performed using with hematoxylin and eosin stains. Antifungal sensitivity testing was screened using Mueller's Hinton Agar for studying the susceptibility of the recovered fungal isolates to the most commonly used antifungal drugs in Egypt, namely amphotericin B, clotrimazole, fluconazole, itraconazole, ketoconazole, and nystatin. The obtained results demonstrated that mold isolation was the highest in the collected samples from birds at 36.84%, followed by drinking water (31.57%). The highest incidence of mold isolation was recorded at the lungs of broilers and baladi birds followed by the air sacs. While in saso birds, the highest incidence was at the air sac. Collectively, 97 mold strains were identified from the lung, 74 from the air sacs, 30 from the liver, 61 from the trachea, and 44 from the crop. In addition, 19 mold isolates were recovered from the bird surroundings. *Aspergillus niger* as well as *Penicillium chrysogenum* were recovered and showed resistance to ketoconazole, while *Cladosporium perangustum* was resistant to fluconazole. All of the isolated molds were sensitive to itraconazole and nystatin except *A. flavus* that was resistant to nystatin. All *Aspergillus spp.* were resistant to fluconazole except *A. niger.* In conclusion, *Aspergillus spp.* was the most associated mold with poultry species and their surroundings in Egypt farms. Itraconazole and nystatin could be applied as proper antifungal drugs the control of for *Aspergillus* infection in birds.

Key words: phenotypic; fungi; poultry; antifungal sensitivity

Introduction

The poultry industry has grown at a rate of more than 5% annually over the past three decades, and its proportion of global meat production has also expanded from 15% to 30% at the moment (1). One of the primary agricultural sectors in Egypt is the poultry sector, which has received over 18 billion LE in investment. There are over 1.5 million permanent employees and roughly 1 million temporary employees in the labor market. The sector contributes significantly to the nation's supply of animal protein (white meats and eggs) (2).

Poultry production is affected by several fungal diseases. Due to the abuse of broad spectrum antibiotics, corticosteroids, and immune-suppressive medications, as well as the rise in the number of patients who are terminally ill and unwell, a fungal infection has recently become a global health concern and a significant cause of respiratory infections in poultry. Fungi are heterotrophic eukaryotes that can be single cells

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or multicellular. They obtain their sustenance through nutrient absorption and can reproduce asexually, sexually, or both (3).

The source of infection to poultry farms occur either via using of a moldy litter or from hatcheries when one day-old bird have retained conidia arrived at the farms. Other sources of farm contamination might occur through poor quality feed stuffs, improper bedding management, the entrance of air loaded with conidia. In poultry farms, humidity, and temperature conditions stimulate the hyphae growth and multiplication of fungi leading to the huge production of conidia, which are subsequently spread and inhaled by the birds (4).

Through the creation of mycotoxins, which have the potential to inflict economic losses, fungal illnesses result in a direct or indirect infection. Some fungi are pathogenic for birds, and they also cause fatal diseases in humans (5). The fungi cause certain pathological changes in the host, including inflammation, lesions, and illness that can result in death. It mostly affects the neurological and respiratory systems of chickens. Their saprophyte lifestyle results in propagation and dispersion; infection is a dead end, with the exception of favus (Dermatophytosis), as mycoses are not contagious (3).

Aspergillosis, candidiasis, favus, and cryptococcosis are examples of fungal illnesses that affect chicken farms. Additionally, both aspergillosis and candidiasis were the most significant ones. These species are frequently discovered in soil, decomposing organic matter, animal feed, dried grains, and others. Additionally, fungal species are in charge of the biodegradation of materials, the rotting of food and beverages, and the production of harmful mycotoxins. This attracts the attention for investigating the source and reservoir of those fungi (6).

It was reported that symptoms of aspergillosis depend on the organs affected and whether infection is localized or disseminated. Aspergillosis appears to be more significant in confinement situations where stress factors may be involved or where moldy litter or grain is present. *Aspergillus fumigatus* is considered as a major pathogen in birds. Other species like *A. flavus*, *A. niger, A. nidulans*, and *A. terreus* may also be isolated from avian cases of aspergillosis (sometimes in mixed infections) but much less frequently than *A. fumigatus* (2).

Traditional microscopic and cultural features are still often utilized to identify fungi. Conidial and mycelial color, colony reverse color, colony diameter, soluble pigments, and exudate production are used to make macro-morphological identification. Microscopic identification depends primarily on seriation, vesicle shape and size, conidia and stipe morphology (7).

The aim of this study was to identify the phenotypic characteristics of some fungi isolated from poultry and their surroundings.

Materials and methods

Sample collection

A total of 210 birds with a history of respiratory distress were collected randomly from broiler chicks (n = 180), Baladi breeds (n = 5), and Saso chickens (n = 25) from sporadic different private farms and hatcheries in El–Gharbia Governorate, Egypt. These farms showed different mortality rates during the period from November 2020 to July 2021. The birds were sacrificed; out of 1050 tissue specimens from lung, air sacs, liver, crop and trachea were collected under complete aseptic condition. Besides, a total of 87 samples of the surroundings of the poultry were collected (40 samples of poultry ration, 14 bedding materials, 4 air samples and 29 water samples).

The mycological examination

The prepared samples were cultured on to Sabouraud's Dextrose agar plates with a sterile glass rod. The cultured plates were incubated at 25°C and examined daily for five days. Mould colonies were picked up and subcultured on slope agar for further identification. The mould cultures were purified by sub-culturing on Sabouraud's agar plates, incubated at 25°C for 3-5 days and examined for macro and micromorphological characters.

Samples for histopathological examination originated from lung nodules of the affected birds were collected for histopathological examination (8).

Preparation of sample homogenates according to (9)

The sacrificed birds were aseptically eviscerated and each sample was taken directly to a sterile blender containing 90 ml of peptone water. Then ten grams from each sample (lung, liver, trachea, air

sacs, and crop) were aseptically placed in a sterile blender with 90 ml of 0.1% sterile peptone water.

For the bedding material and poultry ration samples

The mixture was homogenized for three minutes at 2500 rpm using sterile homogenizer (Type M-P3-302, Mechanic, Precyzina, Poland), then allowed to stand 2 minutes at room temperature and the contents were mixed by shaking before applying to the media.

Colony count of fungal cell in ration samples

Serial dilutions (10^{-1}:10^{-8}) were made by adding 1 g of each feed sample to 9 ml of sterile physiological saline (10). One milliliter was inoculated onto Sabouraud's Dextrose Agar (SDA) using spread plate method and incubated at 35 °C for 3–7 days. Single colonies of fungal growth were identified on the basis of morphologically and microscopically by using the stain lacto phenol cotton blue (11).

For air sampling

using passive sampling technique as described (12), the sterilized labelled plates of SDA were taken to the farms with history of bad condition of ventilation, all the openings of the farm were closed (for indoors result), air flow was allowed, then open in each corner of the farm the plates were opened and left for a few minutes then closed and placed in tightly closed sterilized plastic bag then transported to the lab under aseptic condition for incubation at 25°C for five days and examined daily.

For water sampling

by using a sterile syringe 100 ml from each sample were taken and placed in a sterile cooled container for delivery to the laboratory without delay, and directly 10 ml of the sample was added to 90 ml of peptone water 1%, sub-samples of 0.1 ml were then plated and spread evenly on to SDA plates and incubated at 25°C for five days and examined daily for identification and enumeration (direct plating technique (13).

Isolation of the mould genera from examined poultry samples

From the previously diluted poultry-originated samples, 0.1 ml was spread evenly on to SDA. The inoculated plates were incubated at 25°C and examined daily for five days. Colonies were picked up and subcultured on slope agar for further identification.

Mould identification

The mould cultures were purified by sub-culturing on Sabouraud's agar plates, incubated at 25°C for 3-5 days and examined for macro and micromorphological characters (14) as follows:

Macromorphological characters

The morphological examination included rate of growth, texture changes in color of the surface and reverse sides of the cultures.

Micromorphological characters

A small portion of the periphery of fresh culture was picked and placed on a clean slide with a drop of lactophenol cotton blue stain and examined microscopically to detect septation of hyphae, roughness or smoothness of conidiophores, shape of the vesicles, arrangement and number of the rows strigmata.

Histopathological examination

Samples for histopathological examination originated from lung nodules of the affected birds were collected from different groups, then fixed in 10% neutral buffered formalin. After dehydration and clearance, the tissues were embedded in paraffin and sectioned in 5 µm thickness. The serial sections were subjected to staining with hematoxylin and eosin (8).

Antifungal sensitivity test

Plates were prepared with Mueller Hinton Agar, modified according to CLSI guides for antifungal sensitivity testing using antifungal discs. Sterile non-toxic cotton swabs were dipped into the standardized inoculum. The entire agar surface of the plate was streaked with the swab three times, turning the plate at 60° angle between each streaking. Allow the inoculum to dry for 5-15 minutes with lid in place. The discs were applied using a septic

technique. Deposit the discs with centers at least 24 mm apart. The plates were inverted and place in an incubator set to 35°C within 15 minutes after the discs were applied. Each plate was examined after 20-24 hours of incubation. The results were read at 48 hours only when insufficient growth was observed after 24 hours. Six antifungal discs were used; Amphotericin-B, Clortrimazole, Fluconazole, Itraconazole, Ketoconazole, and Nystatin.

Results

Incidence of moulds in poultry samples

From 210 birds with 1050 tissue pieces, 306 fungal spp. were isolated and identified, as showed in Table 1. The highest incidence was in lungs among the broiler and Baladi birds followed by the air sacs then trachea. In Saso birds, the highest incidence was in air sacs followed by the lungs then the trachea.

Total number and percentage of moulds isolated from lung samples

As shown in Table 2, the mycological examination of 210 lung samples revealed the isolation of 97 strains of mould spp., which were identified as (*Aspergillus spp.,* and *Peniciillium spp.*). The highest incidence was for *A. flavus* (50.5%) followed by *Penicillium chrysogenum* (23.71%).

Total number and percentage of moulds isolated from air sacs samples

As shown in Table 3, the mycological examination of 210 air sac samples revealed the isolation of 74 mould stains were identified as (*Aspergillus spp.* and *Peniciillium spp.)* The highest incidence was for *A. flavus* (43.24%) followed by *Penicillium chrysogenum* (25.68%).

Total number and percentage of moulds isolated from liver samples

As showed in Table 4, from 210 liver samples, 30 mould stains were identified as (*Aspergillus spp.*, and *Penicillium spp.*). The highest incidence was for *Penicillium chrysogenum* (40%)., followed by *A. flavus* (36.66%).

Total number and percentage of moulds isolated from trachea samples

As showed in Table 5, from 210 trachea samples, 61 mould stains were identified as (*Aspergillus spp.*, and *Penicillium spp.*). The highest incidence was for *A. flavus* (40%) followed by *A. niger* (27.87%).

Total number and percentage of moulds isolated from crop samples

As showed in Table 6, from 210 crop samples 44 mould strains were identified as *Aspergillus spp.*, and *Peniciillium spp.* The highest incidence was for *Penicillium chrysogenum* (65.9%), followed by *A. flavus* (25%)

Incidence and percentage of mould isolated from the surroundings of poultry

Mycological examination of 87 surroundings samples revealed the isolation of 19 strains of mould spp. as shown in Table 7.

Total number and percentage of moulds isolated from ration samples

From 40 ration samples, 7 mould isolates were identified (*Aspergillus spp., Penicillium spp.,* and *Cladosporium spp.)* as shown in Table 8.

Total number and percentage of moulds isolated from beddings samples

From 14 bedding material samples, 4 mould isolates were identified (*Aspergillus spp.* and *Penicillium spp.)* as shown in Table 9.

Total number and percentage of moulds isolated from air samples

From 4 air samples, 2 mould isolates were identified (*Aspergillus spp.* And *Penicillium spp.*) as shown in Table 10.

Total number and percentage of moulds isolated from water samples

From 29 water samples, 6 mould isolates were identified (*Aspergillus spp*) as shown in Table 11.

Table 1: Incidence of moulds isolated from the poultry

Type of birds	Broiler (180)		Baladi (5)		Saso (25)	
Organ	No. of +ve	%	No. of +ve	%	No. of +ve	%
Lung	77	25	3	0.9	17	5.5
Air sac	52	16.44	3	0.9	19	6.2
Liver	21	6.86	1	0.3	8	2.6
Trachea	44	14.37	2	0.6	15	4.4
Crop	32	10.4	2	0.6	10	3.2
Total strains	306					

*The percentage of +ve samples = the numbers of +ve strain samples divided on the total numbers of +ve strains multiplied by 100.

Table 2: Total No. and percentage of fungi isolated from lung samples

Mould Species	No. of +ve isolates	%	Total No. of examined samples
A. flavus	49	50.5	210
A. fumigatus	13	13.4	
A. niger	12	12.37	
Total *Aspergillus* species	74	76.24	
Penicillium chrysogenum	23	23.71	

*The Percentage of +ve isolates calculated according to total number of the +ve isolates of all mould strains isolated from lung (97).

Table 3: Total No. and percentage of fungi isolated from air sacs samples

Mould Species	No. of +ve isolates	%	Total No. of examined samples
A. flavus	32	43.24	210
A. fumigatus	11	14.86	
A. niger	12	16.22	
Total *Aspergillus species*	55	74.32	
Penicillium chrysogenum	19	25.68	

*Percentage of +ve isolates calculated according to total number of the +ve isolates of all mould strains isolated from air sacs (74).

Table 4: Total No. and percentage of fungi isolated from liver samples

Mould Species	No. of +ve isolates	%	Total No. of examined samples
A. flavus	11	36.66	210
A. fumigatus	2	6.66	
A. niger	5	16.66	
Total *Aspergillus species*	18	59.98	
Penicillium chrysogenum	12	40	

*The Percentage of +ve isolates calculated according to total number of the +ve isolates of all mould strains isolated from liver (30).

Table 5: Total No. and percentage of fungi isolated from Trachea samples

Mould Species	No. of +ve isolates	%	Total No. of examined samples
A.flavus	25	40	210
A.fumigatus	7	11.48	
A.niger	17	27.87	
Total *Aspergillus species*	49	79.35	
Penicillium chrysogenum	12	19.67	

*The Percentage of +ve isolates calculated according to total number of the +ve isolates of all mould strains isolated from trachea (61).

Table 6: Total No. and percentage of fungi isolated from crop samples

Mould Species	No. of +ve isolates	%	Total No. of examined samples
A. flavus	11	25	210
A. fumigatus	2	4.55	
A. niger	2	4.55	
Total *Aspergillus species*	15	34.09	
Penicillium chrysogenum	29	65.9	

*The Percentage of +ve isolates calculated according to total number of the +ve isolates of all mould strains isolated from trachea (44).

Table 7: Total No. and percentage of fungi isolated from surroundi

	No. of +ve samples	%of +ve samples	Total number of samples(87)
Air	2	10.52	4
Bedding	4	21	14
Ration	7	36.84	40
Water	6	31.57	29
Total strains	87		19

*The percentage of +ve samples = the numbers of +ve samples divided on the total numbers of +ve strains multiplied by 100.

Table 8: Total No. and percentage of fungi isolated from ration samples

Mould Species	No. of +ve isolates	% of +ve isolates	Total No. of ration samples
A. fumigatus	2	28.57	40
A. niger	2	28.57	
P. chrysogenum	2	28.57	
Cladosporium	1	14.29	

*The Percentage of +ve isolates calculated according to total number of the +ve isolates of all mould strains isolated from ration (7).

Table 9: Total No. and percentage of fungi isolated from bedding material samples

Mould Species	No. of +ve isolates	%	Total No. of bedding material samples
A. flavus	2	50	14
A. niger	1	25	
P. chrysogenum	1	25	

*The Percentage of +ve isolates calculated according to total number of the +ve isolates of all mould strains isolated from bedding materials (4).

Table 10: Total No. and percentage of fungi isolated from air samples

No. of +ve isolates	Mould Species	%	Total No. of air samples
1	*A. flavus*	50	4
1	*P. chrysogenum*	50	

*The Percentage of +ve isolates calculated according to total number of the +ve isolates of all mould strains isolated from air (2).

Table 11: Total No. and percentage of fungi isolated from water samples

No. of +ve isolates	Mould Species	%	Total No. of water samples
4	*A. flavus*	66.6	29
2	*A. fumigatus*	33.3	

*Percentage of +ve isolates calculated according to total number of the +ve isolates of all mould strains isolated from water (6).

Table 12: Total mould count of the examined ration and drinking water samples:

Type of sample	No. of examined samples	% of +ve samples	Min. mould count (cfu\g or 100 mL)	Max. mould count (cfu\g or 100 mL)	Mean count (cfu\g or 100 mL)	Standard error
Ration	40	17.5	6×	12	1.01×10^{7}	4.6×10^{6}
Drinking water	29	20.68	12	118	30.6	7.8

Table 13: Antifungal susceptibility testing of the recovered mould species

Mould Species	AmP	CLC	FLC	ITC	KTC	NYT
P. Chrysogenum	S	S	S	S	R	S
Cladosporium perangustum	S	S	R	S	S	S
P. Chrysogenum	S	S	S	S	R	S
A. flavus	R	S	R	S	R	R
A. flavus	S	R	R	S	R	S
P. chrysogenum	S	S	S	S	S	S
P. chrysogenum	S	S	S	S	R	S
A. flavus	S	R	R	S	S	R
A. niger	S	S	S	S	R	S

AmP: Amphotericin B (100 µg/disc), CLC: Clotrimazole (10 µg/disc), FLC: Fluconazole (100 µg/disc), ITC: Itraconazole (10 µg/disc), KTC: Ketoconazole (100 µg/disc), NYT: Nystatin (10 µg/disc), S: sensitive, R: resistant.

Histopathological findings

Nodular lesions on lung and pleural surface were examined. The examined nodules showed central massive liquifactive necrosis involving wide area of the alveolar and bronchial tissues with presence of hyphal elements, with characteristic basophilic septated hyphae, with about 45 angle degree of branched manner, which might be consistent with Aspergillosis (Figures 1-4).

Total mould count of the examined ration and drinking water samples:

The obtained result revealed that the total mould count in poultry feed samples ranged from 6 x 10^{3} to 12 x 10^{7}cfu/g and this indicated that the ration is highly contaminated with mould, on the other hand it was found that the total mould count of the examined drinking water ranged from 12 to 118 cfu/100 ml and also this indicates that

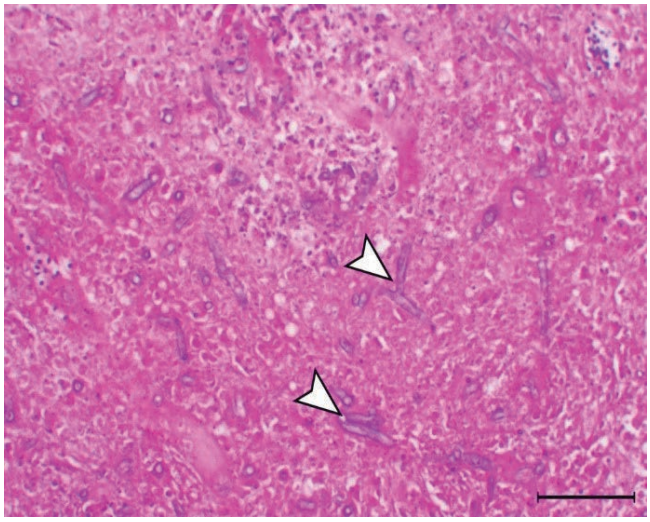


Figure 1: The lung of infected bird with *Aspergillosis* showing nodule associated with liquifactive necrosis with presence of branched hyphae (arrowheads). H&E, X200, bar= 50 µm

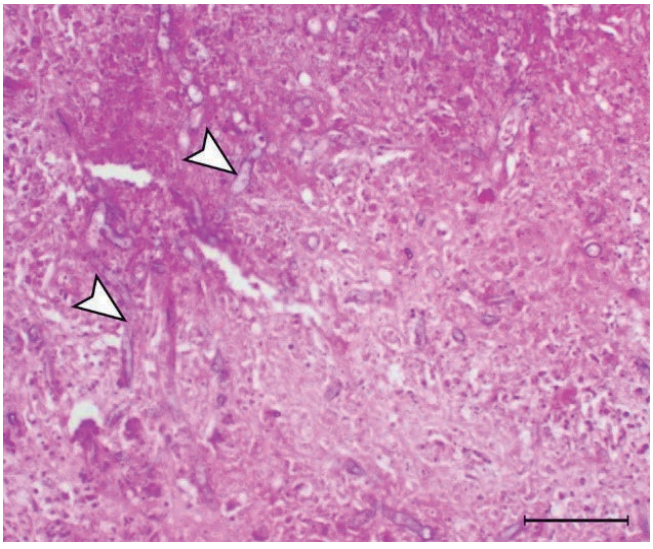


Figure 2: The lung of infected bird with Aspergillosis showing basophilic septated hyphae (arrowheads). H&E, X200, bar= 50 µm

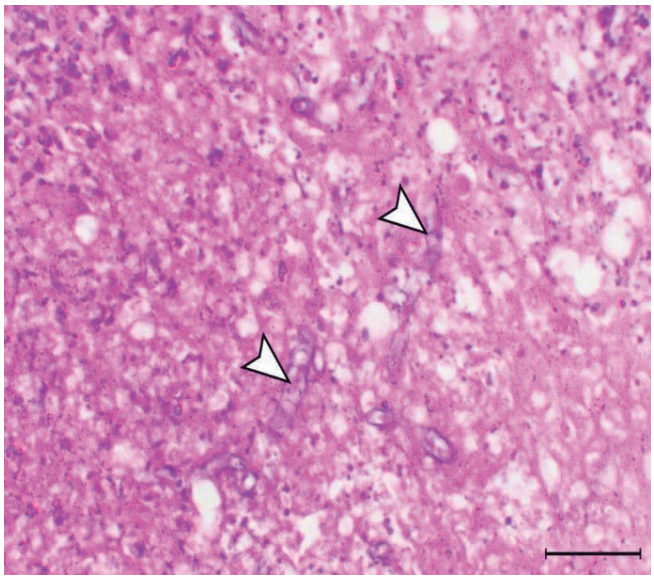


Figure 3: The lung of bird showing liquefactive necrosis with presence of basophilic branched hyphae (arrow-heads). H&E, X200, bar= 25 µm

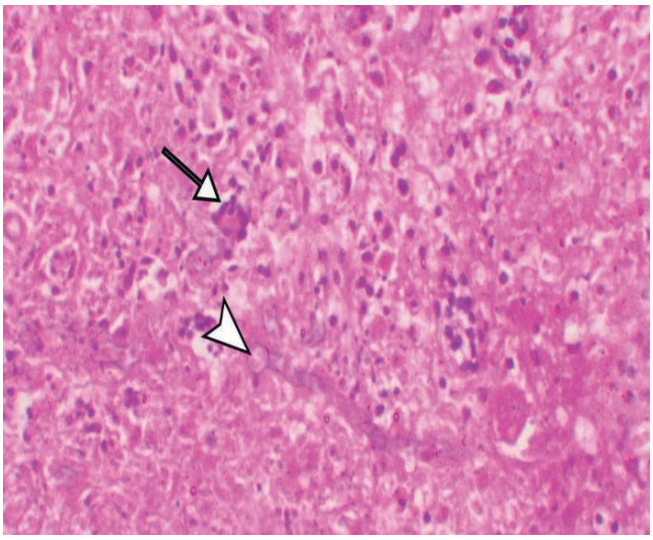


Figure 4: The lung of bird showing liquefactive necrosis with presence of basophilic branched hyphae (arrowhead) and infiltration of multinucleated giant cell (arrow), H&E, X200, bar= 25 µm

the drinking water was contaminated with mould, as shown in Table 12.

Result of antifungal susceptibility test of moulds isolated from poultry:

As shown in Table 7, *A. niger* as well as *P. chrysogenum* was resistant to Ketoconazole, while *Cladosporium perangustum* was resistant to Fluconazole. All of the isolated mould was sensitive to Itraconazole, and Nystatin except *A. flavu*s that was resistant to Nystatin. All *Aspergillus spp.* were resistant to Fluconazole except *A. niger.*

Discussion

Fungal infections have their own significance among infectious diseases and appear to be one of the biggest challenges for chicken breeders (15). The combination of bedding material, chicken waste, and litter makes the fungal infection one of the primary poultry infections (16). The present study was conducted aiming to the phenotypic characterization of some fungi isolated from broiler, Baladi, and Saso chicken from different farms in Egypt.

The results of the present study showed that moulds were isolated from the broilers at the highest rate. In addition, the lungs as well as air sacs were the highest organs for mould invasion. These results agreed with a previous report (5, 17) who isolated *A. niger,* and *A. fumigatus* at a high rate from broiler farms. The results of fungal isolation from the studied chicken showed that the highest mould species isolated from the lung, air sacs, liver and trachea were *Aspergillus species* (76.24%, 74.32%, 60%, and 80.32%, respectively), with the most frequent was *A. flavus* compared to *A. fumigatus,* and *A. niger*, while the most frequent fungi isolated from the crop was *Penicillium chrysogenum* (65.9%). These findings were in line with (19) who reported that *Aspergillus spp.*, *Mucor*, and *Penicillium species* were the most prevalent fungal species detected in poultry. In another study that conducted in Egypt, the most prevalent fungal species were *A. niger, C. albicans, A. fumigatus, A. terreus,* and *P. corylophilum* at prevalence rates of 26.6% (45/169), 22.5% (38/169), 20.7% (35/169), 14.8% (25/169), and 15.4% (26/169), respectively (5). The results also were in line with other reports (19, 20) who reported that *Aspergillus species* were the most prevalent fungi, particularly, *A. fumigatus* (2.2%), *A. flavus* (2.6%), *A. niger* (4.4%). Besides, it was reported that the fungal isolates that recovered from bird ceca were *Aspergillus species* (18.4%), followed by *Penicillium* (15.6%), *Verticillium* (6.2%), *Sporidiobolus* (5.2%). In addition, in the ceca, *A. fumigatus* was the most frequent species identified (10.6%), followed by *A. flavus* (5.4%) (21). Aspergillosis, commonly known as brooder's pneumonia, is caused mainly by *Aspergillus fumigatus*, most pathogenic fungi

affecting poultry, but *A. flavus* has also been the culprit associated with many cases (22). It was also reported that the most frequent isolated fungi from chickens were *A. fumigatus* (21.7%) followed by *A. flavus* (19.4%), and *A. niger* (17.1%) (17). In another report (23), it was revealed that *A. fumigatus* was recovered at 28.0%, followed by *A. niger* at 18.6%, and *Penicillium species* at 8.5%.

The present study also revealed that the fungal isolation was the highest in ration (50%), followed by the bedding (28.5%). Unlikely, it was found that chicks' litter and fecal droppings had the highest rate of fungal isolation, followed by drinkers, feeders, air, as well as feeds (5). It was found that the litter (bedding materials) is one of the most contributive factors to fungal contamination in poultry farms (24). In addition, *Aspergillus species*, especially *A. flavus* was the most frequent fungal species isolated from bedding material, water, and air samples (50%, 66.6%, and 50% respectively), followed by *Penicillium chrysogenum* which was recovered from ration, bedding material, and air samples (28.57%, 25%, and 50%, respectively). However, *A. niger* was found at the highest rate in chick droppings, followed by feeds, feeder swabs, and chick litter (5). Meanwhile, the rate found in air samples and drinkers' swabs was higher compared to the rate found in tap water and attendants' hand swabs, which was the lowest. The dispersion of *A. fumigatus* in air samples and feeds, oppositely, was discovered at the highest rate followed by chicks dropping and their litter. It was also reported that the recovered moulds from broiler chicks' surroundings were 6.4% for *A. fumigatus*, 33.3% for *A. flavus*, 2.1% for each of *A. niger*, *A. nidulans* and *A. terrus* as well as 23% for Zygomycetes (19). Likewise, It was stated that there were some of the fungal isolates more frequently identified in the study (24) including *Penicillium* and *Aspergillus species* in fresh, new, and aged litter. In addition, *Cladosporium species* was also frequently isolated in the new litter. Furthermore, air was assessed from poultry habitats for mould contamination (25). The genera *Penicillium species*, *Aspergillus species* were also found in the litter. The existence of fungi in chicken environments indicates poor management approaches, which is the primary source of mould infection of broilers, either in a direct way by fungi or by an indirect way by the fungal mycotoxins. Humidity and temperature conditions seen in chicken farms encourage hyphal growth and effective asexual multiplication, resulting in a large number of easily airborne hydrophobic conidia that are disseminated and inhaled by the birds (26). Increased fungal infections in chicks' environments could be due to increased contamination of the environment with organic waste and sewage, demonstrating poor hygienic procedures and poor ventilation inside the poultry buildings (27).

The histopathological examination of lung nodules of the affected birds revealed central massive liquefactive necrosis involving wide area of the alveolar and bronchial tissues with presence of hyphal elements, with characteristic basophilic septated hyphae, with about 45 angle degree of branched manner, which might be consistent with aspergillosis, and this agrees with previous reports (22, 28, 29, 30).

The present study also showed that the total mould count in the poultry feed samples was higher than that of drinking water samples, where the total mould count in feed samples ranged from 6×cfu/g to 12cfu/g while it ranged from 12 cfu/100 ml to 118 cfu/100 ml in drinking water. These findings were in agreement with a previous study (31), who found that the total mould count (log 10 cfu/g) in poultry feed samples ranged from 1.8 to 4.4 (log 10 cfu/g) while in drinking water samples, it was ranged from 1 to 1.8 (log 10 cfu/ml). Also, it was revealed that the majority of feed samples analyzed contained 1-9 × 10^4 cfu/g (32).

The antifungal susceptibility testing of moulds isolated from poultry was investigated for the *in vitro* efficacy of antifungals against the representative fungal isolates. The isolated fungal species revealed variable degrees of resistance to the antifungal drugs used in the present study. It was shown that *A. niger* as well as *P. chrysogenum* was resistant to Ketoconazole, while *Cladosporium perangustum* was resistant to Fluconazole. In addition, all the isolated moulds were sensitive to Itraconazole and Nystatin except *A. flavus* that was resistant to Nystatin. All *Aspergillus spp.* were resistant to Fluconazole except *A. niger*. These results were in agreement with (5) where the susceptibility of all fungal isolates to the medication Itraconazole was determined (100 %), while *Aspergillus spp.* (*A. niger, A. fumigatus, and A. terreus*) showed no susceptibility to fluconazole. In contrast to *A. niger*, which was highly resistant to Nystatin (100%), the other fungal species were very sensitive. Also, it was reported that

Aspergillus species were resistant to fluconazole but were sensitive to Nystatin and Voriconazole (33). Unlike the findings of the present study, it was reported that generally all isolates were resistant to fluconazole and Nystatin (4). Highly active triazoles except fluconazole were recommended as the first-line therapy to treat aspergillosis, but their effectiveness is challenged by the emergence of drug resistance (34, 35). The variable degrees of the observed resistance by the *Aspergillus species* could be attributed to the concentration of the antifungal drug applied. It is possible that higher concentrations or higher doses of the drug may be required for effective antifungal effects. It is also likely that some of the strains of the *Aspergillus species* tested in this investigation have developed resistance to the tested antimicrobials.

Conclusion

It is concluded that *Aspergillus species* is associated with poultry farms in Egypt. Fungi was mainly isolated from ration followed by drinking water. Itraconazole and Nystatin could be applied as proper antifungals for *Aspergillus* infection except for *A. flavu*s that was resistant to Nystatin.

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Original Research Article
Veterinary Medicine and The One Health Concept

HISTAMINE LEVEL AND HISTAMINE-PRODUCING BACTERIA ISOLATED FROM SALTED AND FREEZE SARDINE FISH (*Sardina* spp.)

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Abstract: Histamine is the biogenic amine responsible for scombroid poisoning in various types of fish and fish products. The study evaluated histamine content and bacteriological quality of salted and freeze sardine samples. All fish samples were cultured on Reinforced Closterdial medium; mannitol salt (MS) agar and violet red bile glucose (VRBG) agar, the positive colonies were sub-cultured on histamine-forming bacterium agar, followed by biochemical and PCR identification. Production of histamine in the fish muscles was measured by high performance liquid chromatography. The mean histamine values were about; 8.25 mg/100g. The mean of total aerobic counts was; $4\times10^4 \pm 2.1\times10^4$ while, the total anaerobic count; $5\times10^4 \pm 1.2\times10^3$ Log CFU/g. *Enterobacteriacea spp.* about; $5\times10^4 \pm 2.5\times10^4$ Log CFU/g while, the mean value of *Staphylococcus spp.* about; $3\times10^4 \pm 3.7\times10^4$ Log CFU/g. The mean value of histamine-forming bacterial were; 3.3×10^6 Log CFU/g. Molecular Identification For Enterobacteriaceae were *Klebsiella pneumonia*, *Staphylococcus xylosus*, *Escherichia coli* and *Enterobacter cloacae*. We concluded that during storage, there was a positive relationship between the histamine levels and the histamine-forming bacteria (HFB) numbers, while, histamine and the bacteria forming histamine were recorded in all examined sardine samples.

Key words: chistamine; *Escherichia coli*; *Klebsiella pneumonia*; molecular identification; scombroid; *Staphylococcus xylosus*

Introduction

Sardine, *Sardina pilchardus* is one of small pelagic oily fish, which is characterized by dark-colored flesh and strong flavors and affordable price. Fish spoilage changes occur as soon as fish dies, and they take place in a successive process, its consumption might result in some foodborne illnesses and outbreaks (1). Scombroid and other dark muscle fish such as tuna, mackerel, blue fish, sardine, carangids, herring, and anchovies are prone to form histamine, as their muscles are histidine-rich (2). Histamine ($C_5H_9N_3$) is one of the biogenic amines which leading to food-borne intoxication which is named as histamine poisoning. Histamine has pharmacological effects e.g. inflammation & vasodilation by allergic reaction mediation (2). The symptoms of scombroid poisoning appear within a few minutes after eating fish of Scombridae family and related species. The first symptoms are cutaneous, with flush, pruritus, and erythema of the face and trunk having an urticarial appearance, together with faintness. Gastrointestinal symptoms include nausea, vomiting, abdominal cramps and occasionally diarrhea (3). The

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major bacterial species reported in fish, which can form histamine, are: *Klebsiella pneumonia, Staphylococcus xylosus, Escherichia soli, Morganella morganii, Hafnia alvei, Enterobacter aerogenes, Enterobacter cloacae, Proteus mirabilis, Serratia liquefaciens, Serratic fonticola, Citrobacter freundii* and *Raoultella ornithinolytica* (4). These types of bacteria naturally present on the gills, external surfaces and in the gut of live saltwater fish with no harm to the fish. Up on death, the defense mechanism of the fish no longer inhibits bacterial growth in the muscle tissue and histamine forming bacteria may start to grow resulting in the formation of biogenic amines (5). Inadequate cooling following harvest promotes bacterial histamine production and can result in outbreaks of scombroid poisoning (6) Specific conditions are needed for preservation of fish and avoid the chemical intoxication hazardous (7). Freshly caught fish does not contain histamine however, once fish starts to decay or is subjected to temperature abuse; histamine can start to accumulate. The production of histamine in fish is generally believed to result from the growth of Gram negative, rod shaped bacteria capable of producing pyridoxal phosphate dependent decarboxylase enzymes (8). This process can start as soon as the fish dies and kept at temperature above 4°C for an extended period (FAO/WHO, 2012). Histamine forming bacteria produce histidine decarboxylase (HDC) enzyme, once the HDC enzyme is synthesized, it continues to produce histamine even if the bacteria get inactivated (EFSA, 2011; FDA, 2011). Cooking can inhibit the HPB and the HDC, while histamine cannot be degraded by cooking, smoking, or canning of fish, indicating that both raw and cooked fish might cause HPB (2). Fish is very important source of protein especially in Egypt. Fish and fish products are one of the most important food stuffs as they are one of the cheapest sources of animal protein (9). So far, little information is available about HPB in Egypt. Therefore, this study was conducted for evaluation of the histamine content in addition to bacteriological quality of sardine fish samples.

Materials and methods

Samples collection: A total of 50 sardine samples were collected from various commercial fish retails and markets at Ismailia city, Egypt. Samples were kept in icebox during transportation to the laboratory and stored at −20 °C until further analysis. bacteriological evaluation was determined in the all samples.

Determination of pH value, salt content, water activity and total volatile nitrogenous compound (TVN): The muscles of sardine fish samples (15 g) were blended in mixer for 10 min at 6000 rpm with 30 ml distilled water to make thick slurry. The salt content of the fish muscle was determined by the method of AOAC procedures (1995)(10. 10 ml of the filtered extract (D: dilution coefficient) were pipetted into 100ml Erlenmeyer and 1ml of K_2CrO_4 (IN) was added. The samples were titrated with 0.1NAgNO3 standard solution (f: factor) until the first perceptible pale red brown appeared (T: titration volume).

Calculation: Concentration of sodium chloride based on sodium can calculated by the formula: NaCl (%)= 0.00585× T × D × ---------- × F. Water content was detected by Hygrometer electric instrument at 27 °C. The pH was measured using pH meter. The (TVN) was measured by dissolving 100g of the sardine fish sample in 200ml (7.5%) aqueous solution of trichloro-acetic acid, then filter the mixture and mixing 25ml of the extract in a distillation flask with 6ml 10%NaOH. The TVN condensed is mixed with 10ml (4%) boric acid and 0.04ml of methyl-red and bromocresol green indicator which turns green when alkalinized by the TVN (11).

Calculation: The TVN content was calculated by the formula: TVB-N = (14 × a × b × 300) / 25 (mg/100g). Where: (a) is a volume of sulphuric acid (ml) and (b) is the normality of sulphuric acid (%).

Microbiological analysis: The following Bacterial counts and Isolation of histamine-forming bacteria were carried out according to (12), the flesh of fish sample transferred to a homogenizer to mince the sample using Moulinex Food Processor (La Kiloulinette, type D56, Paris, France), 25g of homogenized sample was added to 225 mL of 0.1% peptone water using plastic bag, put in a Seward stomacher (4001R/LIK) for 2 min. prepared serial dilutions a final dilution of (10-7) by peptone water.

Total aerobic bacterial count: Standard plate count agar by spread plate method were used for the determination of aerobic plate counts, and then incubated at 30±1°C/72±3 hrs.

Total anaerobic colony counts: The same procedures as for the previous sample preparation were incorporated using Reinforced Closterdial

medium agar (Oxoid; CM151), incubated in an anaerobic jar (Gaspak plus anaerobic system) at 37°C/48 hours.

Staphylococci spp.: Staphylococci spp. were determined on Baird Parker Agar (Oxoid CM 275) supplemented by egg yolk tellurite at 37 °C for 24–48 h. Typical black colonies with zones around and atypical black colonies were considered as Staphylococcus spp.

Total Enterobacteriaceae Counts: Enterobacteriaceae were detected using violet red bile glucose agar (VRBGA) which were incubated at an inverted position at 30-35°C/24 hrs.

Measurement of Biogenic amine content in fish muscles using HPLC: Fish samples were subjected to HPLC to analyses the contents of histamine, putrescine, cadaverine, serotonin, phenylethylamine, spermidine and tyramine. In brief, 5g muscle from each sample was homogenized separately using l0mL trichloroacetic acid extracting solution (Thermo Fischer Scientific, Waltham, MA) and centrifugation. The supernatant was filtered, mixed with lmL of 1M NaOH (Thermo Fischer Scientific), and incubated at RT for 5 min. One milliliter of ophthalaldehyde (Acros Organics, Geel, Belgium) and 3nnL ethyl acetate (Thermo Fischer Scientific) were then added, and the homogenate was precipitated by centrifugation. The pellets were dried on a rotatory evaporator, then re-suspended in 1 ml acetonitrile (Thermo Fischer Scientific). A histamine dihydrochloride standard solution (Sigma- Aldrich, St. Louis, MO) was serially diluted in 0.1M HCI (Thermo Fischer Scientific) to obtain the following concentrations: 0.5, 1, 2, 4, 8, 16, 32, and 64 mg/L. The standard and the processed samples were injected into intersil C18 columns (Agilent Technologies, Waldbron, Germany); each determination was injected twice. The chromatographic separation was performed using 1050 HPLC (Agilent Technologies) according to (13).

Determination of total histamine-forming bacterial counts: Spread 0.1ml aliquot of diluted sample on histamine-forming bacterium isolation agar (HBI agar) mixed with L¬histidine. Incubation of agar plates for 4 days/35°C, counted the purple and blue colonies, picked on trypticase soy agar (Difco) to identification by pure cultures (14).

Identification histamine-forming bacteria: Bacterial isolates were identified using the Vitek instrument (bio-Merieux Vitek Inc.,Hazelwood, Mich., U.S.A.). Additional biochemical tests were carried out for definitive species identification by screening on (TSI) triple sugar iron agar, Vogs-proskauer (VP) test, Oxidase test, Fermentation test, Methyl-Red test Citrate-utilization test, Urease test, Indole production test (15, 16).

PCR Identification: DNA extraction was done using marketed kit for DNA extraction (Presto Mini-DNA Bacteria Kit. Ltd. USA). Preparation of DNA: using 0.05 N NaOH and 0.25% sodium dodecyl sulfate. PCR product analysis performed using a 1% agarose substance by electrophoresis apparatus and observed under the UV trans-illuminator. Amplification and sequence determination of 16S rDNA.

Statistical analysis; Data are presented as mean±SEM. Analysis was performed using GraphPad Prism 7.03 software (GraphPad Software, San Diego, CA, USA). Statistical differences over two groups were verified by Student's *t*-test

Results

The obtained results in Table 1 represented the values of the salt content, pH, total volatile nitrogen (TVN), water activity. The salt content, pH value, TVN and water activity in all samples ranged from 0.01 to 9.6%, 6.1 to 7.6, 6 to 7.8 and 21.4 to 35.7 mg/100 g respectively. The average TVN level (24.3 mg/100 g) of frozen samples were significantly ($P < 0.05$) lower than those of salted samples. The levels of TVN in most fish samples were below the Egyptian regulatory standard of 25 mg/100 g, 6 (Table 1).

TVN, including NH3, dimethylamine (DMA) and trimethylamine (TMA) is used as indicators for the fish spoilage and quality. In this study, the TVN values in salted sardines were significantly higher than those obtained in frozen samples. Bacteriological profiles of total coliforms, Aerobic, anaerobic, *Enterobacteriacea spp. E. coli* and *Staphylococcus spp.* counts were detected in table 2. Statistical analysis for Different Types of Microorganisms (Log CFU/g) in the Sardine samples were; the total aerobic counts reported about; 8×10^3, 6×10^4, $4\times10^4 \pm 2.1\times10^4$ as minimum, maximum, mean ±SE values respectively while, about; 5×10^3, 6×10^4, $5\times10^4 \pm 1.2\times10^3$ Log CFU/g were detected as minimum, maximum, mean ±SE values respectively in case of total anaerobic count. *Enterobacteriacea spp. s*howed about; 4×10^3,

6.0×10^5, $5\times10^4 \pm 2.5\times10^4$ Log CFU/g as minimum, maximum, mean ±SE values respectively while, about; 3×10^3, 4×10^5, 3×10^4 $\pm 3.7\times10^4$ Log CFU/g were detected as minimum, maximum, mean ±SE values respectively in case of *Staphylococcus spp.* counts. The lower water content (<0.92) and higher salt concentration (>4.0%) in these salted sardine samples apparently had some inhibitory effect on coliform bacterial growth.

Table 3 reported the contents of biogenic amines in the frozen and salted sardine fish. The average content of each of the seven biogenic amines in all samples was less than 10 mg/100 g. Based on the results, it is cleared that higher levels of histamine were found in salted and frozen sardine fish was 8.25 and 0.64 mg/100g. Among them, higher level of biogenic amines in salted sardine samples as follow, the average cadaverine (2.82 mg/100 g), tyramine (4.17 mg/100 g), putrescine (4.72 mg/100 g) and agmentine (4.57 mg/100 g) this results of salted sardine samples were significantly ($p < 0.05$) higher than those of frozen samples.

Figure 1 and 2 show the identity and Frequency distribution of histamine-forming bacterial isolates of frozen sardines' samples as determined by 16S rDNA sequences. They were identified as *Citrobacter freundii* (thirteen strain), *Enterobacter aerogenes* (ten strain), *Enterobacter cloacae* (twelve strain), *Klebsiella pneumoniae* (eleven strain), *Micrococcus spp* (fourteen strain), *Proteus vulgaris* (thirty strain), *Pseudomonas aeruginosa* (seventeen strain), *Staphylococcus xylosus* (sixteen strain), In this study, that the highest incidence was recorded by *Proteus vulgaris* spp. which represented 12 (20%) and 18 (29%) in both frozen and salted samples respectively. On the other side, the lowest incidence in frozen samples was recorded by *Micrococcus spp.* and *Staphylococcus xylosus* spp. which represented 4 (7%). Although, the lowest incidence in salted samples was recorded by *Enterobacter aerogenes* spp. which represented 4 (5%) then *Citrobacter freundii* and *Enterobacter cloacae* which represented 4 (6%).

Table 1: Values of the salt content, pH, water content, total volatile nitrogen (TVN) in Frozen and salted Sardine sam-ples

Samples	Salt content (%)	pH	Water content	TVN(mg/100 g)
Frozen Sardine	0.01-0.12 (0.05 ± 0.01) A	6.2-7.2(6.6 ± 0.1) A	48–78 (63.4 ± 2,9)A	21.4-30.4 (24.3 ± 0.93)B
Salted Sardine	4.5-9.6(7.6 ± 0.5)A	6.1-7.6 (6.75 ± 02.)A	6-17 (13.6 ± 1.3)A	23.1–35.7 29.9 ± 1,59)A

Mean ± SD. Values in the same column with different letters are statistically different ($P < 0.05$).

Table 2: Microbial content and percentage of evidence of aerobic and Anaerobic plate count (APC), total coliform (TC), *Escherichia coli* (*E. coli*), *Enter. cloacae* and *Staph. xylosus* in Frozen and salted Sardine samples

Sam-ples	APC		TC		E. coli(		Anaerobic plate count		Enter. cloacae		Staph. xylosus	
	Percent %	(log CFU/g)	Percent %	(MPN/g)	Percent %	(MPN/g)	Percent %	(log CFU/g)	Percent %	(log CFU/g)	Percent %	(log CFU/g)
Frozen Sardine	25	$2X10^3 \pm$ 0.9 B	5	<3	25	<2	75	$2X10^2 \pm$ 0.9 B	50	$3X10^3 \pm$ 1.2 B	25	$3X10^3 \pm$ 2.2 B
Salted Sardine	100	$8X10^3$-6 $X10^4$(4X $10^4 \pm$ 1.8)A	20	<3	50	<3	100	$5X10^3$-6 $X10^4$(5X $10^4 \pm$ 1.2)A	100	$4X10^3$-6 $X10^5$(5X $10^4 \pm$ 2.5)A	50	$3X10^3$-$4X10^5$(3X $10^4 \pm$ 3.7)A

Table 3: Contents of biogenic amines in in Frozen and salted Sardine samples

Samples	No. of samples	Cad	Tyr	His	Agm	Put	Phe	Spd
Frozen Sardine	50	0.26 (0.01 ± 0.05)A	0.97 (0.37 ± 0.22)A	1.64 (0.13 ± 0.1)A	ND-0.15 (0.03 ± 0.01)A	0.52 (0.05 ± 0.14)A	ND	ND
Salted Sardine	50	2.82 (0.69 ± 0.48)A	4.17 (1.72 ± 1.21)B	8.25 (0.89 ± 1.62)A	4.57 (5.13 ± 4.79)B	4.72 (2.18 ± 2.00)B	ND	ND

Cad: cadaverine; Tyr: tyramine; His: histamine; Agm: agmatine; Put: putrescine; Phe: 2-phenylethylamine; Spd: spermidine.

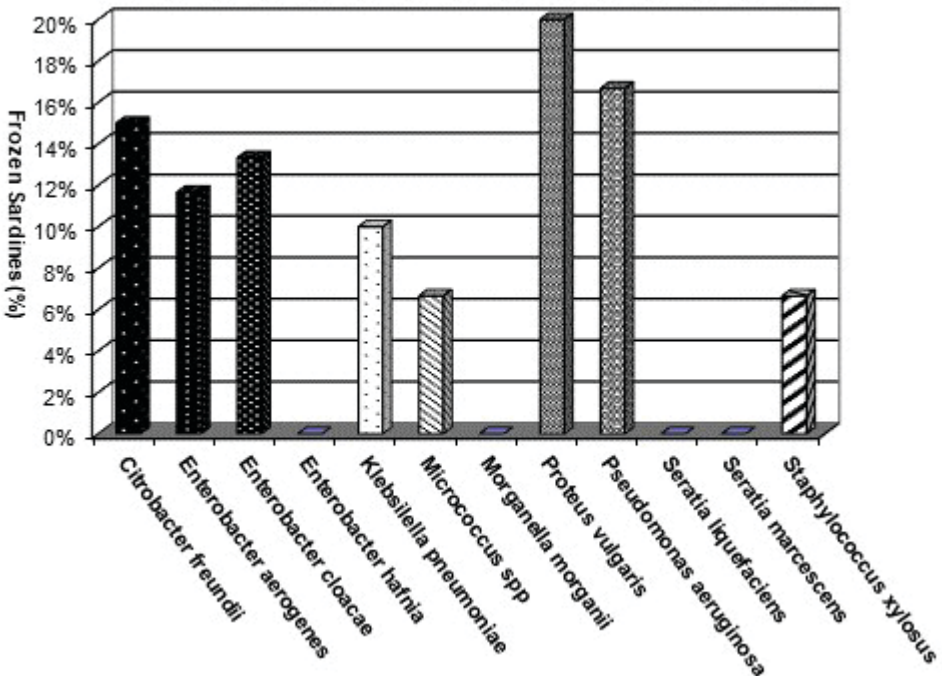


Figure 1: Frequency distribution of histamine-forming bacterial isolates of frozen sardines' samples

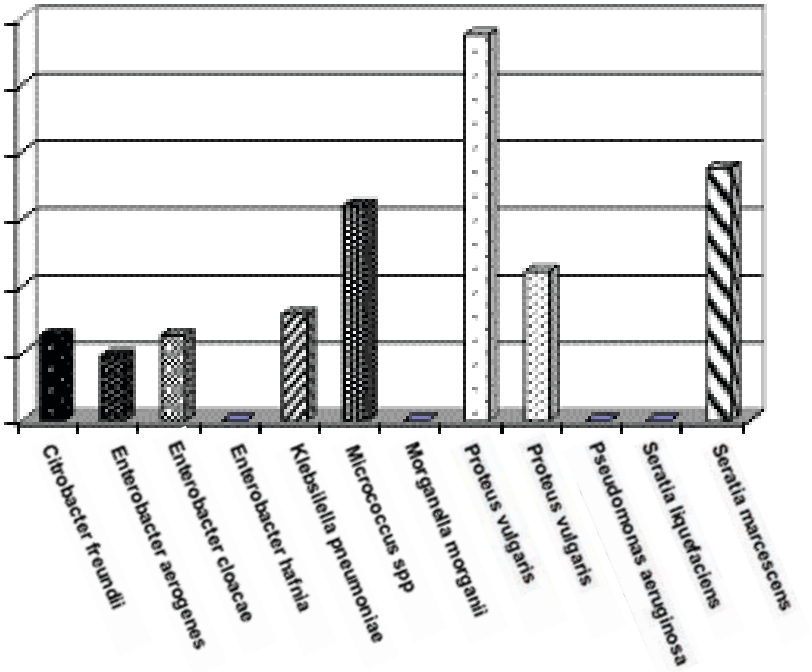


Figure 2: Frequency distribution of histamine-forming bacterial isolates of salted sardines' samples

Discussion

Scombroid is a type of food poisoning experienced around the world, with a higher reported prevalence in Japan, England and the United States. This could be attributed to the high consumption of scombroid fish (17). Histamine presence in food items reflect the food quality, bad storage conditions one of the major inducers of histamine formation and aggregation as the histamine-forming bacteria can produce the enzyme "histamine–decarboxylase" which converts histidine amino acid in fish tissues in histamine even at low temperatures as 5°C. formation of

histamine not degraded by freezing or heating (18). Based on the finding that the higher levels of TVN noticed in dried salted fish samples, we explained that these samples have been seriously contaminated during food preparation and processing (19). The obtained results of TVN in frozen sardine samples were nearly like those were submitted by (19). Although, lower TVN results were obtained by (20) confirmed that the TVN values of frozen sardine samples were ranged from 15 to 17mg/100g. El-Dengawy et al. (21) studied that mean TVN values of frozen sardine samples were 13.34±0.00 and 16.23±0.00mg/100g. (22) reported that the TVN values of frozen sardines were ranged from 11.2 to19.9mg/100g. Similar results with TVN (43 mg/100 g) and higher salt concentration (5.6%) in salted fish product were reported (23). The total aerobic, anaerobic and *Enterobacteriacea spp.* were approximately 100% of the total sample count, while only 50% of tested samples had *Staphylococcus spp.* Similar results in the total aerobic count were detected by (24,25) during their assessment of the bacteriological quality of the bacteriological quality of some meat products in the Egyptian retail markets. Also, nearly similar results observed by (26) during their bacteriological evaluation of different fish samples of Dharan markets as; (54%) in case of *staphylococcus sp.* Lower results reported by (27) during their microbiological quality survallaince in Istanbul as (96.67%). While, only about (24%) of *Enterobacteracea sp.* and (8%) of *staphylococcus sp.* was estimated by (28). Similar results found by Salem, *et al.* (28) whome reocrded about $5.61x10^5$ Log CFU/g of total aerobic counts. Ragab, *et al.* (24) detected about ($6.6x\ 10^8$ Log CFU/g) of total aerobic counts. While, (29) also recorded about (4.27×10^3 Log CFU/g) of *Enterobacteracea sp.* and 5.6×10^5 Log CFU/g of *staphylococcus sp.* was detected by (30). Lower result observed by Shaltout, *et al.* (20) who obtained about 8.03×10^4 Log CFU/g of total aerobic count while, in case of *Enterobacteracea sp.* they recorded about (2.02×10^2 Log CFU/g) and isolated about (2.67×10^2 Log CFU/g) of *staphylococcus*. Most aerobic of these bacteria would die or at least stop growing as the salt content of the fish was increased to 6–8%. (26) also reported similar findings with the unclean market and storage temperatures could have contributed to the high contents of TVN in samples (26). The observed results of histamine in frozen *sardine* samples were parallel to those were pointed out by Lee *et al.* (31). Biji *et al.* (32) confirmed that histamine levels in *sardines* were ranged from 1.0 to 2.6 mg/100g. Also, Martin *et al.* (33) studied that mean values of histamine were ranged from 0.21±0.37 to 1.04±0.82 mg/100g in samples. On the other hand, higher histamine results were reported by El Hariri, *et al.* (34) estimated the level of histamine in the *S. pilchardus* muscle at the rejection time (12 days) preserved in ice was 16.2 mg/100g. Li *et al.* (35) reported that mean histamine content of samples was16.4±2.6 mg/100g. Aruna *et al.* (36) reported that histamine level of frozen sardines were ranged from 12 to 32 mg/100g. The strong evidence exists that the toxic effects of histamine affected and increased by the biogenic amines in fish tissue by inhibiting intestinal histamine-metabolizing enzymes such as diamine oxidase (13). Histamine-forming bacteria species which isolated in this study have been previously recorded by different investigators (37-39). However, the *Enterobacter* strains were isolated from many scombroid fish species (40). This increment in histamine concentration for sardine samples caused by consumptions of poor-quality raw food and bad handling techniques, improper storage and defective processing methods (17). Salting techniques can remove the bacterial contamination but unable to destroy histamine (scombroid) toxin of fish poisoning (41). If histamine levels in fish and its products are more than 50mg/100g, it is reported as the hazard action level of histamine (42). According to, U.S. Food and Drug Administration (U.S.FDA) histamine should be found at 50ppm of histamine to be enough to cause the scombroid poisoning signs, While, the Egyptian Organization for Standardization "EOS" (2005); permits histamine to be found at 20mg/100g (9, 43). The higher levels of fish histamine affected by certain factors such as natural microorganisms which depend on habitat, storage temperature after caught and improper handling on board the harvest vessel. Naturally, live fish contains different bacterial species on; external surfaces, gills, and in the intestinal content but after the death of fish, the defense mechanisms of fish can't inhibit the growth of bacteria which allowing the replications of microorganisms. Sabry et al.,(44) showed the occurrence of histamine producing bacteria associated with histamine production in muscles of retail sardine and mackerel in Egypt. The sardine samples showed a relatively high level of histamine than the legal limit, possibly

due to temperature abuse. The habitats in marine water differ and can be affected by the origin of fish contamination. The main approach after fish catching depend on the melting temperature in addition to the presence of some conditions which controlling the speed of reaching these temperatures such as fish size, fish initial temperature and the ice content of fish (45-47).

Conclusion

Results in this study indicated to that histamine and the bacteria forming histamine were recorded in all examined sardine samples. Temperature, salt concentration and water activity greatly influenced the histamine production in Sardine samples. The rate of histamine accumulation was rapid in salted fish. Increase of bacterial counts and histamine content slowed down with the decrease of storage temperature.it is referred that hygienic conditions and storage time/ temperature can significantly affect the frozen fish quality. bacteriological quality of anaerobic, *Enterobacteriacea spp. E. coli* and *Staphylococcus spp.* counts with no coliforms, was $8\times10^{3,}$ $5\times10^{4,}$ 5×10^{4} , 3×10^{4} log CFU/g, respectively. The histamine content in all samples was less than 10 mg/100 g USFDA guideline value. Molecular Identification of Sardines samples declared as following; 25/50 (50%), 10/50 (20%), 10/50 (20%), 5/50 (10%) for *Klebsiella pneumonia, Staphylococcus xylosus, Escherichia coli* and *Enterobacter cloacae* respectively. The isolated histamine-forming bacterial species which have been demonstrated in the examined sardine samples. There must be an attention to the fishing process which should be carried out under good hygiene to decrease the initial bacterial load.

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Original Research Article
Veterinary Medicine and The One Health Concept

HEAVY METALS CONTENT IN CHEESE: A STUDY OF THEIR DIETARY INTAKE AND HEALTH RISK ASSESSMENT

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Abstract: Cheese represents an important source of essential amino acids, vitamins, and trace elements such as copper (Cu), and zinc (Zn). Cheese can be contaminated with toxic heavy metals due to the use of contaminated ingredients, or during the manufacture process, and distribution. This study was taken to estimate the residual contents of some toxic metals including lead (Pb), cadmium (Cd), arsenic (As), and mercury (Hg), and trace elements such as zinc (Zn) and copper (Cu) in five of the most consumed cheese types in Egypt including Domiati, Feta, Karish, Mish, and Rumi. Moreover, calculation of the dietary intakes, and health risk assessment among children and adults were conducted. The obtained results revealed that Mish cheese had the highest residual content of Pb, and As. Karish cheese had the highest content of Cd, and Zn. Mercury was not detected in Feta and Domiati cheese. Calculation of the estimated daily intakes, hazard ratio, and hazard index revealed that consumption of cheese among Egyptian adults and children would not pose health risks. However, such data should be handled carefully as consumption of cheese will provide considerable concentrations of the total buildup of heavy metals to the Egyptian consumers. In addition, consumption of cheese will provide part of the human needs of the essential trace elements including Zn, and Cu.

Key words: heavy metals; trace elements; cheese; health risk assessment; dietary intakes

Introduction

Cheese is one of the dairy products that is consumed the most in the world. This is possibly due to its great nutritional content, distinct aroma and flavor, and availability of several varieties. The essential amino acids, vitamins, and trace minerals including calcium magnesium, copper, and zinc are all abundant in cheese (1, 2). The cheese Domiati, Feta, Karish, Mish, and Rumi, and are only a few of the varieties created from raw or pasteurized milk in Egypt. These kinds go through various processing steps with the potential for being contaminated with large varieties of biological contaminants and chemical contaminants such as heavy metals, mycotoxins, and biogenic amines (2).

Living animals can be exposed to a vast array of xenobiotics including heavy metals and other environmental pollutants. Such metals can accumulate in the different tissues of the animals and can cross the milk barrier (3). Milk also can be contaminated with heavy metals during the milking process. Metals can be leached from the milking tanks to the milk. Such contaminated

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milk might lead to contamination of the resultant dairy products from the processing of such milk (4). Dairy products such as cheese can be contaminated with heavy metals during the manufacture process (5), storage in tanks made from tin, and during marketing as in the case of Karish cheese which is sold open to air with street vendors (6).

Heavy metals such as lead (Pb), cadmium (Cd), arsenic (As), and mercury (Hg) are of no physiological functions for living organisms. Such metals are characterized by their bioaccumulation, and biomagnification nature, and find their way to the human body via ingestion of contaminated food and water leading to several toxicological implications. For instances, Pb is implicated in many cases of deaths among children as reported in Nigeria, and Zambia (7, 8). Moreover, Pb is also associated with the reduction in cognitive development and intellectual performance (9). Cadmium is the prime cause of itai-itai disease that is characterized by softening of bone and renal failure (10). Moreover, Cd is classified as an endocrine disruptor and associated with the occurrence of breast cancer (11). Another poisonous metal, Hg, was once widely employed in dentistry, antiseptics, and gold mining (12). However, chronic exposure to mercury was linked to serious neurological conditions, also known as Minamata illness. Arsenic is associated with dermal, nervous, respiratory mutagenic and carcinogenic effects (13).

Zinc serves as a crucial trace element for the catalytic activity of more than 100 enzymes in the body. It is also required for the regulation of the gene expression of many different cell components. Zinc is also necessary for maintaining the cell wall. In many underdeveloped countries, Zn deficiency is a serious problem that can result in anemia, and decreased immunity (14). Copper is an essential element that has a profound impact on the biochemistry and physiology of living creatures as a co-factor for many enzymes. Furthermore, Cu is essential for cellular respiration. However, prolonged contact with Cu may result in oxidative damage to the cell's organelles (15).

In sight of the previous facts, this study was taken to estimate the residual content of Pb, Cd, As, Hg, Zn, and Cu in five cheese types including Domiati, Feta, Karish, Mish, and Rumi retailed in Egypt. In addition, dietary intakes and health risks associated with the consumption of such cheese types were calculated among Egyptian children and adults.

Material and methods

The used reagents in the present study were of analytical grades and purchased from Merck, Darmstadt, Germany, unless specified.

Collection of samples

A hundred cheese samples including Domiati, Feta, Karish, Mish, and Rumi (20 of each) were collected from the grocery stores and street vendors in Zagazig city, Egypt. Samples (50 g of each) were packed separately and transferred in plastic tubes to the laboratory for heavy metals' extraction and measurement.

Sample preparation and extraction

One gram from each cheese sample was digested in a 10 mL liquid mixture composed of 3 parts of HNO3 and 2 parts of HClO4. The sample mixture was left overnight for digestion at room temperature. Then the resultant mixture was heated in a water bath at 70°C with continuous swirling for 3 h (16). The digested mixture was filtered, and the concentrations of the heavy metals (Pb, Cd, Zn, and Cu) were directly measured using the atomic absorption spectrophotometer (Shimadzu AAS 6800, Shimadzu, Japan) using hollow cathode lamps with an air-acetylene flame. While hydride generation/cold vapor atomic absorption spectroscopy was used to evaluate Hg, and As concentrations (Shelton, CT, USA). Based on standard curves created for each of the analyzed metals, the concentrations of the detected heavy metals were calculated. A wet weight (ww) basis was used to record the results, which were presented as μg/g.

Quality assurance

The accuracy of the analysis was established by measuring the samples twice and using the recognized reference material IAEA-142/TM (muscle homogenate), Vienna, Austria. The average recoveries for the metals under investigation ranged from 95 to 105%. The recovered concentrations of the certified samples were within 3% to 5% of the certified limits. All of the tools and supplies used in the

study were cleaned with diluted nitric acid to avoid external contamination with heavy metals.

Dietary intakes of heavy metals

The estimated daily intake (EDI) (µg/kg/day) values for the tested metals were calculated using the equation suggested by US Environmental Protection Agency, US EPA) (17):

$$EDI = C*F_{IR}/BW$$

Where C is the concentration of the tested metal in the sample (µg/g wet weight); F_{IR} is the food ingestion rate in Egypt, according to the global per capita consumption of cheese by nation in 2016, the average daily ingestion rate of cheese in Egypt was calculated to be 11.51 g/day based on an annual consumption of 4.2 kg of cheese (18). BW is the body weight of Egyptian consumers, which was set at 70 kg for adults, and 30 kg for children.

Health risk assessment

The non-cancer risks among the Egyptian consumers that associated with the consumption of cheese contaminated with metals were calculated using the guidelines recommended by the US EPA (17). The EDI was compared with the recommended reference doses (RfD) (0.001 mg/kg/day for Cd, 0.004 mg/kg/day for Pb, 0.0004 for As, and 0.3 mg/kg/day for Zn) (17), to generate the hazard ratio (HR) as following:

$$HR = EDI/RfD*10^{-3}$$

The hazard ratios can be summed to calculate a hazard index (HI) for estimation of the health risks associated with the mixed heavy metals.

$$HI = \sum HRi$$

where *i* represents each metal

A potential risk to human health is indicated if the HR and/or HI value is greater than one, whereas a result of one or less indicates no risk.

Statistical analysis

For statistical comparisons, the Tukey-Kramer HSD difference test (JMP) (SAS Institute, Cary, NC, USA) was applied ($p < 0.05$).

Results

The obtained results in Table 1, and Fig. 1A showed that 70%, 40%, 90%, 100%, and 80% of Domiati, Feta, Karish, Mish, and Rumi cheese samples were contaminated with Pb. Mish cheese had significantly ($p < 0.05$) the highest Pb residues, followed by Karish, Rumi, Domiati, and Feta with average Pb residual contents (µg/g ww) of 0.25±0.008, 0.14±0.009, 0.13±0.02, 0.07±0.01, and 0.02±0.004, respectively. It is evident from the obtained results 90%, 40%, 80%, 75%, and 65% of Domiati, Feta, Karish, Mish, and Rumi cheese samples were contaminated with Cd. Feta cheese had significantly ($p< 0.05$) the lowest Cd residues, however, no significant difference was observed in Cd residues among the other cheese kinds. Cd residual concentrations (µg/g ww) in the examined cheese kinds were 0.06±0.01, 0.08±0.01, 0.05±0.01, 0.07±0.007, 0.01±0.006 in the examined Mish, Karish, Rumi, Domiati, and Feta cheese samples, respectively (Table 1, Fig. 1B).

Arsenic was also detected at 100%, 30%, 100%, 100%, and 100% of Domiati, Feta, Karish, Mish, and Rumi cheese samples. The recorded As contents (µg/g ww) in the examined cheese kinds were 0.34±0.02, 0.13±0.003, 0.11±0.005, 0.16±0.06, and 0.01±0.003 in the examined Mish, Karish, Rumi, Domiati, and Feta cheese samples, respectively (Table 1, Fig. 2A). Mercury was not detectable in Feta and Rumi cheese but detected at 60%, 75%, and 65% of Domiati, Karish, and Mish cheese samples. The recorded concentrations (µg/g ww) of Hg were 0.02±0.001, 0.03±0.004, and 0.002±0.001 in the examined Mish, Karish, and Domiati cheese samples, respectively (Table 1, Fig. 2B).

In the case of trace elements, both Zn and Cu were detectable in all examined cheese types (Table 1). The average concentrations of Zn were 1.55±0.07, 3.35±0.07, 2.15±0.04, 2.18±0.08, and 2.55±0.08 in the examined Mish, Karish, Rumi, Domiati, and Feta cheese samples, respectively (Fig. 3A). While the average Cu residual contents were 0.05±0.008, 0.06±0.006, 0.09±0.003, 0.06±0.004, and 0.04±0.004 in the examined Mish, Karish, Rumi, Domiati, and Feta cheese samples, respectively (Fig. 3B). However, the recorded concentrations of Zn and Cu did not exceed the established maximum permissible limits. The recorded results in Table 2 showed that consumption of cheese among the Egyptian population could contribute to the daily intake of the measured heavy metal and subsequently to their buildup in the human body. However, calculation of the hazard ratio and hazard index for the tested metals recorded values far below 1 in both Egyptian adults and children (Table 2).

Table 1: Percentage of cheese samples contaminated with the tested heavy metals and that exceeding maximum per-missible limits (MPL)

	Domiati		Feta		Karish		Mish		Rumi	
	Positive	**Exceeding MPL**	**Positive**	**Exceeding MPL**	**Positive**	**Exceeding MPL**	**Positive**	**Exceeding MPL**	**Positive**	**Exceeding MPL**
Pb	70	70	40	35	90	90	100	100	80	80
Cd	90	85	40	5	80	80	75	55	65	50
As	100	20	30	0	100	15	100	95	100	10
Hg	60	0	0	0	75	45	65	30	0	0
Zn	100	0	100	0	100	0	100	0	100	0
Cu	100	0	100	0	100	0	100	0	100	0

MPL of Pb is 0.02, Cd is 0.05, As is 0.1, Hg is 0.01, Zn is 50, and Cu is 5 µg/g according to EU (2006)

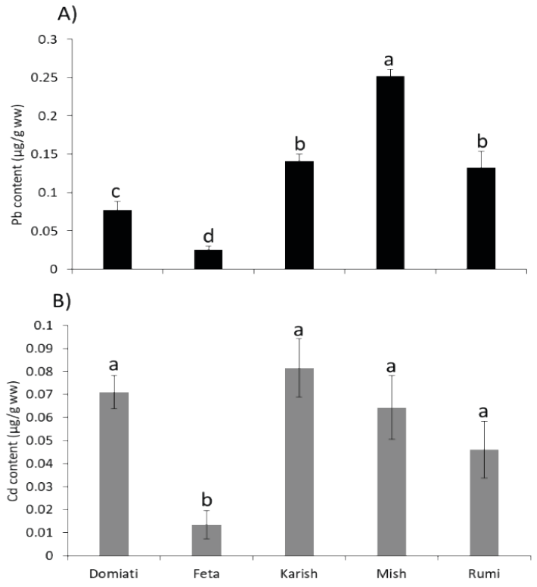


Figure 1: Toxic metals content A) lead (Pb), and B) cadmium (Cd) in the examined cheese samples. Values repre-sent mean ± SD (µg/g ww). Columns with different letter are significantly different at $p < 0.05$

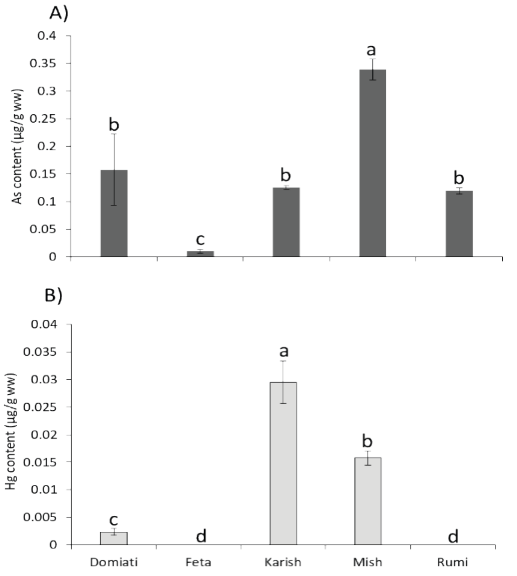


Figure 2: Toxic metals content A) arsenic (As), and B) mercury (Hg) in the examined cheese samples. Values repre-sent mean ± SD (µg/g ww). Columns with different letter are significantly different at $p < 0.05$

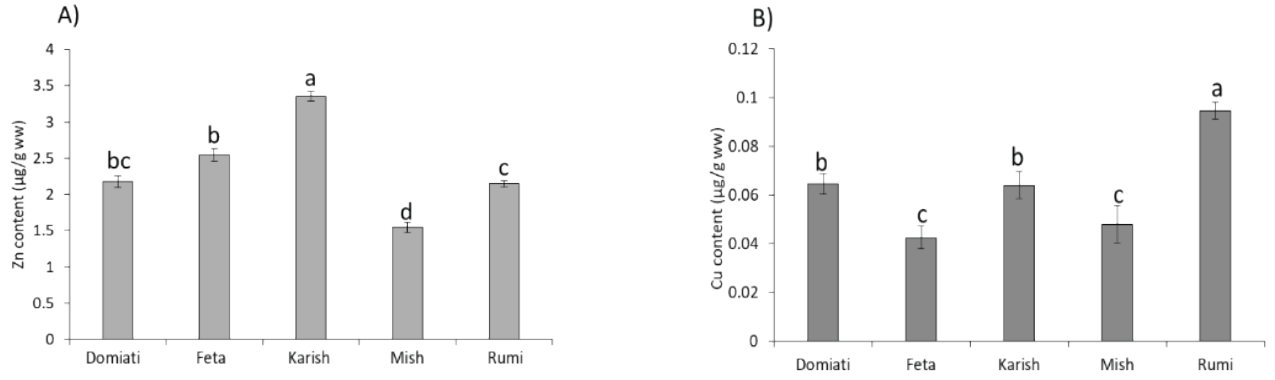


Figure 3: Trace elements content A) zinc (Zn), and B) copper (Cu) in the examined cheese samples. Values repre-sent mean ± SD (µg/g ww). Columns with differ-ent letter are significantly different at $p < 0.05$

Table 2: Estimated daily intake and health risk assessment of consumption of different cheese kinds

	EDI		HR	
	Adult	**Children**	**Adult**	**Children**
Domiati cheese				
Pb	0.013	0.029	0.003	0.007
Cd	0.012	0.027	0.017	0.027
As	0.026	0.061	0.086	0.201
Hg	0.001	0.001	0	0
Zn	0.361	0.835	0.001	0.003
Cu	0.011	0.025	0	0
HI			0.102	0.239
Feta cheese				
Pb	0.003	0.008	0.001	0.002
Cd	0.002	0.005	0.002	0.005
As	0.001	0.004	0.005	0.013
Hg	0	0	0	0
Zn	0.418	0.977	0.001	0.003
Cu	0.007	0.016	0	0
HI			0.010	0.023
Karish cheese				
Pb	0.023	0.054	0.006	0.013
Cd	0.013	0.031	0.013	0.031
As	0.021	0.048	0.069	0.161
Hg	0.005	0.011	0	0
Zn	0.551	1.286	0.002	0.004
Cu	0.011	0.024	0	0
HI			0.090	0.210
Mish cheese				
Pb	0.041	0.096	0.010	0.024
Cd	0.011	0.025	0.010	0.025
As	0.056	0.130	0.186	0.434
Hg	0.003	0.006	0	0
Zn	0.251	0.593	0.001	0.002
Cu	0.008	0.018	0	0
HI			0.208	0.484
Rumi cheese				
Pb	0.022	0.051	0.005	0.013
Cd	0.008	0.017	0.007	0.017
As	0.019	0.045	0.065	0.153
Hg	0	0	0	0
Zn	0.353	0.823	0.001	0.008
Cu	0.016	0.036	0	0
HI			0.079	0.186

Discussion

Heavy metals are characterized by their bioaccumulation and biomagnification nature. In addition, heavy metals are hardly affected by heat treatment. The obtained results of the present study revealed contamination of different cheese types retailed in Egypt with the toxic metals such as Pb, Cd, As, and Hg. Mish cheese had significantly the highest Pb residues followed by Karish, and Rumi cheese, while Feta cheese had the lowest Pb residues. Feta cheese also had the lowest Cd residues among the examined cheese types. Lead residues exceeded the established MPL (0.02 µg/g) in 100%, 90%, 80%, 70%, and 35% of Mish, Karish, Rumi, Domiati, and Feta cheese, respectively. Cadmium concentrations recorded in the current investigation exceeded the established MPL of Cd (0.05 µg/g) (19) in 85%, 80%, 55%, 50%, and 5% of Domiati, Karish, Mish, Rumi, and Feta cheese, respectively. Similarly, Pb and Cd were detected at higher levels that exceeded MPL in 48% & 44%, 64% & 68%, and 72% & 64% of soft, Ras, and processed cheese retailed in Ismailia governorate, Egypt (20). However, Pb and Cd were detected at lower levels that were within MPL in the soft cheese Sold in Ogbomoso, Southwestern Nigeria (21). Lead and Cd were detected at higher levels in cheese samples retailed in Arak, Iran (22), and in Karish cheese retailed in Egypt (6). Comparable levels of Pb were recorded in ranchero cheese in Mexico (23). Lower Cd residues (0.0001-0.007 ppb) were detected in milk, butter milk, cream, cheese, and yoghurt samples from different regions in Poland (24).

Arsenic was also detected in all examined cheese types in the present study, where 95%, 20%, 15%, 10%, and 0% of Mish, Domiati, Karish, Rumi, and Feta cheese exceeded the established MPL. While Hg was found at higher levels in Karish (45%), and Mish (30%), respectively. For both As, and Hg, Feta cheese had the lowest residual contents. Mercury was within the acceptable levels in the soft, Ras, and processed cheese retailed in Ismailia governorate, Egypt (20). While both As, and Hg exceeded the EU established MPL (19) in cheese samples retailed in Arak, Iran (22).

Detection of such toxicants in the different cheese kinds indicate contamination of the used milk in the processing of cheese with such toxic metals. Animals are exposed during their lifetime to a vast array ofxenobiotics including heavy metals which by turn can cross the milk barrier and find their way to the milk. Animals can be exposed to heavy metals via inhalation or ingestion of contaminated feed and water (4). Besides, As is commonly used in livestock farmsas a feed additive to increase the feed conversion ratio. This could explain the detected concentrations of As in the present study (25). Raw milk was found to be contaminated with heavy metals such as Pb, Cd, As, and Hg in several reports worldwide (6, 22, 24).

Zinc and Cu are trace elements that are routinely added for livestock and in dairy farms to avoid nutritional disorders. In the present study, Zn and Cu were detected in all examined samples but with no samples exceeded MPL of Zn and Cu. Relatively comparable Zn and Cu levels were recorded in dairy products including milk, and Karish cheese retailed in Beni-Suef, and Zagazig cities, Egypt (6, 26). Besides, Zn and Cu were recorded at higher levels in the marketed dairy products in Poland (24). Copper exceeded the MPL in 80%, 100% and 60% of soft, Ras, and processed cheese samples, respectively that sold in Ismailia, Egypt (20).

The EDI (µg/kg/day) values for Pb among the Egyptian consumers ranged between 0.003 (Feta cheese) and 0.09 (Mish cheese); for Cd ranged between 0.002 (Feta cheese) and 0.03 (Karish cheese); for As ranged between 0.001 (Feta cheese) and 0.130 (Mish cheese); for Hg ranged between 0.001 (Domiati cheese) and 0.011 (Karish cheese); for Zn ranged between 0.251 (Mish cheese) and 1.286 (Karish cheese); and for Cu ranged between 0.007 (Feta cheese) and 0.036 (Rumi cheese).

For both Egyptian adults and children, the computed non-carcinogenic hazard ratios and hazard indices were much below one, indicating that there were no potential hazards related to the consumption of the tested cheese varieties. The examined heavy metals' EDIs were comparable to those previously reported for consumption by Egyptian populations (6). Compared to Ghana (27), and Egypt (28), the reported HR and HI values were lower. The examined metals have been linked to several adverse health impacts, including cancer risk, neurological abnormalities, and toxic effects (4). As a result, even very low quantities of the tested hazardous metals will build up over time and produce a variety of toxicological effects.

It also notes worthy to mention that different cheese types tested in the present study were proven to afford at least part of the human needs of the essential trace elements such as Zn, and Cu.

Conclusion

The obtained results of the present study showed that different cheese kinds consumed by Egyptian population contained considerable concentrations of toxic and trace elements. Calculation of the HR and HI values for different cheese kinds demonstrated no potential non carcinogenic risks associated with the consumption of such cheese types. However, such results should be handled carefully as cheese also supports the total buildup of heavy metals with other food sources for the Egyptian population.

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This study was done according to the guidelines of Zagazig University, Egypt. This study did not use any experimental animals nor human subjects.

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Original Research Article
Veterinary Medicine and The One Health Concept

EPIDEMIOLOGICAL STUDY OF HUMAN BRUCELLOSIS IN THE KINGDOM OF SAUDI ARABIA; PRE- AND DURING COVID-19 PANDEMIC

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Abstract: Brucellosis is one of the most reported zoonotic diseases that affects human health with subsequent economic losses. Brucellosis is an endemic disease in countries in many regions such as the Kingdom of Saudi Arabia (KSA) in the Middle East. On 30 Jan 2020, the Director-General of the World Health Organisation (WHO) declared the novel coronavirus outbreak (later named COVID-19) a public health emergency of international concern. In KSA, on 2 March 2020, the first case of COVID-19 was confirmed. To contain the disease outbreak, partial and full suspension of public activities/curfew started in March and April. In this study, characterization of brucellosis in KSA from 2013-2019 (pre-pandemic), the year 2020 (pandemic control interventions'" year), and the year 2021is presented. Incidence data were obtained from the Ministry of Health's (MoH) records for laboratory-confirmed cases. Extracted incidence data were analysed according to the nationality of the patients (Saudi and non-Saudi), gender (male and female), age, health regions (20 throughout KSA), and months. From 2013-2019, there were 28073 (mean; 4010) cases of human brucellosis reported. In 2020 and 2021, there were 2372 and 2400 cases of brucellosis reported, respectively. The decrease in 2021, which continued after easing PCI restrictions, is worth noting. This represents a decrease of 59.15% of reported cases compared to incidence means for the period 2013-2019. Compared to other studies, misdiagnosis, delayed diagnosis, patients not seeking medical examination, and dedicating much of the health sector for COVID-19 patients are likely reasons for brucellosis reporting reduction. However, and due to lack of additional necessary data, it is not possible currently to conclude that PCI have (or have not) influenced reducing brucellosis in KSA. Additionally, and in comparison, to reported regional and global incidence rates of the disease, reported rates from 2013-2019 in KSA are likely to be underreported.

Key words: brucellosis; incidence; epidemiology; Saudi Arabia; COVID-19 pandemic

Introduction

Worldwide, in human, it is estimated that *Brucella* spp. cause 832000 cases (range: 338000-19500000) of brucellosis every year (1). Brucellosis is one of the most reported zoonotic diseases in Northern Africa, the Middle East, Central, and Southern America, Southern Mediterranean countries, and Central and South Asia. Incidences of more than 100 cases/100000 persons-year have been reported in many Middle Eastern countries (2, 3). Globally, brucellosis infections in livestock and human are caused by *Brucella abortus* (infecting cattle), *Brucella melitensis* (infecting small ruminants), and *Brucella suis* (infecting swine) (4).

In livestock, brucellosis is a highly contagious disease resulting in the transmission of infection to other species. The infection can occur through contact with aborted foetuses and its liquid and membranes (5). Brucellosis infection in human can be contracted via the consumption of raw milk and its products, aerosols inhalation, and direct contact with infected animals/discharges. Consumption of undercooked meat, breastfeeding,

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and sexual contact are less common routes of infection (6).

Acute brucellosis is characterized by undulant fever, sweating, fatigue, weakness, headache, rash, and myalgia with recovery in less than 60 days, and may precede to a milder sub-acute phase lasting 2 months to a year. Brucellosis can be in chronic form with symptoms that may include recurrent fever, fatigue, arthritis, spondylitis, and endocarditis (6, 7).

In brucellosis endemic regions, the disease has direct and indirect effects on animals farming and humans. Visible direct effects on animals include reduced milk production, reduce weight, veterinary care, and losses due to culling (8). Invisible effects include reduced fertility and transmission of disease to offspring and other animals. Indirect effects are related to trade losses, vaccination campaigns, and control programs. (9).

On human, and in addition to physical pain and emotional suffering, direct effects include health care costs and non-health care costs (e.g., accommodation and transportation). Indirect effects are on individuals (e.g., loss of productivity due to premature death, fertility complications, loss of workdays), and on population (decrease in workforce ability and decrease in food security) (9).

The novel coronavirus, which originated in Wuhan, China was declared a Public Health Emergency of International Concern by WHO on January 30th, 2020. The virus, which was named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has infected almost 48 million people by the 1st week of November 2020. The pandemic caused by the virus (named COVID-19 pandemic) resulted in about 634 million confirmed cases and 6.594 million deaths by mid-November 2022. (10). Symptoms in patients with COVID-19 include influenza-like symptoms, headache, dry mouth, fatigue, and sore throat. Although that asymptomatic state has been reported for 20 % of individual infected with virus, up to 18% can develop severe disease needing intensive medical care (11).

For countries to contain the spread of the pandemic, WHO has recommended strategic response and control pillars. Detailed description of the application of the WHO's recommendations in KSA has been reviewed elsewhere (12). Measures taken to control and prevent additional COVID-19 infections in KSA included suspension of social gatherings (in prayer, in schools/colleges, malls/restaurants, and wedding/parties) and travel within, to and from the Kingdom. It also included mandatory mask waring and vaccination. Other social activities that bring people and animals (camel) to place of activity (festival, races) were also suspended.

The Kingdom of Saudi Arabia (KSA) is the largest country in the Middle East with 2 million square kilometres. As of 2017, the population of KSA is estimated at 32.55 million of which 37.3% are non-Saudis (13). In 2016, KSA imported food products, animals, and vegetables valued at $ 19.48 billion representing 15% of total imports. Food animals and poultry are estimated to be 13849779 and 53139159, respectively. In 2017, 7173776 local and 4888494 imported food animals were slaughtered for meat consumption (13). The demands for red meat and fishery products due to population growth are increasing yearly.

Human brucellosis is a notifiable disease in KSA. This descriptive epidemiological study was carried out to analyse human brucellosis trends in the country from 2013-2019 (pre-COVID-19 pandemic) and during the pandemic.

Materials and methods

Incidence data source

Brucellosis-related incidence data were obtained from the Ministry of Health's records (MoH) for the period 2013 to 2020 (14).

Incidence data according to nationality (Saudi and non-Saudi), gender (male, female), age (< 1, 1-4, 5-14, 15-44, and ≥ 45 years old), months, and health provinces were extracted, grouped, and analysed. Data analysis was based on five-year population averages of 21.12, 11.06, 18.36, and 13.83 million, Saudi, non-Saudi, male, and female, respectively.

Case definition

Only laboratory-confirmed cases and reported as "brucellosis" were considered in this study.

Health provinces information

The population data for health provinces in KSA were obtained records of the General Authority

for Statistics (13**)**. Riyadh, Jeddah, the Eastern, Makkah, and Medinah are the largest provinces in KSA with 63% of the Kingdom's total population. Makkah, Medinah, and Jeddah are in western KSA where Riyadh is in the central.

Literature search

The databases (PubMed, ScienceDirect, SpringerLink, and Google Scholar) were searched in September 2022, for brucellosis- related data for the period 1997 to 2022.

The search was performed to obtain articles that contain the terms Saudi Arabia and *Brucella* or brucellosis; prevalence, incidence, in articles' title and/or abstract.

Additional search was performed for "brucellosis", "human brucellosis" and "coronavirus", "COVID-19" for the period from April 30, 2020, to September. 30, 2022.

Statistical Analysis

Statistical analysis was perform using SPSS software version 20. In addition to analysing data to calculate means, percentages, and incidence rate, the two-tailed t-test was performed to test if there is statistical significance (at p-value: <0.05) differences in incidences according to nationality, gender, and age.

Results

In KSA, during 2013-2019, there were total reported cases of 28073 of human brucellosis. This represents average incidence rates (I.R) over 7 years period of 12.83 per 100000 people (Table 1) of the disease in the general population. The I.R for 2020 and 2021, were 7.59 and 7.68 per 100000 people, respectively. From 2013-2019, there were fluctuations in brucellosis incidences recording the highest numbers in 2018. A sharp drop of reported incidences was observed by 59% in 2020 and 2021.

Nationality and Gender

According to the nationality of patients, I.R for brucellosis was 11.97 for Saudi patients and 11.78 for non-Saudi patients (p-value: 0.0159) (Table 1).

Among male and female population, I.R for brucellosis was 16.78 and 6.71, respectively (p-value; 0.00001). Generally, brucellosis affected Saudi males (mean; 1663) and non-Saudi males (mean; 1419) more than Saudi females (mean; 857) and non-Saudi females (mean;70) (p-value; 0.00001) (Table 1).

Health Provinces

Total cases, means, I.R for brucellosis in KSA 2013-2019, and reported cases in 2020 and 2021 by health provinces are shown in table 2. For brucellosis, the highest I.R were 91.1, 62.19, and 58.38 in Northern border, Najran, and Bishah, respectively. It is worth noting that Riyadh, Jeddah, the Eastern, Makkah, and Medinah are the largest provinces representing 63% of KSA's population.

Table 1: Total human brucellosis cases, mean, and incidence rate according to nationality, gender, and age group in KSA

	2013	2014	2015	2016	2017	2018	2019	Mean	I.R	*P*-value	**2020**	2021
MALE	2318	2372	2583	3217	3598	4218	3271	**3082**	16.78	<.00001	**1847**	1795
FEMALE	946	738	650	845	1094	1237	986	**928**	6.71		**525**	605
Saudi	2186	1983	1783	2314	2802	3759	2870	**2528**	11.97	<.00159	**1432**	1811
Non-Saudi	1078	1177	1450	1748	1890	1696	1387	**1489**	11.78		**940**	589
Saudi male	1338	1243	1184	1539	1780	2615	1946	**1663**		<.000922	**945**	1234
Saudi female	848	690	599	775	1022	1144	924	**857**			**487**	577
Non-Saudi male	980	1129	1399	1678	1818	1603	1325	**1419**		<.00001	**902**	561
Non-Saudi female	98	48	51	70	72	93	62	**70**			**38**	28
<15	504	387	366	428	541	785	750	**537**	5.97	<.00001	**296**	377
≥15	2760	2723	2867	3634	4151	4670	3507	**3473**	15.6		**2076**	2023

Table 2: Reported human brucellosis cases by health regions, means of 2013-2019, and incidence rates (I.R) in KSA

Health region	Reported	Mean	2020	2021	I.R
Riyadh	2552	364	366	504	4.63
Makkah	1090	155	134	308	7.18
Jeddah	1063	151	128	158	3.48
Taif	2499	357	301	318	28.13
Medinah	1525	218	114	202	10.69
Qassim	4081	583	234	186	42.06
Eastern	1943	277	233	154	8.99
Ahsa	342	49	23	20	4.07
HafrBaten	1193	170	72	39	38.82
Aseer	1898	271	64	61	15.32
Bishah	1504	214	141	100	**58.38**
Tabouk	636	91	29	42	10.18
Hail	1411	201	219	120	29.68
Northern border	2296	328	52	32	**91.1**
Jazan	175	25	7	9	1.66
Najran	2486	355	188	87	**62.19**
Baha	819	117	20	10	25.16
Jouf	308	44	34	37	13.25
Qurayat	187	27	2	0	16.28
Qunfatha	66	9	11	12	3.09

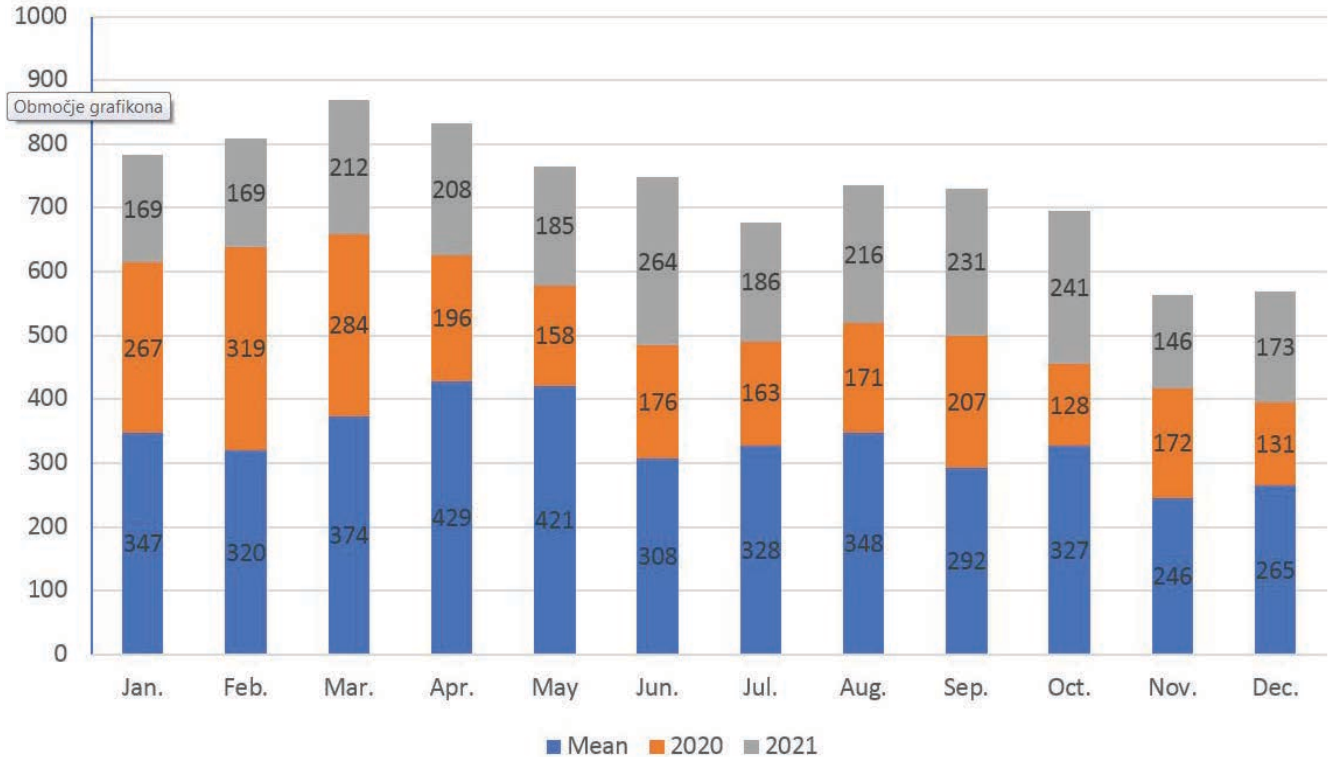


Figure 1: Means of monthly human brucellosis from 2013-2019 and reported cases in 2020 and 2021 in KSA

Age

Total reported cases according to age in 2013-2021, means and I.R (for 2013-2019) are shown in table 1. For children < 15 and population ≥ 15, I.R for brucellosis were 5.97 and 15.6 (p-value: 0.00001), respectively.

Seasonality

Means of monthly brucellosis incidence for 2013-2021 in KSA are shown in figure 1. The highest incidences for brucellosis were reported in March, April, and May with fluctuation in other month's incidences. The hottest months in 2013-2019 in KSA with average high temperatures over 40 c were June, July, and August. The average temperatures recorded were 24.7, 36.9, 40.9, 29 C for Jan-Mar., Apr.-Jun., Jul.-Sept., and Oct.-Dec. yearly quarters, respectively (13).

Literature search

A large brucellosis seroprevalence study covering different regions of KSA reported prevalence of 20%, 18.3%, 14.6%, 14%, and 11.6% in the northern, southern, central (15), eastern and western regions, respectively (16). Other studies showed a high prevalence in the northern (17, 18) and southern regions (19, 20, 21, 22). Active brucellosis cases ranged from 2.3-9.8% in seropositive patients (15, 21, 23). The estimated national brucellosis seroprevalence in KSA is 15% (15).

For effect of COVID-19 on diagnosis and reporting of human brucellosis incidence, few studies have been reported (24, 25). However, none of these studies had the necessary data to statistically conclude that COVID-19 control measures reduced the incidence of brucellosis.

Discussion

Sporadic cases were reported in KSA from 1956 to 1970 (26). With economic growth in the late 1970s, high numbers of cattle and sheep farms were established accompanied with the increase of animals import. The veterinary care at that time was not advanced to carry out required testing of imported animals and on the farms. This led to an increase in brucellosis in KSA (27). Serological testing for revealed that high numbers of the imported animal during Hajj season were infected (28). In 1980s, an increasing pattern of patients diagnosed with brucellosis was reported in main hospitals throughout KSA (29). This pattern was concurrent with an increase in brucellosis in farm animals. Unregulated animal imports, the spread of animal farms, raw milk consumption were the main factors contributing to this increase (30, 31). In 1999, a report about brucellosis in the Middle East ranked KSA as the highest country in the region affected by human brucellosis (32).

Regions most affected by brucellosis include the Middle East, the Mediterranean, Central Asia, Latin America and sub-Saharan African nations (2, 33). In Saudi Arabia, brucellosis is considered an endemic illness, whereas consumption of unpasteurized milk products is considered a risk factor (34).

In this study, total reported brucellosis cases were 28073 for the period 2013-2019 (Saudi; 58.9%, non-Saudi; 41.1%) and I.R was 12.83. The male (69.9 %): female (30.1%) ratio was 2.32:1. Most of the animals' farms' labour, veterinarians, and abattoirs workers in KSA are males. Males were generally affected by the disease more than females. This might be explained by the nature of brucellosis as an occupational disease and that males represent most workers dealing with animals (33). However, 30.1%% of the cases were among females. In KSA, in rural areas, female also participate in animal husbandry practices and may be exposed to contact with infected animals. Drinking of raw milk is another potential risk factor in such areas (33).

Among age groups, 15.46% of brucellosis cases were reported for < 15 years old population, and 84.54% for ≥ 15 years old. Infections in children might be as a sequence of contact with infected animals and consumption of raw milk in rural areas of KSA (33). This observation is similar to the reported prevalence in age groups in other studies where the disease affect > 15 years old population. (35, 36, 37).

Geographically, and within KSA, brucellosis I.R were higher in three health regions in the northern, central, and southern regions characterized by the presence of high animals farming projects and tribal population involvement in animals' husbandry. Demographic, occupational, and socioeconomic factors contribute to brucellosis incidence variations even within the same country (32). In KSA, disease's

full epidemiological and populations at risk may not receive the required characterization especially in small rural areas (33).

Consistent with other studies (17,18, 36), there were seasonal fluctuations in brucellosis incidence in KSA with an increase from February to May. During these months, there is an increasing pattern in birth in sheep and goat herds in the country.

Through a literature search, brucellosis seroprevalence in KSA is 15% (15). Brucellosis reported prevalence in neighbouring countries were 8% in Jordan (37), 6.7% in Yemen (39), 12% (40) and 24.8% in Kuwait (41).

Although brucellosis causes economic losses in livestock and adverse and prolonged health effects on human, the disease in patients is not easily recognised. The disease in human is characterised by non-typical influenza-like symptoms, fever, sweating, and fatigue (6, 7). Interestingly, such non-typical symptoms are observed in patients with COVID-19. Due to such symptoms similarity and pressure of time and resources dedication to COVID-19 patients, cases of brucellosis being initially diagnosed as COVID-19 have been reported (23, 24). Therefore, the decrease in disease reporting, during the pandemic might be attributed to misdiagnosis situations.

Brucellosis in human can develop a chronic form accompanied by recurrent fevers, arthritis, neuropathies, and myocarditis (23, 24). Delayed diagnosis and subsequent timely treatment may result in the proceeding into chronic brucellosis.

Brucellosis diagnosis was delayed by up to 52 days (42). Patients' education, income, insufficient diagnostic capability, and age of patients are factors leading to delay of diagnosis (43). These factors are thought be more complicated during COVID-19 pandemic. This is due toa added pressure on health systems and patients not seeking medical assistance because of contracting the viral infection. Therefore, reported incidence of brucellosis may not accounted for underreporting due to delayed and/or misdiagnosed.

In brucellosis endemic developing countries, national passive surveillance data, which rely on laboratory confirmation and clinical definition, may underestimate the disease true health burden. Underestimations by up to 18 folds have been reported (2, 44). The situation of underestimation extends to developed countries where disease cases are misdiagnosed, mismanaged, or not included in the official system database (33).

To estimate a disease incidence in a country or a region, numerical values (multiplier) are synthesized to construct a surveillance pyramid. The values are assigned for asymptomatic infection, not patients not seeking medical care, underdiagnosis, and underreporting. The accuracy of obtained values depends in part on the availability of laboratory infrastructures, trained personnel, and on data supplied by authorities investigating the disease in question. To estimate a disease 's incidence, and where a country-specific's multiplier is not available, it is possible to apply multiplier of neighbouring country or of the region to calculate a more accurate disease incidence (42,43).

A systematic review of disease frequency study (33) reported wide variations in brucellosis incidence in the Eastern Mediterranean Region (EMR) region including KSA with a reported incidence of 100 and 134/100,000 (3, 32). Another data synthesis study (1) estimating the burden of brucellosis among other 21 diseases at the regional and global level reported brucellosis median rate of 33/100,000 people (range: 10-187) and 6 (range: 2-132) as estimations for the EMR and globally, respectively. To estimate brucellosis incidence in EMR including KSA and some neighbouring countries, and to account for underreporting, a mean of 5.4 (range: 1.6-15.4) was used.

The disability-adjusted life years (DALY) metric measures the burden of disease (BoD) in a population. It accounts for not only premature mortality but also disability caused by a specific disease or injury. DALY, which is a time-based indicator, measures years of life lost because of premature mortality combined with years of life lost because of time lived suffering from the disease (45). Attempting to measure DALY for brucellosis in KSA based on the reported incidence rates will result in measurement (3.69 DALY/100000 people) which is well below regional (median 23 DALY/100000 people) values for the disease (1).

Conclusions

During 2020, and toward 2022, reported incidences of human brucellosis in KSA were 59% compared to means of 2013-2019. To statistically describe the reduction, there is a need for more incidence data for at least the coming year of 2022 and 2023. In KSA, brucellosis prevention measures

as in countries where the disease is endemic shall focus on disease eradication and prevention in livestock and human. Diagnosing, treatment/culling, and vaccinating animals help in reducing the spread of disease. Local authorities in KSA will need to collaborate with academic institutes to plan and co-supervise prevention campaigns and carry out research to accurately estimate the disease' burden throughout the country.

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Original Research Article
Veterinary Medicine and The One Health Concept

PREVALENCE, PHENOTYPIC-GENOTYPIC RESISTANCE AND BIOFILM FORMATION OF *Staphylococcus aureus* IN CHICKEN MEAT WITH REFERENCE TO ITS PUBLIC HEALTH HAZARD

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Abstract: The purpose of this study was to determine the prevalence, antimicrobial resistance in *S. aureus* and presence of enterotoxigenic as well as biofilm-forming genes in *S. aureus* bacteria isolated from broiler meat from traditional shops and supermarkets in Sharkia, Egypt. For this determination, two hundred fresh raw chicken meat cotton swabs were collected from the breast and thighs (100 each). *S. aureus* was highlighted through a coagulase test, and then phenotypic and genotypic characterizations were studied. Uniplex PCR was used to identify the occurrence of enterotoxin genes in the selected isolates. Finally, they were subjected to a biofilm formation test using 96-well flat bottom polystyrene microtiter plates; besides, biofilm-forming genes were investigated. Nineteen isolates out of the 200 samples tested positive for *S. aureus* (9.5 percent), No Vancomycin resistance strains were obtained, nor did any ciprofloxacin isolates for *S. aureus* while, all isolates were 100 % resistant for streptomycin, amoxicillin- clavulanic acid and sulfamethoxazole-trimethoprim *S. aureus* isolates were found resistant to at least one antibiotic. The multiple antibiotic resistance (MAR) index of *S. aureus* isolates was ranged from 0.3 to 0.7 with an average of 0.4. In The mecicillin resistant *S.aureus* (MRSA) strains carried *mec*A gene with a percentage of 5%, while the *bla*Z gene was distributed with a percentage of 5.5%, 11/200). The obtained isolates gave 60% strong biofilm formation and 40% were non-formers, with nil results for moderate or weak production isolates. The *ica*A gene was 100%, in comparison to *ica*D which was zero. It should be noted that strong biofilm former strains were only 100% positive for *sea* and *seb*. This study pointed out the higher prevalence of MDR isolates of *S. aureus* isolates in chicken meat due to inadequate handling and insufficient sanitary equipment and post microbial contamination. This finding highlighted the importance of broiler meat as a reservoir for antimicrobial-resistant strains of *S. aureus* and biofilm-forming of *Staphylococcus aureus*.

Key words: prevalence; antimicrobial resistance; *Staphylococcus aureus*; biofilm producer

Introduction

Owing to its low cost, lack of religious obstructions and high quantity of required amino acids, poultry meat production and consumption have grown rapidly all over the world. Moreover, given the obvious scarcity of red meat, broiler meat is seen as a valuable source of protein (1). The annual chicken meat production in Egypt is estimated around 1.5 million tons in 2020 and represented a slight increase to reach 1.55 million tons in 2022. To inspect microbiological security, cleanliness conditions throughout processing, besides product storage conditions, *S. aureus* had been tested in meat as well as fowl products (2). Bacterial foodborne illness accounted for almost 68 percent of all incidences in the last ten years, with *S. aureus* being the third most common pathogen after *Salmonella* (3). Staphylococci have been categorized into 50 dissimilar species and subspecies based on their competency to make coagulase thus far. Coagulase-positive staphylococci (CPS) are

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those that produce coagulase, while coagulase-negative staphylococci (CNS) are those that do not. Only CPS strains, on the other hand, have been linked to food poisoning (4). Food poisoning caused by *S. aureus* is an intoxication triggered by the intake of foodstuffs containing adequate preformed enterotoxins (one or more). Nausea, intense vomiting, stomach cramps, with or without diarrhoea, are all symptoms of it, which appear quickly (2–8 hours). The sickness is normally self-limiting and disappears within 24–48 hours of commencement. It is frequently serious enough to necessitate hospitalization, especially when newborns, the aged, or disabled are involved (5).

Staphylococcus aureus bacterium's foodborne disorders are caused by the presence of multiple enterotoxins, that are produced by staphylococci and streptococci that are functionally related and have similar sequences (6). These bacterial proteins are known to be pyrogenic; in addition, they are interrelated to serious human illnesses like food poisoning and toxic shock syndrome. Although further species have been shown to be enterotoxigenic , such as: *S. hyicus*, *S. intermedius*, *S. epidermidis* and *S. xylosus* (7,8). The majority of these toxins are produced by *S. aureus* (7). Staphylococcal enterotoxins (SEs) are single-chain, low-molecular-weight proteins that have high heat tolerance. They are characterized into twenty-three types. The most critical types with severe clinical implications are SEA, SEB, SEC, SED, and SEE(6)

Antimicrobial resistance (AMR) is a significant hazard to global health security and has emerged as a significant concern in clinical practice and healthcare. Higher costs and reduced efficacy of therapy for common diseases are seen in healthcare settings as a result of antibiotic resistance and a lack of synthesis of newer antibiotic drugs (9). Through carrying the *mec*A gene, which encodes the penicillin binding protein 2a (PBP2a), Methicillin Resistant *S. aureus* (MRSA) obtains resistance to penicillin and other β-lactam drugs (10). In clinical testing, most MRSA strains were found to be multidrug resistant (MDR) (11). The occurrence of *S. aureus* and MRSA in retail meat has gotten a lot of attention recently (10). Between May 2002 and August 2003, researchers looked for MRSA in 444 raw chicken flesh samples (165 thighs and breasts, as well as other organs) sold in 145 stores across 47 prefectures in Japan. *S. aureus* was found in 292 (65.8%) of the 444 samples and 131 of the 145 markets (12).

Biofilms, as opposed to planktonic cells, increase bacterial resistance to environmental stresses such as cleaning, disinfection, and inhibition, allowing these microorganisms to remain on surfaces and processing facilities (13). *S. aureus* biofilms are known to be one of the bacteria's main virulence factors as well as a major source of clinical infection. The most essential step in *S. aureus* biofilm establishment is the synthesis of the polysaccharide intercellular adhesin (PIA), which modulates the adhesion of bacterial cells to one another in the biofilm (14). The icaADBC operon encodes the enzymes necessary for PIA production. The ica operon was discovered and investigated extensively in *S. epidermidis* before being discovered in *S. aureus*. In spite of indications to the contrary, most *S. aureus* strains appear to contain the whole ica operon (15). Therefore, the aim of this study was to investigate the prevalence, phenotypic-genotypic antimicrobial resistance, and existence of biofilm-forming genes in *S. aureus* bacteria isolated from broiler meat.

Materials and methods

Sample Collection

From September to November 2021, 200 cotton swabs of broiler chicken meat from the breast and thigh regions (100 each) were acquired from retail outlets in Alsharqia province, including traditional open markets and supermarkets. The obtained swabs were immediately transferred under aseptic conditions to pre prepared buffered peptone water (0.1 %), then to the laboratory of the Faculty of Veterinary Medicine, Zagazig University, in an icebox under the same conditions for bacteriological analysis.

Bacterial isolation and identification

All samples were examined bacteriologically for the presence of *S. aureus*. Isolation and identification of coagulase-positive Staphylococci was done according to standard methods (16)

Antimicrobial susceptibility testing

The disk diffusion test, as described by the Clinical and Laboratory Standards Institute was performed using Mueller-Hinton agar plates with disks containing the following 10 antimicrobial agents for *S. aureus*. The interpretive class for each isolate (resistant, intermediate, or susceptible) was obtained according to the CLSI recommendations (17). Isolates showing resistance to three or more antibiotics were defined as MDR isolates.

Molecular detection of antimicrobial resistance and virulence genes

The genomic DNA was extracted from 19 biochemically identified *S. aureus* isolates with phenotypic resistance and isolated from poultry meat using the QIAamp DNA mini kit (Takara Kit, Catalogue no. 51304, Japan). From Table 1, The selected primer sequences and cycling conditions were carried out to amplify the resistance genes to methicillin (*mec*A) and beta-lactamase (*blaZ*) in *S. aureus* isolates using uniplex PCR (18,19). Moreover, the oligonucleotides sequences and PCR cycling conditions to identify enterotoxin genes (*sea*, *seb, sec*, *sed*, and *see*) in *S. aureus* were performed by multiplex PCR (20). The electrophoresis of PCR products was performed on a 1.5% agarose gel with ethidium bromide (0.5 µg/ml) and a gel pilot 100 bp ladder (QIAGEN, USA). A positive control isolate of *S. aureus* was included in each reaction.

Detection of Biofilm Formation Ability and Biofilm Genes

Five *S. aureus* isolates (n = 5) to generate biofilms were tested and studied using 96-well flatbottom polystyrene microtiter plates. (Techno Plastic Products, Switzerland) as formerly termed (21). Each isolate's fresh culture in TSB (200 µL) was injected into wells of a sterile microtiter plate and incubated at 37°C for 24 hours. Negative and positive controls were TSB without bacteria *and S. aureus* ATCC 25923, respectively. On the way to eliminate non-adherent cells, the contents of every well were removed and splashed three times with 200 µL of phosphate buffer saline (PBS, pH 7.3).

For 15 minutes, the plates were drained and air-dried. The biofilms were dyed for 30 minutes with 150µL of 0.1 percent crystal violet (Fluka AG, Buchs, Switzerland), then rinsed twice with PBS and air-dried. The dye bound to the cells was resolubilized for 45 minutes in 150 µL of 95 percent ethanol, and the optical density (OD) was quantified using an ELISA reader at a wavelength of 570nm. (Awareness Technologies stat fax 2100, CA, United States). The samples were examined in triplicate, and the procedure was repeated three times. As a result, the average OD values and standard deviations (SD) for the tested isolates and negative controls were estimated. For evaluation of biofilm development, the cut-off value of the OD (ODc) was calculated as follows: ODc = average OD of negative control + (3× SD of negative control), and the isolates were classified as follows: strong biofilm producers (4× ODc< OD), moderate (2× ODc< OD≤ 4×ODc), weak (ODc< OD≤ 2× ODc) and non-producer (OD≤ ODc). Additionally, Biofilm producing isolates were further investigated for biofilm-related genes (*ica*A and *ica*B) (22, 23).

Results

Prevalence of S. aureus in broiler meat

Table (1) showed the prevalence of *S. aureus* in the chicken samples. *S. aureus* was found in 19 of the tested 200 samples (9.5 %).The broiler thigh had slightly higher incidence (10%) of *S. aureus* compared with the broiler breast (9%).

Among the total recovered isolates of *S.aureus*, the distribution of *S.aureus* in the thigh region was the highest (52.63%, 10 out of 19) in comparison to that distribution in chicken breast (47.36%, 9 out of 19).

Determination of enterotoxin genes in S. aureus

The ratio of enterotoxin-encoding genes *sea, seb*, *sec*, and *sed* in *S. aureus* isolates were screened by using multiplex PCR. Each of *sea* and *seb* genes exhibited the highest distribution (31.57%, 6 out of 19) among the recovered isolates of *S.aureus*; while all recovered isolates were negative for other enterotoxin genes (*sec*, *sed* &*see*) as illustrated in Table (2). The PCR products of *sea* and *seb* genes were 102 and 164 bp, respectively (Figure 1).

Phenotypic resistance of S. aureus

The present study revealed that all isolates of *S.aureus* showed the peak resistance (100%) to methicillin and amoxicillin- clavulanic acid followed by moderate resistance percentage (63.15%) to doxycycline (Table 3). A lower resistance (26.31%) was detected to sulfamethoxazole-trimethoprim followed by gentamicin, clindamycin (15.78%, each) and chloramphenicol (10.52%). The resistance of *S. aureus* isolates to imipenem was the lowest (5.26%). Neither vancomycin nor ciprofloxacin resistant isolate were found (Table 3).

The multiple antibiotic resistance was determined for 19 isolates of *S.aureus* based on results of disc diffusion method. The MAR index of the isolates was ranged from 0.3 to 0.7 with an average of 0.4 (Table 4). The predominant MAR index (0.3) was found in 4 isolates of *S. aureus isolates* which were resistant to 3 antibiotics. One isolate of S. aureus of chicken meat origin was found to have the highest MAR index of 0.7 which was resistant to 7 antimicrobials out of 10 tested antibiotics. Moreover, slightly higher MAR index (0.5) was detected in one isolate of S. aureus as this strain was resistant to 5 out of 10 tested antimicrobials (Table 4).

Genotypic resistance of S. aureus

The phenotypic resistance of *S. aureus* finding was confirmed through molecular detection of antimicrobial resistance genes (*blaZ*, *mec*A and *van*A). Concerning *mec*A gene, 10 out of 19 *S. aureus* isolates was positive to *mec*A gene with distribution percentage of 52.6%; while the distribution of *blaZ* gene was 57.89% (11/19) (Table 5). It was noticed that all *S. aureus* isolates were negative for *van*B gene. It was shown from that *blaZ* gene was amplified with PCR product of 610 bp (Figure 2). The PCR product of *mec*A gene was 310 bp (Figure 3).

Analysis of biofilm formation by the micro-titer plate method

This study showed three out of five isolates were strong biofilm producers, which were the same isolates that produce *sea* and *seb*, representing 60% of the total, while the other 40% were non-biofilm producers. A zero percentage was obtained for both weak and moderate biofilm producer isolates (Table 6)

Determination of biofilm formation encoding genes.

S. aureus isolates were screened for the total distribution of biofilm-related genes *ica*A and *ica*D. The biofilm-related gene *ica*A was found in 100% of the highly biofilm-producing *S. aureus* strains. However, the *ica*D gene was not negative in all isolates (Figure 4).

Table 1: Prevalence of *S. aureus* in chicken meat (n=19)

Source	No. of examined samples	No. of infected samples	Percent of infection (%)
Broiler breast	100	9	9
Broiler thigh	100	10	10
Total	200	19	9.5

Table 2: Distribution of pathogenic enterotoxin genes in *Staphylococcus aureus* of chicken meat (n=19)

Enterotoxin genes	No of positive isolates	% of distribution
sea	6	31.57
seb	6	31.57
sec, sed, see	0	0

Table 3: Phenotypic resistance of *S. aureus* strains isolated from broiler meat using disk diffusion method (n= 19)

Antimicrobials (disc concentration/µg)	R		S		I	
	NO	%	NO	%	NO	%
ME (10)	19	100	0	0	0	0
AMC(30)	19	100	0	0	0	0
CN (10)	3	15.78	16	84.21	0	0
DA (2)	3	15.78	16	84.21	0	0
IPM(10)	1	5.26	18	94.73	0	0
CIP(5)	0	0	16	84.21	3	15.78
SXT (25)	5	26.31	12	63.15	2	10.52
C (30)	2	10.5	14	73.68	2	10.52
VA (30)	0	0	18	94.73	1	5.26
DO(30)	12	63.15	3	15.78	4	21.05

R: resistant; I: intermediate; S: sensitive, N: Number of examined isolate, NO: Number of positive, %: Percentage; Data were represented by No (%); ME: methicillin; AMC: Amoxicillin- clavulanic acid; CN: gentamicin; DA: clindamycin; CIP: ciprofloxacin; SXT: sulfamethoxazole-trimethoprim; C: chloramphenicol; VA: vancomycin; IPM: Imipenem; DO: Doxycycline.

Table 4: Multiple antibiotic resistance profile (MAR index) of *S. aureus* isolated from chicken meat (n=19)

Resistance pattern	Antimicrobial resistance profile	Number of isolates	Number of antibiotics	MAR index
I	ME, AMC ,CN,DA, SXT, IMP, DO	1	7	0.7
II	ME, AMC, CN, DA, DO	1	5	0.5
II	ME, AMC, DA, SXT	1	4	0.4
IV	ME, AMC, CN	1	3	0.3
V	ME, AMC, SXT	3	3	0.3
VI	ME, AMC, DO	10	3	0.3
VII	ME, AMC, C	2	3	0.3

Table 5: Distribution of antimicrobial resistance genes in *S. aureus* stains (n=19)

Gene	No. of isolates	Percentage%
mecA	10	52.63
blaZ	11	57.89
vanA	0	0

Table 6: Analysis of biofilm formation in *S. aureus* isolates by the microtiter plate method (n=5)

Degree of biofilm formation	No. of isolates	Percentage%
None	2	40%
Weak	0	0
Moderate	0	0
Strong	3	60%

Discussion

The purpose of this study was to look for the incidence, antimicrobial resistance patterns in *S. aureus* in addition to studying enterotoxins and the distribution of biofilm-forming genes in *S. aureus* bacteria obtained from broiler meat. Food products of animal source, particularly poultry, have been recognized as the primary transmitters of *Salmonella* illnesses in individuals in epidemiological data (24). *Staphylococcus aureus* is an opportunistic human and animal pathogen that causes food poisoning and a wide range of infections, from the skin and soft tissue infections to catastrophic illnesses like endocarditis, septicemia, osteomyelitis, and pneumonia (25). Inadequate handling, unsuitable storage conditions, insufficient sanitary procedures, and post-production microbial contamination could all contribute to *S. aureus* contamination of food (26).

S. aureus was found in 9.5% of the investigated samples, with 47.36 % in the broiler breast and 52.63 % in the thigh region. It is, however, less common than the *S. aureus* bacterium found

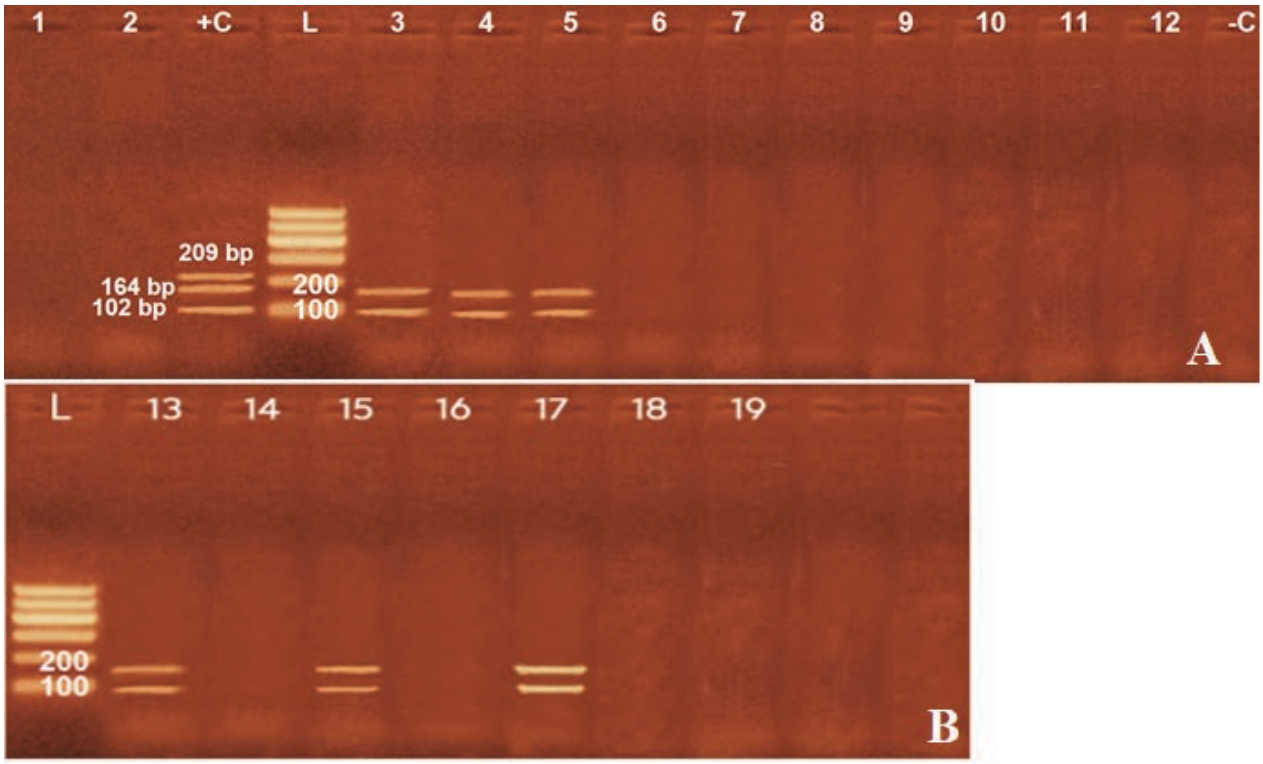


Figure 1: Agarose gel electrophoresis of multiplex polymerase chain reaction for detection of enterotoxin genes in *Staphylococcus aureus* isolated from chicken meat. Enterotoxin genes of *S. aureus*; L: Ladder: 100bp. Lanes (3, 4, 5, 13, 15 and 17) were positive strains for *sea* gene (102 bp) and *seb* (164 bp)

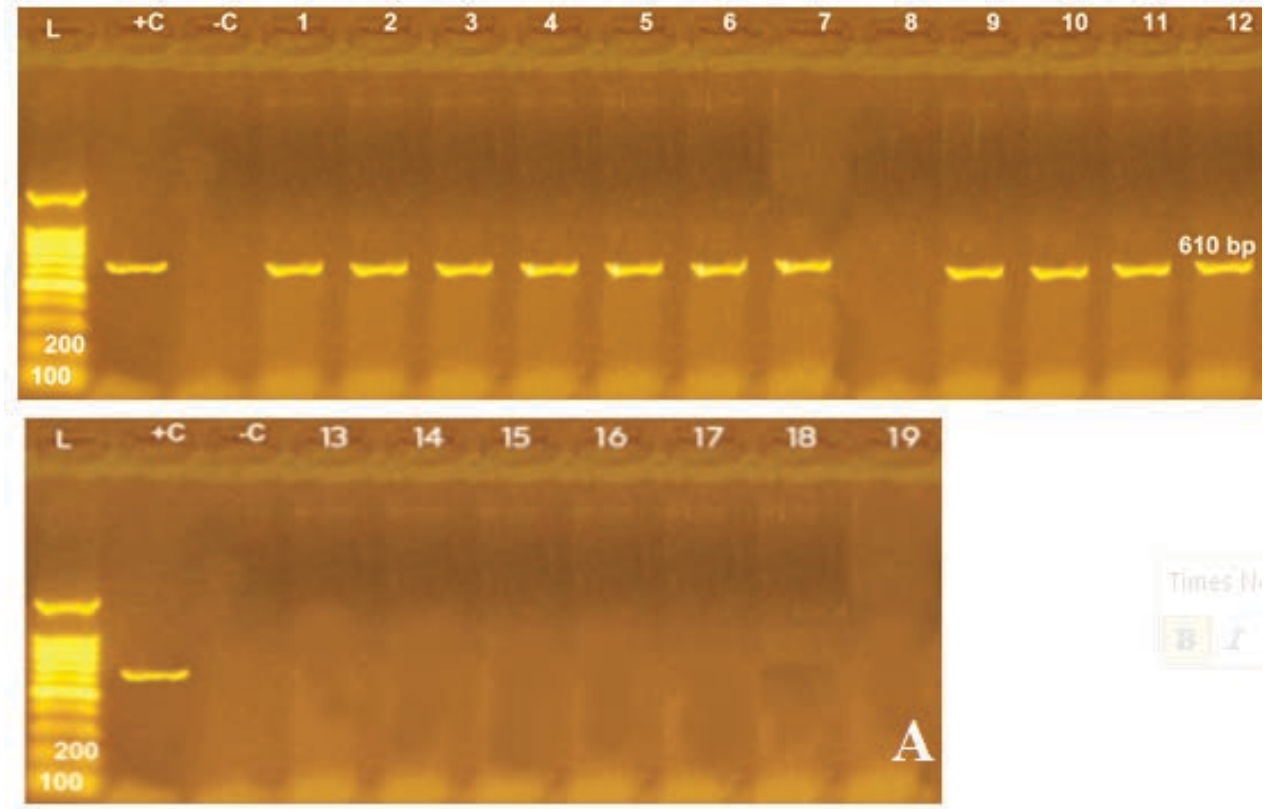


Figure 2: Uniplex PCR to amplify *blaZ* gene in 19 *S. aureus* strains from poultry meat. C+: positive control isolate; C-: negative control isolate of *S. aureus*; L: Ladder 100 bp; lanes 1,2,3,4,5,6,7,9,10,11 and 12 were positive PCR product at 610 bp

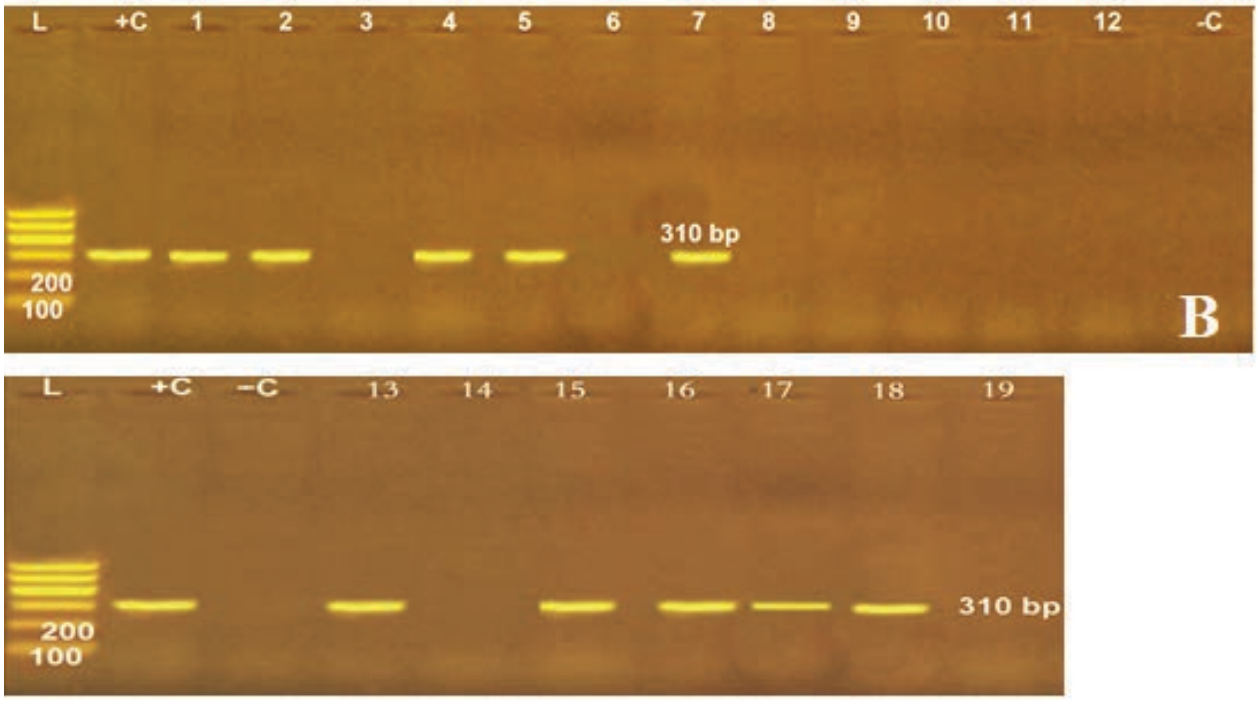


Figure 3: Uniplex PCR to amplify *mec*A gene in 19 *S. aureus* strains from poultry meat. C+: positive control isolate; C-: negative control isolates of *S. aureus*; L: Ladder 100 bp. Lanes 1,2, 4,5, 7,13,15,16, 17 and 18 were positive PCR product at 310 bp

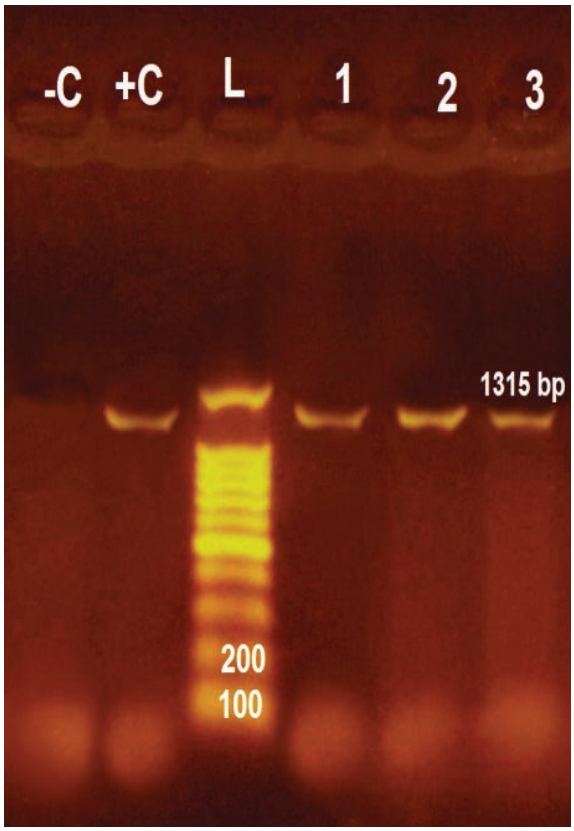


Figure 4: Gel electrophoresis indicating the presence of *ica*A gene in *S. aureus* isolates. C+: Positive control isolate; C-: Negative control; L: Ladder 100 bp; lanes(1,2&3) were positive for *ica*A gene

in raw broiler meat samples; 70% and 80% for the breast and thigh meat, respectively (27). Although, lower percentage of thighs (27.5%) and breasts (22.5%) were obtained (28). Furthermore, Abd El Tawab *et al.* (29) found that 34.3% of the fresh broiler samples were contaminated with *S. aureus*. Another study stated a much higher prevalence of *S. aureus* reached 52% of coagulase *S. aureus* detected in fresh chicken portions (30). The disparity between the estimated isolation rates, including those previously recorded in Egypt could be ascribed to the identification of *S. aureus* isolates in the abovementioned investigations utilizing just biochemical identification. Staphylococci phenotypic identification has already been documented to be inadequate, inconsistent, and unreliable (31). Thighs always had a larger contamination proportion than the breasts, which could be related to the higher quantity of fat (6gm vs. 4gm in the breast). Also, Saadati *et al.* (6) found that *S. aureus* was present in 12 % of the investigated chicken specimens in Iran, according to**,** which nearly matched our findings. Additionally, Momtaz *et al.* (32) reported that 28.05% of *S. aureus* was detected in fresh raw chicken flesh in the same country. In Japan, however, Kitai *et al.* (12) found 41.2 percent of

S. aureus in chicken thighs and 37.3 % in chicken breasts. It's worth noticing that the findings in Portugal indicated that *S. aureus* was the most common infection found in 38.5 % of the poultry flesh (33). *S. aureus* can be found in a variety of environments, involving human body parts that can contaminate food. It is regarded as one of the most important pathogenic species for foodborne diseases. When it's found in food, it's a sign of reduced cleanliness and bad storage (34).

An additional goal of this study was to use specific primers to determine the prevalence of SEs in fresh poultry meat. Because of its propensity to create enterotoxins, *Staphylococcus aureus* is key in poultry meat hygiene (28). The existence of one or more types of enterotoxigenic genes at the same time is a secondary but crucial result of the current study. SEs generated by coagulase-positive staphylococci (CPS), primarily *Staphylococcus aureus*, exhibit super-antigenic and emetic properties, resulting in toxic shock syndrome and staphylococcal food poisoning. Therefore, SEs are deemed to represent a concern to public health, and reporting food poisoning outbreaks has been mandatory (35). The seriousness of food poisoning signs depends on the amount of SE consumed in food and the human health level. Individuals of all ages typically suffer from more sophisticated ailments (36). Among the *S. aureus* bacteria isolated from raw poultry specimens, the furthermost commonly found enterotoxigenic genes were *sea* and *seb*. The prevalence of *sea* and *seb* was 31.57% each in combination. SEC, SED, or SEE were not found in any of the isolates in our research, either alone or in combination. Compared to Darwish *et al*. (37) who concluded that *sea* was the most common gene (64.28 %), afterward *sed* (57.14 %), *sec* (50%), and lastly *seb* (7.14 %). However, Fasiku *et al*. (38) declared a higher percentage for *seb*, which was 75% without combinations of more than one of the tested *se* genes (38).

In India, however, *seb* was the most prevalent gene in isolates from chicken flesh, accounting for 80.95% of the isolates, while none of the isolates in the study had *sea* or *see* (39). Furthermore, Sundararaji *et al*. (40) reported that four isolates from chicken samples tested positive for *sea* (40%), and three tested positive for *seb* as well as *sec* gene (30%). In Iran, on the other hand, Madahi *et al*. (41) who achieved in their study that, eight *S. aureus* isolates (33.33 %) generated *sea*, one (4.16 %) *seb*, three (12.50 %) *sec*, two (8.33 %) *sed,* three (12.50 %) *sed,* and no *see* was detected from chicken nuggets in their study. Staphylococcal enterotoxins cause the signs of staphylococcal foodborne illness and might be linked to other staphylococcal illnesses. These enterotoxins are resistant to heat, and once released, they are difficult to eliminate, even after heat processing. As a result, momentous hygiene precautions ought to be taken once handling and processing chicken flesh (37).

The unregulated use of medications such as growth boosters in animals has been linked to the establishment of antimicrobial-resistant S. aureus; thus, usage should be limited to the field scale (31). The antibiotics chosen were routinely used in humans and animals to treat infections caused by S. aureus and Salmonella. For instance, Penicillin (e.g., amoxicillin-clavulanic acid and methicillin) are b-lactam antibiotics, and their mode of action involves inhibiting bacterial peptidoglycan layer formation (42). As a result , It is challenging to handle infections generated by resistant strains (11). In our investigation, the antimicrobial resistance profile for *S. aureus* strains isolated from fresh chicken meat revealed that the strains exhibited the highest antibiotic resistance to Methicillin and Amoxicillin-clavulanic acid; moreover, all the isolates were resistant to at least one antibiotic, except for Vancomycin and Ciprofloxacin, which had zero resistance results. Besides, the most of the isolates, in this recent study, were multi drug resistant especially to methicillin, amoxicillin-clavulanic acid and doxycycline. In line with our results, El Bayomi *et al*. (31) demonstrated that no isolates were vancomycin resistant; nevertheless, penicillin was only 53.3% resistant, and ciprofloxacin resistant isolates were 33.3% in contract to ours, which was negative (37). In Pakistan, results vary for gentamicin and chloramphenicol being 13.15% and 21.05%, correspondingly , as stated by Akbar and Anal (34), which contradict our results. Further, Safarpoor *et al* (39) featured that the highest prevalence of resistance were to penicillin (60 %) and trimethoprim–sulfamethoxazole (50%), which was obtained from chicken-containing foods. Additionally, resistance to chloramphenicol was found to be (50%) from foods of chicken origin (39). Also, Kim *et al* (43) in Korea said that penicillin resistance was found in a high percentage of

S. aureus isolates (51% percent) isolated from chicken meat. The present study revealed that MAR index of *S. aureus* isolates was ranged from .3 to 0.7 with an average of 0.4. In Comparison with a previous study in India, MAR index of different S. aureus isolates indicates an overall range from 0.06-0.56 where in raw chicken meat and ready to eat (RTE) chicken products range differs from 0.06 to 0.56 and 0.06-0.37, respectively (44). Our finding confirmed that S. aureus isolates revealing more than 0.2 MAR index exhibits that these are from a high-risk source of contamination where antimicrobials are frequently used (45).

Methicillin-resistant *S. aureus* (MRSA) is a pathogen that colonizes and attacks a variety of host species. It has been discovered in the avian farming system, increasing worries regarding probable transfer from farm to plate (46). All MRSA strains have a genetic determinant called *mec*A or *mec*C, which encodes for low affinity penicillin binding proteins called PBP2a. The *mec*A gene is found on the Staphylococcal cassette chromosome mec (SCCmec), a genomic island that contains resistance genes to -lactam ATBs in addition to other resistance genes (11). In the present study, 52.63% of the phenotypic resistant isolates were MRSA and 57.89% had the *bla*Z encoding virulence gene, whereas, *mec*A represents 5% of the total samples and *bla*Z is 5.5%. 100% of the tested strains. This finding was coordinated with Abd El tawab *et al.* (29). However, Saadati *et al.* (6) obtained 5% of MRSA and 100% for *bla*Z encoding gene. The subgroup evaluation done by Ribeiro *et al.* (46) revealed a pooled occurrence of 5% of MRSA in chicken meat. Two years later, Abolghait *et al.* (47) showed that MRSA was found in 5.5 % of the chicken samples (8/144) and the *seb* gene was found in most of MRSA isolates (75 %).

Microorganisms use biofilm development as an environmental adaptation method besides the production of it, is aided by high humidity or moisture content in the environment. Consequently, it is frequently difficult to be eliminated using standard disinfection methods, such as detergents or sanitizing agents (48). In our study, most of the MRSA strains are biofilm producers (60 %, 3/5) and harbored 76% *ica*A biofilm-forming gene. Results that obtained by Saber *et al.* (25) revealed that 50 (83.3 %) of the 60 MRSA isolates produced biofilms, while 10 (16.6 %) were non producer. In china, Chen *et al.* (49) stated that biofilms were generated by around 72% of the isolates. Precisely, 54.6 % of these isolates created weak biofilms, whereas 14.4 % and 3.09 % developed moderate as well as strong biofilms, correspondingly with 100% *ica*A positive. Meanwhile, Ou *et al.* (50) avowed that, 64.8% of all the 165 isolates had strong biofilm formation ability and 20.0% were moderate producers. Two years before, Wang *et al.* (51) reported that, six isolates obtained from chicken meat were classified as strong biofilm formers and none were negative producer in contrast to our conclusions. The importance of isolated *S. aureus* strains was boosted by the *ica*ABCD gene development genes, which encode a polysaccharide intercellular adhesion (PIA), resulting in significant protection of *S. aureus* bacteria against adverse environmental conditions such as the presence of antimicrobials and antiseptic chemicals, as well as immunological responses (52). In A study revealed that 50% of *S.aureus* isolates from chicken meat were positive for *ica*A gene as was previously reported by Abbasi *et al.* (52). A nearly parallel results to our finding was achieved by Eftekhar and Dadaei (15), who declared that by colony morphology, 53.3 % of clinical MRSA isolates demonstrated the potential to produce biofilm, with 75 % carrying the ica operon. Biofilms can prevent antibiotics from reaching bacteria and boost bacterial resistance, that bacterium in biofilms exhibiting 10 to 1500 times' greater antibiotic resistance than bacteria in free cells (53). As a result, a better knowledge of the molecular evolution of staphylococcal biofilms is required to create innovative solutions for biofilm associated contamination.

Conclusion

This study confirmed higher prevalence of multidrug resistant isolates of *S. aureus* isolates in chicken meat due to inadequate handling and insufficient sanitary equipment and post microbial contamination. A higher MAR index of *S. aureus* from chicken meat origin more than 0.2 exhibits that these isolates are derived from a high-risk source of contamination where antibiotics are frequently used. The strong biofilm former strains were only 100% positive for *sea* and *seb* genes. This study highlighted the importance of broiler meat as a reservoir for antimicrobial-resistant strains foe *S. aureus* and biofilm-forming of

Staphylococcus aureus, and these MDR isolates represents a hazard for food safety and public health. Further studies are required to create innovative solutions for biofilm-associated MDR isolates of *Staphylococcus aureus*.

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Original Research Article
Veterinary Medicine and The One Health Concept

PREVALENCE OF *Staphylococcus aureus* AND *Salmonella* SPECIES IN CHICKEN MEAT PRODUCTS RETAILED IN EGYPT

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Abstract: Chicken meat products represent an important source of animal derived proteins, vitamins, and minerals. However, chicken meat products might act as potential sources of human exposure to foodborne pathogens such as *Staphylococcus aureus (S. aureus)* and *Salmonella* species. The objectives of the present study were first to investigate the prevalence rates of *S. aureus* and *Salmonella* species in the retailed chicken meat products at Zagazig city, Egypt. Second, serological identification of the isolated bacteria was followed. Third, screening of *S. aureus* enterotoxin coding genes (*sea seb, and sed*) as well as *Salmonella* virulence associated genes including *invA*, *Salmonella hyper-invasive locus* (*hil*A), and *Salmonella* enterotoxin (*stn*) was done using PCR. The obtained results revealed isolation of *S. aureus* from the examined chicken meat products at 22%, and *Salmonella* spp., at 6.66%. Where *S. aureus* was isolated at 33.33%, 36.66%, 13.33%, 6.66%, and 20% from the examined chicken burger, fillet, luncheon, nuggets, and panne, respectively. *Salmonella* spp. could be isolated only from chicken burger and fillet at 10%, and 23.33%, respectively. The recovered *S. aureus* isolates harbored enterotoxin coding genes (*sea,* and *seb*). Likely the recovered *Salmonella* spp. isolates harbored virulence associated genes such as *invA, hilA,* and *stn.* Moreover, antimicrobial sensitivity testing of the recovered isolates showed multidrug resistance profiling. In conclusion, chicken meat products retailed in Egypt might be potential sources for the spread of multidrug resistant *S. aureus* and *Salmonella* spp. Therefore, strict hygienic measures should be adopted during manufacturing of such meat products.

Key words: chicken meat products; *Staphylococcus aureus*; *Salmonella* species; Egypt

Introduction

Chicken meat products make a substantial contribution as a reasonably affordable substitute for red meat, which is severely undersupplied in Egypt. Such meat source is rich in high-quality animal protein, essential amino acids, and trace elements. Additionally, a number of chicken meat products, including chicken burgers, chicken fillets, chicken luncheons, chicken nuggets, and chicken panne, were produced and released into the chicken meat markets as a result of the quick advancements in food processing and technology. Such important products are distinguished by their distinct flavor and aroma, which draws the attention of customers, especially kids (1, 2).

Chicken meat products may get contaminated by microbes at any stage of production, including the preparation of the raw materials, the manufacturing process, distribution, and storage. As a result, products made from chicken are thought to be a possible source for the spread of bacteria that cause food poisoning, including

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Staphylococcus aureus (*S. aureus*) and *Salmonella* spp., (3, 4).

The major cause of food foodborne intoxication cases is consuming foods contaminated with *S. aureus*-enterotoxins, which are known for their quick onset (between one and six hours after ingesting contaminated foods), nausea, vomiting, stomach cramps, and diarrhea (5). *S. aureus* and staphylococcal enterotoxins (SEs) were identified and found in chicken meat and giblets sold in Egypt (3, 6), and US markets (7), and chicken breast and thigh sold in Cambodian markets (8).

Non-typhoidal salmonellosis is a major foodborne infection caused by consumption of foods contaminated with *Salmonella* spp. The disease is characterized by 12 to 36 hours incubation period post ingestion of contaminated foods, nausea, vomiting, fever, severe diarrhea, abdominal cramps, and malaise (9). Several studies had reported isolation of *Salmonella* spp. from chicken meat and meat products as reported in Vietnam (10), Japan (11), and Egypt (3).

Antimicrobials are extensively used in poultry farms for the purposes of prevention and control of bacterial diseases, as feed additives for improving the feed conversion ratio, and as preservatives in meat products' industry (12). However, the uncontrolled usage of such antimicrobials had led to development of drug resistance among foodborne pathogens (13).

Taken the previous notes, this study was conducted to investigate the prevalence rates of *S. aureus*, and *Salmonella* spp. in retailed chicken meat products including chicken burgers, chicken fillets, chicken luncheons, chicken nuggets, and chicken panne retailed in Egypt. Furthermore, detection of enterotoxin coding genes and virulence associated genes was done using PCR. Antimicrobial susceptibility of the recovered *S. aureus*, and *Salmonella* spp. isolates was carried out using the disk diffusion method.

Material and ethods

Sampling and samples preparation:

A hundred and fifty chicken meat product samples including thirty each of chicken burger, fillet, luncheon, nuggets, and panne were collected from retail markets and grocery stores in Zagazig city, Egypt. The samples were transferred cooled without delay to Animal Health Research Institute, Zagazig branch for bacteriological examination.

The method of APHA (14) was followed during preparation of samples for bacteriological examination. In short, 10 g from each sample was a-septically homogenized in 90 mL of sterile peptone water 1% (Oxoid CM9, UK) and blended for 3 min at 3000 rpm. Serial dilution from each sample was also prepared.

Isolation and identification of S. aureus

The method of APHA (14) was used for isolation and identification of *S. aureus*. In brief, the surface spreading method was used to inoculate 0.1 mL of each prepared serial dilution to a Baird Parker agar (Oxoid, UK) plate supplemented with egg yolk emulsion using. The plates were left in the incubator at 37°C for 48 h on inverted positions. The colonies of *S. aureus* appeared as circular black, shiny with narrow white margin and surrounded by a clear zone extending into the opaque medium. From each cultured plate, five suspected *S. aureus* colonies were picked up and purified on nutrient agar slopes. Identification of *S. aureus* colonies was done using morphological, biochemical, and serological characteristics (14).

Isolation and identification of Salmonella spp.

The method of ISO 6579 (15) was followed during the isolation and identification procedures of *Salmonella* spp. Ten mL from each of the previously prepared meat homogenate were incubated at 37°C for 18 ± 2 h as pre-enrichment. Rappaport Vassiliadis soya broth (Oxoid, UK) was used as a selective enrichment medium. Enriched cultures were incubated at 41.5°C for 24 ± 2 h. The surface streaking method was used to inoculate a loopful from that enriched culture on the surface of xylose lysine desoxycholate (XLD) agar (Oxoid, UK) plate and incubated 37°C for 24 ± 2 h. Suspected colonies (red with or without black centers) were purified and sub-cultured onto nutrient agar slopes and incubated at 37°C for 24 h. The purified colonies were subjected to morphological, biochemical, and serological identification (15).

Molecular identification of Staphylococcal enterotoxins and Salmonella-associated virulence genes:

A Genomic DNA extraction kit (Alliance Global, Dubai, UAE) was used for DNA isolation from the cultured and identified bacteria according to the instructions of the manufacturer. Oligonucleotide pairs for *S. aureus*-specific enterotoxin genes (*Sea, Seb,* and *Sed)*, and *Salmonella-specific hilA, invA,* and *stn* virulence associated genes were bought from Metabion International, Gmbh, Germany, and presented in Table 1.

PCR amplifications were done according to the previously published method (18) on a Thermal Cycler (Master cycler, Eppendorf, Germany) using PCR technique. The cycling conditions started with an initial denaturation at 95°C for 2 min, followed by 35 amplification cycles that consisted of, for each cycle, denaturation for 15 sec at 95°C, annealing for 30 sec at 50°C for *S. aureus* targets, and 60°C for Salmonella targets, and an extension for 1 min at 72°C. A final extension was followed for 7 min at 72°C and ended by holding at 4°C. The resultant PCR products were electrophoresed on agarose gel 1.5% (AppliChem, GmbH, Germany) in 1x Tris Borate EDTA (TBE) buffer stained with ethidium bromide. PCR products were visualized on a UV transilluminator.

Statistical analysis:

All microbial counts were transferred into log 10 of CFU/g. Data were expressed as means ± SD. Data were analyzed using a one-way ANOVA procedure, followed by Tukey's multiple comparison tests (SPSS Inc., Chicago, Illinois, The USA), with a *P*-value of 0.05.

Table 1: Oligonucleotide primer sequences used in the study

Target	Primer sequence (5'-3')	Product size (bp)	Reference
S. aureus genes			
sea	F- GGTTATCAATGTGCGGGTGG	102 bp	**(16)**
	R- CGGCACTTTTTTCTCTTCGG		
seb	F- GTATGGTGGTGTAACTGAGC	164 bp	
	R- CCAAATAGTGACGAGTTAGG		
sed	F- CCAATAATAGGAGAAAATAAAAG	278 bp	
	R- ATTGGTATTTTTTTTCGTTC		
Salmonella genes			
invA	F- GTGAAATTATCGCCACGTTCGGGCAA	284 bp	**(17)**
	R- TCATCGCACCGTCAAAGGAACC		
stn	F- TTGTGTCGCTATCACTGGCAACC	617 bp	
	R- ATTCGTAACCCGCTCTCGTCC		
hilA	F- CGGAAGCTTATTTGCGCCATGCTGAGGTAG	854 bp	**(18)**
	R- GCATGGATCCCCGCCGGCGAGATTGTG		

Results

The obtained results revealed isolation of *S. aureus* from the examined chicken meat products at 22% (33 out of 150 samples), and *Salmonella* spp., at 6.66% (10 out of 150 samples). Where *S. aureus* was isolated at 33.33%, 36.66%, 13.33%, 6.66%, and 20% from the examined chicken burger, fillet, luncheon, nuggets, and panne, respectively. Salmonella spp. was not isolated from chicken luncheon, nuggets and panne but could be isolated from chicken burger and fillet at 10%, and 23.33%, respectively (Table 2).

Total *S. aureus* counts were 3.80±0.22, 3.58±0.20, 3.71±0.20, 3.39±0.19, and 3.60±0.16 log 10 CFU/g from the examined chicken burger, fillet, luncheon, nuggets, and panne, respectively (Table 3).

Three *Salmonella* species were recovered from the examined chicken fillet namely, *S.* Typhimurium, *S.* Infantis, and *S.* Kentucky. *S.* Typhimurium and *S.* Kentucky were isolated both at 28.57%, and *S.* Infantis at 42.86%. *S.* Infantis was the only recovered serotype from chicken burger at 100% (Table 4).

PCR testing of 15 randomly selected *S. aureus* isolates for harboring *Staphylococcal* enterotoxins

revealed detection of *sea* in 7 out of 15 of the tested isolates and *seb* was detected in 10 out of 15 of the tested isolates, while *sed* was not detected at any isolate. Similarly, PCR testing of four selected *S.* Typhimurium (2), *S.* Infantis (1), and *S.* Kentucky (1) isolates for harboring *invA*, *hilA*, and *stn* virulence associated genes revealed detection of *invA* in all tested isolates, *hilA* in the two tested *S.* Typhimurium isolates, and one *S.* Infantis isolate, while *stn* was detected in only one each of *S.* Typhimurium and *S.* Infantis isolate (data are not shown).

Antimicrobial sensitivity testing of the recovered *S. aureus* isolates towards the most commonly used antimicrobials for treatment of *S. aureus* infections revealed resistance of the recovered isolates to methicillin (100%), erythromycin (75.75%), cefotaxime (72.72%), chloramphenicol (57.57%), norfloxacin (9.09%), doxycycline (3.03%), ciprofloxacin (3.03%), and ofloxacin (3.03%), respectively (Table 5).

Regarding antimicrobial sensitivity of the recovered Salmonella isolates, they showed resistance to ampicillin (100%), cefoxitin (100%), trimethoprim/sulfamethoxazole (80%), tetracycline (80%), ciprofloxacin (70%), chloramphenicol (70%), gentamicin (60%), azithromycin (50%), and amoxicillin/clavulanic acid (40%) (Table 6).

Table 2: Prevalence of *S. aureus* and *Salmonella spp.* in the examined chicken meat products (n=30, for each).

Bacterial types / Sample Types	*S. aureus*		*Salmonella* spp.	
	No.	%	No.	%
Chicken burger	10	33.33	3	10
Chicken Fillet	11	36.66	7	23.33
Chicken luncheon	4	13.33	0	0
Chicken nuggets	2	6.66	0	0
Chicken Panne	6	20	0	0
Total	33	22	10	6.66

Table 3: *S. aureus* count (log 10 CFU/g) in the positive examined samples

Sample type	Min	Max	Mean± SE
Chicken burger	3	4.48	3.80 ± 0.22^{a}
Chicken Fillet	3	4.20	3.58 ± 0.20^{ab}
Chicken luncheon	3.30	4.26	3.71 ± 0.20^{ab}
Chicken nuggets	3	3.70	3.39 ± 0.19^{b}
Chicken Panne	3.30	4	3.60 ± 0.16^{ab}

Values carrying different superscript letter within the same column are significantly different at $P < 0.05$

Table 4: Serological identification of *Salmonella* spp. isolated from the examined samples

Salmonella serotypes	Chicken burger (n=3)		Chicken fillet (n=7)		Total (n=10)		Group	Antigenic Structure	
	No.	%	No.	%	No.	%		O	H
***S.* Typhimurium**	-	-	2	28.57	2	20	B	1,4,5,12	i : 1,2
***S.* Infantis**	3	100	3	42.86	6	60	C1	6,7,14	r:1,5
***S.* Kentucky**	-	-	2	28.57	2	20	E1	8,20	$i:Z_6$
Total	3	100	7	100	10	100			

Table 5: Antimicrobial resistance profiling of the recovered *S. aureus* isolates

Antimicrobial agents (AMA)	Resistant (R)		Intermediate (I)		Sensitive (S)	
	NO.	%	NO.	%	NO.	%
Methicillin (ME)	33	100	-	-	-	-
Nitrofurantoin (F)	-	-	-	-	33	100
Norfloxacin (NOR)	3	9.09	2	6.06	28	84.84
Erythromycin (E)	25	75.75	2	6.06	6	18.18
Doxycycline (DO)	1	3.03	1	3.03	31	93.93
Ciprofloxacin (CIP)	1	3.03	3	9.09	29	87.87
Chloramphenicol (C)	19	57.57	-	-	14	42.42
Ofloxacin (OFX)	1	3.03	1	3.03	31	93.93
Gatifloxacin (GAT)	-	-	-	-	33	100
Cefotaxime (CTX)	24	72.72	7	21.21	2	6.06

Table 6: Antimicrobial resistance profiling of the recovered Salmonella spp., isolates

Antimicrobial agents (AMA)	Resistant(R)		Intermediate(I)		Sensitive(S)	
	NO.	%	NO.	%	NO.	%
Trimethoprim/ Sulfamethoxazole(SXT)	8	80	-	-	2	20
Ampicillin (Am)	10	100	-	-	-	-
Cefoxitin (FOX)	10	100	-	-	-	-
Azithromycin (AZM)	5	50	-	-	5	50
Amoxicillin/clavulanic acid (AMC)	4	40	3	30	3	30
Tetracycline (TE)	8	80	1	10	1	10
Ciprofloxacin (CIP)	7	70	3	30	-	-
Gentamicin (CN)	6	60	2	20	2	20
Meropenem (MEM)	-	-	-	-	10	100
Chloramphenicol (C)	7	70	1	10	2	20

Discussion

Products made from chicken meat can become contaminated by various pathogens from a variety of sources starting with pre-processing, processing processes, and post-processing during packing, marketing, and storage. The chicken products are rendered unsafe for consumption by humans due to these pathogenic bacteria (19). Contamination of chicken meat products with *S. aureus* was confirmed in the present study, which reflects the poor hygienic measures adopted during processing of such products. Contamination of chicken meat and its products with *S. aureus* was also reported in several studies around the world as in Iowa, USA (20), Germany (21), Thailand (22), Iran (23), and Egypt (19). *S. aureus* is implicated in many cases of foodborne intoxications (24, 25). Such intoxication cases occur via production of enterotoxins such as *Sea, Seb,* and *Sed* (26, 27). Such enterotoxins were clearly detected in *S. aureus* isolates recovered from the current study. Likely, enterotoxins were detected in *S. aureus* recovered from chicken meat products sampled in Italy (28), Iran (29), and Egypt (3).

The recovered *S. aureus* isolates in the present study showed clear multiple drug resistance profiling. Such drug resistance was developed possibly via the uncontrolled usage of antimicrobials in poultry farming. Similar observation was reported in *S. aureus* isolated from chicken meat in Iran (23), chicken giblets

sampled from Egypt (3), and poultry and poultry products retailed in South Africa (30).

Similar to *S. aureus*, some examined chicken burgers and chicken fillet in the present study were found contaminated with *Salmonella* spp. This agrees with other reports which showed contamination of retailed chicken meat products with *Salmonella* spp. as reported in chicken fillet in Egypt (4), in retail chicken meat in Hanoi, Vietnam (10), Japan (11), and Malaysia (31).

Salmonella was also implicated in many cases of foodborne infections which are characterized by high fever, abdominal pain, diarrhea. Foodborne outbreaks of *Salmonella* were reported in the United States (32), Switzerland, during May-June 2008 (33), and in the United Kingdom during 2010 (34). In addition, three Australian states and territories reported seven *S.* Typhimurium outbreaks (35). Salmonella enterotoxin (*Stn*) and hyper-invasive locus (*hilA*) coding were shown to be two virulence factors in the isolated *S.* Typhimurium. *Salmonella* serovars Typhi, Typhimurium, and Enteritidis have been found to contain *Salmonella* enterotoxin (*Stn*), which contributes to the pathogenicity process of Salmonella and predominantly causes diarrhea (36). A regulator that triggers the expression of invasion genes in response to both environmental and genetic regulatory cues is encoded by the *hilA* gene (37). Moreover, the recovered *Salmonella* spp. were found to have multidrug resistance profiling. This could be attributed to the massive uncontrolled usage of antimicrobials in poultry farms (13, 38).

Conclusion

This study indicated contamination of chicken meat products retailed in Egypt with two important foodborne pathogens, namely, *S. aureus* and *Salmonella* spp. Therefore, strict hygienic precautions should be adopted during processing of such products to avoid cross contamination with pathogens of public health significances.

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Original Research Article
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AMELIORATIVE EFFECTS OF VITAMIN E AND SELENIUM ON BLEOMYCIN-INDUCED MALE INFERTILITY

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Abstract: Bleomycin (BL) is a well-known anticarcinogenic chemotherapy that is used for the remedy of numerous varieties of most cancers. However, the use of BL became connected to the prevalence of several unfavorable results together with skin and lung fibrosis and testicular cancer. Dietary micronutrients such as vitamin E, and selenium (Se) are gambling critical roles in maintaining the health of human and animals and preserving the cell redox status homeostasis through their antioxidant roles. The objectives of the current investigation had been to investigate BL-induced male infertility in Albino rats. The protecting effects of either vitamin E, or Se towards such detrimental outcomes had been additionally examined. Moreover, the potential mechanisms behind such adverse effects had been evaluated. The obtained results indicated significant reduction in the testosterone, FSH, and LH levels in the BL-treated group. Likely, sperm characteristics as sperm count and motility had been significantly reduced in BL-treated group. Such changes have been associated with a significant reduction within the antioxidant enzymes including CAT, GPx, SOD, and MDA. Interestingly, co-exposure of experimental rats to either BL with vitamin E, or Se had notably parameters relatively close to the normal levels suggesting the ameliorative effects of vitamin E and Se towards BL-induced male infertility.

Key words: prevalence; Bleomycin; selenium; vitamin E; vale fertility

Introduction

Human cancer is frequently treated with bleomycin (BL), a glycopeptide antibiotic produced from the bacteria *Streptomyces verticillus*. In the past 20 years, early identification and improved cancer treatment protocols have significantly boosted the percentage of men who survive cancer at a young age; now, more than 75% of young cancer patients are long-term survivors. However, after receiving chemotherapy and radiation treatment, fertility is commonly compromised (1). In this context, BL is linked to several incidences of male infertility in both humans and animals (2-5).

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By reacting with free radicals to generate a tocopheryl radical, which is then reduced by a hydrogen donor and returns to its reduced state, vitamin E functions as an antioxidant by acting as a peroxyl radical scavenger and limiting free radical proliferation in tissues. Due to its solubility, it is absorbed into cell membranes, preventing oxidative damage (6). Due to the high amount of polyunsaturated fatty acids (PUFA) in their membranes, spermatozoa and germ cells are particularly susceptible to oxidation. Vitamin E is a strong lipophilic chain-breaking antioxidant that shields tissue PUFA from peroxidation (7, 8).

For good spermatogenesis, spermatozoa motility, -and testicular development, selenium (Se) is an essential mineral. In both humans and animals, selenium predominantly guards cellular

membranes and organelles against oxidative damage by functioning as an antioxidant through the Se-dependent enzyme glutathione peroxidase (9). A slight deviation, either a lack or an excess of Se in the testis, causes the male reproductive tissue, which depends on an appropriate amount of Se in the testis, to develop improperly. When spermatozoa mature, selenium helps selenoproteins like GPx1, GPx3, mGPx4, cGPx4, and GPx5 protect them from oxidative damage (10).

Infertility affects 15% of couples and in 30% of these cases, the male partner's abnormalities—known as male infertility—are to blame. The oxidative damage caused by an increase in reactive oxygen species is now being linked to numerous cases of male infertility that were previously assumed to be idiopathic. This type of infertility is indicated to be treated with oral Se, and vitamin E supplementation (11).

In sight of the previous facts, this study aimed to investigate the adverse effects of BL on the male infertility using Albino rats as a model. Besides, the ameliorative effects of Vitamin E and Se against BL-induced male infertility in Albino rats had been evaluated. Moreover, the possible mechanisms behind such effects were also examined.

Material and methods

All experiments using animals were done according to the animal use guidelines of Zagazig University, Egypt. This study received an ethical approval number ZU-IACUC/2/F/115/2021.

Animals:

Forty albino male healthy rats, each weighing 180±10 g, were utilized in this investigation. Animals were acquired from the Animal House at Faculty of Veterinary Medicine, Zagazig University, Egypt. Before beginning the research work, the animals were kept under observation and given a week to get used to the lab setting. They were housed in metal cages under hygienic conditions, fed laboratory animal ration during the trial, and given free access to water.

Experimental design, Animals, and treatment:

Animals were grouped into four groups (n = 10 animals/group). Animals in group 1 received distilled water via intraperitoneal injection at a dose of 0.2 mL/animal and served as a control group. Animals in group 2, animals received bleomycin at 1.5 IU/kg BW via intraperitoneal injection twice weekly for three successive weeks. Animals in group 3 received Vitamin E (100 mg/kg BW) orally two hours prior to the administration of bleomycin at 1.5 IU/kg BW via intraperitoneal injection twice weekly for three successive weeks. Animals in group 4 received selenium (0.2 mg/kg BW) orally two hours prior to the administration of bleomycin at 1.5 IU/kg BW via intraperitoneal injection twice weekly for three successive weeks. Animals had given a free excess to feed and water. The environmental conditions of the animal room were at 23–25 °C, 12 hr light/dark cycle, and 50 ± 10% relative humidity. Pentobarbital sodium (40 mg/kg, 0.036 g/mL) was used to anaesthetize each animal before it was weighed and sacrificed. The serum was separated by centrifuging blood samples for 10 min. at 1500 rpm and 4 °C. Blood samples were taken from the retro-orbital sinus. After that, serum was kept at -80 °C for upcoming biochemical tests. The cauda epididymis of the left testis was dissected and transferred to sterilized Petri dish containing 2 ml warm normal saline at 37 °C, then macerated by sterilized scissor to obtain a suspension of the epididymal content (12).

Estimation of reproductive hormones:

Serum testosterone level was estimated by using rat ELISA kit (Cusabio, China), while LH, and gametogenic hormone (FSH) levels were determined by using rat ELISA kit purchased from MY–BIO Source (San Diego, CA) according to the manufacturer's protocol (13).

Analysis of sperm characteristics:

Sperm characteristics including count, motility and sperm abnormalities were analyzed according to the established methods (14, 15).

Measurement of oxidant/antioxidant enzyme assays in the testicular tissue:

Measurement of the antioxidant enzymes including catalase (CAT), glutathione peroxidase (GPX), malondialdehyde (MDA), and superoxide

dismutase level in the testicular tissue was done using specific Biodiagnostic kits according to the manufacturer's guides (16).

Statistical analysis:

The results were shown as means ± standard error (SE). The data were subjected to a one-way analysis of variance (ANOVA) at a 95% level of confidence using the statistical package for social sciences (SPSS-16: Chicago, IL, USA) software followed by Tukey's Kramer HD test, where $P < 0.05$ was considered as significant.

Results

The recorded results in Table 1 showed that BL significantly reduced reproductive hormones including testosterone, FSH, and LH after both 30 and 60 days of treatment. While co-exposure of rats to either vitamin E and BL, or Se and BL could significantly restore the reproductive hormones to their normal levels or even increase their secretion.

The obtained results in Table 2 demonstrated that BL caused significant reduction in the sperm counts and motilities with a significant increase in the total abnormalities. Interestingly, co-exposure of rats to either vitamin E and BL, or Se and BL could significantly increase the sperm count and motility and reduce the total abnormalities.

The recorded results in Table 3 showed that BL caused significant reduction in the testicular antioxidant enzymes including CAT, GPx, and SOD with significant increase in MDA production. Unlikely, co-exposure of rats to either vitamin E and BL, or Se and BL could significantly increase the CAT, GPx, and SOD levels with significant reduction in the MDA levels.

Table 1: Effect of Vitamin E (100 mg/kg BW) and Selenium (0.2 mg/kg BW) orally twice weekly for three successive weeks on serum testosterone, FSH and LH level in adult male rats treated with bleomycin (1.5 IU/kg BW I/P twice weekly for three successive weeks) after 30 and 60 days

Groups	Testosterone (ng/mL)		FSH (mLu/mL)		LH (mLu/mL)	
	After 30 days	After 60 days	After 30 days	After 60 days	After 30 days	After 60 days
Control	4.65 ± 0.29^{b}	5.43 ± 0.57^{b}	2.49 ± 0.29^{b}	2.63 ± 0.32^{a}	11.27 ± 0.37^{b}	11.93 ± 0.64^{ab}
BL	0.53 ± 0.02^{c}	0.45 ± 0.05^{c}	0.76 ± 0.03^{c}	0.63 ± 0.06^{b}	6.53 ± 0.85^{c}	8.50 ± 1.51^{b}
Vit. E + BL	8.74 ± 0.32^{a}	8.55 ± 0.33^{a}	4.33 ± 0.49^{a}	4.10 ± 0.59^{a}	15.17 ± 0.66^{a}	15.10 ± 0.67^{a}
Se + BL	5.63 ± 0.37^{b}	6.21 ± 0.43^{b}	2.60±0.35^{b}	3.13 ± 0.19^{a}	11.40 ± 0.36^{b}	13.20 ± 0.44^{a}

Values within the same column carrying different superscript letter are significantly different at P< 0.05

Table 2: Effect of Vitamin E (100 mg/kg b. wt) and Selenium (0.2 mg/kg b. wt) orally twice weekly for three successive weeks on sperm characters (Sperm count, sperm motility and sperm abnormalities) in adult male rats treated with bleomycin (1.5 IU/kg b. wt I/P twice weekly for three successive weeks) after 30 and 60 days

Groups	Sperm count × 10^{6}/mL		Motility (%)		Total abnormalities (%)	
	After 30 days	After 60 days	After 30 days	After 60 days	After 30 days	After 60 days
Control	61.67 ± 4.41^{b}	60.00 ± 5.77^{b}	46.00 ± 8.89^{b}	47.50 ± 3.04^{b}	9.53 ± 1.12^{b}	13.83 ± 1.17^{b}
BL	35.00 ± 2.52^{c}	29.00 ± 2.08^{c}	24.10 ± 3.16^{c}	17.63 ± 2.86^{c}	12.90 ± 0.59^{a}	19.33 ± 0.67^{a}
Vit. E + BL	76.67 ± 3.33^{a}	86.67 ± 3.33^{a}	71.67 ± 4.33^{a}	81.73 ± 2.91^{a}	7.90 ± 0.10^{b}	10.07 ± 0.64^{b}
Se + BL	75.67 ± 2.96^{a}	78.33 ± 1.67^{a}	67.47 ± 4.17^{a}	71.83 ± 2.46^{ab}	8.57 ± 0.52^{b}	12.67 ± 1.30^{b}

Values within the same column carrying different superscript letter are significantly different at P< 0.05

Table 3: Effect of Vitamin E (100 mg/kg b. wt) and Selenium (0.2 mg/kg b. wt) orally twice weekly for three successive weeks on antioxidant/oxidant status (CAT, GPX, SOD and MDA) in adult male rats treated with bleomy-cin (1.5 IU/kg b. wt I/P twice weekly for three successive weeks) after 30 and 60 days

	CAT (u/g)		GPx (mu/mL)		SOD (u/mg)		MDA (nmol/g)	
	After 30 days	After 60 days	After 30 days	After 60 days	After 30 days	After 60 days	After 30 days	After 60 days
Control	1.43 ± 0.07[b]	1.63 ± 0.09[a]	6.10 ± 0.09[a]	6.10 ± 0.09[a]	77.33 ± 2.73[a]	78.00 ± 3.21[a]	0.40 ± 0.04[c]	0.43 ± 0.01[b]
BL	0.71 ± 0.15[c]	0.67 ± 0.16[b]	3.43 ± 0.23	2.90 ± 0.38[b]	41.67 ± 0.88	40.67 ± 0.88[d]	4.64 ± 0.52[a]	5.66 ± 1.1[a]
Vit. E + BL	2.13 ± 0.06[a]	2.13 ± 0.07[a]	4.47 ± 0.27	4.97 ± 0.35[a]	52.67 ± 1.45	56.00 ± 1.15[c]	2.07 ± 0.09[b]	2.07 ± 0.23[b]
Se + BL	1.67 ± 0.09[b]	1.83 ± 0.17[a]	4.80 ± 0.17	4.94 ± 0.20[a]	59.33 ± 0.88	62.33 ± 2.33[b]	2.00 ± 0.25[b]	1.87 ± 0.23[b]

Values within the same column carrying different superscript letter are significantly different at P< 0.05

Discussion

Bleomycin is a glycopeptide antibiotic that is frequently used to treat human cancer. However, there are numerous instances of both human and animal male infertility that have been connected to BL. In the present study, BL caused significant reduction in the reproductive hormones such as testosterone, FSH, and LH. Besides, BL caused significant decrease in the sperm count and motility with an increase in the total sperm abnormalities. Such reproductive changes were associated with significant reduction in the testicular antioxidant enzymes with an increase in the oxidative damage as declared by the high MDA levels in the treatment groups compared with the control. Several cancer types have been treated with bleomycin, either alone or in conjunction with other chemotherapy drugs including etoposide and cis-platinum. In this regard, adult male and female Sprague-Dawley rats were treated with the therapeutically adequate dose levels of bleomycin, etoposide, and cis-platinum (BEP) in three cycles of 21 days each. Serum hormone levels, testicular histopathology, PCNA and transferrin expression, sperm characteristics, fertility, and testicular histopathology were all evaluated after the course of treatment. BEP caused sperm motility to decrease, sperm motility to decrease, abnormalities to rise, and tubular atrophy. elevated levels of inhibin B, but transferrin, FSH, and testosterone were lowered (17). In another study, the effects of BL on sperm parameters and the production of MDA in rats were evaluated. BL markedly increased the quantity of immature sperm, sperm with damaged DNA, and MDA concentration in comparison to the control group. On the other hand, BL markedly reduced the quantity, viability, and motility of sperm (2). In a different study, Balb/c mice in the BL-treated group received 10 mg/kg of BL intraperitoneally every day for 35 days. The results showed that BL significantly increased intermediate and immature non-progressive movement and immotile sperm while significantly decreasing sperm count, viability, morphology, maturation, and progressive movement, testosterone, the ratio of testis weight to body weight, the number of spermatogonia, spermatocytes, and Sertoli cells per tubule (18).

By interacting with free radicals to generate a tocopheryl radical, which is then reduced by a hydrogen donor and returns to its reduced state, vitamin E functions as an antioxidant and scavenger of peroxyl radicals, inhibiting their growth in tissues. Selenium is most likely an important substance that actively participates in a range of metabolic processes and has a number of significant physiological functions. Participation in different enzyme systems and antioxidant cellular processes are a couple of these roles. Vitamin E and selenium are very necessary micronutrients for the normal functions of the reproductive system. In co-exposure groups with either vitamin E and BL or Se and BL, significant improvements were achieved in the reproductive functions compared with BL-treated group. Interestingly the groups that received vitamin E and Se had higher levels of the reproductive hormones, sperm counts and motility. Such ameliorative effects were associated with a significant increase in the antioxidant enzymes in the testes. Similarly, the zona binding test shows that vitamin E taken orally greatly enhances the *in vitro* performance of human spermatozoa (19). Likely, in a study by Keskes-Ammar et al. (20) Vitamin E and selenium

supplements resulted in a considerable drop in MDA concentrations and an improvement in sperm motility. The findings support the use of vitamin E and selenium in the treatment of male infertility because they have protective and advantageous effects on the quality of semen. Furthermore, Se caused a significant improvement in the semen quality and secretion of testosterone in a human trial (21).

Conclusion

The current investigation demonstrated occurrence of BL-induced toxic effects on male reproductive organs leading to occurrence of male infertility as demonstrated by the significant reductions in the male hormones, and deviations in the sperm characteristics, such changes were associated with the induction of oxidative stress in the testicular tissue. Interestingly, vitamin E and selenium could ameliorate such adverse effects, possibly via upregulation of the testicular antioxidant enzymes.

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Authors declare that they have no conflict of interest.

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Original Research Article
Veterinary Medicine and The One Health Concept

RABBIT MEAT AS A POTENTIAL SOURCE OF *Staphylococcus aureus* AND *Salmonella spp.*

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Abstract: Rabbit meat and offal are considered valuable sources of high biological value animal protein. Rabbit meat is rich in essential amino acids, low in cholesterol, and contains considerable amounts of trace elements such as calcium, magnesium, and zinc. However, the potential contribution of rabbit meat and offal in the transmission of foodborne pathogens such as *Staphylococcus aureus* (*S. aureus*) and *Salmonella* spp. is neglected. Therefore, this study was conducted first to investigate the prevalence rates of *S. aureus* and *Salmonella* spp. in the retailed rabbit meat at Sharkia Governorate, Egypt. Second, serological identification of the isolated bacteria was followed. Detection of *S. aureus*-enterotoxins and Salmonella-virulence-associated genes was also done using PCR. Antimicrobial susceptibility testing of the recovered bacterial isolates was additionally examined. The acquired results showed that 17% of the investigated samples of rabbit meat contained *S. aureus*. Where *S. aureus* was isolated from the investigated rabbit's breast, thigh, liver, and kidney at 20%, 24%, 12%, and 12% of each, respectively. *Salmonella* spp. was isolated at 13%. *Salmonella* spp. was isolated from the investigated rabbit thigh, liver, breast, and kidney at 16%, 16%, 12%, and 8%, respectively. Four different strains of *Salmonella* spp namely, *S.* Typhimurium, *S.* Kentucky, *S.* Virchow, and *S.* Infantis were recovered in the current study. The recovered *S. aureus* and *Salmonella* spp. harbored enterotoxins and virulence attributes with multidrug resistance. Therefore, strict hygienic measures should be followed during the processing and handling of rabbit meat and offal.

Key words: rabbit meat; offal; *Staphylococcus aureus*; *Salmonella spp.*; Egypt

Introduction

Most Mediterranean countries produce a significant amount of rabbit meat, and many others have a thriving rabbit meat business. Good muscle-to-bone ratios, quick growth rates, high feed efficiency, early marketing age, and a low land area demand are all characteristics of rabbits (1). This suggests that the rabbit has a real future in large-scale livestock production. Studies have shown that rabbit meat is a great food for humans to consume because it is high in protein and low in sodium, fat, and cholesterol (2).

Rabbits have historically been bred in Egypt in small colonies in backyards to augment the family income, but in more recent years, the practice has emerged as a distinctive source of meat. Due to their rapid feed conversion rates, brief life cycles, and short gestation periods, rabbits are regarded as the best animals for producing meat. Low manufacturing costs and a

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constrained breeding area set it apart. Similar to how rabbits make excellent research animals for experiments and gain economically. Because it contains lean meat with high biological value, as well as high quantities of unsaturated fat and low cholesterol content, consuming rabbit meat would be beneficial to human health (3). Rabbit meat is rich in amino acids content as recently reported in the New Zealand White rabbit in the following order: lysine > isoleucine > valine > methionine > threonine > histidine > phenylalanine > leucine, such amino acids in California rabbits were in the following order: lysine > leucine > valine > threonine > isoleucine > phenylalanine > histidine > methionine (4). Even though rabbit meat has a high nutritional value, it is also linked to the spread of various foodborne infections, which can have serious negative health effects (5, 6).

Microbes can contaminate rabbit meat at any point during the production process, including the slaughtering procedure, dressing, evisceration, and storage. As a result, tainted rabbit meat has been linked to the spread of microorganisms that cause food poisoning, such as *Salmonella* spp. and *Staphylococcus aureus* (*S. aureus*) (7).

Consuming foods contaminated with *S. aureus*-enterotoxins is the primary cause of foodborne poisoning cases. These enterotoxins are well-known for their rapid onset (between one- and six hours), nausea, vomiting, stomach cramps, and diarrhea (8). *S. aureus* was isolated from both rabbit meat and offal sold in Egypt (9, 10) and Spain (6).

Salmonella spp. causes a serious foodborne illness brought on by eating items tainted with these bacteria. The illness is distinguished by an incubation period of 12 to 36 hours following the consumption of infected foods, as well as by nausea, vomiting, fever, severe diarrhea, abdominal cramps, and malaise (11).

Antimicrobials are widely used in the meat industry as preservatives, as feed additives to increase feed conversion ratios, and in rabbit farms for the prevention and management of bacterial infections (12). But uncontrolled use of such antibiotics had resulted in the emergence of drug-resistant foodborne bacteria (13).

In the sight of the previous facts, the current study was undertaken to investigate the prevalence of *S. aureus* and *Salmonella* spp. in rabbit meat retailed in Egypt. Virulence attributes and antimicrobial resistances among the recovered isolates were additionally screened.

Material and methods

Sampling and samples preparation:

A hundred rabbit meat parts including 25 each of breast, thigh, liver, and kidney were collected from rabbit butchery shops at Sharkia Governorate, Egypt. The samples were transferred and cooled without delay to Animal Health Research Institute, Zagazig branch for bacteriological examination.

The procedure outlined by APHA (14) was adhered to while preparing samples for bacteriological analysis. In brief, 90 mL of sterile peptone water 1% (Oxoid CM9, UK) were blended for 3 min at 3000 rpm with 10 g of each sample after being aseptically homogenized. Each sample's serial dilution was also prepared.

Count, isolation, and identification of S. aureus:

S. aureus was isolated and identified using APHA's (14) technique. In short, 0.1 mL of each generated serial dilution was added using the surface spreading method to a Baird Parker agar (Oxoid, UK) plate that had been supplemented with an egg yolk emulsion. 48 hours were spent with the plates inverted in the incubator at 37°C. The *S. aureus* colonies were spherical, shiny, and black, with a thin white edge, and they were encircled by a transparent zone that extended into the opaque medium. Total *S. aureus* (TSC) was counted according to the following equation:

TSC/g = average No. of colonies × reciprocal of the dilution

Counted colonies expressed as log 10 cfu/g.

Five potential *S. aureus* colonies were selected from each cultivated plate and purified on nutrient agar slopes. Utilizing morphological, biochemical, and serological traits, *S. aureus* colonies were identified.

Isolation and identification of Salmonella spp.:

During the *Salmonella* spp. isolation and identification processes, the ISO 6579 (15) method was used. Pre-enrichment was carried out by

incubating ten mL of each of the previously produced meat homogenates for 18 ± 2 hours at 37 °C. As a selective enrichment medium, Rappaport Vassiliadis soy broth (Oxoid, UK) was employed. For 24 ± 2 hours, enriched cultures were incubated at 41.5°C. A loopful of that enriched culture was inoculated using the surface streaking technique on the xylose lysine desoxycholate (XLD) agar (Oxoid, UK) plate, and it was then incubated at 37°C for 24 ± 2 hours. Purified and sub-cultured suspected colonies (red with or without black centers) were placed on nutrient agar slopes, and they were then incubated at 37°C for 24 hours. The purified colonies underwent morphological, biochemical, serological, and genetic analyses.

Molecular identification of Staphylococcal enterotoxins and Salmonella-associated virulence genes:

According to the manufacturer's recommendations, genomic DNA was extracted from the recovered bacterial isolates. The oligonucleotide pairs for the enterotoxin genes (*sea,* and *seb*) specific to *S. aureus* and the virulence-associated genes (*hilA, invA*, and *stn*) specific to *Salmonella* spp. were purchased from Metabion International, Gmbh, Germany, and were prepared according to previous reports (16-18). PCR amplifications were carried out on a Thermal Cycler (Master cycler, Eppendorf, Germany) following the previously described method (17). The initial denaturation at 95°C for two minutes was followed by 35 amplification cycles, each of which included a denaturation step at 95°C for 15 seconds, an annealing step at 50°C or 60°C for targets of *S. aureus* or *Salmonella* spp., and an extension step at 72°C for one minute. Following a final extension for seven minutes at 72°C, a holding period at 4°C was followed. The resulting PCR products were electrophoresed on a 1.5% agarose gel in 1x Tris Borate EDTA (TBE) buffer and stained with ethidium bromide (AppliChem, GmbH, Germany). With the use of a UV transilluminator, PCR products were visualized.

Antimicrobial sensitivity testing:

Antimicrobial susceptibility of the recovered isolates to the most commonly used antimicrobials in the rabbit farms was performed following the Clinical and Laboratory Standards Institute's standards (19).

Statistical analysis:

All microbial counts were converted into log 10 cfu/g for statistical analysis. The data were presented as means ± SE. One-way ANOVA was used to evaluate the data, and Tukey's multiple comparison tests were performed (SPSS Inc., Chicago, Illinois, USA), with a P-value of 0.05.

Results

The obtained results indicated the isolation of *S. aureus* from the examined rabbit meat samples at 17% (17 out of 100 samples). Where *S. aureus* was isolated at 20%, 24%, 12%, and 12% from the examined rabbit breast, thigh, liver, and kidney, respectively. The average *S. aureus* counts (log 10 cfu/g) in the examined samples were 2.76 ± 0.09, 2.64 ± 0.10, 2.54 ± 0.11, and 2.39 ± 0.06 in the examined breast, kidney, liver, and thigh respectively (Table 1). The overall isolation rate of *Salmonella* spp. was 13% (13 out of 100 samples). Where *Salmonella* spp. was isolated at 16%, 16%, 12%, and 8% from the examined rabbit thigh, liver, breast, and kidney, respectively (Table 2). Four *Salmonella* species were recovered from the examined rabbit samples namely, *S.* Typhimurium, *S.* Infantis, S. Virchow, and *S.* Kentucky. *S.* Typhimurium was isolated from the liver and thigh at 50% and 25%, respectively. *S.* Kentucky was isolated from kidney, liver, and thigh samples at 50%, 25%, and 25%, respectively. S. Virchow was isolated from the breast and thigh samples at 66.66%, and 25%, respectively. *S.* Infantis was isolated from kidney, breast, liver, and thigh samples at 50%, 33.33%, 25%, and 25%, respectively. (Table 3).

PCR testing of randomly selected colonies revealed the detection of *S. aureus* enterotoxins *sea, seb*, and salmonella virulence attributes at variable rates (data are not shown).

Antimicrobial susceptibility testing of the recovered *S. aureus* isolates towards the most used antimicrobials for treatment of *S. aureus* infections revealed resistance of the recovered isolates to cefotaxime (100%), chloramphenicol (88.2%), erythromycin (82.4%), norfloxacin (29.4%), cipro-

floxacin (17.6%), and doxycycline (5.9%) (Table 4). While the recovered Salmonella isolates showed resistance to ampicillin (76.9%), tetracycline (76.9%), azithromycin (69.2%), sulfamethoxazole/ trimethoprim (61.5%), ciprofloxacin (38.5%), chloramphenicol (38.5%), gentamicin (7.7%), and Meropenem (7.7%) (Table 5).

Table 1: Prevalence and total *S. aureus* count (log 10 cfu/g) in the examined rabbit samples

	Positive samples		Min.	Max.	Mean ± SE
	No.	%			
Breast	5	20	2.00	3.40	± 0.09[a]2.76
Thigh	6	24	1.70	2.90	2.39 ± 0.06[a]
Liver	3	12	1.30	3.48	2.54 ± 0.11[a]
Kidney	3	12	2.00	3.60	2.64 ± 0.10[a]

Values within the same column carrying the same superscript letter are not significantly different at P< 0.05.

Table 2: Prevalence of *Salmonella* spp. in the examined rabbit samples

Sample	Positive samples	
	No.	%
Breast	3	12
Thigh	4	16
Liver	4	16
Kidney	2	8

Table 3: Serological identification of the recovered *Salmonella* spp.

***Salmonella* serotypes**	**Breast (n=3)**		**Thigh (n=4)**		**Liver (n= 4)**		**Kidney (n= 2)**		**Antigenic Structure**	
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**O**	**H**
***S.* Typhimurium**	-	-	1	25	2	50	-	-	1,4,5,12	i: 1,2
***S.* Kentucky**	-	-	1	25	1	25	1	50	8,20	i: Z6
***S.* Virchow**	2	66.66	1	25	-	-	-	-	6,7,14	r: 1,2
***S.* Infantis**	1	33.33	1	25	1	25	1	50	6,7,14	r: 1,5
Total	2	100	4	100	2	100	2	100		

Table 4: Antimicrobial susceptibility of the recovered *S. aureus* isolates (N=17

Antimicrobial agent	**S**		**I**		**R**	
	No.	**%**	**No.**	**%**	**No.**	**%**
Cefotaxime (CXT)	-	-	-	-	17	100
Chloramphenicol (C)	2	11.8	-	-	15	88.2
Ciprofloxacin (Cip)	11	64.7	3	17.6	3	17.6
Doxycycline (DO)	9	52.9	7	41.2	1	5.9
Erythromycin(E)	3	17.6	-	-	14	82.4
Gatifloxacin (GAT)	16	94.1	1	5.9	-	-
Nitrofurantion (F)	12	70.6	5	29.4	-	-
Norfloxacin (NOR)	12	70.6	-	-	5	29.4
Ofloxacin (OFX)	13	76.5	4	23.5	-	-

Table 5: Antimicrobial susceptibility of the recovered *Salmonella* spp. isolates (N=13)

Antimicrobial agent	S		I		R	
	No.	%	No.	%	No.	%
Amoxicillin (AMC)	11	84.6	1	7.7	1	7.7
Ampicillin (AM)	3	23.1	-	-	10	76.9
Azithromycin (AZM)	4	30.8	-	-	9	69.2
Cefoxitine (FOX)	12	92.4	1	7.7	-	-
Chloramphenicol (C)	7	53.8	1	7.7	5	38.5
Ciprofloxacin (Cip)	5	38.5	3	23.1	5	38.5
Gentamycin (CN)	8	61.6	4	30.8	1	7.7
Meropenem (MEM)	12	92.3	-	-	1	7.7
Sulfamethoxazole trimethoprim (SXT)	5	38.5	-	-	8	61.5
Tetracycline (TE)	1	7.7	2	15.38	10	76.9

Discussion

There is no doubt that each year, foodborne pathogenic bacteria lead to significant human and financial costs due to the numerous illnesses and fatalities they cause. The microbiological quality of red meat, poultry, and their products has been the subject of numerous research in Egypt (8, 11, 20, 21). However, there is some question as to the microbiological quality of rabbit meat, which, like many other raw meals of animal origin, might be contaminated with a vast array of microorganisms including food poisoning organisms such as *S. aureus* and *Salmonella* spp. The obtained results of the present study demonstrated isolation and identification of enterotoxigenic *S. aureus*, and *Salmonella* spp. that harbor several virulence attributes. In agreement with the current study *S. aureus* and *Salmonella* spp. were isolated from processed rabbit carcasses retailed in grocery stores in Beni-Suef city, Egypt (9). Initial counts of Enterobacteriaceae, psychrophilic bacteria, aerobic mesophilic bacteria, molds, and yeasts were found to be relatively high in rabbit meat samples, with mean log10 counts of 6.021, 5.888, 4.785, and 4.886 cfu/g, respectively. This represents the potential for cross-contamination during slaughter, which significantly affects the bacterial status of the carcass (22). Additionally, the rabbit's cooled muscles contained Enterobacteriaceae (5.72 ± 0.26 log10 cfu/g), Staphylococcus (5.32 ± 0.24 log10 cfu/g), and aerobic plate count (7.82 ± 0.34 log10 cfu/g) (10). Besides, Mahmoud et al. (23) recently examined the microbiological condition of rabbit carcasses sold at retail in Zagazig City, Sharkia Governorate, Egypt. They collected 80 random samples of fresh chops of rabbit meat (shoulder, loin, ribs, and thigh regions). Aerobic plate count (APC), Enterobacteriaceae, as well as the isolation and identification of *E. coli* and *Salmonella* Spp., were examined. The mean APC and Enterobacteriaceae values for the shoulders were $1.1x10^6$ and $4.7x10^4$ cfu/g, respectively. The values for the ribs were $9.6x10^5$ and $5.7x10^4$ cfu/g, the loin samples were $1.0x10^6$ and $5.1x10^4$ cfu/g, and the thigh samples were $1.2x10^6$ and $6.0x10^4$ cfu/g. Salmonellae were isolated from the shoulder, ribs, loin, and thigh regions at 6 (30%), 7 (35%), 6 (30%), and 4 (20%), respectively. On the other hand, *E. coli* was isolated at 18 (90%), 16 (80%), 15 (75%), and 19 (95%) of the aforementioned samples. The findings highlighted the significance of stringent sanitary procedures during slaughtering and revealed that fresh rabbit meat might contain a diversity of spoilage and food poisoning bacteria. Globally, Enterotoxigenic *S. aureus* and *Salmonella* spp. were isolated from rabbit carcasses retailed in Spain (24).

The uncontrolled usage of antimicrobials in rabbit farming is a major reason for the emergence of multidrug-resistant foodborne pathogens as was apparent in the current study. Therefore, strict observation of the withdrawal times of the antimicrobials, besides the use of antimicrobials at the minimum requirement is of significance to avoid the development of drug resistance.

Conclusion

The current study revealed the isolation of multidrug-resistant *S. aureus* and *Salmonella*

spp. from rabbit meat and offal, suggesting that these products might act as potential sources for transmission of such foodborne pathogens.

Acknowledegments

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Authors declare that they have no conflict of interest.

This study was done according to the guidelines of Zagazig University, Egypt. This study did not use any experimental animals or human subjects.

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