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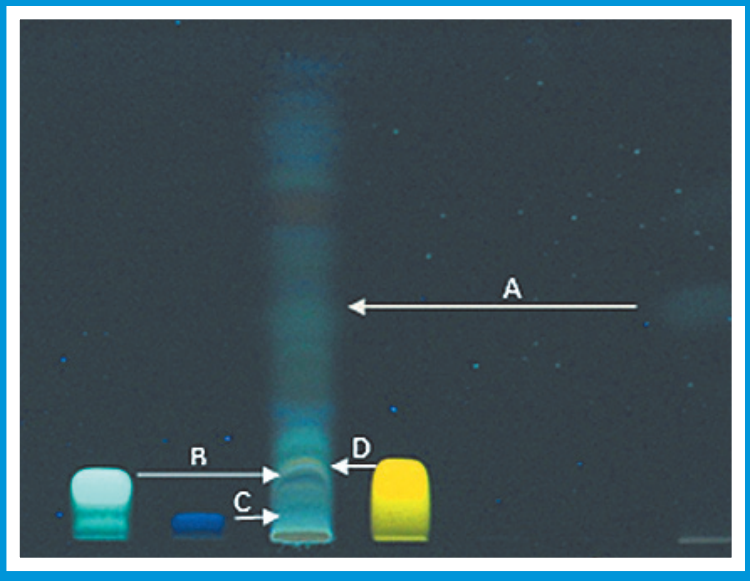

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Address: Veterinary Faculty, Gerbičeva 60, 1000 Ljubljana, Slovenia
Naslov: Veterinarska fakulteta, Gerbičeva 60, 1000 Ljubljana, Slovenija
Tel.: +386 (0)1 47 79 100, Fax: +386 (0)1 28 32 243
E-mail: slovetres@vf.uni-lj.si

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Original Research Article

ANTI-AGEING EFFECTS OF DIETARY BEE PRODUCTS AND CALORIE RESTRICTION ON SEMEN PRODUCTION AND OXIDATIVE DAMAGE IN OLDER BROILER BREEDER MALES

Çiğdem Şeremet Tuğalay*, Özge Altan

Department of Animal Science, Faculty of Agriculture, Ege University, 35040, Bornova, Izmir, Turkey

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

Abstract: This study was conducted to investigate the effects of calorie restriction and dietary bee products (apilarnil plus royal jelly) supplementation on reproductive and oxidative responses and to determine the possibilities that these treatments may be used in retarding the reproductive ageing of broiler breeder males. At 52 weeks of age, broiler breeder males were assigned to four treatment groups. The control group was fed on restricted feed as recommended by the breeder company throughout the study; the *ad libitum* group was fed *ad libitum* for a four-week period; the bee products group was fed similar to the control group except that their diet was supplemented with apilarnil and royal jelly for a four-week period and in the last group calorie restriction (45 % of standard diet) was applied for a four-week period. After a four-week adaptation period, the experiment was continued for 18 weeks. The results obtained in the present study have demonstrated that the percentage of dead sperm was the most affected semen characteristic by reproductive ageing. Long-term moderate feed restriction could not prevent age-related declines in sperm production. Dietary bee products supplementation or calorie restriction for a four-week period positively affected the semen characteristics, and these beneficial effects could be maintained to some extend up until 72 weeks of age. Calorie restriction enhanced antioxidant defence for the first four-week period; however, this beneficial effect could not be sustained until the end of the experiment.

Key words: broiler breeder males; ageing; semen characteristics; oxidative stress; bee products; calorie restriction

Introduction

Although domestic roosters may live for more than 10 years, the fertility of commercial breeder strains declines during the last phase of their reproductive life, so they are kept for a much shorter period. Commercial broiler breeders have a relatively shorter reproductive life span, and their reproductive performance decreases, particularly after 45 weeks of age (1, 2). Therefore it is recommended to keep broiler breeder flocks in production until 61 to 64 weeks of age. The decline of fertility in ageing roosters was accompanied by a reduction in spermatozoa production and by a decrease in motility and viability of spermatozoa (3, 4, 5). In contrast, the demand for spermatozoa increases with ageing because of the changes in the oviduct conditions, especially in sperm storage tubules (6, 7). Therefore, one of the most important goals in the poultry industry is to extend the reproductive life span and retard signs of reproductive senescence, both in terms of animal welfare and production costs.

Calorie restriction (CR) has been shown to extend the life span and retard the onset of many age-related disorders in a variety of animal models (8, 9, 10). Short-term calorie or food restriction has been routinely used by the poultry industry for many years to extend the reproductive life span and delay the maturation of laying hens (11, 12).

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However, it has been suggested that severe CR caused a significant decrease both in testis weight and in plasma testosterone levels and an increase in plasma corticosterone levels (13, 14).

The cumulative oxidative damage to macromolecules caused by reactive oxygen species (ROS) is considered to be related to cellular senescence, life span, and fitness (15, 16). It is suggested that in avian cells the accumulation of ROS-induced damage would be slower due to lower ROS production, better resistance to oxidative stress, enhanced antioxidant capacity, and better DNA repair (16, 17, 18). If ageing is caused by ROS as described by the free radical theory or oxidative damage theory of ageing, supplementation of exogenous antioxidants may enhance the antioxidant defence capacity, slow down ageing, and prolong the reproductive life span.

Sperm membranes have a higher content of PUFAs (polyunsaturated fatty acids); therefore, they are more susceptible to oxidative damage. Oxidative stress may be reduced by antioxidant supplementation of broiler breeder diets (19).

Therefore, in this study, the natural bee products apilarnil and royal jelly have been used to retard the ageing process because of their antioxidant and antiaging activities.

Royal Jelly (RJ) is a functional food secreted by the hypopharyngeal and mandibular glands of worker bees. RJ has many properties, including antitumor, antibacterial, antioxidant, antiaging, hypotensive, growth-stimulating, and anti-inflammatory activities (20). It is believed that RJ can prolong life span because it prolongs the longevity of queens comparing to worker bees. In a mice model study, it is suggested that a 16-week RJ supplementation prolonged the average life span protecting the DNA and lowering oxidative stress (21).

Apilarnil is mainly a drone larvae extract that also contains small amounts of royal jelly, bee bread, honey, and propolis. Apilarnil has many pharmacological activities, such as anabolism stimulator, antiviral, immunomodulator, biostimulator (22, 23). Additionally, because it comes from a "male-like" structure, apilarnil is very rich in male-like hormones, so it stimulates the spermatogenesis (22, 24) and regulates the human endocrine system (25, 26, 27). It also has high levels of 10-HDA (10-hydroxy-2-decenoic acid), and many vitamins and minerals (27, 28) affecting antioxidant capacity. (29) reported that both the total antioxidant potential and activity of capturing the free radicals of RJ and drone larvae are sufficiently high, and their combination leads to extremely valuable products.

The present study was conducted to determine the possibilities that calorie restriction or dietary apilarnil and royal jelly supplementation may be used in retarding the reproductive ageing of broiler breeder males. We evaluated the effects of those dietary manipulations on the aging of roosters by quantifying lipid peroxidation, some antioxidant activities, and semen quality parameters. Moreover, these age-related parameters were compared with those of the *ad libitum* feeding group.

Materials and methods

The ethical committee approval of Ege University (2011-092) was granted in order to conduct this study.

Experimental design and diets

A total of 160 broiler breeder males (Ross 308) at 52 weeks of age were used in the present study. Males were identified with a leg tag and randomly assigned to 4 treatment groups with four replications. The treatments were 1) males were fed a restricted feed (135 g/day) as recommended by the breeder company (Control-CONT); 2) males were fed *ad libitum* for a four-week experimental period (*Ad libitum*-ADL), 3) males were fed similar to the CONT group except that diet was supplemented with bee products (apilarnil 5 g/d/male and royal jelly 200 mg/d/male) (Bee products-BP); 4) calorie restriction was applied at about 45 % of the standard diet for a four-week experimental period (Calorie restriction-CR). After a four-week adaptation period, the experiment was continued for 18 weeks. Males were placed in floor pens and fed a standard diet (Table 1) except for the four-week experimental period.

1: Allzyme SSF, *Aspergillus niger* (CBS 114.94) amylase, cellulase, phytase, xylanase, beta-glucanase, pectinase, protease.

2: 2 kg mineral mixture, antioxidant, 125.000 mg; copper, 10.000 mg; calcium D pantothenat, 15.000 mg; zinc, 100.000 mg; D-Biotin, 250 mg; iron, 60.000 mg; folic acid, 2.000 mg; iodine, 2.000 mg; cobalt, 500 mg; manganese, 80.000 mg; niacin, 55.000 mg; selenium, 250 mg.

3: 1 kg vitamin mixture, retinol-acetate, 13.000.000

IU; thiamine, 3.000 mg; cyanocobolamin, 40 mg; riboflavin, 12.000 mg; pyridoxine, 4.500 mg; cholecalciferol, 3.000.000 IU; α-tocopherol acetate, 100.000 mg; menadione, 5.000 mg.

4: Toxin binder, probiyotic, vitamin D_3

Table 1: Nutrient composition and analysis of standard and calorie restriction diets (g/kg)

Ingredients	**Standard diet (g/kg)**
Corn	748.49
Full fat soybean	40.00
Soybean cake	12.42
Sunflower cake	148.76
Acid oil	10.00
Limestone	23.53
MCP-22.7	4.28
Sodium sulphate	1.21
Salt	2.40
DL-Methionine	0.89
Vitamin C	0.20
L-Lysine sulphate	0.32
Colin chloride liquid	0.50
Enzyme mixture[1]	0.20
Trace Mineral mixture[2]	0.50
Vitamin mixture[3]	2.00
Other additives[4]	4.80
Analysed composition	**(g/kg)**
Dry matter	875.70
Crude protein	133.40
Crude fat	50.00
Crude fibre	50.70
Crude ash	56.60
ME (MJ kg^{-1})	12.01

Sample collection and analysis

Blood samples for biochemical analysis were collected from 10 males of each treatment group at the 4th, 8th, and 18th weeks of the trial. Lipid peroxidation (LPO) was ascertained by the formation of malondialdehyde (MDA), which was estimated using the thiobarbituric acid (TBARS) method (30). Superoxide dismutase (SOD) activity was determined using the commercially available enzyme kit (Ransod, RANDOX/SD-125). Glutathione peroxidase (GSH-Px) activity was determined using a Ransel kit (RANDOX/RS-504). The Randox Uric Acid (UA) Enzymatic Colorimetric method kit was used for uric acid analysis. Total antioxidant capacity (TAC) was measured using an Abbott Architect Analyzer commercial kit (Abbott Lab. Illinois, USA).

Semen collection and evaluation

Semen samples were collected using the abdominal massage technique (at 2nd, 6th, 10th, and 16th weeks of trial) and evaluated in 20 minutes using Sperm Vision System (Minitüb Abfüll und Labortechnik Gmbh&Co.KG). Semen volume, motility (%), dead sperm (%), progressive motility (%) were determined in each semen sample.

Statistical analysis

The data were analysed using a one-way analysis of variance (ANOVA) with the General Linear Models (GLM) procedure of the SAS software (31). Significant differences between groups were determined by Student's t-test. Differences were considered to be significant at $P<0.05$ and the results were presented as the mean and standard error of the mean (SEM).

Results and discussion

The effects of dietary manipulations on semen quality parameters are presented in Table 2. As seen prior to treatments, there were no significant differences among groups for semen quality parameters. Dietary treatments caused dramatic changes in all semen parameters throughout the experimental period. At the 2nd week, BP supplementation positively affected semen quality. These males had the highest progressive sperm motility and semen volume. However, CR treatment adversely affected semen quality. Lower semen volume, lower sperm motility and higher dead sperm were observed in CR males compared to the other groups. After a four-week CR period, the detrimental effects of CR on semen production were gradually alleviated by increasing feed allocation.

Table 2: Semen quality characteristics in broiler breeder males during the experimental period (±SEM)

GROUP	Pretreatment				2 wk				6 wk				10 wk				16 wk				Pre vs Post-treatment			
	Semen volume (ml)	Motility (%)	Progressive sperm motility (%)	Dead sperm (%)	Semen volume (ml)	Motility (%)	Progressive sperm motility (%)	Dead sperm (%)	Semen volume (ml)	Motility (%)	Progressive sperm motility (%)	Dead sperm (%)	Semen volume (ml)	Motility (%)	Progressive sperm motility (%)	Dead sperm (%)	Semen volume (ml)	Motility (%)	Progressive sperm motility (%)	Dead sperm (%)	Semen volume	Motility	Progressive sperm motility	Dead sperm
CONTROL	0.23 ± 0.02	98.06 ± 0.71	87.62 ± 0.75	2.13 ± 0.34	0.27 ± 0.01^{c}	95.21 ± 0.47^{b}	88.21 ± 0.52^{b}	9.56 ± 0.92^{a}	0.25 ± 0.01^{b}	88.39 ± 0.70^{b}	84.69 ± 0.92^{b}	11.27 ± 1.07^{a}	0.18 ± 0.02^{b}	85.36 ± 0.86^{b}	78.25 ± 0.97^{c}	16.32 ± 1.83^{b}	0.18 ± 0.01^{c}	84.26 ± 0.88^{b}	80.39 ± 0.93^{c}	16.92 ± 1.48^{a}	NS	***	**	***
ADL	0.31 ± 0.04	98.12 ± 0.55	84.97 ± 1.89	2.07 ± 0.68	0.39 ± 0.02^{b}	98.56 ± 0.53^{a}	87.21 ± 0.59^{b}	2.06 ± 0.62^{b}	0.21 ± 0.02^{bc}	82.11 ± 1.39^{c}	69.32 ± 1.03^{d}	8.66 ± 1.55^{a}	0.25 ± 0.04^{b}	78.21 ± 0.83^{c}	60.10 ± 1.06^{d}	24.34 ± 1.49^{a}	0.33 ± 0.04^{b}	80.95 ± 2.21^{b}	72.69 ± 2.03^{d}	20.48 ± 2.43^{a}	NS	***	**	***
BP	0.30 ± 0.07	97.51 ± 0.73	84.41 ± 2.31	1.73 ± 0.36	0.46 ± 0.03^{a}	95.31 ± 0.54^{b}	91.27 ± 1.31^{a}	2.33 ± 0.50^{b}	0.72 ± 0.10^{a}	98.96 ± 0.16^{a}	94.32 ± 0.49^{a}	0.41 ± 0.11^{b}	0.61 ± 0.02^{a}	99.26 ± 0.11^{a}	93.85 ± 0.30^{a}	0.79 ± 0.13^{d}	0.43 ± 0.01^{a}	97.89 ± 0.12^{a}	93.00 ± 0.35^{a}	1.51 ± 0.21^{b}	NS	NS	**	NS
CR	0.36 ± 0.05	97.64 ± 0.74	87.23 ± 2.32	2.45 ± 0.29	0.11 ± 0.01^{d}	88.21 ± 0.72^{c}	81.51 ± 0.73^{c}	7.89 ± 0.42^{a}	0.07 ± 0.01^{c}	81.58 ± 0.43^{c}	80.63 ± 1.31^{c}	9.23 ± 0.73^{a}	0.27 ± 0.02^{b}	87.15 ± 0.68^{b}	84.53 ± 0.45^{b}	5.51 ± 0.48^{c}	0.39 ± 0.01^{ab}	96.25 ± 0.66^{a}	89.33 ± 0.91^{b}	4.62 ± 0.94^{b}	NS	NS	NS	*
X±SEM	0.30 ± 0.02	97.83 ± 0.32	86.06 ± 0.94	2.09 ± 0.21	0.31 ± 0.03	94.32 ± 0.90	87.05 ± 0.89	5.46 ± 0.81	0.31 ± 0.06	87.76 ± 1.64	82.24 ± 2.10	7.39 ± 1.05	0.33 ± 0.04	87.50 ± 1.76	79.18 ± 2.85	11.74 ± 2.18	0.33 ± 0.02	89.84 ± 1.77	83.85 ± 1.89	10.88 ± 1.96				
Probability	NS	NS	NS	NS	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***				

[abc]dMean values within the same column sharing a common superscript letter are not statistically different at P<0.05.*: P<0.05; **: P<0.01; ***: *: P<0.05; **: P<0.01; ***: P<0.0001; NS: Not Significant (P>0.05). ADL: *Ad libitum*; BP: Bee Products; CR: Calorie Restriction.

At the end of the experiment (16th week), all semen quality parameters of CR and BP males were significantly better than those of CONT and ADL. Semen volume was higher in ADL males than that of CONT, and there were significant differences in sperm motility and dead sperm between these groups.

The semen characteristics significantly changed throughout the experimental period (Table 2). The percentage dead sperm of ADL males markedly increased from 2.07 % at the beginning to 20.46 % on the 16th week of the experiment. Similarly, in CONT males, the percentage of dead sperm increased from 2.13 % to 16.92 % during the experimental period. At the end of the experiment, a 13-fold increase in percentage dead sperm of ADL males occurred in comparison with the BP males. It is noteworthy that dead sperm (%) was one of the semen characteristics most affected by age and dietary treatments.

At the end of the experiment, the ADL, BP, and CR males maintained semen volume similar to those observed at the beginning of the experiment while long-term restricted males (CONT) produced lower semen volume than the other groups did.

The results of the present study showed that sperm motility and progressive sperm motility significantly decreased while dead sperm dramatically increased in CONT males during the experimental period. These data imply that long-term feed restriction (135 g/d) recommended by the breeder company for males could not prevent age-related declines in semen production. Although during CR treatment period, all semen quality parameters were adversely affected, calorie restriction at 45 % of the standard diet for weeks retarded onset of age-related decline in semen quality. The effect of CR treatment observed in the present study was in agreement with previous studies reporting inadequate ME intake could be detrimental to semen production (4, 32, 33) and these detrimental effects could be revised by increasing male feed allocation (34, 35).

(36) suggested that there were no significant differences in sperm production, semen volume or sperm concentration between full-fed and feed restricted males. Moreover, they reported that while overfeeding might assist a male in semen production for the short-term, the long-term effects of being over-weight are negative ones. In agreement with these findings, we obtained no significant differences in sperm motility and dead sperm between ADL and CONT males, but semen volume was higher in ADL males.

In the BP group, progressive motile sperm significantly increased while there were no negative changes in the other semen characteristics at the end of the experiment compared to the pretreatment period, suggesting that age-related declines in semen production could be alleviated with dietary BP supplementation. Congruent with these results, it was reported that RJ administration caused an increase in sperm production, sperm motility, and higher testosterone levels in lab animals (37, 38, 39). (40) suggested that the androgenic effect of apilarnil on chickens was higher than its anabolic effect. Supporting this report, (41) obtained that apilarnil administration at an early age increased testicular weights and testosterone production and stimulated comb growth in broiler males. (42) also reported that RJ administration has a positive effect on libido, semen quality, sperm output, testosterone level, and fertility of heat-stressed male rabbits. Controversially, (43) suggested that high dose RJ (800 mg/kg) administration for four weeks adversely affected the reproductive system of pubescent male rats, but these detrimental effects are alleviated to some extend by the cessation of RJ.

The effects of dietary manipulation on some antioxidant activities and MDA levels are presented in Table 3. On the 4th week, dietary treatment significantly affected TAC and UA levels, but no significant effects were observed in MDA, SOD, and GSH-Px levels. The TAC and UA levels were significantly higher in the CR group than in others. At the end of the experiment, there were no significant differences in antioxidant capacity and MDA levels among the treatment groups.

Table 3: Some biochemical parameters of broiler breeder males during the experimental period (±SEM)

GROUP	UA (mg/dL)	TAC (mmol/L)	SOD (U/L)	GSH-Px (U/L)	MDA (umol/L)
			4 wk		
CONTROL	10.18±1.09[b]	0.76±0.00[b]	1.27±0.26	22983.17±1710.69	1.92±0.10
ADL	7.22±1.02[b]	0.78±0.14[b]	1.59±0.18	23553.67±2851.07	1.87±0.19
BP	10.28±1.24[b]	1.04±0.14[b]	1.62±0.24	28788.20±3675.19	1.72±0.24
CR	25.38±1.58[a]	1.62±0.07[a]	1.42±0.20	20808.80±3371.93	2.02±0.37
Probability	***	**	NS	NS	NS
			8 wk		
CONTROL	9.80±0.99	0.78±0.05	1.70±0.08	24845.67±2707.96ab	2.58±0.83
ADL	7.94±1.12	0.96±0.10	1.57±0.16	30443.83±2994.62[a]	2.62±0.49
BP	8.56±1.18	1.08±0.26	1.25±0.17	19736.00±910.62[b]	1.58±0.19
CR	8.98±0.99	0.97±0.12	1.41±0.21	18596.00±1869.43[b]	1.22±0.16
Probability	NS	NS	NS	**	NS
			18 wk		
CONTROL	7.30±0.50	0.74±0.07	1.16±0.38	28829.17±4625.01	1.88±0.17
ADL	8.14±0.84	0.89±0.11	1.64±0.21	28227.33±1525.50	2.37±0.65
BP	8.10±0.87	0.77±0.00	1.54±0.17	24122.00±2406.23	3.40±1.21
CR	8.33±1.08	0.81±0.08	1.66±0.15	29366.00±6130.38	1.14±0.21
Probability	NS	NS	NS	NS	NS

[ab]Meansvalues within the same column sharing a common superscript letter are not statistically different at P<0.05.
*: P<0.05; **: P<0.01; ***: P<0.0001; NS: Not Significant (P>0.05). ADL: *Ad libitum*; BP: Bee Products; CR: Calorie Restriction. UA: Uric Acid; TAC: Total Antioxidant Capacity; SOD: Superoxide Dismutase; GSH-Px: Glutathione Peroxidase; MDA: Malondialdehyde.

Overexpression of antioxidant enzymes has been considered to be a protective response to oxidative stress (44, 45, 46). Therefore, both increased antioxidant activity and no significant increase in MDA level of CR males may be interpreted as a protective response to LPO damage. Supporting our results, previous studies showed that in calorie-restricted animals, free radical generation and LPO decreased (47, 48) antioxidant defence capacity was enhanced (49), DNA damage decreased (50), and the rate of the ageing process was modulated by retarding many age-related physiological declines (51). (52) suggested that a-10 day fasting treatment can be used to decrease oxidative stress-mediated injury in aged hens without affecting the welfare of hens from previous fasting experiments.

After a four-week treatment period, it was observed no significant difference among treatment groups in MDA levels and antioxidant capacity except GSH-Px activity on the 8th week, suggesting that all groups had similar LPO responses and that there were no significant differences in possible age-related oxidative stress.

It is reported that RJ has high antioxidant activity and scavenging ability against free radical (20, 29, 39). However, in the present study, BP supplementation (royal jelly plus apilarnil) for a four-week period could not enhance the antioxidant capacity, indicating that the antioxidant properties of these products were probably not adequate to affect the oxidative status of males. Supporting this result, (53) reported that RJ and its bioactive component 10-HDA, did not scavenge any ROS; dietary RJ might have protective effects against tissue damage through other mechanisms other than ROS scavenging. There was no report focusing on the antioxidant effect of apilarnil in poultry; therefore, the present results could not be compared with other studies in the literature.

As a result, it was obtained that the ageing effect was most pronounced in the percentage of dead sperm, especially in males fed ad libitum and restricted as recommended by the breeder companies. Royal jelly plus apilarnil supplementation or calorie restriction for a

four-week period positively affected the semen characteristics of broiler breeder males at 56 weeks of age, and these beneficial effects could be maintained to some extent until 72 weeks of age. Supporting these results, (54) determined that antioxidant diet supplementation resulted in a higher percentage of normal sperm cells in male broiler breeders older than 50 weeks.

Calorie restriction has been enhanced antioxidant defence for the first four-week period, indicating a protective mechanism against oxidative stress. However, after the calorie restriction period, this positive effect could not be sustained. A four-week calorie restriction period may not be long enough to induce a long-lasting oxidative response; a longer time may be required to obtain long term antiaging and antioxidative effects.

In conclusion, it seems to be possible that dietary bee products supplementation or calorie restriction can be used to slow down the rate of the ageing process and extended reproductive life span of broiler breeder males by retarding an age-related decline in semen production. However, the long-term moderate feed restriction recommended by the breeder companies could not prevent age-related decline in semen quality parameters.

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The authors declare that they have no conflict of interest.

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VPLIV ČEBELJIH PRIDELKOV IN OMEJEVANJA KALORIJ NA PROIZVODNJO SEMENA IN OKSIDATIVNI STRES PRI STAREJŠIH SAMCIH PLEMENSKIH BROJLERJEV

Ç. Ş. Tuğalay, Ö. Altan

Povzetek: Študija je bila izvedena z namenom raziskovanja učinkov omejevanja kalorij in dodajanja prehranskih čebeljih pridelkov (apilarnil in matični mleček) na reprodukcijske in oksidativne odzive ter ugotoviti možnosti uporabe prehranskih dodatkov za zaviranje reproduktivnega staranja samcev plemenskih brojlerjev. Pri starosti 52 tednov so bili samci plemenskih brojlerjev razporejeni v štiri skupine. Kontrolna skupina je bila ves čas študije krmljena z restrikcijsko krmo po priporočilih podjetja, ki se ukvarja z gojenjem plemenskih broilerjev; skupina *ad libitum* je bila štiri tedne hranjena *ad libitum*; skupina, pri kateri so bili dodani čebelji pridelki je bila krmljena podobno kot kontrolna skupina, le da je bila njihova prehrana štiri tedne dopolnjevana z apilarnilom in matičnim mlečkom, zadnja skupina pa je štiri tedne dobivala kalorično omejeno hrano (45 % običajne prehrane). Po štiritedenskem prilagoditvenem obdobju se je poskus nadaljeval še 18 tednov. Rezultati, pridobljeni v tej študiji, so pokazali, da je bila najbolj prizadeta značilnost staranja povišan odstotek mrtvih semenčic v ejakulatu. Dolgoročna zmerna omejitev krme ni preprečila starostnega zmanjšanja proizvodnje smenčic. Dodatek prehranskih čebeljih pridelkov ali omejevanje kalorij v obdobju štirih tednov je pozitivno vplival na značilnosti semena. Ti blagodejni učinki so se ohranili vse do starosti do 72 tednov. Omejitev kalorij je okrepila tudi antioksidativno obrambo v prvih štirih tednih raziskave; vendar pa se je ta ugodni učinek kasneje izgubil.

Ključne besede: samci plemenskih brojlerjev; staranje; značilnosti semena; oksidativni stres; čebelji proizvodi; omejitev kalorij

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Original Research Article

CHEMICAL COMPOSITION OF TUALANG HONEY AND ITS EFFECT ON THE LUNG SURFACTANTS AND HISTOLOGY OF MALE RATS EXPOSED TO CIGARETTE SMOKE

Chinedum Eleazu[1,4], Aminah Che Romli[1], Wan Faiziah Wan Abdul Rahman[2], Zaida Zakaria[1], Zaidatul Akmal Othman[1,5], Mahaneem Mohamed[1,3]*

[1]Department of Physiology, [2]Department of Pathology, [3]Unit of Integrative Medicine, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia,[4]Department of Chemistry/Biochemistry and Molecular Biology, Alex Ekwueme Federal University Ndufu-Alike, Ikwo, Ebonyi State, Nigeria,[5]Unit of Physiology, Faculty of Medicine, Universiti Sultan Zainal Abidin, 20400, Kuala Terengganu, Terengganu, Malaysia

*Corresponding author, E-mail: maheneem@usm.my

Abstract: This study reported the chemical composition of Tualang honey and its effect on the lung surfactants and histology of male rats exposed to cigarette smoke. Thirty-two adult male Sprague-Dawley rats were used and they were randomly divided into 4 groups of eight rats each: control, honey-treated (Honey), cigarette smoke-exposed (CS) and honey-treated plus CS (Honey+CS). Rats in control and CS groups received distilled water (0.5 mL/day) while rats in Honey and Honey+CS groups received honey (1.2 g/kg body weight/day) by oral gavage. Furthermore, rats in CS and Honey+CS groups were exposed to CS in a chamber for 8 minutes (3 times/day). Exposure of rats to cigarette smoke significantly altered their phosphatidyl choline/phosphatidyl glycerol (PC/PG) ratio but not their surfactant protein A levels with increased number of alveolar macrophage containing carbon particles. Administration of Tualang honey to CS exposed rats resulted in modulation of most of these parameters and which results were coroborrated by histology. The colour intenstity, pH and moisture content of the honey were obtained as 272.75 mili-absorbance unit, 3.43 and 17.38%, respectively. Screening for phenolic compounds in Tualang honey using High Performance Thin Layer Chromatography showed the presence of 18 compounds while only five were identified which possessed strong in vitro antioxidant capacity as seen from their 2,2,diphenyl-1-picryl hydrazyl radical scavenging ability. The study showed the promising potentials of Tualung honey in protecting lung surfactants from the deleterious action of cigarette smoke which may be associated with its antioxidant phenolic compounds.

Key words: tualang honey; surfactant; inhalation toxicology; phenolic compounds

Introduction

Lung surfactant is a mixture of approximately 90 % of phospholipids and 10 % proteins (Sulfactant Proteins-A, B, C, and D) that are seen in the epithelial lining fluid in the internal surface of the lung alveoli (1, 2). Synthesis and release of these surfactants are important functions of type II pneumocytes (3). The normal production of surfactant is necessary to maintain alveolar stability and lung function (4) including protection of the type II pneumocyte against oxidants (5). Therefore, the absence of lung surfactants results in the development of Respiratory Distress Syndrome, which is characterized by collapse of the alveoli and reduced gas exchange and an increased effort to breathe (2).

Cigarette smoking is one of the most pervasive habits that is practiced by several people in different countries of the world and which has been assoiated with several health risks such as

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lung cancers, heart disease, asthma, tuberculosis and others (2, 6, 7, 8). Previous reports have it that there are numerous cigarette smokers all over the world and cigarette smoking was reported to account for over 5 million deaths yearly world wide within the last decade (9). Furthermore, the chronic and insidious nature of smoking-related diseases can reduce the quality of life (10). It has been reported that tobacco smoke from cigarette interacts with the epithelial lining fluid (that contains antioxidants and lung surfactant) (2, 11, 12, 13). Through such interactions, the oxidants in tobacco smoke release free radicals and reactive oxygen species which can denature or degrade the components of the epithelial lining fluid, one of which is the lung surfactant. Furthermore, being that the epithelial lining fluid is thinner in the lower airways and alveoli, and contains lower concentration of antioxidants than the upper airways, damage from oxidants such as tobacco smoke also negatively affects the lung surfactants (2, 15).

Honey is a natural product that is produced by honeybees (*Apis mellifera*) from the nectar of flowers or from the secretions of living parts of plants (15, 16). Several health beneficial properties have been credited to it such as: antidiabetic (17, 18), anti-inflammatory (19), antibacterial (20), antioxidant (21, 22) and anticancer properties (23). In our previous study (22), we reported the protective effect of honey in cigarette smoke-induced testicular damage in rats. However, the action of honey on lung surfactants exposed to cigarette smoke has not been reported in humans or animals. This therefore led to this study which aimed at investigating the chemical composition of Tualang honey and its effects on the lung surfactant profiles and lung histology of male rats exposed to cigarette smoke.

Materials and methods

Animal study

Thirty-two adult male Sprague-Dawley rats weighing 270-320 g were used for this study and they were obtained from the Laboratory Animal Research Unit, Health Campus, Universiti Sains Malaysia and as such, permissions were not needed for the sampling. The study protocol was approved by the Animal Ethics Committee of the Universiti Sains Malaysia (PPSG/07(A)/044/2007[32]) and the animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals by National Institute of Health. The rats were kept in a polycarbonate cage, and were maintained on a 12-h light/dark cycle at 20-24°C. Following acclamitization to their diet and water which they had access to *ad libitum*, they were randomly divided into 4 groups of eight rats each. Group I served as the control (C). Group 2 served as the honey group (Honey); Group 3 served as the negative control or cigarette smoke-exposed (CS) while Group 4 was the treatment group or honey-treated plus CS-exposed (Honey+CS).

Rats in control and CS groups received distilled water (0.5 mL/day) while rats in Honey and Honey+CS groups received honey (1.2 g/kg body weight/day) by oral gavage with distilled water as the carrier. Rats in CS and Honey+CS groups were exposed to cigarette smoke in a chamber for 8 minutes (3 times/day) (22). Water and food were given to the rats *ad libitum*. The study was approved by the animal ethical committee of the Universiti Sains Malaysia and the animals were handled in line with the Guide for the Care and Use of Laboratory Animals as given by the National Institute of Health. Following 13 weeks of treatment, the rats were anesthetized with sodium thiopental and euthanized. Their lungs were harvested and a portion was used for histology study. For each of the remaining portions, the trachea was tied to a syringe containing 10 ml 0.9 % NaCl, sucked out after 1 min, transferred to a bottle and infused with 10 ml 0.9 % NaCl. Thereafter, it was sucked out again and the bronchoalveolar lavage fluid (BALF) was transferred to another bottle and stored at -80^{o}C until it was analyzed for the lung surfactants-phosphatidyl choline, phosphatidyl glycerol and phosphatidyl choline/phosphatidyl glycerol ratio (using Thin Layer Chromatography and Malachite Green assay kit), surfactant protein A levels respectively using (ELISA kit).

Histology

A section of the lungs from each of the rats was cut, fixed in 10 % formalin and histology assays were carried out following the method of Dunn (24) and Parveen et al (25) respectively. For hematoxylin and eosin (H&E) staining, 5-µm-cut sections of the lungs were stained in hematoxylin for 5 min and washed with water. Thereafter,

they were stained in eosin for 2 min, dehydrated with alcohol, passed through xylene 1 and 2 and mounted for viewing under a microscope. The average number of alveolar macrophages containing carbon particles per field at 40x magnification was analysed. A total of 5 fields per slide for each rat was assessed and counted in a blinded fashion.

Physical analysis of Tualang honey
Evaluation of colour intensity

Tualang honey was diluted to 50 % (w/v) with warm distilled water (40-50°C) and vortex-mixed for 5 min. Thereafter, it was filtered (0.45 µm pore size) to eliminate large particles. Colour intensity of the honey was calculated as the difference between spectrophotometric absorbance at 450 and 720 nm respectively. This measurement was done in triplicate and the colour intensity was expressed as mili-absorbance unit (mAU) (26).

Evaluation of pH

Ten grams of honey were dissolved in 75 mL of deionized water in a beaker. Thereafter, the pH of the honey solution was measured using a pH meter (27).

Determination of the moisture content

Briefly, a weighed amount of honey sample was dried in an oven at 105°C for 3 hrs or until a constant weight was achieved. Samples were analysed in triplicate and the moisture content was calculated as a percentage.

Preparation of honey extract for screening for phenolic compounds

Natural phenolic compounds that are present in the honey were extracted using ethyl acetate to separate them from dominant sugars as reported earlier with some modifications (28, 29). Briefly, 10 g of honey was dissolved in 10 mL of deionized water. Then 10 mL of ethyl acetate was added and the setup was mixed well in a rotary shaker for 10 min. The solution was centrifuged at 2500 rpm at 4°C for 10 min and the supernatant was transferred into a tube. This ethyl acetate extraction procedure was repeated twice. The extracts were combined and dried under a nitrogen flow at room temperature. The dried extract was then dissolved in 0.4 mL of methanol and screened for the phenolic profile using high performance thin layer chromatography (HPTLC).

Screening for the presence of phenolic compounds:

The screening for the presence of phenolic compounds was performed twice and divided into two phases: phase 1 was without the standards whereas phase 2 was with the standards.

Phase 1

Briefly, 2 µL of honey extract was spotted on a thin layer chromatography (TLC) plate (10 cm x 10 cm) pre-coated with silica gel at the starting position. The phenolic compounds in the honey extract migrated through this thin sorbent layer (stationary phase). The solvent mixtures, n-hexane/ethyl acetate (4:6, v/v) were used as the mobile phase to separate these compounds. This mobile phase solvent ratio was selected as it could produce the highest number of separated compounds on the TLC plate after optimization was carried out using different ratios of solvents. The movement of the compounds depends on their different affinities to these two phases (30). The phenolic compounds were detected as coloured spots after spraying the plates with 1 % 2-aminoethyl diphenylborinate solution (dissolved in methanol). This reagent reacts with phenolic compounds which became visible as blue, green, yellow and orange fluorescent spots or bands. After 5 min, the plate was visualized under ultraviolet (UV) light at 366 nm using a TLC visualizer (29).

Another chromatogram was prepared and sprayed with 0.02 % 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical solution (prepared in ethanol) to assess the *in vitro* antioxidant property of the compounds. The DPPH radical reacts with the compounds that have antioxidant activity. In this reaction, the purple-coloured DPPH radical turned into yellow-coloured DPPH spots. After an hour, the chromatogram was examined under white light (29).

Phase 2

In this phase, 2 µL of honey extract and 1 µL of phenolic standards such as benzoic acid,

caffeicacid, gallic acid, luteolin, *trans*-cinnamic acid and vanillic acid (1 mg mL^{-1} in methanol) were spotted on a TLC plate (10 cm x 10 cm) pre-coated with silica gel. Another plate was also prepared with honey extract and phenolic standards (1 mg mL^{-1} in methanol) such as catechin, hesperitin, kaempferol, naringenin, naringin, *p*-coumaric acid and syringic acid. The plates were developed, sprayed with 1 % 2-aminoethyl diphenylborinate solution and visualized under UV light at 366 nm.

Statistical Analysis

Statistical analysis using SPSS version 24 was carried out. Data are expressed as mean ± SEM. One-way analysis of variance (ANOVA) was used for data analysis, followed by Tukey post-hoc test. *P* value < 0.05 was considered statically significant.

Results

Table 1 shows the results of the physical properties of the Tualang honey that was analyzed in this study. As shown in the Table, the colour intensity of the Tualang honey was obtained as 272.75 mAU; the pH was obtained as 3.43 while the moisture content was obtained as 17.38%.

Table 1: Physical properties of Tualang honey

Parameters	Tualang honey	Criteria for quality honey*
Colour intensity (mAU)	272.75(1.50)	-
pH	3.43(0.00)	-
Moisture (%)	17.38(0.44)	< 20

Data are expressed as mean (SEM), n=3. mAU, mili-absorbance unit; *Codex Alimentarius Commision (35)

Figure 1A shows the profile of the chromatogram of the Tualang honey extract (Phase 1 screening) after it was sprayed with 2-aminoethyl diphenylborinate and viewed under UV light at 366 nm. The phenolic compounds in the extract presented as blue, green, yellow and orange bands. A total of 18 coloured bands were seen in the chromatogram which indicates the presence of 18 unidentified phenolic compounds in the extract.

Figure 1B shows the profile of the chromatogram of Tualang honey extract after it was sprayed with DPPH radical and viewed under white light. The presence of compounds that have antioxidant activity became visible as yellow bands.

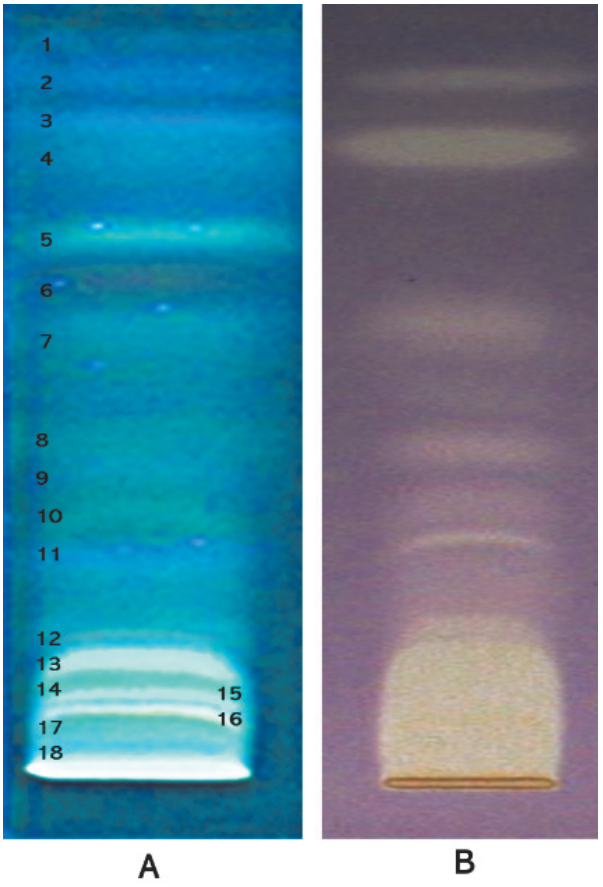

Figure 1: Chromatogram of Tualang honey extract when sprayed with 2-aminoethyl diphenylborinate and viewed under UV light 366 nm (A) and when sprayed with DPPH radical and viewed under white light (B). Chromatogram A shows the presence of 18 unidentified phenolic compounds which became visible as blue, green, yellow and orange bands. Chromatogram B shows the presence of compounds that have antioxidant activity which became visible as yellow bands

Figure 2 shows the findings on the screening of the phenolic compounds in the Tualang honey extract (Phase 2 screening). The chromatogram of Tualang honey extract and the phenolic standards, when they were sprayed with 2-aminoethyl diphenylborinate and viewed under UV light at 366 nm, showed the presence of five identified phenolic compounds namely: caffeic acid, gallic acid, luteolin, kaempferol and *p*-coumaric acid in the honey extract.

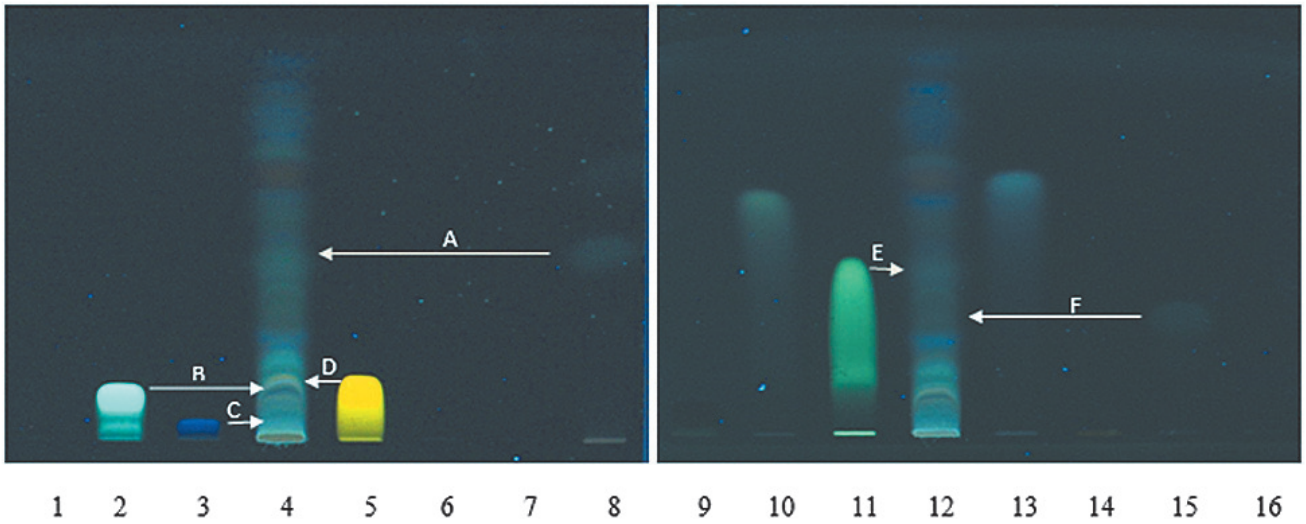

Figure 2: Chromatogram of Tualang honey extract and standards (tracks 1 – 16) when sprayed with 2-aminoethyl diphenylborinate and viewed under UV light 366 nm. Arrows show the presence of 5-hydroxymethyl-2-furfural (HMF; A) and phenolic compounds such as caffeic acid (B), gallic acid (C), luteolin (D), kaempferol (E) and p-coumaric acid (F) in the honey extract. 1, benzoic acid; 2, caffeic acid; 3, gallic acids; 4 and 12, Tualang honey extract; 5, luteolin; 6, trans-cinnamic acid; 7, vanillic acid; 8, HMF; 9, catechin; 10, hesperitin; 11, kaempferol; 13, naringenin; 14, naringin 15, p-coumaric acid; and 16, syringic acid

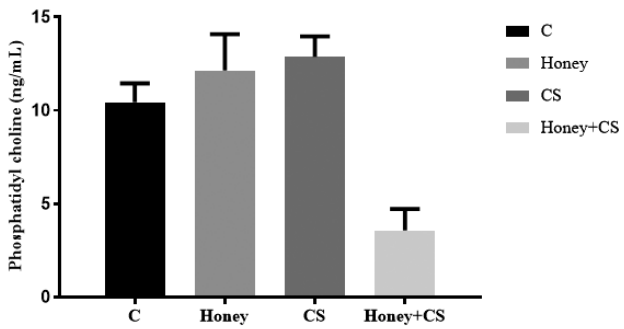

Figure 3: Phosphatidyl choline concentrations of rats. Data are expressed as mean ± SEM. n=8/group. No significant difference were found among all the groups (One-way ANOVA). C: Control, CS: Cigarette smoke

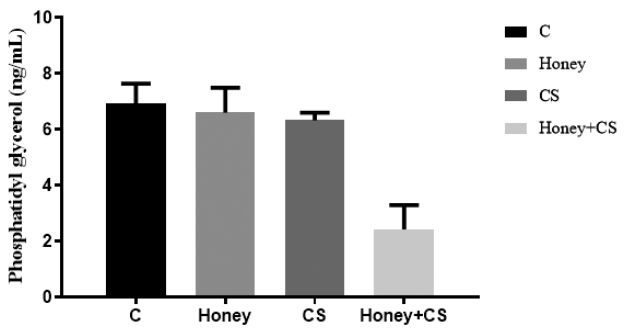

Figure 4: Phosphatidyl glycerol concentrations of rats. Data are expressed as mean ± SEM. n=8/group. No significant difference were found among all the groups (One-way ANOVA). C: Control, CS: Cigarette smoke

Figure 3 presents the phosphatidyl choline concentrations of the rats that were investigated. As shown in the figure, there were no significant differences (P>0.05) in the phosphatidyl choline concentrations of all the rats in the respective groups.

The phosphatidyl glycerol concentrations of the rats that were studied are shown in Figure 4. As shown in the figure, there were also no significant differences (P>0.05) in the phosphatidyl glycerol concentrations of all the rats in the respective groups.

Figure 5 shows the phosphatidylcholine/phosphatidylglycerol ratio of the rats that were investigated in this study. As shown in the Figure, the PC/PG ratio of the normal rats administerd honey did not differ significantly (P>0.05) from that of the control. In contrast, the PC/PG ratio of the rats in the CS group was significantly higher (P<0.05) than the control group. However, administration of Tualang honey to the rats exposed to cigarette smoke resulted in decreased (P<0.05) levels of their PC/PG ratio compared to the CS group.

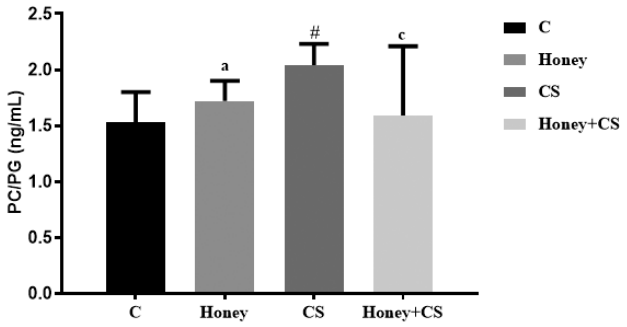

Figure 5: PC/PG ratio of rats exposed to cigarette smoke. n=8/group; [a]P>0.05 as compared to C group; [#]P<0.05 as compared to C group; [c]P<0.05 as compared to CS group (One-way ANOVA followed by Tukey's post hoc test). C: Control, CS: Cigarette smoke, PC: Phosphatidylcholine, PG: Phosphatidylglycerol

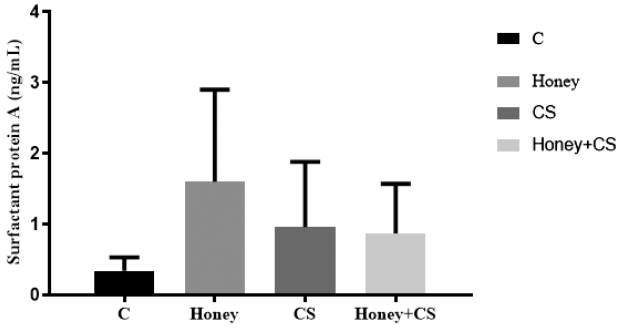

Figure 6: Surfactant Protein A concentrations of rats. Data are expressed as mean ± SEM. n=8/group. No significant difference were found among all the groups (One-way ANOVA). C: Control, CS: Cigarette smoke

Figure 6 shows the surfactant protein A concentrations of the rats that were studied. As presented in the figure, there were no significant differences (*P*>0.05) in the surfactant protein A concentrations of all the rats in the respective groups.

Figure 7 shows the histology of the lungs of the rats that were investigated in this study while the quantitative data that were collected on the number of alveolar macrophages containing carbon particles are presented in Table 2. As seen in the figure presented, the C group had no visible alveolar macrophages containing carbon particles (Figure 7[A]). Similarly, histology of the lungs of the normal rats administered Tualang honey showed no visible alveolar macrophages containing carbon particles (Figure 7[B]). These findings were corroborated with the quantitative data in Table 2. In contrast, histology of the lungs of the CS group revealed the presence of many alveolar macrophages containing carbon particles (Figure 7[C]) compared to C, Honey and Honey+CS groups (Figure 7[D]). Quantitative data showed significant increased number of alveolar macrophages containing carbon particles in CS group compared to C and Honey groups. However, the number of alveolar macrophages containing carbon particles was significantly decreased in the cigarette smoke exposed rats co-administered with Tualang honey (Honey+CS group) compared to CS group although it was significantly increased compared to C and Honey groups (Table 2).

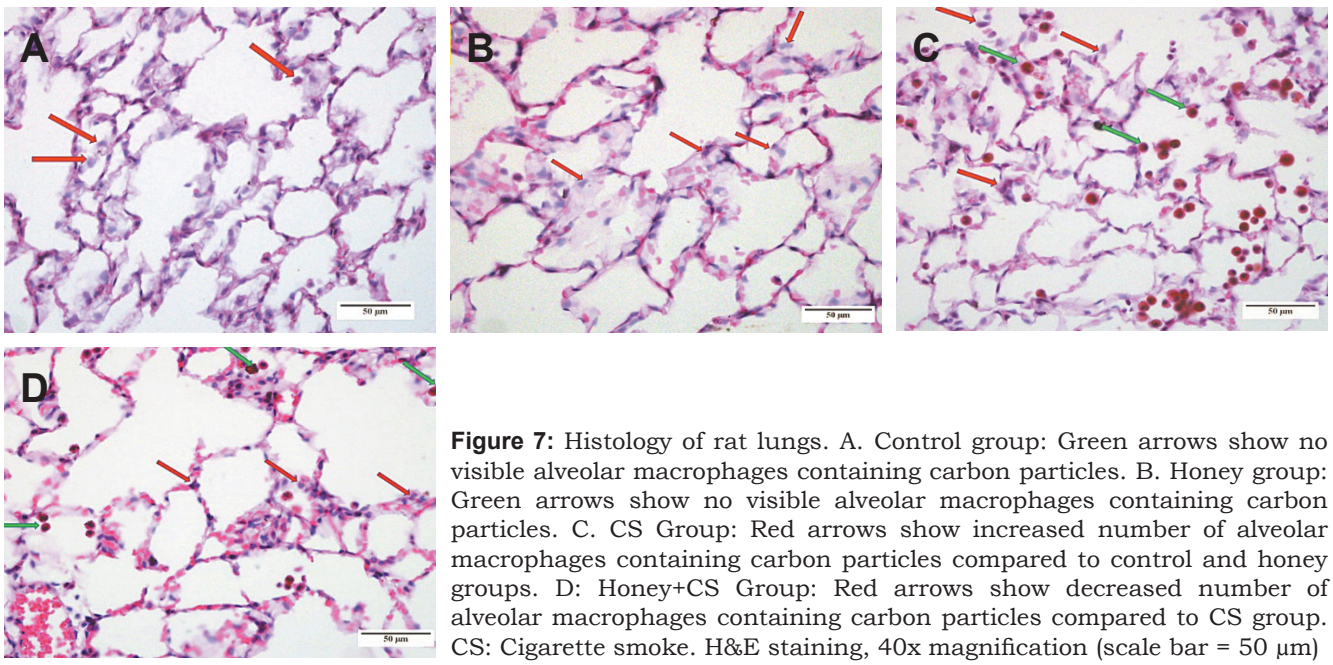

Figure 7: Histology of rat lungs. A. Control group: Green arrows show no visible alveolar macrophages containing carbon particles. B. Honey group: Green arrows show no visible alveolar macrophages containing carbon particles. C. CS Group: Red arrows show increased number of alveolar macrophages containing carbon particles compared to control and honey groups. D: Honey+CS Group: Red arrows show decreased number of alveolar macrophages containing carbon particles compared to CS group. CS: Cigarette smoke. H&E staining, 40x magnification (scale bar = 50 µm)

Table 2: Number of alveolar macrophages containing carbon particles

Groups	Number of alveolar macrophages containing carbon particles
C	0.00±0.00
Honey	0.00±0.00
CS	16.97±0.88[a,b]
Honey+CS	5.83±0.30[a,b,c]

Data are expressed as mean ± SEM. [a]$P < 0.05$ as compared to C group, [b]$P<0.05$ as compared to Honey group, [c]$P<0.05$ as compared CS group (One-way ANOVA followed by Tukey's post hoc test). C: Control, CS: Cigarette smoke

Discussion

Colour intensity of honey can help in classifying different types of honey and it gives an idea about its quality. In fact, colour intensity of honey was previously reported (30) to correlate with its total phenolic contents.

The values we obtained in this study for the colour intensity of the Tualang honey was similar to the values that were reported for Chicory honey (26), suggesing that our Tualang honey may be rich in phenolic constituents.

Honey has been reported to have an acidic pH which helps it in promoting wound healing and also inhibiting the growth of microorganisms (32). The value that was obtained for the pH of Tualang honey was consistent with the values that were reported for floral or blossom honeys (27) and honeys from other countries (33, 34, 35). The moisture content of honey is one of the criteria that determines its shelf life and ability to resist spoilage by microorganisms (32). The Codex Alimentarius Commission (36) gave a standard for water in good and natural honeys (Table 1). Going by this, data that were obtained for the moisture content of Tualang honey make the Tualang honey analyzed to be good honey apart from other properties of this Tualang honey (such as glucose, fructose, maltose, sucrose and 5-hydroxymethyl-2-furfural) that also fell within the standard that was given by the Codex Alimentarius Commission (36) as previously reported (22).

Phenolic compounds have been suggested to be the main antioxidants that contribute to the antioxidant properties of honey (26) and this informed our screening and separation of the phenolic compounds in Tualang honey using HPTLC. Most of the phenolic compounds that appeared as coloured bands (in Phase 1 screening) were found to scavenge DPPH radical as seen from the yellow bands of the chromatogram (when it was sprayed with DPPH radical), indicating the antioxidant properties of the phenolic compounds in Tualang honey. This further affirms our previous reports on the antioxidant properties of Tualang honey (22).

The number of phenolic compounds in Tualang honey that were separated and identified by HPTLC was higher as compared to Buckwheat and Chilean honeys which were reported to have 4 unknown phenolic compounds (29). This difference could be due to the different floral sources of honeys as floral source is one of the important factors that affect the composition of honey (37). The other plausible reason might be due to the different solvent system that was used as mobile phase to separate those compounds. The movement of the compounds depends on their different affinities to the stationary and mobile phases. In the present study, a solvent, n-hexane/ethyl acetate (4:6, v/v) was used to separate the compounds in Tualang honey while another solvent, acetonitrile/water (87:13, v/v) was used to separate the compounds in Buckwheat and Chilean honeys (29). This study confirmed the presence of phenolic compounds in Tualang honey and this chromatogram could be used as HPTLC fingerprint of this Tualang honey for identification and comparison with other types of honeys.

In this study, phospholipids were measured by the phosphatidyl choline and phosphatidy glycerol concentrations of the rats as the later form the hydrophilic and hydrophobic constituents of phospholipids that in turn act as the major components of lung surfactants. As seen in this study, the phosphatidylcholine and phosphatidyl glycerol concentrations of the normal rats administered Tualang honey did not differ from that of the control, suggesting that the administered honey had no impact on the phosphatidylcholine and phosphatidyl glycerol concentrations of the rats. Furthermore, exposure to cigarette smoke appeared not to affect the phosphatidylcholine and phosphatidyl glycerol concentrations of the rats as seen from the non significant changes in the phosphatidyl choline and phosphatidyl glycerol concentrations of the rats exposed to cigarette smoke when compared to the control.

When the phosphatidylcholine/phosphatidylglycerol ratio of the rats was determined, Tualang honey did not also significantly affect this ratio, indicating that it did not negatively impact on the distribution of these phospholipids in the lungs of these groups of rats. On the contrary, exposure to cigarette smoke was found to alter the PC/PG ratio of the rats. Its noteworthy that these phospholipids form essential components of biological membranes and their alteration could lead to increased membrane permeability and tissue damage. Studies have also shown that cigarette smoke contains some free radicals and the soot that arises from cigarette smoke attracts neutrophils to the site which releases more free radicals, leading to peroxidation of these lipids, all of which can result to lung damage (38). Cigarette smoke possibly interferred with the distribution of the phospholipids in the membranes surrounding the lungs of the rats that were exposed to it which led to the increased PC/PG ratio of these rats. Tualang honey demonstrated the ability to protect lung surfactants and biological membranes surrounding the lungs from toxicity of cigarette smoke as seen from the normalization of the PC/PG ratio of the cigarette smoke exposed rats co-administered this honey.

To futher investigate the effect of Tualang honey on lung surfactants exposed to cigarette smoke, surfactant protein A concentration of the rats exposed to cigarette smoke and the effect of administration of Tualang honey was measured. The non significant changes in the surfactant protein A concentrations of the rats in the respective groups as obtained in this study, suggests that exposure to cigarette smoke may not have had any negative effect on the surfactant protein A concentrations of these rats. The implication is that the potential targets for cigarette smoke in the lungs are the distribution of phospholipids.

To further confirm the effect of exposure of lung surfactants to cigarette smoke and the protective action of Tualang honey, a histological assay was performed. Findings from this study showed a reduced number of type II pneumocytes but increased number of alveolar macrophages in the cigarette smoke group. It is possible that the reduced number of type II pneumocytes but increased number of alveolar macrophages in the cigarette smoke group may have exercabated or enhanced the alteration in the PC/PG ratio of their lung surfactants. We therefore postulate that cigarette smoke exerts its deletrious action on the lungs by altering the PC/PG ratio, decreasing the Type II pneumocytes levels and increasing alveolar macrophage levels.

The histology of the lungs of the cigarette smoke exposed rats treated with Tualang honey as seen in this study was found to be similar to the control group with reduced number of alveolar macrophage as compared to cigarette smoke group. These findings which corroborated the results of other assays we performed, therefore indicate the promising potentials of Tualung honey in protecting lung surfactants from the deleterious action of cigarette smoke which may be associated with its antioxidant phenolic compounds as these phenolic compounds have been reported to protect the lungs from cigarette smoke associated lung injury (39).

This study revealed that honey has some protective effects against the cigarette smoke-induced changes in lung surfactant profiles and pneumocytes in male rats.

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KEMIČNA SESTAVA MEDU TUALANG IN NJEGOV VPLIV NA POVRŠINSKO AKTIVNE SNOVI V PLJUČIH TER HISTOLOGIJO PLJUČ SAMCEV PODGAN, IZPOSTAVLJENIH CIGARETNEM DIMU

C. Eleazu, A. C. Romli, W. F. W. A. Rahman, Z. Zakaria, Z. A. Othman, M. Mohamed

Povzetek: Raziskava poroča o kemijski sestavi medu Tualang in njegovem vplivu na površinsko aktivne snovi v pljučih ter histologijo pljuč samcev podgan, izpostavljenih cigaretnemu dimu. V študiji so uporabili 32 odraslih podgajih samcev seva Sprague-Dawley, ki so bili naključno razdeljeni v 4 skupine po osem podgan: kontrolna skupina, skupina, ki je uživala med s prehrano (Honey), skupina, ki je bila izpostavljena cigaretnemu dimu (CS) in skupina, ki je bila izpostavljena cigaretnemu dimu ter je uživala med (Honey+CS). Podgane v kontrolnih skupinah in skupinah CS so dobile destilirano vodo (0,5 ml/dan), podgane v skupinah Honey in Honey + CS pa peroralno med (1,2 g/kg telesne teže/dan). Poleg tega so bile podgane v skupinah CS in Honey + CS izpostavljene v komori cigaretnemu dimu trikrat na dan po 8 minut. Izpostavljenost podgan cigaretnemu dimu je bistveno spremenila razmerje fosfatidilholina/fosfatidilglicerola (PC/PG), ne pa tudi ravni površinsko aktivne snovi A v pljučih in ni vplivala na število alveolarnih makrofagov, ki vsebujejo ogljikove delce. Uporaba medu Tualang pri podganah, izpostavljenih cigaretnemu dimu, je povzročila spremembo večine opazovanih parametrov, katerih rezultati so bili potrjeni s histološko preiskavo. Intenzivnost barve medu je bila 272,75 enote mili-absorbance, pH 3,43, vsebnost vlage v medu pa je bila 17,38 %. Presejalni test na fenolne spojin v medu Tualang s tankoplastno kromatografijo je pokazal prisotnost 18 spojin, od katerih jih je bilo 5 prepoznanih kot spojine z močno in vitro antioksidativno sposobnostjo, kot je razvidno iz njihove sposobnosti odstranjevanja radikala 2,2-difenil-1-pikril hidrazila. Študija je pokazala obetavne potenciale medu Tualung pri zaščiti pljučnih površinsko aktivnih snovi pred škodljivim delovanjem cigaretnega dima, kar je lahko povezano z njegovimi antioksidativnimi fenolnimi spojinami.

Ključne besede: med tualang; surfaktant; inhalacijska toksikologija; fenoli

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Original Research Article

THE EFFICACY OF VACCINATION OF LAYER CHICKENS WITH INACTIVATED FOWL CHOLERA BACTERIN PREPARED FROM LOCAL EGYPTIAN STRAINS OF *Pasteurella multocida*

Wafaa Abd El-Ghany Abd El-Ghany[1]*, Hanan Ali Ahmed[2], Ali Zaher Qandoos[1], Mohamed Abd El-Rahman Bosila[3]

[1]Faculty of Veterinary Medicine, Cairo University, Cairo, [2]Central Laboratory for Evaluation of Veterinary Biologics, Cairo, [3]National Research Center, Cairo, Egypt

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

Abstract: This study was carried out to evaluate the efficacy of vaccination of layer chickens with inactivated FC bacterin prepared from local Egyptian strains of *Pasteurella multocida* (*P. multocida).* A total of 200 layer chickens were divided into 5 equal groups, 40 for each. At the age of 6 weeks, chickens in groups (A) and (B) were vaccinated with *P. multocida* serotypes A:1 and A:3, respectively, booster doses were given after 3 weeks (9 weeks old) and challenge was done with virulent serotypes A:1 and A:3 at 2 weeks later (11 weeks old). Chickens in groups (C) and (D) were not vaccinated, only challenged with *P. multocida* serotype A:1 and A:3, respectively. Birds in group (E) were kept as non-vaccinated and non-challenged. Blood samples were collected weekly from all groups for humoral immune response. All the birds were kept under observation for signs, mortalities, lesions and re-isolation of challenging organism and for histopathological examination. Results of the mean Enzyme Linked Immuno-Sorbent Assay (ELISA) revealed that the highest level was at 5 weeks post vaccination as the titers reached to 3970 in group (A) and 3905 in group (B). The clinical signs, mortality rate and lesions were mild in the vaccinated birds while severe lesions were in non-vaccinated and challenged birds. The protection rates were 85 % and 80 % in groups (A) and (B); respectively, while 10 % and 20 % in groups (C) and (D); respectively. The re-isolation rates of *P. multocida* after challenge were 95 % and 90 % in non-vaccinated-challenged birds with *P. multocida* serotypes A:1 and A:3; respectively, while they were 25 % and 15 % in vaccinated-challenged groups with *P. multocida* serotypes A:1 and A:3; respectively. Histopathological examination of *P. multocida* vaccinated-challenged birds revealed mild to no microscopic lesions when compared with non-vaccinated challenged chickens. In conclusion, the prepared FC inactivated bacterin from the local Egyptian predominant *P. multocida* serovars proved efficacy and protection of layer chickens.

Key words: *Pasteurella multocida;* chickens; immunization; protection; Egypt

Introduction

Fowl cholera (FC) is a bacterial disease of domestic and wild birds that causes high economic losses including; deaths, weight losses and condemnations (1-3). Mortalities related to FC infection in layer chickens may range from few percentages up to 100 % (4). The incidence of FC is more common in mature layer chickens than young broilers because of age factors (5, 6). Infection with FC is caused by *Pasteurella multocida* (*P. multocida*) micro-organism (7). *P. multocida* is considered as Gram-negative coccobacilli, non-motile and non-spore former and capsulated organism (8).

Vaccination against FC is considered as one of the most important worldwide strategy to decrease the incidence of infection (9, 10). Globally, living and inactivated (bacterin) vaccines are being used to immunize birds against FC (4, 11). Living attenuated vaccines have advantages regarding good protection with long immunity as well as cross-protection against *P. multocida* of different serotypes or surface lipopolysaccharide (LPS) structures (12). However, living attenuated FC vaccines may lack of maintainable and sustainable attenuation methods and/or instability which may

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lead to risk of returning their virulence. Therefore, inactivated FC bacterins are widely used as there is no chance of the vaccines reverted to virulence and cause the disease (13). In this regard, inactivated FC bacterins have comparative advantages over the living vaccines and are thus favored to protect chickens against infection caused by homologous *P. multocida* strains (14).

Bacterins used for prevention of FC provide homologous but not heterologous protection (15). Immunogenic local strains of *P. multocida* should be selected as the ideal strains to prepare effective bacterin (16). There are 16 different serovars of *P. multocida* and the most common types associated with FC outbreaks are serovars 1, 3 and 4. Evaluation and quality control of the efficacy of locally prepared FC bacterin are based mainly on inactivation of *P. multocida* serovars, followed by vaccination and challenge test by which the protective indices are measured.

According to FAO report, Egyptian poultry production systems are varying from rural very small-scale, extensive poultry production to highly intensive systems with over 70,000 birds per house in industrial commercial systems. The meat production or broiler sector has a pyramid structure; with grandparents at the top of the pyramid, the broilers at the bottom and the broiler breeders in between the two. Egypt is 100 % self-sufficient from table eggs as it produces nearly 13 billion commercial table eggs annually.

Some studies have been conducted in Egypt considering the situation of *P. multocida* infection in layer, breeder and broiler chicken flocks. Researches indicated that the most common circulating *P. multocida* serotypes that circulating in Egyptian flocks are types A:1 and A:3 causing severe economic losses in back yard as well as layer and breeder chicken flocks (17-20).

There is available commercial inactivated oil adjuvant bacterin used for vaccination of Egyptian layer and breeder chicken flocks against FC. This bacterin was prepared from local *P. multocida* serotypes A:1 and A:3 strains and used at age of 8-10 weeks and boostered at 16-17 weeks. However, the bacterin doesn't confer complete protection of flocks against *P. multocida* infection. Therefore, from time to another, trials have been done to prepare and test the efficacy of using locally prepared FC bacterin from the predominant circulating *P. multocida* serovars in chickens (21-23).

Accordingly, this work was designed to evaluate the efficacy of vaccination of layer chickens with inactivated FC bacterin prepared from local Egyptian strains of *P. multocida*.

Materials and methods

Experimental design:

A total of 200, day-old layer chickens was obtained from local hatcheries and reared on thoroughly cleaned and disinfected semi closed houses for 13 weeks. Birds were vaccinated using standard protocol for vaccination. Feed and water was given *ad libitum*. At 6 weeks old chickens, birds were divided into 5 equal groups 40 for each. Groups (A) and (B) were vaccinated with 0.5 ml I/M with *P. multocida* serotypes A:1 and A:3; respectively, booster doses were given after 3 weeks (9 weeks old), and challenge was done orally with virulent serotypes A:1 and A:3 at 2 weeks later (11 weeks old). Chickens in groups (C) and (D) were not vaccinated and challenged with *P. multocida* serotype A:1 and A:3; respectively. Birds in group (E) were kept as non-vaccinated and non-challenged. The experiment was done according to the National Guidelines and Regulations on Animal Welfare and Institutional Animal Ethical Committee (IAEC) of Cairo University with approval number (CU II F 100 18).

Preparation of inactivated bacterin from the predominant P. multocida local strains

Local *P. multocida* strains serotypes (A:1 and A:3) that were isolated from different Egyptian governorates were used. The used *P. multocida* strains were previously serologically and molecularly identified (20). Inactivated bacterin was prepared according to method described by Borkowska-Opacka et al. (24). Simply, *P. multocida* strains were grown in brain heart infusion broth at 37°C for 16-24 hr to obtain a dense culture containing approximately 10^8 CFU of each strain. Formalin was added to the culture in final concentration of 0.2 % and the formalized culture was re-incubated at 37°C for 24 hr to ensure complete bacterial inactivation. Two percent of aluminium hydroxide gel was added in concentration of 20 % and was mixed well with the culture. Cultures were equally mixed

together. The bacterin was standardized to contain 10^8 CFU/0.5ml dose. Finally, the bacterin was preserved with 0.01 % of thiomersal and stored at 4 C° until use. The quality control parameters of the locally prepared bacterin were sterility, safety and potency test (25). Briefly, the prepared bacterin was inoculated on bacteriological and mycological media to prove its sterility or purity from any bacterial or fungal contaminations. In addition, 0.5 ml of the prepared bacterin was inoculated in 5 chickens and the birds were kept under observation for a weeks to ensure that the bacterin induced no adverse effects like signs, lesions or mortalities. The potency test was applied by collection of blood samples and measuring of immunological parameters.

Vaccination scheme

Primary vaccination was done at the age of 6 weeks, 0.5 ml of the prepared inactivated *P. multocida* bacterin containing 10^8 CFU/0.5 ml was inoculated intramuscular (I/M) into the thigh region in chickens of groups (A) and (B). Booster vaccination was done at 9 weeks of age (3 weeks after primary vaccination) (24).

Challenge test with virulent P. multocida

Serovars of *P. multocida* that were used for bacterin preparation were used for chickens challenge. Both vaccinated and non-vaccinated chickens (groups A, B, C and D) were challenged I/M with virulent *P. multocida* serotypes A:1 and A:3 separately at 2 weeks after booster vaccination (11 weeks old) in a dose of 0.1 ml of bacterial suspension/bird containing 10^7/ml that inoculated I/M (26).

Parameters for evaluation of inactivated P. multocida bacterin in chickens

Immunological parameters:

Detection of humeral immune response of vaccinated and challenged birds was done using Enzyme Linked Immuno-Sorbent Assay (ELISA). Commercial ELISA kit (ID-VET) was used and the test was done as manufacturer's instructions. Blood samples were collected from the wing vein of birds in each group pre-vaccination and weekly after vaccination till the end of the study to determine the serum antibody titer of chickens. The results of antibody titers were determined from S/P ratio

$$\frac{S}{P} = \frac{\text{(Sample mean – negative control)}}{\text{(positive control-negative control)}}$$

$$\text{Log}_{10}\ \text{Titer} = 1.09\ (\log_{10}\ S/P) + 3.36$$

Clinical parameters

The clinical signs, mortality rate and post-mortem lesions specific for *P. multocida* were recorded in groups two weeks post challenge for measuring bacterin protection rate.

$$\text{The Protection rate (\%)} = \frac{\text{(Number of living birds)}}{\text{(Total number of birds)}}$$

Re-isolation rate

Liver was collected from dead as well as sacrificed living birds at the end of the study in each group for *P. multocida* re-isolation. It was done by inoculation of the liver on blood agar media and then by morphological and biochemical identification (27-29).

Histopathological examination

Three chickens from each group were sacrificed by cervical dislocation at the end of study (13 weeks old) for histopathological examination and tissue specimens were collected from heart, liver and spleen and then fixed in 10% formol saline solution. After proper fixation, the specimens were dehydrated in ascending grades of ethanol from 70 % to 100 %, diluted in alcohol (methyl, ethyl and absolute ethyle), cleared in xylol and manually embedded in paraffin at 56°C in hot air oven for 24 hr. Thin tissue sections, 5 µm thick were cut from paraffin blocks and stained with Hematoxylin and Eosin (HE) for histopathological examination through the light microscope (30).

Results and discussion

Fowl cholera is one of the most important problem facing poultry industry, so vaccination against the disease is practiced as preventive measures in many countries of the world including Egypt (31). Both live and inactivated *P. multocida* vaccines had been attempted to prevent the disease (4). Inactivated *P. multocida* vaccines are widely

used as the organisms do not have chance to be reverted to virulence to cause the disease (13).

In the present work, an inactivated local bacterin was prepared using *P. multocida* field strains and its efficacy was determined. The quality control assessment of the prepared *P. multocida* bacterin showed it was sterile and free from any bacterial or fungal contaminations and it was safe as it didn't produce any local or systemic reactions as well as no mortalities in inoculated birds.

Table (1) shows the potency of the locally prepared *P. multocida* bacterin through determining the humoral immune response using ELISA. In both groups (A) (vaccinated and challenged birds with *P. multocida* type A1) and (B) (vaccinated and challenged birds with *P. multocida* type A3), the mean ELISA antibody titers increased from (80) pre-vaccination level to reach (2260) and (2010) at the 3rd week after primary vaccination; respectively, however, two weeks after secondary vaccination, the antibody titers reach (4350) and (3980) respectively, then declined to (2998) and (2679) one week after challenge then increased to (3970) and (3905) at two weeks after challenge, respectively. Results of the mean ELISA antibody levels in controls groups (C) (non-vaccinated and challenged birds with *P. multocida* type A:1) and (D) (non- vaccinated and challenged birds with *P. multocida* type A:3) were 60-80 before challenge and then increased to (95) at two weeks after challenge. Group (E) (non-vaccinated-non challenged control) showed steady mean ELISA antibody levels (65-80). Solano et al. (32) developed ELISA assay to determine the humoral immune response of chicken to *P. multocida* and compared the results with indirect haemagglutination (IHA) test as they concluded that the antibody titers measured by ELISA was at least twice as sensitive as IHA. Furthermore, Avakian et al. (33), Perelman et al. (9) and Esmaily et al. (34) recorded that polyvalent FC oil-based bacterin induced a high antibody titer in broiler breeder hens measured by ELISA technique. In addition, Jabbri and Moazeni Jula (35) stated that inactivated trivalent FC vaccine consisted of serotypes 1, 3 and 4 *P. multocida* strains induced immunogenic response in vaccinated chickens, as ELISA assay showed a considerable increase in antibody titer after twice vaccination of 8 weeks old chickens. Birds vaccinated two or three times between the ages of 7 and 20 weeks became sufficiently immune tolerated to FC challenge, while those were not vaccinated or only vaccinated at the age of 7 weeks were not sufficiently immunized using ELISA (36). The results of humoral immune response obtained in this study were comparable Akhtar et al. (16) who tested a formalin killed FC in 15 weeks old chickens and found an increase in humoral antibody titers after booster vaccination.

The bird's immune response to *P. multocida* bacterin was previously explained (37, 38). The capsule and LPS of *P. multocida* cell surface are considered primary stimulators of immune response and critical determinants of bacterin

Table 1: Mean ELISA antibody titres of sera in chicken groups pre-vaccination; one, two and three weeks after primary vaccination; one and two weeks after secondary vaccination and one and two weeks after challenge

Group	Treatment	*P. multocida* strain	*P. multocida* mean antibody titers/ weeks after vaccination							
				Weeks after primary vaccination (6th wks old)			Weeks after secondary vaccination (9th wks old)		Weeks after challenge (11th wks old)	
			Pre-V*	1	2	3	1	2	1	2
A	Vaccinated- challenged	A:1	80	687	1760	2260	3400	4350	2998	3970
B		A:3	80	654	1590	2010	3085	3980	2676	3905
C	Non vaccinated-challenged control	A:1	80	70	70	65	70	80	95	95
D		A:3	80	75	60	60	75	70	90	95
E	Non vaccinated- non challenged control	–	70	70	80	75	65	80	75	65

Pre V*: Pre-vaccination

protective efficacy (39). Both play key roles in a range of interaction between the bacteria and the hosts they colonize or infect. Gong et al. (40) demonstrated that outer membrane proteins (H and A) of *P. multocida* are the major immunogene antigens which play an important role in confer resistance against infections. The Omps promote adherence to host cell surfaces and are therefore likely involve in *P. multocida* virulence (41).

Our results revealed that, the clinical signs of *P. multocida* vaccinated and challenged chicken groups were mild depression, off food, diarrhea, septicaemia and congested mucous membrane of conjunctiva and buccal cavity. Severe signs were observed in non-vaccinated challenged controls groups, while no signs appeared in the non-challenged control group during the observation period. These results were in agreement with Levy et al. (42) who recorded signs of depression dullness, anorexia, greenish diarrhea and labored breathing in *P. multocida* challenged non vaccinated chickens, while the vaccinated birds did not show clinical signs except dullness and depression. Also, signs of FC in commercial layer flocks were recorded as depression, anorexia with ruffled feathers, mucous discharge from mouth and nares and cyanotic comb and wattles (43).

The mortality rates in different groups that recorded here were 15 % and 20 % in vaccinated and challenged birds with *P. multocida* type A:1 and A:3; respectively, however, in non-vaccinated and challenged controls, they were 90 % and 80 % for *P. multocida* type A:1 and A:3; respectively (Table 2). These results were in a partial agreement with others (44, 45). The results showed that vaccinated chickens were resistant to challenge with *P. multocida* A:1 and A:3 strains, where the protection rates were 85 % and 80 % respectively, while they were 10 % and 20 % in the non-vaccinated-controls, respectively (Table 2). Two doses of prepared bacterin induced good protection (80-90 %) against challenge with *P. multocida* of homologous immunogenic type but low protection (10-30 %) against heterologous challenge (46). An Egyptian study concluded that locally prepared polyvalent bacterins should be used in cases of FC outbreaks, and the capsular antigen plays a little role in immunization when compared with the somatic antigen (47). Inactivated trivalent FC vaccine consists of serotype 1, 3 and 4 *P. multocida* provided 70-80 % protection in chickens against challenge with homologous strains (35). Also, some other Egyptian trials revealed that adjuvented local FC vaccine gave 100 % protection in chickens against challenge with virulent strains of *P. multocida* types A and D (48) as well as 95 % and 90 % for types A:5 and D:2; respectively (49). Furthermore, the protection rate was 100 % in chickens vaccinated twice with alum-precipitated FC vaccine (50).

Post-mortem examination of chickens revealed mild and severe lesions in *P. multocida* vaccinated -challenged and non-vaccinated-challenged control groups, respectively. The lesions included septicaemia, congestion of internal organs, enlarged liver with sub-capsular hemorrhage, pericarditis and enlarged and congested spleen. These lesions were in accordance with other study (45) where the lesions of birds vaccinated with double doses of *P. multocida* local bacterin were congested heart and

Table 2: Results of protection rates in different chicken groups after vaccination with local *P. multocida* bacterin and challenge with the same serovars

Group	Chicken groups	Challenge *P. multocida* Strain	No. of chickens	No. of survived birds	No. of dead birds	Mortality rate (%)	Protection Rate (%)
A	Vaccinated-challenged	A:1	40	34	6	15	85
B		A:3	40	32	8	20	80
C	Non vaccinated-challenged control	A:1	40	4	36	90	10
D		A:3	40	8	32	80	20
E	Non vaccinated-non challenged control	_	40	40	0	0	100

slight congestion of the liver and spleen, however the lesions in non-vaccinated-challenged birds were congestion of the subcutaneous blood vessels, dark red muscles, enlarged and congested liver and spleen and pericarditis. In other study, severe congestion, hemorrhages, pericardial and peritoneum exudations, enlargement of spleen and liver and white necrotic foci over liver were reported in chickens vaccinated with type A:1 of *P. multocida* at 14 day post-challenge (46).

Table 3 reveals that *P. multocida* could be re-isolated from liver, heart blood and spleen of the chickens with high rate (90-95 %) and low rate (15-25 %) in non-vaccinated and vaccinated-challenged birds; respectively. These findings supported by Egyptian researcher Mahmoud (51) who found that the incidence of isolation of *P. multocida* was higher from non-vaccinated flocks than those from vaccinated ones. Partial agreement with ours revealed that *P. multocida* could not be recovered from immunized and challenged survived chickens, while it could be isolated from all dead or diseased birds (35).

In this study, Table 4 and Figures 1-4 demonstrate the histopathological findings in different examined organs at the end of the experiment. Figure 1 shows the microscopic lesions of chickens vaccinated and challenged with *P. multocida* serotype A:1. Mild congestion of hepatic central vein and some inflammatory cells infiltrate (A), normal myocardium (B) and congested red pulp of spleen (C) were the observed lesions. In chickens vaccinated and challenged with *P. multocida* serotype A:3 (fig. 2) showed hydropic degeneration of hepatocytes (A), slight congestion of the myocardium (B) and hyperplasia of splenic lymphoid follicle (C). The previous results were in a partial agreement who noticed congestion with presence of some degenerative changes of liver, mild depletion of splenic lymphoid cells and slight myocarditis in *P. multocida* vaccinated and challenged chickens (45, 52).

Table 3: Results of re-isolation of *P. multocida* among chicken groups after vaccination with local *P. multocida* bacterin and challenge with the same serovars

Group	Chicken groups	Challenge *P. multocida* Strain	No. of chickens	Re-isolation % of *P. multocida*
A	Vaccinated- challenged	A:1	40	6/40 (15%)
B		A:3	40	10/40 (25%)
C	Non vaccinated-challenged control	A:1	40	38/40 (95%)
D		A:3	40	36/40 (90%)
E	Non vaccinated-non challenged control	_	40	0/40 (0%)

Table 4: Results of histopathological examination of chicken groups after vaccination with local *P. multocida* bacterin and challenge with the same serovars

Point of comparison	Group			
	A	B	C	D
Liver				
Congestion of central vein	+	-	-	+
Inflammatory cells infiltrate	+	-	+	-
Coagulative necrosis	-	-	+	-
Hydropic degeneration	-	+	-	+
Myocardium				
Congestion	-	+	-	-
Inflammatory cells infiltrate	-	-	+	-
Endocardial haemorrhages	-	-	-	+
Spleen				
Red pulb congestion	+	-	+	-
Lymphoid follicle hyperplasia	-	+	-	+
Focal area of necrosis	-	-	-	+

+ = Present - = Absent

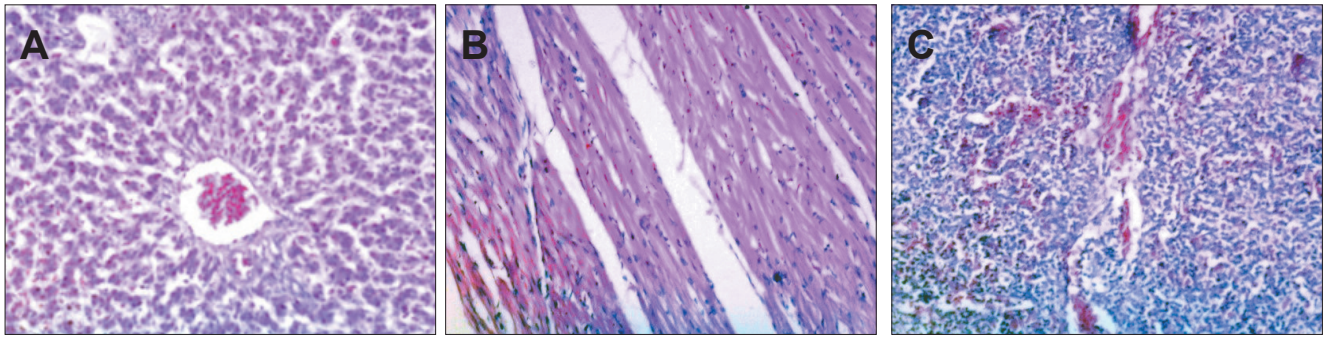

Figure 1: Chickens vaccinated and challenged chickens with *P. multocida* serotype A:1 (group A) (H&E. X 200): A: Liver shows mild congestion of central vein and some inflammatory cells infiltrate, B: Heart showing normal myocardium, C: Spleen showing congested red pulp

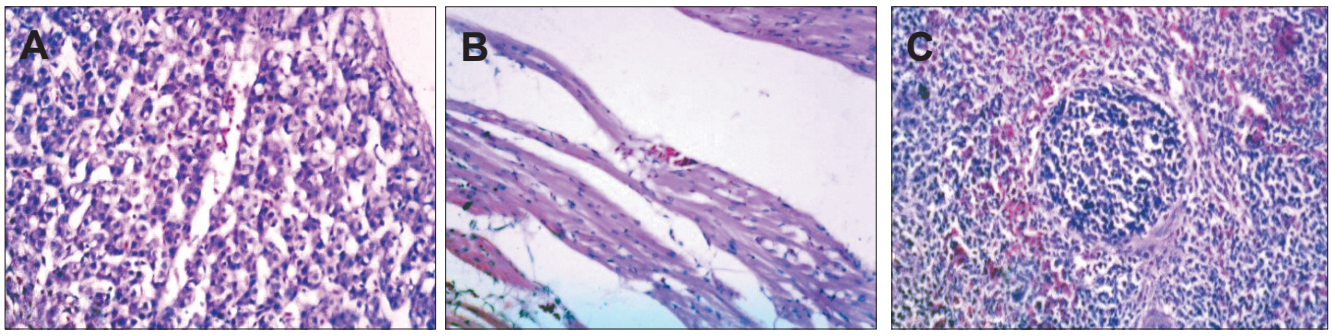

Figure 2: Chickens vaccinated and challenged chickens with *P. multocida* serotype A:3 (group B) (H&E. X 200): A: Liver showing hydropic degeneration of hepatocytes, B: Heart shows slight congestion of the myocardium, C: Spleen showing hyperplasia of lymphoid follicle

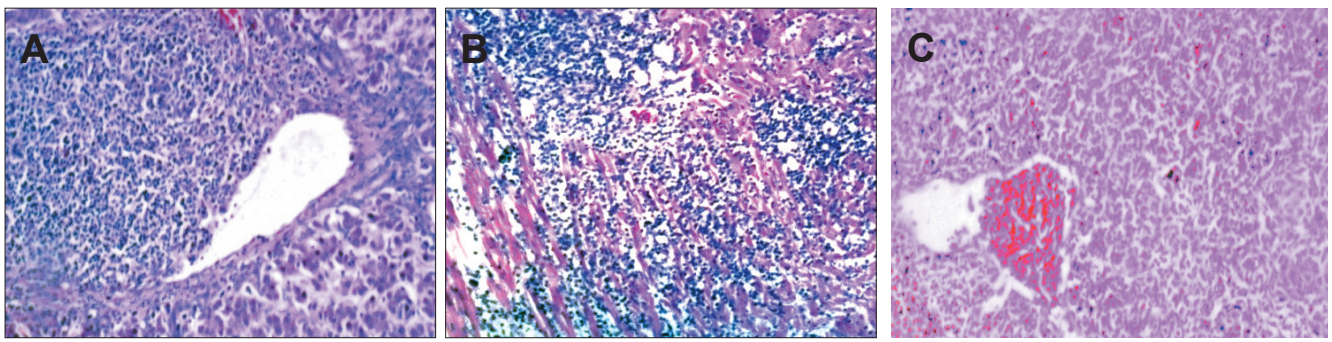

Figure 3: Chickens experimentally challenged with *P. multocida* A:1 strain (group C) (H&E. X 200): A: Liver showing area of coagulative necrosis infiltrated with heterophils, B: Heart showing severe inflammatory cells infiltration of myocardium, C: Spleen shows congestion of red pulp and splenic artery

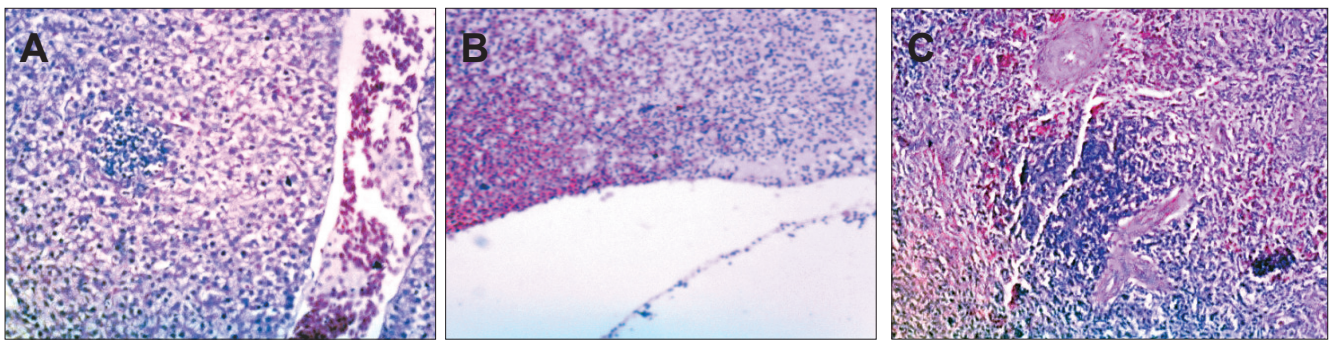

Figure 4: Chickens experimentally challenged with *P. multocida* A:3 strain (group D) (H&E. X 200): A: Liver showing congestion of portal vein and hydropic degeneration of cytoplasm, B: Heart sub endocardial haemorrhage with heterophilic infiltration, C: Spleen shows severe congestion and focal area of necrosis (arrow)

Histopathological lesions in chickens challenged with *P. multocida* A:1 strain (fig. 3) showed area of hepatic coagulative necrosis infiltrated with heterophils (A), severe inflammatory cells infiltration of myocardium (B) and congestion of splenic red pulp and artery (C). Moreover, in chickens experimentally challenged with *P. multocida* A:3 strain (fig. 4), the liver showed congestion of portal vein and hydropic degeneration of cytoplasm (A), the heart revealed sub endocardial haemorrhage with heterophilic infiltration (B) and the spleen demonstrated severe congestion and focal area of necrosis (C). Similarly, histopathological changes of local isolates of *P. multocida* A:1 in chickens were congestion, hemorrhages and mild degeneration of liver associated with necrotic changes involving groups of hepatic parenchymatous cells with prominent heterophilic infiltration (53, 54).

These lesions could be attributed to the direct effect of the endotoxin and ischemia which resulted from the bacterial emboli.

From the above mentioned results, it could be concluded that the prepared FC inactivated bacterin from the local Egyptian predominant *P. multocida* serovars proved efficacy and protection of layer chickens.

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The authors declare that they have no conflict of interest.

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UČINKOVITOST CEPLJENJA KOKOŠI NESNIC Z INAKTIVIRANO BAKTERIJO KOLERE PERJADI, PRIPRAVLJENE IZ LOKALNIH EGIPTOVSKIH SEVOV BAKTERIJE *Pasteurella multocida*

W. A. Abd El-Ghany, H. A. Ahmed, A. Z. Qandoos, M. A. Bosila

Povzetek: Raziskava je bila izvedena z namenom ocenitve učinkovitosti cepljenja kokoši nesnic z inaktivirano bakterijo FC, pripravljeno iz lokalnih egiptovskih sevov bakterije *Pasteurella multocida (P. multocida).* Skupno 200 kokoši nesnic je bilo razdeljenih v 5 enakih skupin. V vsaki skupini je bilo 40 kokoši. Pri 6 tednih smo kokoši v skupinah A in B cepili s serotipoma *P. multocida* A:1 in A:3, po 3 tednih, ko so bile živali stare 9 tednov, so dobile poživitvene doze cepiva. Po dveh tednih (v starosti 11 tednov) so bile kokoši okužene z virulentnima serotipoma A:1 in A:3. Piščanci v skupinah C in D niso bili cepljeni temveč samo okuženi s serotipoma A:1 in A:3. Kokoši v skupini E niso bile niti cepljene, niti okužene. Vzorci krvi so bili odvzeti pri vseh skupinah tedensko za preverjanje humoralnega imunskega odziva. Vse kokoši smo stalno opazovali in beležili prisotnost bolezenskih znakov, različnih ran in umiranje kokoši. Pri poginulih kokoših smo osamili bakterije ter opravili histopatološki pregled. Rezultati encimsko-imunskega testa (ELISA) so pokazali da je bila najvišja stopnja zaščite dosežena 5 tednov po cepljenju, saj so titri dosegli 3970 v skupini A in 3905 v skupini B. Klinični znaki, stopnja umrljivosti in rane so bili pri cepljenih kokoših blagi, hude rane pa so bile vidne pri necepljenih in okuženih kokoših. Stopnja zaščite je bila v skupinah A in B 85- oziroma 80-odstotna, v skupinah C in D pa 10- oziroma 20-odstotna. Stopnje ponovne izolacije *P. multocida* po okužbi so bile 90 in 95 odstotkov pri kokoših, ki niso bile cepljene, medtem, ko so bile v skupinah, ki so bile okužene s *P. multocida* serotipa A:1 in A:3 15- in 25-odstotkov. Histopatološki pregled cepljenih in okuženih kokoši je pokazal popolno odsotnost ali prisotnost blagih mikroskopskih poškodb, medtem ko so imele necepljene okužene kokoši bolj obsežne histopatološke poškodbe. Pripravljena inaktivirana bakterija FC iz lokalnih egiptovskih prevladujočih serovarov *P. multocide* se je izkazala za učinkovito zaščito kokoši nesnic.

Ključne besede: *Pasteurella multocida*; kokoši; imunizacija; zaščita; Egipt

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Original Research Article

CHICKEN ANAEMIA VIRUS IMPAIRS NITRIC OXIDE PRODUCTION IN HD11 CHICKEN MACROPHAGES

Katja Ester[1]*, William L. Ragland[2†]

[1]Laboratory of Experimental Therapy, Division of Molecular Medicine, Ruđer Bošković Institute, Bijenička 54, 10000 Zagreb, Croatia, [2]Zagreb Biotek d.o.o., 21400 Supetar, Croatia

[†]Deceased in March 2019

*Corresponding author, E-mail: kester@irb.hr

Abstract: Immunosuppressive viruses cause substantial economic losses to the poultry industry. Chicken anaemia virus (CAV) causes severe disease in young chickens, whereas subclinical infection in older birds causes immunosuppression. In this study, we addressed the ability of CAV to interfere with production of antimicrobial molecule nitric oxide (NO) by macrophages. NO production in chicken macrophage cell line HD11 was induced using both Toll-like receptor 4 agonist, bacterial lipopolysaccharide, and an immune modulator, interferon-γ. In addition, we treated macrophages with CAV propagated in chicken lymphoblastoid cells. The levels of NO were measured by the Griess reaction. Addition of CAV decreased both the interferon-γ and the lipopolysaccharide associated induction of NO. Observed effect was not caused by CAV-related cytotoxicity, as no decrease in number of viable cells was observed. Although CAV could not completely abrogate NO production, attenuation of NO induction was clearly present. We have previously shown that CAV interferes with the expression of interferons in chickens during subclinical infection. Since the signalling pathways of expression of interferons and type 2 nitric oxide synthase, enzyme involved in NO formation, overlap, we conclude that measured decrease in NO levels is a consequence of CAV interference with interferon and NO synthase signalling. Regardless of the fact whether the attenuation of NO serves as a viral primary defence, or is only a secondary effect, it could impair the immune response to other pathogens and contribute to the global immunosuppression in chicken houses.

Key words: chicken; immunosuppression; chicken anaemia virus (CAV); macrophage; nitric oxide (NO)

Introduction

The consumption of poultry, according to the USDA Foreign Agricultural Service, will increase worldwide, especially in China, The European Union (EU), Brazil and India (1). EU produced 15.2 million tonnes of poultry meat in 2018, which represents a new high and a cumulative rise since 2010 (2). The extraordinary performance of the world production and consumption of poultry meat reflects the modest and decelerating growth in world per capita consumption of red meat, which has been taking place for a wide variety of reasons. For the high-income countries, the reasons include the near saturation of consumption (e.g. in the EU and Australia), policies of high domestic meat prices and/or preference for fish (Japan and Norway), and health and food safety reasons everywhere. The production of poultry is increasing, and the overall profit in USA only, based on the domestic consumption and on a large export to the other markets, was $46.3 billion in 2018 (3). Extensive vaccination and disease monitoring are the most important strategies that make this global increase possible.

Immunosuppressive viruses of poultry, widely present in chicken houses all around the world, have devastating effects on the poultry industry. Immunosuppression and increased mortality are

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caused by these viruses, as they interfere with vaccination, and moreover, as in the case of Chicken anaemia virus (CAV), they often cause subclinical infections with no obvious clinical signs.

CAV is a non-enveloped virus that contains a circular, single-stranded 2.3-kb DNA genome contained within an icosahedral capsid, 25 nm in diameter (4). It belongs to the Gyrovirus genus, Anelloviridae family (5). The CAV genome encodes three open reading frames: VP1 - a major structural protein, VP2 - a scaffolding protein, and VP3 - a non-structural protein, named apoptin, which is able to induce apoptosis selectively in tumour cells (6).

CAV causes severe disease in young chickens, characterized by a generalized lymphoid atrophy, severe anaemia, development of subcutaneous and intramuscular haemorrhages, and increased mortality. The important targets for viral infection are haemocytoblasts in the bone marrow and precursor lymphocytes in the thymus. Subclinical infection in older birds also presents an economical problem, since infected birds are often immunosuppressed (7, 8). Infected chickens suffer an increased incidence of secondary bacterial infections and evidence of decreased responsiveness to vaccines (9, 10, 11), all of which brought investigation of CAV pathogenesis back into the focus. Moreover, CAV infection increases susceptibility to viral infections such as avian Infectious bronchitis and Influenza (12). Additional problems arise from the fact that CAV also infects specific-pathogen free (SPF) flocks (13) that are important for vaccine production.

CAV compromises immune response through lymphoid depletion, but immunosuppression persists after repopulation of lymphoid tissues. In a transcriptomic profiling study of CAV infection in an *in vivo* model, Giotis *et al.* pointed that CAV induces a global immune deregulation with emphasis on T-cells suppression in infected host (14). It is also likely that CAV developed subtle strategies to evade immune surveillance, and we previously demonstrated that CAV interferes with transcription of chicken interferons alpha and gamma during subclinical infection (15). McConnell *et al.* (16) reported that CAV displays inhibitory effects on chicken macrophage cells that play a central role in body defences against microbial infections. Macrophages are crucial cell types in both innate immunity, for the clearance of invading microorganisms, and for adaptive immunity as one of the major antigen presenting cells (17). Inhibition of their function severely impairs the host immune response, and consequently compromise vaccination in chicken houses.

To obtain additional insight into CAV biology and its immunosuppressive properties, we have examined CAV effects on commonly used macrophage cell line HD11 (18). Chicken macrophages, when exposed to pathogens or pathogen-associated molecular patterns (PAMPs), are activated to produce pro-inflammatory cytokines, chemokines and antimicrobial reactive oxygen species (ROS) and nitric oxide (NO) (19, 20), as do their mammalian counterparts. NO is the small inorganic radical of nitric oxide, produced by inducible type 2 nitric oxide synthase (NOS2) (21). This potent antimicrobial molecule reacts with DNA, proteins and lipids, and can inhibit replication of both DNA and RNA viruses. When PAMPs bind to Toll-like receptors (TLR), the signalling cascade results in upregulation of NOS2 and interferons. In this study, we used bacterial lipopolysaccharide (LPS) and interferon-γ (IFN-γ) as NO inducing agents, one representing TLR4 agonist and another representing an important mediator of the antimicrobial response, respectively, and compared measured NO levels in HD11 cells with induced NO in cells infected with CAV.

Materials and methods

Reagents

Chicken recombinant IFN-γ (22) was a gift from Dr. J. W. Lowenthal, CSIRO Livestock Industries, Geelong, Australia. Foetal bovine serum and chicken serum were purchased from Eurobio (Les Ulis Cedex B, France). RPMI 1640 medium, LPS, sulfanilamide, N (1-naphthyl) ethylenediamine dihydrochloride, methylene blue, Tryptophan (TRP) and Trypan Blue were purchased from Sigma-Aldrich (Taufkirchen, Germany). All the remaining reagents were purchased from Kemika (Zagreb, Croatia).

Cells and viruses

Chicken cell lines used in this study: avian leukaemia virus MC29-transformed chicken macrophage cell line HD11 (a gift from Dr. Bernd Kaspers, University of Munich, Germany), Marek's

disease virus transformed chicken lymphoblastoid cell line MDCC-MSB1 and spontaneously immortalized quail fibroblast cell line CEC-32 (gift from Dr. Bernd Kaspers). All cells were maintained in RPMI 1640 medium with 8 % foetal bovine serum and 2 % chicken serum, with antibiotics. Cells were kept at 41 °C (optimal temperature for avian cells) in a humidified 5 % CO_2 / 95 % air atmosphere, with regular passages. The DelRoss strain of CAV was propagated and titrated in MDCC-MSB1 cells as described by Yuasa *et al.* (23). The viral titre from MDCC-MSB1 supernatant used to infect cells in CAV experiments was 10^6 $TCID_{50}$ CAV in 0.1 ml.

Effect of CAV on the induction of NO

Cells were seeded at 0.5×10^6/ml as quintuplicates in flat-bottomed 96 well plates. To induce NO, 100 µl of media was mixed with 100 µl of LPS or recombinant ChIFN-γ at selected concentrations (see Results and Discussion). To determine if addition of TRP would influence NO induction/inhibition, 100 µM TRP was added into cell medium. To determine if CAV influences NO induction, cells were infected with CAV (2.8 PFU/cell). Virus was added simultaneously or 1 hr prior to the stimulation of cells. The levels of NO that accumulated in cell culture media in response to various stimuli were determined by the Griess reaction after 24 hr.

Nitric oxide analysis

The quantity of NO produced in stimulated cells was measured by the Griess reaction. Briefly, 100 µl of cell supernatant was incubated with an equal volume of Griess reagent (1 % sulfanilamide, 0.1 % N (1-naphthyl) ethylenediamine dihydrochloride in 2.5 % phosphoric acid) at room temperature for 10 min. Absorbance was measured at 570 nm, using a microtiter plate reader (Multiscan EX, Thermo LabSystems, US). Obtained absorbances were converted to micromolar values using the slope of a calibration curve established by serial dilutions of sodium nitrite from 250 µM to 1.95 µM.

Viability assay

To test whether LPS or CAV will reduce cell number, HD11 cells at 2×10^5/ml were seeded in 75 cm^2 flasks, cells were treated with 0.1 µg/ml and 1 µg/ml LPS and incubated overnight. Cells were also exposed to CAV (2.5 PFU/cell) and incubated for 1 hr prior to the addition of LPS. Viable cells were counted in a haemocytometer the next day using Trypan Blue Exclusion staining. Briefly, cells in medium are resuspended in Trypan Blue in PBS, and only transparent cells were used to determine the percentage of viable cells.

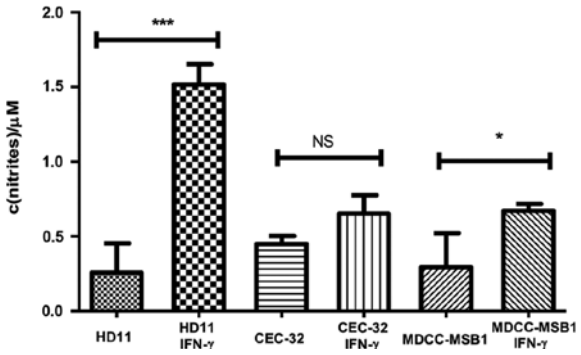

Figure 1: Production of NO in avian cells upon stimulation with IFN-γ. Cells were seeded at 0.5×10^6/ml in 96 well plates, and treated with IFN-γ (70 ng/ml) for 24 hr. The levels of accumulated NO were measured by the Griess reaction. Each bar represents a mean value ± s.d. from at least three individual experiments. One-way ANOVA with Tukey's post-hoc test was used for statistical analysis (NS – non-significant; * - $p < 0.05$; *** - $p<0.001$)

Statistics

Results are represented as mean values from at least three separate experiments, except for dose-response curves and cell viability experiments where data represents mean values from two independent experiments. All graphics with error bars are presented as mean ± s.d. and were generated in GraphPad Prism 5 software. To determine statistical significance between samples, one-way ANOVA with post-hoc Tukey's multiple comparison test was performed in GraphPad Prism (NS – non-significant; * - $p < 0.05$, ** - $p < 0.01$ and *** - $p < 0.001$).

Results

Beside chicken macrophage cell line HD11, we analysed the induction of NO by IFN-γ in chicken lymphoblastoid cell line MDCC-MSB1 and in quail fibroblasts CEC-32. Chicken macrophages HD11 were the most inducible by IFN-γ (Figure 1). In addition, we measured a mild increase in NO production in two other cell lines. CEC-32 cell line was the least inducible in our experiments.

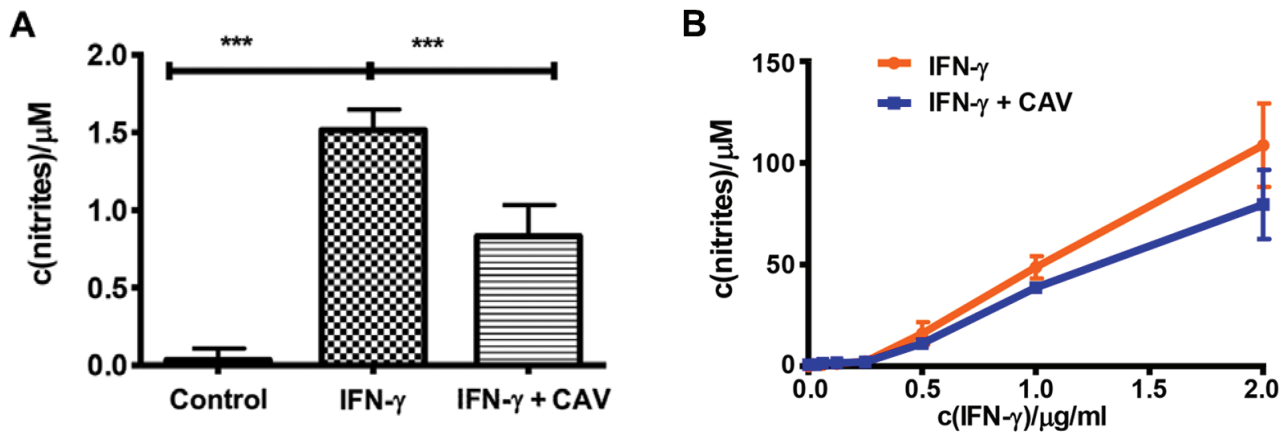

Figure 2: Effects of CAV on NO induction after stimulation with IFN-γ. HD11 cells were seeded in 96 well plates and treated with IFN-γ (70 ng/ml) and 2.8 PFU/cell CAV for 24 hr. The levels of accumulated NO were measured by the Griess reaction. Each bar represents a mean value ± s.d. of at least three individual experiments. One-way ANOVA with Tukey's post-hoc test was used for statistical analysis (***p<0.001) (A). HD11 cells were seeded in 96 well plates and treated with IFN-γ prepared as serial twofold dilutions from 2 µg/ml to 0.03 µg/ml and CAV for 24 hr. The levels of accumulated NO were measured by the Griess reaction. Each point represents a mean value ± s.d. of two independent experiments (B)

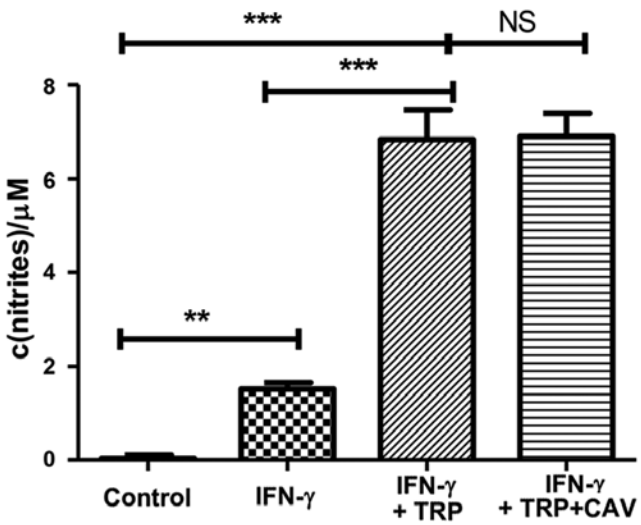

Figure 3: TRP protects cells from CAV-induced decrease in NO induction. HD11 cells were seeded in 96 well plates and treated with IFN-γ (70 ng/ml), TRP (100 µM) and CAV (2.8 PFU/cell) for 24 hr. The levels of accumulated NO were measured by the Griess reaction. Each bar represents a mean value ± s.d. of at least three individual experiments. One-way ANOVA with Tukey's post-hoc test was used for statistical analysis (NS – non-significant; **p<0.01; ***p<0.001)

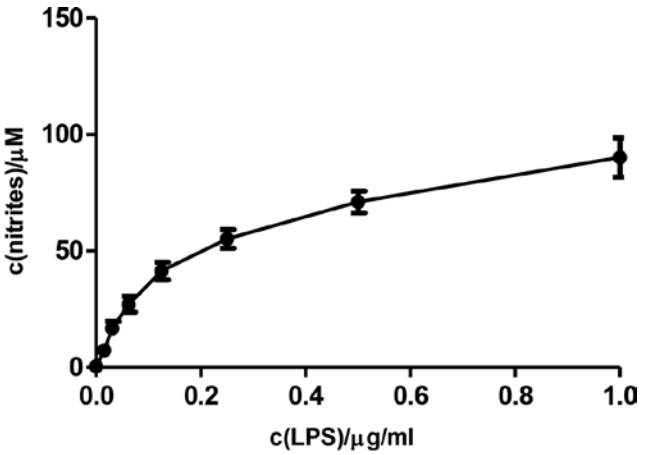

Figure 4: LPS dose-response curve. HD11 cells were seeded in 96 well plates and treated with LPS prepared as 1:2 serial dilutions from 1 µg/ml to 0.0156 µg/ml. The levels of accumulated NO were measured by the Griess reaction. Each point represents a mean value ± s.d. of two independent experiments

To test whether CAV influences NO production by IFN-γ in chicken macrophages, cells were treated with IFN-γ (70 ng/ml) and CAV for 24 hr. The addition of CAV decreased NO production (Figure 2). In addition, we titrated interferon-γ with and without CAV to obtain dose-response curve. The inhibition of NO induction by CAV was minuscule by low dose of IFN-γ, while in higher concentration (c > 1 µg/ml) the inhibitory effect of virus was more evident (Figure 2). Furthermore, we investigated whether addition of TRP will influence NO induction by IFN-γ, and moreover, what effect will CAV have in this model. We added 100 µM TRP into HD11 cell media, and treated cells as described above. Addition of TRP upregulated NO induction by IFN-γ, whereas CAV did not have any impact on NO induction when TRP was included (Figure 3).

Induction of NO by LPS was more robust than by IFN-γ in our experiments, with visible dose response

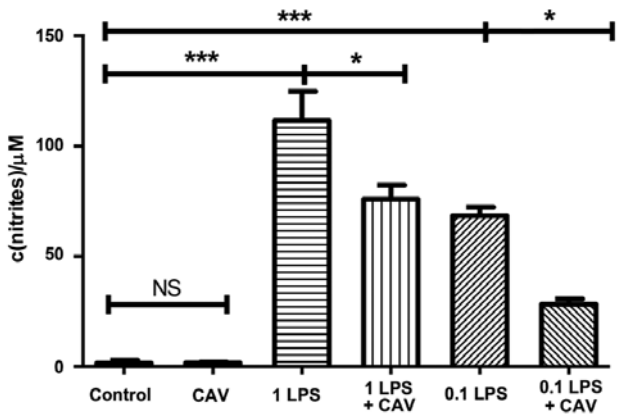

Figure 5. Effects of CAV on NO induction after stimulation with LPS. HD11 cells were seeded in 96 well plates and treated with LPS (0.1 µg/ml and 1 µg/ml) and CAV (2.8 PFU/cell) for 24 hr. CAV was added 1 hr prior to the stimulation of cells. The levels of accumulated NO were measured by the Griess reaction. Each bar represents a mean value ± s.d. of at least three individual experiments. One-way ANOVA with Tukey's post-hoc test was used for statistical analysis (NS – non-significant; **p<0.01; ***p<0.001)

(Figure 4). To test whether CAV influences NO production in chicken macrophages stimulated with LPS, HD11 cells were treated with 0.1 µg/ml LPS and 1 µg/ml LPS and CAV for 24 hr. CAV showed inhibitory effect on the NO induction by LPS (Figure 5), with the effect being stronger in cells treated with higher concentration of LPS. The virus itself does not induce NO production (Figure 5).

To exclude cytotoxic effects of CAV that may be responsible for decrease in measured NO levels, we performed viability assay. Incubation of cells with the virus did not reduce number of viable cells, using virus alone or in the combination with LPS (Table 1). However, we observed reduction in number of viable cells incubated with LPS alone.

Discussion

IFN-γ is important mediator of the immune response, and one of its effector functions is activation of macrophages and subsequent induction of antimicrobial mediator NO (24). Differential nitric oxide production in avian cells after stimulation with interferon-γ revealed chicken macrophages HD11 as the most inducible by IFN-γ, what was expected according to their biological role and vast literature data. We measured a mild increase in NO production in MDCC-MSB1 cells. These cells are widely used for propagation of CAV and are well characterised in the context of host response to infection (23, 25). Giardi et al. (26) also reported that MDCC-MSB1 cells are able to produce low levels of NO after IFN-γ stimulation. Regarding induction of NO production in CEC-32 cells, we showed that these cells produce very low levels of NO. To the best of our knowledge, no report on CEC-32 cells NO production is available, which can be attributed to the fact that CEC-32 cells are rarely used, possibly due to the fact that they were characterised as a problematic cell line, previously misidentified as a chicken cell line (27).

Although CAV did not completely abrogate NO production in macrophages in our experiments, attenuation of NO induction by both LPS and IFN-γ was clearly present. NO is not only an effector molecule, but also an important player in the signalling cascade affecting both the adaptive and innate immunity (21, 28). It is not surprising that avian viruses, which are targeted by the macrophages/NO system, have adapted strategies to evade NO production (29, 30). Here we showed that CAV, as a part of its vast arsenal of immunosuppressive properties, inhibits IFN-γ/macrophages/NO axis, although not to a non-induced levels.

Since it is known that production of NO interplays with several biochemical pathways that eventually lead to depletion of Tryptophan (31, 32), we included TRP in our measurements and showed that addition of TRP upregulated NO induction by IFN-γ. Our findings are consistent with the previously published data on murine cells where addition of TRP caused a 12-fold increase in NO synthesis after IFN-γ stimulation (32). Interestingly, CAV did not have any impact on NO induction when TRP was added, leading

Table 1: Cell viability assay. Chicken macrophages HD11 were seeded in 75 cm^2 flasks at 2×10^5/ml and incubated with 1 µg/ml or 0.1 µg/ml LPS for 24 hr. Cells were also exposed to CAV (2.5 PFU/cell) alone or in combination with LPS. Numbers represent mean values ± s.d. from two experiments

Treatment	0.1 LPS	1 LPS	0.1LPS CAV	1 LPS CAV	CAV	HD11
Cell number after 24 hr × 10^5/ml	1.9 ± 0.1	1.5 ± 0.2	2.1± 0.2	1.5 ± 0.3	3.9 ± 0.1	4.2 ± 0.1

to the conclusion that boost in NO production by addition of TRP protected cells from the inhibitory effect of the virus.

Induction of NO by LPS was stronger than by IFN-γ in our experiments. Our findings correlate with previously published results by He *et al.* (33), who reported that chicken IFN-γ itself isn't strong NO inducer in macrophages as microbial TLR4 agonist LPS.

Observed cytotoxic effect of LPS on chicken macrophages has been previously documented by others (34). There is an association between NO induction by LPS and cell death, and moreover, LPS triggers apoptosis in murine macrophages (35).

It is still not clear whether CAV is able to infect and replicate in macrophages, although it impairs their function (16). Viral proteins could bind to receptors on the macrophage membrane, but there is no data on CAV ability to bind to TLR receptors. Based on our data, we could speculate that virus doesn't bind to TLR4, since it couldn't trigger NO production by itself. Nonetheless, one plausible explanation of CAV attenuation of NO induction by other stimuli is that virus interferes with signalling pathways related to NOS2 expression in macrophages.

In general, viruses that interfere with expression of type 1 interferons often impair expression of NOS2, since these signalling pathways overlap, and as for CAV we previously showed that it interferes with both IFN-α and IFN-γ expression (15). For effective NO induction by bacterial LPS, viral priming via IFN-γ is often crucial. Hence, based on our findings, CAV interferes with both steps for effective NO induction upon bacterial infections while at the same time it interferes with interferon and NO based antiviral response. Subclinical CAV infection in chicken houses clearly involves impediment in host defence against bacteria and other viruses, and based on our previous and recent data, interference with both NO and IFN systems is involved. We can't speculate at the moment whether NO attenuation serves as a viral primary defence, or is just a secondary effect, where CAV may additionally impair NO induction on its way to evade interferon response. More importantly, virus is able to impair chicken immune response to other pathogens and to reduce effectiveness of vaccines, all of which will contribute to the global immunosuppression in chicken houses and to substantial economical losses.

We have shown for the first time that CAV interferes with NO induction in chicken macrophages. Among other avian cells, immortalised chicken macrophages HD11 were the most inducible by IFN-γ to produce NO. Addition of CAV into cell media decreased levels of NO induced by IFN-γ. When TRP was added into media, NO induction by IFN-γ was upregulated and CAV didn't have any impact. Induction of NO by bacterial LPS was more robust than by IFN-γ in HD11 cells, where CAV decreased induced NO levels upon LPS stimulation. Attenuation of NO production was not a consequence of CAV cytotoxicity, since we observed no decrease in cell viability after CAV addition. We have previously shown that CAV interferes with expression of both type 1 and type 2 interferons in chicken during subclinical infection. Since IFNs and NOS2 signalling cascades overlap, we hypothesize that CAV interferes with IFNs/NO signalling in chicken macrophages. The effect observed may contribute to the general immunosuppression in chicken houses, because NO, IFNs and their interplay are important effectors and mediators in defence against viruses and bacteria.

Acknowledgments

The authors declare no conflict of interests.

This paper is dedicated to William L. Ragland, pioneer of molecular immunology, R&D enthusiast, mentor and friend.

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VIRUS PIŠČANČJE ANEMIJE VPLIVA NA PROIZVODNJO DUŠIKOVIH OKSIDOV V MAKROFAGIH PIŠČANEV HD11

K. Ester, W. L. Ragland

Povzetek: Imunosupresivni virusi povzročajo velike gospodarske izgube v perutninski industriji. Virus piščančje anemije (CAV) pri mladih piščancih povzroča hudo bolezen, medtem ko subklinična okužba pri starejših pticah povzroča oslabljen imunski odziv. V tej raziskavi je bil spremljan vpliv CAV na proizvodnjo dušikovih oksidov (NO) v makrofagih. Proizvodnja NO v piščančjih makrofagih v celični liniji HD11 je bila sprožena z uporabo agonista Toll-u podobnega receptorja 4, bakterijskega lipopolisaharida in imunskega modulatorja interferona-γ, makrofagi pa so bili okuženi s CAV, razmnoženim v piščančjih limfoblastoidnih celicah. Ravni NO so izmerili po Griessovi reakciji. Prisotnost CAV je zmanjšala proizvodnjo NO, spodbujeno tako z interferonom-γ, kot z lipopolisaharidom. Opaženega učinka ni povzročila citotoksičnost, povezana s CAV, saj ni bilo opaziti zmanjšanja števila živih celic. Čeprav CAV ni popolnoma zavrla nastajanja NO, je bilo očitno prisotno zmanjšanje nastajanja NO. Pred tem so pokazali, da CAV moti izražanje interferonov pri piščancih med subklinično okužbo. Ker se poti znotrajceličnega prenosa urejanja izražanja interferonov in sintaze dušikovih oksidov tipa 2, encima, ki sodeluje pri tvorbi NO, prekrivajo, predvidevamo, da je izmerjeno znižanje ravni NO posledica motenj CAV pri znotrajceličnem prenosu sporočila interferona do sintaze dušikovih oksidov. Ne glede na to, ali zaviranje nastajanja NO služi kot primarna virusna obramba ali je le sekundarni učinek, lahko poslabša imunski odziv na druge patogene in prispeva k splošnemu zmanjšanju imunskega odziva v kurnikih ali na kokošjih farmah.

Ključne besede: piščanci; zmanjšanje imunskega odziva; virus piščančje anemije (CAV); makrofagi; dušikov oksid (NO)

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