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The Effect of *in ovo* Feeding Compared with Dietary Feeding of Betaine on Performance, Immunity and Liver Activity of Broiler Chickens Exposed to High Temperatures

Research Article

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ABSTRACT

This experiment was conducted to investigate the effects of *in ovo* feeding and dietary feeding of betaine on performance, immunity, liver activity, blood cation-anion balance and bone parameters of chickens exposed to high temperatures. A total of 600 fertile eggs from a broiler breeder strain (Ross 308) were used for *in ovo* feeding of 0 and 1 g/L betaine solution at 17.5 d of incubation. After hatching, 192 male chicks were transferred to the experimental cages. The diets consisted of two types; one of them without betaine and the other one containing 1 g/kg of betaine. Then chicks were exposed to elevated temperatures 4 ˚C above optimum from 7-28 d of age for 4 h per d and after 28 d they were kept at optimum temperature. The chicks were divided into 4 treatments as a completely randomized 2 × 2 factorial design. There was no effect of *in ovo* feeding of betaine on performance, immunity parameters, liver activity, blood cation–anion balance and bone parameters. Chickens fed on the diet containing the betaine had the higher feed intake, daily weight gain and final body weight ($P<0.05$), but not significantly different feed conversion ratio. At 28 d, the weights of spleen and bursa of birds in the treatment groups fed betaine were higher ($P<0.01$). The betaine-fed group had higher antibody titer against infectious bursal disease ($P<0.05$), and lower heterophile/lymphocyte ratio, alanine aminotransferase and aspartate aminotransferase enzymes and C-reactive protein ($P<0.01$). In birds fed on the betaine in the diet, blood cation–anion balance was lower ($P<0.01$), whereas tibia length ($P<0.05$), and tibia ash percentage were higher at 28 ($P<0.05$) and 42 d ($P<0.01$). These results indicated that dietary feeding of betaine resulted in an improvement in performance, immunity parameters, liver activity, blood cation–anion balance and bone parameters and decreased the adverse effects of the high temperatures.

KEY WORDS antibody titer, cation-anion balance, immune organs, WBC count.

INTRODUCTION

Climatic factors are among the most crucial factors limiting animal production in hot areas. Nowadays, chickens are more susceptible to heat stress due to higher production and feed conversion efficiency. In addition, global warming makes high temperature-related problems more highlighted (He *et al.* 2015). High temperatures may cause decreased feed intake and body weight gain, higher feed: conversion ratio, reduced metabolic rate and intestinal microbial dysbiosis (Dai *et al.* 2011; He *et al.* 2015). Several methods have been proposed for reducing the negative effects of high temperatures in poultry, such as managing the environment and/or nutrition, like feed additives in the diet. Diet modifications that promote a higher intake can maintain electrolytic and water balance during heat stress (Heidari *et al.* 2013; He *et al.* 2015).

Natural betaine is a feed additive derived from sugar beet. Betaine is used in poultry diet to reduce methionine and choline requirement as a methyl donor, as well as decrease performance deficiencies as an osmoprotectant in stressful conditions, such as heat and hyperosmotic stress (Ratriyanto *et al.* 2009; Dos Santos *et al.* 2019). Being an osmotic balancer, betaine can be effective in preventing dehydration in broilers under heat stress and maintaining their optimum performance (Tucker and Remus, 2001). It has been shown that betaine improves water transit through the enterocyte, helps villus growth of broilers and decreases the impact of a coccidial challenge (Kettunen *et al.* 2001). Also, enterocyte apoptosis is reduced (Alfieri *et al.* 2002) and inflammatory responses like the phagocytosis activity of macrophages and nitrogen oxide release are improved by betaine usage (Klasing *et al.* 2002). The data are contradictory, as for instance Matthews and Southern (2000) did not observe any effect when betaine was fed during a coccidial challenge, and also, some studies have shown that performance results are inconsistent (Mahmoudi *et al.* 2018; Dos Santos *et al.* 2019).

In ovo injection technology shows that the post hatching physiological state is improved by supplementing eggs with nutrients (Gholami *et al.* 2015; Youssef *et al.* 2017). Johnston *et al.* (1997) demonstrated that poultry's protection against pathogens is better using *in ovo* technology. It has been observed that solutions like amino acids, carbohydrates, prebiotics and synbiotics injected into the amniotic fluid during late embryogenesis in domestic fowl, can be digested and absorbed by the embryonic intestine before hatching (Uni *et al.* 2005; Foye *et al.* 2006; Youssef *et al.* 2017).

Also, *in ovo* administration affects growth performance, carcass traits and meat quality (Maiorano *et al.* 2012) as well as immune system development (Slawinska *et al.* 2014). The problem of limited egg nutrients can be solved by the help of *in ovo* feeding of supplemental nutrients (Foye *et al.* 2006; Gholami *et al.* 2015).

Few studies on *in ovo* feeding of betaine are available. It has been reported that betaine enhances young chicks' performance quality through the transition period after hatching. Administration of betaine via *in ovo* nutrition regulates hepatic cholesterol metabolism (Hu *et al.* 2015) and improves hatching weight, final weight and feed conversion of chickens at 42 d of age (Gholami *et al.* 2015).

Therefore, this study aimed to evaluate the effect of *in ovo* feeding compared with dietary feeding of betaine on performance, immunity, liver activity, blood cation–anion balance and bone parameters of broiler chickens exposed to high temperatures.

MATERIALS AND METHODS

In ovo feeding

In ovo solutions were prepared by dissolving 1 g of betaine (Betafin S1, 96% Betaine anhydrous, feed grade; Danisco Animal Nutrition, UK) in 1 L distilled water as solvent (w/v). For *in ovo* feeding (IOF) of betaine, a total number of 600 fertile eggs from (Ross-308) broiler breeder strain at 43 weeks of age were obtained. Eggs were incubated at 37.8 ˚C and 63% RH, and unfertilized eggs were discarded after the eggs were candled at day 17.5 of incubation. The fertilized eggs were weighed and distributed into two groups (each group 300 eggs) with an equal weight variation of 55 ± 1 g. The eggs were washed and sanitized using iodine tincture before the injection, then 1 mL of the prepared betaine solution was injected into the amniotic fluid using a 21-gauge needle. Distilled water was injected into the betaine-free group to even out the conditions. The IOF procedure was applied as described by Tako *et al.* (2004). Afterwards, molten paraffin was used to seal the injection holes, and the eggs were put in hatching trays. Finally, all the eggs were returned to the hatcher up to the hatching day.

Birds and diets

The experiment was conducted at the poultry farm of Rezvan Agriculture Faculty of Kerman, Iran in 30˚ 17' 24" N, 57˚ 3' 36" E Geographical coordinates. After hatching, 192 male chicks were randomly chosen and reared according to the instructions for Ross 308 broilers (Aviagen, Newbridge, Scotland, UK). At day 1 of age, chicks were individually weighed and assigned to four groups (48 chicks/ treatment) with four replicates (12 birds per pen). Broiler chicks were housed in floor pens covered with wood shavings and were given *ad libitum* access to water and diet (Table 1). Based on NRC (1994) recommendations, the diet was formulated including two types: a diet without betaine and another diet containing 1g/kg of betaine. The experimental factors included two levels of 0 and 1 g/L of betaine injected *in ovo*, and two levels of 0 and 1g/kg of betaine in the diet after hatching. The treatments were as follows:

1) *In ovo* injection of no betaine solution (distilled water) with a control diet (without betaine).
2) *In ovo* feeding of no betaine solution (distilled water) with a diet containing 1 g/kg of betaine.
3) *In ovo* feeding of 1 g/L betaine solution with a control

diet (without betaine).

4) *In ovo* feeding of 1 g/kg betaine solution with a diet containing 1g/kg of betaine.

The high temperature conditions (optimum temperature + 4 ˚C, based on Morovat *et al.* (2016) study was set for 4 h per d (12:00 h-16:00 h) during d 7-28 of the experiment for all broilers. The high temperature conditions were discontinued after day 28, but the diets were used until day 42. The birds were kept according to the Iranian Council of Animal Care guidelines (1995).

Sample collection

During the experiment, body weight (BW), feed intake, and feed conversion were recorded every week. All chicks were vaccinated against infectious bursal disease (IBD) (cevac sant animal) on d 16 and Newcastle disease (ND) (Lohmann Animal Health Gmbh and Co. KG) on days 8 and 22 via drinking water. Two chicks from each pen were slaughtered, plucked and eviscerated at days 28 and 42 of age, after birds were starved for 6 h. Afterwards, the relative weights (% of BW) of immunity organs (liver, bursa and spleen) of broilers were recorded, and one blood sample was taken from the neck artery in each replicate. The blood samples were centrifuged at 1500 g for 10 min and the serum was transferred into vials and stored at -20 ˚C. Later, the serum samples were analyzed for antibody titers, Na^+, K^+, Cl^-, liver enzymes [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)] and C-reactive protein (CRP) levels.

Blood cation–anion balance (CAB) was calculated by using Na + K – Cl level formula (mEq/L). Also, 4 blood samples containing anti-coagulant were taken per each treatment for evaluating blood cell counts. Hemagglutination Inhibition (HI) test (Brugh *et al.* 1978) and ELISA method were used for measuring antibody titer against Newcastle disease vaccine (NDV) and IBD, respectively.

In addition, the length of the left tibia of each carcass was measured by a caliper (in mm), after separation from meat. For measuring the ash percentage, bones were placed in an oven with 80 ˚C temperature for 24 h, then their fat was extracted by Soxhlet apparatus and again the fat-free bones were placed in the oven for 24 h. The initial weight of samples was in fact the weight of dry fat free bones. Subsequently, bones were crushed and placed in an electrical oven with 600 ˚C temperature for 8 h for obtaining the white ash. Then, samples were weighed again and the ash weight (g) was measured. Finally, the ash percentage was calculated by the following formula:

Bone ash (%) = (ash weight (g)/dry sample weight (g)) × 100

Statistical analysis

To determine statistical differences among the treatments, data were analyzed using the generalized linear model (GLM) procedures (SAS, 2005). This experiment was designed for all parameters in a 2 × 2 factorial trial within a completely randomized design. Differences among the treatments were compared by the Tukey test, and the values were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

Table 2 shows that betaine injection had no significant effect on broilers performance parameters, but betaine feeding significantly increased feed intake, daily weight gain and final body weight of broilers ($P<0.05$). However, feed conversion ratio (FCR) in betaine-fed broilers was not significantly different from not fed betaine broilers. Also, the interaction of betaine feeding and injection did not cause any significant difference in any of the performance parameters of broilers.

According to Table 3, the weight of immune organs was not affected by betaine injection, but betaine feeding significantly increased bursa and spleen weight at 28 days of age ($P<0.01$) but had no significant effect on liver weight. Weight of none of the immune organs was affected by betaine feeding at 42 days of age. Interaction of injecting and feeding betaine at day 28 was significant on bursa ($P<0.01$) and spleen weight ($P<0.05$).

Betaine-consumed groups had the highest bursa weight at day 28, which was significantly different from the control group ($P<0.01$). Betaine-fed and -injected group had the highest spleen weight at day 28, which was significantly different from the control and betaine-injected groups ($P<0.05$). Interactions between experimental factors had no significant effect on liver weight at day 28 and liver, bursa and spleen weight at day 42.

It is observed in Table 4 that betaine injection had no significant effect on any of the humoral immune parameters, liver enzymes and CRP. Betaine feeding had no effect on antibody titers against Newcastle vaccine but increased antibody titers against IBD vaccine ($P<0.05$). Total WBC count was not affected by betaine feeding. But, the ratio of heterophils to lymphocytes decreased significantly ($P<0.01$) as a result of betaine feeding. Antibody titers against Newcastle and IBD vaccines, as well as white blood cells (WBC) counts, were not affected by the interactions of experimental factors.

Betaine feeding treatments produced significantly lower heterophil to lymphocyte ratios ($P<0.05$) than other treatments. Also, feeding betaine significantly decreased liver enzymes and CRP levels ($P<0.01$).

Table 1 Ingredients and calculated nutrient compositions of the basal diets (as fed basis)

Ingredient (g/kg)	0-21 d	21-42 d
Corn	547	622.5
Fish meal	30. 0	20. 0
Soybean meal	355	297.3
Soybean oil	35	30
Oyster shell-flour	12	12.5
Dicalcium phosphate (DCP)	11.2	9
Sodium chloride	3.9	3
Methionine	1.40	0.7
Trace mineral-vitamin permix[1]	5	5
Calculated values (g/kg)		
Metabolisable energy (kcal/kg)	3016	3081
Crude protein	216.8	192.6
Ca	9.43	8.67
Available phosphorus	4.24	3.37
Na	1.89	1.44

[1] Supplied per kg of diet: Antioxidant (ethoxyquin): 100 mg; Biotin: 0.2 mg; Calcium pantothenate: 12.8 mg; Cholecalciferol: 60 mg; Cyanocobalamin: 0.017 mg; Folic acid: 5.2 mg; Menadione: 4 mg; Niacin: 35 mg; Pyridoxine: 10 mg; Trans-retinol: 3.33 mg; Riboflavin: 12 mg; Thiamine: 3.0 mg; DL-tocopheryl acetate: 60 mg; Choline chloride: 638 mg; Co: 0.3 mg; Cu: 3 mg; Fe: 25 mg; I: 1 mg; Mn: 125 mg; Mo: 0.5 mg; Se: 200 mg and Zn: 60 mg.

Table 2 The effect of *in ovo* and dietary feeding of betaine on performance of broiler chickens under high temperature conditions for 7-42 d

Item (7-42 d)	Feed intake (g/day)	Daily weight gain (g)	Feed conversion ratio	Final body weight (g)
***In ovo* injection**				
0 ppm injection	90.81	46.88	1.94	1931.52
1000 ppm injection	91.05	47.41	1.92	1953.23
SEM	0.233	0.237	0.010	9.786
Significance	NS	NS	NS	NS
Feeding				
Diet without betaine	90.44^{b}	46.65^{b}	1.94	1921.98^{b}
Diet with 1000 ppm betaine	91.41^{a}	47.64^{a}	1.92	1962.76^{a}
SEM	0.234	0.237	0.009	9.786
Significance	*	*	NS	*
Interaction				
0 ppm injection × no feeding	90.29	46.45	1.94	1913.93
0 ppm injection × 1000 ppm feeding	91.33	47.31	1.93	1949.12
1000 ppm injection × no feeding	90.59	46.85	1.93	1930.05
1000 ppm injection × 1000 ppm feeding	91.50	47.97	1.91	1976.42
SEM	0.330	0.336	0.013	13.839
Significance	NS	NS	NS	NS

The means within the same column with at least one common letter, do not have significant difference (P>0.05).
* (P<0.05) and ** (P<0.01).
NS: non significant.
SEM: standard error of the means.

The interaction between betaine injection and feeding on liver enzymes and CRP was significant. Betaine fed and injected group had the lowest ALT and the difference between this group and the control and only injected groups was significant (P<0.01). The AST and CRP levels were also affected by the interaction effects of the treatments. Betaine-treated groups had significantly lower AST and CRP levels than control and betaine-injected alone groups (P<0.01).

As shown in Table 5, the effect of betaine injection on blood cation-anion balance was not significant. But the effect of betaine feeding at 28 days of age significantly decreased the blood Na^+ and K^+ levels and increased the blood Cl^- level (P<0.01). At 42 days of age, blood Na^+ (P<0.05) and K^+ (P<0.01) levels were reduced by betaine feeding and Cl^- level (P<0.01) was elevated. As a result, the CAB levels of the blood were reduced by betaine feeding at both 28 and 42 days of age (P<0.01). Interaction of experimental factors on blood CAB was also significant. At day 28, the betaine-fed groups had significantly lower Na^+ and K^+ and higher Cl^- levels, resulting in lower CAB level (P<0.01).

At day 42, Na^+ level was not affected by the interaction of the studied factors, but a decrease in blood K^+ level and an increase in blood Cl^- level resulted in a significant decrease in CAB level in betaine fed groups (P<0.01).

Table 3 The effect of *in ovo* and dietary feeding of betaine on relative weights (% of live weight) of immune organs of broilers under high temperature conditions after 28 and 42 days

Item	28 d			42 d		
	Liver	Bursa	Spleen	Liver	Bursa	Spleen
***In ovo* injection**						
0 ppm injection	2.59	0.204	0.08	2.82	0.09	0.13
1000 ppm injection	2.74	0.211	0.09	2.81	0.09	0.14
SEM	0.072	0.003	0.004	0.095	0.003	0.005
Significance	NS	NS	NS	NS	NS	NS
Feeding						
Diet without betaine	2.63	0.196^{b}	0.075^{b}	2.77	0.10	0.14
Diet with 1000 ppm betaine	2.71	0.219^{a}	0.096^{a}	2.85	0.08	0.13
SEM	0.072	0.003	0.004	0.095	0.003	0.005
Significance	NS	**	**	NS	NS	NS
Interaction						
0 ppm injection × no feeding	2.51	0.19^{c}	0.07^{c}	2.78	0.10	0.13
0 ppm injection × 1000 ppm feeding	2.67	0.22^{a}	0.09^{ab}	2.85	0.09	0.14
1000 ppm injection × no feeding	2.74	0.21^{b}	0.08^{bc}	2.76	0.10	0.14
1000 ppm injection × 1000 ppm feeding	2.74	0.22^{a}	0.10^{a}	2.85	0.08	0.12
SEM	0.102	0.005	0.006	0.134	0.008	0.007
Significance	NS	**	*	NS	NS	NS

The means within the same column with at least one common letter, do not have significant difference (P>0.05).
* (P<0.05) and ** (P<0.01).
NS: non significant.
SEM: standard error of the means.

Table 4 The effect of *in ovo* and dietary feeding of betaine on humoral immunity and liver activity of broilers under high temperature conditions after 42 days

Item	Anti-body titer		Blood cell count		Liver enzymes		
	NDV	IBDV	WBC	Het/lymph	ALT (U/mL)	AST (U/mL)	CRP (mg/L)
In ovo injection							
0 ppm injection	2.38	3940.88	5.34	0.53	8.33	183.12	0.57
1000 ppm injection	2.38	4128.38	5.29	0.53	8.15	181.54	0.56
SEM	0.191	88.281	0.213	0.016	0.086	3.004	0.005
Significance	NS	NS	NS	NS	NS	NS	NS
Feeding							
Diet without betaine	2.25	3728.37^{b}	5.44	0.58^{a}	8.51^{a}	191.43^{a}	0.64^{a}
Diet with 1000 ppm betaine	2.50	4340.88^{a}	5.19	0.49^{a}	7.97^{b}	173.24^{b}	0.48^{b}
SEM	0.191	88.281	0.213	0.016	0.086	3.004	0.005
Significance	NS	*	NS	**	**	**	**
Interaction							
0 ppm injection× no feeding	2.25	3597.50	5.48	0.58^{a}	8.56^{a}	192.74^{a}	0.65^{a}
0 ppm injection× 1000 ppm feeding	2.50	4284.25	5.20	0.49^{b}	8.10^{bc}	173.49^{b}	0.49^{b}
1000 ppm injection× no feeding	2.25	3859.25	5.40	0.57^{a}	8.47^{ab}	190.11^{a}	0.64^{a}
1000 ppm injection× 1000 ppm feeding	2.50	4397.50	5.18	0.49^{b}	7.84^{c}	172.98^{b}	0.48^{b}
SEM	0.270	227.46	0.302	0.023	0.122	4.249	0.007
Significance	NS	NS	NS	*	**	**	**

NDV: Newcastle disease vaccine; IBDV: Infectious bursal disease; WBC: white blood cells; ALT: alanine aminotransferase and AST: aspartate aminotransferase and CRP: C-reactive protein.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
* (P<0.05) and ** (P<0.01).
NS: non significant.
SEM: standard error of the means.

Based on Table 6, betaine injection had no significant effect on bone parameters. Betaine feeding resulted in a significant increase in tibia length at 28 and 42 days of age (P<0.05) and tibia ash at day 28 (P<0.05) and 42 (P<0.01). It was observed the interaction effect on bone parameters at 28 days of age. At day 42, tibia length was not affected by the treatments, but tibia ash was significantly higher in the betaine-treated groups (P<0.01).

Researchers have reported that stress causes reduced feed intake and weight gain, as well as lower digestibility and higher FCR, which were linked to a decrease in nutrients available for growth under heat stress (Deeb and Cahaner, 2001). It seems that improvement in the performance of broilers fed betaine may be due to the involvement of betaine in protecting the intestinal epithelium against osmotic disorder. Therefore, the effects of betaine on performance improvement in the present study are probably because of its two important roles as methyl donor and osmolality in cell homeostasis (Konca and Kinkpinar, 2008). Also, Shakeri *et al.* (2018) stated that betaine use improved the permeability of nutrients through the jejunal wall in chicks under heat stress.

There have been few studies on the effects of betaine injection on broiler performance. In this study, betaine injection did not affect the chick performance. Youssef *et al.* (2017) reported that injecting 5 mg of betaine into Fayoumi eggs on day 18 resulted in improvement in final chick weight, but the FCR did not change significantly over the 8-weeks. A study by Gholami *et al.* (2015) showed an improvement in final chick weight and FCR by injecting betaine on day 12 of incubation. Hu *et al.* (2015) stated no significant difference in birth weight with 2.5 mg of betaine injection in eggs before incubation.

In this study, betaine feeding increased feed intake, daily weight gain and final weight of chickens affected by high temperatures, but FCR did not change significantly. In agreement with the findings of this study, Sakomura *et al.* (2013) also reported increasing feed intake, but the FCR change was not significant by adding 0.05 and 0.075% betaine to diets containing low levels of methionine and choline. Singh *et al.* (2015) and Ismail and Ahmad (2017) studies showed similar results by adding betaine to the diet. Chand *et al.* (2017) also observed an increase in average daily gain and final weight gain by adding 0.1 and 0.2% betaine to broiler diets under heat stress, but only 0.2% betaine level improved the FCR. Improvement in performance parameters was reported by He *et al.* (2015) and Shakeri *et al.* (2018) using betaine for broilers under heat stress, Al-Sultan *et al.* (2019) replacing betaine with methionine for heat-stressed chickens, Park and Kim (2017) using betaine in heat-stressed ducks diets, and Park and Zammit (2019) who combined the use of betaine and vitamin C for feed-restricted ducks under heat stress conditions. In contrast, Dos Santos *et al.* (2019) reported that different levels of betaine in the diets containing fiber did not impact feed intake, live weight and FCR at day 14. Also, the study by Mahmoudi *et al.* (2018) and Sahebi Ala *et al.* (2019) indicated no significant effect on performance parameters by replacement of methionine with betaine in diets of chickens exposed to heat stress.

Kusandi and Djulardi (2011) stated that the weight of immune organs (bursa, liver and spleen) decreased significantly under heat stress, which could be a consequence of reduced feed intake and thus fewer nutrients for proper growth of these organs. Bursa fabricius and spleen are the first and second lymphoid organs, respectively, that are directly related to the immune system and play a role in both humoral and cellular immunity (Esmailzadeh *et al.* 2013). Betaine can enhance the immune system due to its beneficial effects on lymphocyte synthesis in the internal mucosal layer and thickness of muscle layer of intestine and the role of these layers in the avian immune system (Hamidi *et al.* 2010). The results of this study showed a positive effect of betaine feeding on immunity parameters in broilers under high temperature conditions, while betaine injection did not affect these parameters. Similarly, Youssef *et al.* (2017) reported that Newcastle titer did not significantly change with betaine injection in Fayoumi eggs. Also, Gholami *et al.* (2015) study showed no effect of betaine injection on IgG and IgM antibody titers against sheep red blood cells (SRBC) at 21 and 42 days of age. Moreover, Hu *et al.* (2015) stated that betaine injection did not affect the liver weight at hatching time.

Feeding betaine resulted in increased antibody titers produced against the IBD vaccine as well as increased bursa and spleen weight. Alahgholi *et al.* (2014) also reported an increase in bursa weight and antibody titers against influenza vaccine, using betaine. Furthermore, Attia *et al.* (2016) observed that betaine supplemented in laying hens diet under heat stress resulted in spleen and liver weight increase. In contrast, Mahmoudi *et al.* (2018) noted that liver, spleen and bursa weight were not affected by adding betaine instead of methionine in the broilers diet under heat stress. In the study of Ismail and Ahmad (2017), betaine did not affect the liver and spleen weight of broilers. Differences observed in these studies may be due to differences in the amount of environmental stress applied.

Additionally, in agreement with our results, adding betaine caused a reduction in the ratio of heterophils to lymphocytes in broilers under heat stress (Chand *et al.* 2017; Al-Sultan *et al.* 2019) and high levels of peripheral ammonia in laying hens (Gudev *et al.* 2011), which indicates the desirable effects of betaine on reducing environmental stress.

In the present experiment, the decrease in the level of ALT, AST and CRP indicates the positive impact of betaine on reducing the effects of heat stress on liver oxidation and damages. Zulkifili *et al.* (2004) showed that heat stress decreases antibody synthesis which contributes to the synthesis of inflammatory cytokines under stress conditions that stimulate corticosterone production and release from adrenal glands. Awad *et al.* (2014) reported that hepatic enzymes ALT and AST significantly decreased at levels of 0.1 and 1.5% betaine in broilers diet.

Table 5 The effect of *in ovo* and dietary feeding of betaine on blood cation–anion balance (mEq/L) of broilers under high temperature conditions after 28 and 42 days

Item	28 d				42 d			
	Na	K	Cl	CAB	Na	K	Cl	CAB
***In ovo* injection**								
0 ppm injection	141.25	23.05	0.36	163.94	140.88	18.64	0.40	159.12
1000 ppm injection	140.13	23.15	0.36	162.91	140.75	18.80	0.41	159.15
SEM	0.648	0.304	0.002	0.623	0.234	0.181	0.003	0.305
Sig	NS	NS	NS	NS	NS	NS	NS	NS
Feeding								
Diet without betaine	144.63[a]	25.88[a]	0.35[b]	170.16[a]	141.25[a]	21.04[a]	0.39[b]	161.90[a]
Diet with 1000 ppm betaine	136.75[b]	20.33[b]	0.38[a]	156.70[b]	140.38[b]	16.40[b]	0.42[a]	156.36[b]
SEM	0.648	0.304	0.002	0.624	0.234	0.181	0.003	0.305
Sig	**	**	**	**	*	**	**	**
Interaction								
0 ppm injection × no feeding	145.25[a]	25.58[a]	0.34[b]	170.48[a]	141.25	20.99[a]	0.39[b]	161.86[a]
0 ppm injection × 1000 ppm feeding	137.25[b]	20.53[b]	0.37[a]	157.40[b]	140.50	16.29[b]	0.41[a]	156.38[b]
1000 ppm injection × no feeding	144.00[a]	26.18[a]	0.34[b]	169.83[a]	141.25	21.09[a]	0.40[b]	161.95[a]
1000 ppm injection × 1000 ppm feeding	136.25[b]	20.13[b]	0.38[a]	155.99[b]	140.25	16.51[b]	0.42[a]	156.34[b]
SEM	0.916	0.429	0.003	0.882	0.331	0.256	0.004	0.431
Significance	**	**	**	**	NS	**	**	**

CAB: blood cation–anion balance
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
* (P<0.05) and ** (P<0.01).
NS: non significant.
SEM: standard error of the means.

Table 6 The effect of *in ovo* and dietary feeding of betaine on bone parameters of broilers under high temperature conditions after 28 and 42 days

Item	28 d		42 d	
	Length (cm)	Ash (%)	Length (cm)	Ash (%)
***In ovo* injection**				
0 ppm injection	6.77	47.18	8.49	45.11
1000 ppm injection	6.93	46.27	8.51	44.23
SEM	0.104	0.486	0.079	0.265
Significance	NS	NS	NS	NS
Feeding				
Diet without betaine	6.67[b]	45.77[b]	8.37[b]	43.55[b]
Diet with 1000 ppm betaine	7.02[a]	47.68[a]	8.63[a]	45.79[a]
SEM	0.104	0.486	0.079	0.265
Significance	*	*	*	**
Interaction				
0 ppm injection × no feeding	6.59	46.09	8.31	43.98[b]
0 ppm injection × 1000 ppm feeding	6.95	48.28	8.66	46.24[a]
1000 ppm injection × no feeding	6.76	45.45	8.43	43.12[b]
1000 ppm injection × 1000 ppm feeding	7.10	47.09	8.60	45.34[a]
SEM	0.146	0.687	0.112	0.375
Significance	NS	NS	NS	**

The means within the same column with at least one common letter, do not have significant difference (P>0.05).
* (P<0.05) and ** (P<0.01).
NS: non significant.
SEM: standard error of the means.

Attia *et al.* (2005) also observed that supplementation of 0.1 and 0.2% betaine resulted in a significant decrease in ALT in broilers. However, Zayed (2012) reported that ALT and AST enzymes were slightly reduced with betaine supplementation in turkeys' diet. Attia *et al.* (2005) and Yusuf *et al.* (2008) stated that the addition of betaine led to a significant decrease in ALT but did not have a significant effect on other liver enzymes. On the other hand, Hassan *et al.* (2005) showed that the addition of 0.072-0.144% betaine did not influence ALT and AST enzymes in broilers under heat stress. The study by Mahmoudi *et al.* (2018) also showed that under heat stress, the levels of ALT, AST and ALP enzymes were not affected by using betaine instead of methionine in broilers diet.

Contradictions between the results of the present study and some previous findings regarding the effects of betaine on ALT and AST might be due to the differences of conditions in the experiments. Possibly, heat stress alters the response of liver enzymes to betaine supplementation. Also, to the best of our knowledge, no data are available regarding CRP expression by using betaine, but it was reported that heat stress caused an increase in CRP level in broilers (Sohail *et al.* 2010; Awaad *et al.* 2018). In this research, feeding betaine decreased the CRP level during heat stress condition which confirms the beneficial effect of betaine in elevated temperatures.

Brees *et al.* (1989) reported that the pH of poultry blood increased at elevated temperatures, which is because of panting. According to Teeter *et al.* (1999), panting leads to respiratory alkalosis resulting in altered blood flow patterns and water distribution in the body as well as disruption in minerals and ions balance (Smith and Teeter, 1993). Among the ions, Na^+, K^+ and Cl^- play a role in osmotic pressure and acid-base balance (Honarbakhsh *et al.* 2007).

In this study, the decrease in blood cation–anion balance due to betaine consumption indicates a positive effect of betaine on broilers under heat stress. Based on the study by Pereira *et al.* (2018), betaine increases the osmotic pressure of the cytoplasm during heat stress. Adding betaine to poultry diet or water can prevent body dehydration and maintain the optimum production level at elevated temperatures. Researchers reported that betaine reduces respiration rates in chickens under heat stress (Singh *et al.* 2015; Shakeri *et al.* 2018). The study by Khattak *et al.* (2012) showed that betaine feeding has no effect on blood Cl^- level but reduces blood Na^+ and K^+ levels in broilers which lowers the cation–anion balance. According to Alahgholi *et al.* (2014), blood Na^+, K^+ and Cl^- concentrations were not changed significantly by betaine feeding alone, but the interaction of betaine and water total dissolved solids (TDS) affected plasma electrolytes, which may count as beneficial effects of betaine during stress. Also, Park and Zammit (2019) and Park and Kim (2017) reported changes in electrolyte levels and reduction in blood pH under heat stress, by adding a betaine and vitamin C combination during feed restriction and adding betaine only to duckling diets, respectively.

High ambient temperatures have been observed to decrease tibia, femur and humerus growth in broilers (Bruno *et al.* 2000; Bruno *et al.* 2007). The positive impact of betaine on tibia features is probably due to the effects of betaine on reducing heat stress (Zhan *et al.* 2006; Panda *et al.* 2009), stimulating gastrointestinal growth (Norouzian *et al.* 2018), increasing the length of the intestinal villi (Alahgholi *et al.* 2014; Park and Zammit, 2019) and improving the permeability of materials through the intestinal wall (Shakeri *et al.* 2018) under stress conditions, which may lead to better absorption of nutrients including minerals.

CONCLUSION

These results indicate that dietary betaine improved performance, immunity parameters, liver activity, blood cation–anion balance and bone parameters and reduced the adverse effects of the high temperatures, but *in ovo* feeding of the betaine did not have any effect. Taking into account all the findings obtained through this study, primary nutritional parameters after hatching as well as different levels of betaine IOF are recommended for future research.

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