Effect of feed restriction with or without betaine supplementation on immune response, blood cation-anion balance, body temperature and bone characteristics of broiler chickens under heat stress

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Abstract

This experiment was conducted to determine the effects of feed restriction and betaine supplementation on immune response, blood cation-anion balance, bone characteristics and body temperature of broiler chickens under heat stress. A total of 160 one-day old chicks (Ross 308) in a 2×2 factorial experiment within a completely randomized design were divided into 4 treatments with 4 replicates in each. The treatments included two levels of feed restriction (none and 80% of ad libitum diet from day 7 to day 21) and two levels of dietary betaine (0 and 1 g/kg). The results indicated that betaine supplementation had no effects on antibody titer against Newcastle disease vaccine, but it caused an increase in bursa ($P<0.01$) and spleen ($P<0.05$) weights, as well as the antibody titer against Infectious bursal disease ($P<0.05$). Feed restriction levels had no effects on immune responses of broilers. Using betaine caused blood cation-anion balance to decrease significantly ($P<0.01$). Blood cation-anion balance was also decreased at day 42 by feed restriction ($P<0.05$). Only on day 14 of age and under stress, feed restriction caused a meaningful reduction ($P<0.01$) in cloacal temperature, but no significant difference was observed at days 21 and 28. Betaine supplementation reduced the cloacal temperature under heat stress at days 14 and 21 ($P<0.01$) and day 28 ($P<0.05$), but there was not any meaningful differences before heat stress at these days. Feed restriction led to a decrease in tibia length of broilers at days 28 ($P<0.01$) and 42 ($P<0.05$). Tibia ash showed no significant difference among treatments, but betaine supplementation increased tibia length ($P<0.01$) and tibia ash percentage ($P<0.05$). Interaction between these factors indicated that supplementing betaine and applying feed restriction together can be used as management strategy against heat stress.

Key Words: Feed restriction; Betaine; Immune response; Cation-Anion Balance; broiler

Introduction

Heat stress is considered as a serious problem in poultry industry. High environmental temperature can cause osmolytic changes in cells and disorder in water balance by changing cell water content through dehydration, so it can disrupt cellular activity (Tucker and Remus, 2001). Reports show that birds reduce their feed intake by an increase in environmental temperature (Konca and Kirkpinar, 2008). Therefore, using nutritional supplements in poultry feeding is considered as a solution to improve feed consumption by these birds especially under heat stress. One of these supplements is betaine which is being used increasingly. Betaine that was found in beet for the first time is a tri-methyl derived from glycine amino acid (Ueland *et al.* 2005). This plant extract has basically two important metabolic and physiological roles, because it acts as a methyl donor and also regulates cell osmotic pressure (Sun *et al.* 2008). Betaine as an osmotic balancer can help the birds suffering from heat stress by preventing body water loss (dehydration) and also providing great help to maintain optimum performance (Tucker and Remus, 2001).

In neutral temperature zone, heat production by poultry is in the lowest rate. At this time, the energy required to regulate body temperature is low and the net energy expending for production is high. Moreover, heat production in body is increased by environmental temperature ascendance above critical zone which leads to birds' exposure to heat stress. One of the most effective nutritional strategies to reduce the adverse effects of heat stress is feed restriction (Zulkifli *et al.* 2000). Research has indicated that feed restriction during hot hours of the day reduces heat increment and death, so it is recommended as a nutritional solution under heat stress condition. Since the energy is a restricting factor of growth under heat stress, the best solution is feed elimination and therefore reducing heat production by birds; for example, feed elimination at 10 A.M and feeding at 5 P.M, so that birds consume feed in cooler times of the day (Leeson and Summers, 2009).

The aim of this study is to evaluate the effects of dietary betaine and feed restriction on improving the immune system performance and homeostasis of broiler chickens under heat stress.

Materials and methods

Location and time of experiment

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 in March and April, 2016 with environmental temperature range of 3- 31°C.

Birds and treatments

A total of 160 one-d-old male broiler chicks (Ross 308) were housed in floor pens covered with wood shavings and fed on experimental treatments until the 42nd day of age (Table 1). At day 1 of age, chicks were individually weighed and assigned to 16 floor pens (10 birds per pen) in an environmentally controlled room with continuous fluorescent lighting.

Table 1: Ingredient composition and calculated values of the basal diets (as fed basis)

Ingredient (g/Kg)	0 – 21 d	21 - 42 d
Corn	547.0	622.5
Fish meal	30.0	20.0
Soybean meal	355.0	297.3
Soybean oil	35.0	30.0
Oyster shell-flour	12.0	12.5
Dicalcium phosphate	11.2	9.0
Sodium chloride	3.9	3.0
Methionine	1.4	0.7
Trace mineral+vitamin permix[1]	5.0	5.0
Calculated values		
Metabolisable energy (KJ/Kg)	12.60	12.88
Crude protein (g/Kg)	216.8	192.6
Calcium (g/Kg)	9.43	8.67
Available phosphorus (g/Kg)	4.24	3.37
Sodium (g/Kg)	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 g; 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 g; Zn, 60 mg.

The diet was formulated on the basis of NRC recommendations (1994) and included two types; one without betaine and the other containing 1 g/kg of betaine (Betafin S_1, 96% Betaine anhydrous, feed grade; Danisco Animal Nutrition, UK). The total chicks were divided into four groups (40 chicks/ treatment) with four replicates per each. The treatments included two levels of feed restriction (none and 80% of ad libitum diet from day 7 to day 21) and two levels of dietary betaine (0 and 1 g/kg). The experimental treatments were as follows:

1- Ad libitum diet without betaine (control group).
2- Ad libitum diet with 1 g/kg betaine in diet.
3- Feed restricted (80% ad libitum) diet without betaine.
4- Feed restricted (80% ad libitum) diet with1 g/kg betaine in diet.

For applying feed restriction, feed was removed from target groups for 4 hours a day while chickens were exposed to heat stress from day 7 to day 21. The temperature of experimental room was set on 32°C during the first week, then was decreased 3°C each week until the 5th week, and after that it was constantly 20°C for the rest of experiment. The elevated temperature condition (optimum temperature + 4ºC) was set during days 7 to 28 for all chickens for 4 hours a day (12-16 o'clock). This condition was discontinued after 28 days, but the diets were not changed. The birds were maintained according to the Iranian Council of Animal Care guidelines (1995).

Data collection

At the end of each week (days 14, 21 and 28), the cloacal temperature of two birds from each pen was measured by a digital cloacal thermometer (±0.01 ºC) at two different times: before (at 10:00 o'clock) and during (at 14:00 o'clock) the high temperature condition.

All chickens were vaccinated against Infectious Bursal Disease (IBD) (Cevac Sant Animal) on day 16 and Newcastle Disease (Lohmann Animal Health Gmbh & Co. KG) on days 8 and 22 via drinking water. At days 28 and 42 of age, birds were starved for 6 h before slaughtering and then two chicks from each pen were slaughtered, plucked and eviscerated. After that, the relative weights (% of BW) of immunity segments (liver, bursa and spleen) of chickens were recorded. Also, after slaughtering at days 28 and 42 of age, one blood sample was taken from the neck artery in each replicate. The 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 titer, Na^+, K^+ and Cl^- levels. Antibody titer was measured using Hemagglutination Inhibition (HI) test against Newcastle Disease Vaccine (NDV) (Brugh *et al.* 1978) and ELISA method against IBD.

In addition, the left tibia of each carcass was separated completely from meat and its length was measured by a caliper (in mm). Also for measuring the ash percentage, first bones were placed in an oven with 80° C temperature for 24h, then their fat was extracted by Soxhlet apparatus and again the free fat bones were placed in the oven for 24h. The weight of these dry free fat bones was the initial weight of samples. Afterward, bones were crushed and placed in an electrical oven with 600 °C temperature for 8h. When the bones became white, samples were weighed again and the ash weight (g) was obtained. Finally the ash percentage was calculated by the following formula:

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

Statistical analysis

Data were analyzed by using the GLM procedures (SAS, 2005) to determine statistical differences among the treatments. 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 Tukey test, and the values were considered statistically significant at $P < 0.05$.

Results

Immune response

The results of comparing antibody titers affected by main and interaction factors (Table 2) showed no significant effect on antibody titers against NDV, but using betaine significantly increased the antibody titer against IBD ($P<0.05$). Feed restriction and interaction between betaine and feed restriction had no effect on antibody responses of chickens exposed to heat stress.

Comparing the weights of immune organs at the age of 28 and 42 days (Table 3) showed that the liver weight was not influenced by treatments, but the weight of bursa of fabricius was significantly increased by betaine feeding ($P<0.01$); also, the relative weight of the spleen increased at day 42 by supplementing of betaine ($P<0.05$). Furthermore, the interaction effects showed that the weight of bursa of fabricius at days 28 ($P<0.01$) and 42 ($P<0.05$) were significantly higher in chickens fed diet containing betaine.

Table 2: Effect of feed restriction and betaine supplementation on antibody titer response against NDV (log_2 HI) and IBD (ELISA titer) in broilers under heat stress

Treatment	Antibody Titer (log_2) Against NDV[1]	Antibody Titer (ELISA titer) Against IBD[2]
Feed Restriction		
Ad libitum Diet	2.50	3941.00
Feed restricted Diet	2.66	4064.16
SEM	0.204	122.17
P-value	NS	NS
Betaine Supplementation		
Without Betaine	2.33	3704.16[b]
1 g/Kg Betaine in diet	2.83	4301.00[a]
SEM	0.204	122.17
P-value	NS	*
Interactions		
Ad libitum Diet × Without Betaine	2.33	3597.66
Ad libitum Diet ×1 g/Kg Betaine in diet	2.66	4284.33
Feed restricted Diet × Without Betaine	2.33	3810.66
Feed restricted Diet ×1 g/Kg Betaine in diet	3.00	4317.66
SEM	0.288	172.78
P-value	NS	NS

[1] Newcastle Disease Vaccine; [2] infectious Bursal Disease; [a,b] Within the same column, means with different superscripts differ significantly.; NS: Not Significant; * P < 0.05; ** P < 0.01.

Table 3: Effect of feed restriction and betaine supplementation on relative weights (% of live weight) of immune organs of broilers under heat stress

Treatment	Day 28			Day 42		
	liver	bursa	spleen	liver	bursa	spleen
Feed Restriction						
Ad libitum Diet	2.73	0.24	0.09	2.80	0.09	0.13
Feed restricted Diet	2.54	0.24	0.08	2.84	0.08	0.13
SEM	0.088	0.008	0.006	0.130	0.003	0.005
P-value	NS	NS	NS	NS	NS	NS
Betaine Supplementation						
Without Betaine	2.61	0.20[b]	0.08	2.91	0.07[b]	0.12[b]
1 g/Kg Betaine in diet	2.66	0.27[a]	0.09	2.74	0.09[a]	0.14[a]
SEM	0.088	0.008	0.006	0.130	0.003	0.005
P-value	NS	**	NS	NS	**	*
Interactions						
Ad libitum Diet × Without Betaine	2.73	0.21[b]	0.08	2.85	0.08[b]	0.12
Ad libitum Diet ×1 g/Kg Betaine in diet	2.73	0.27[a]	0.10	2.76	0.10[a]	0.14
Feed restricted Diet × Without Betaine	2.49	0.20[b]	0.08	2.96	0.07[b]	0.11
Feed restricted Diet ×1 g/Kg Betaine in diet	2.59	0.28[a]	0.09	2.72	0.09[ab]	0.14
SEM	0.125	0.012	0.008	0.183	0.005	0.007
P-value	NS	**	NS	NS	*	NS

[a,b] Within the same column, means with different superscripts differ significantly. NS: Not Significant; * P < 0.05; ** P < 0.01.

Blood cation-anion balance

The main and interaction effects of experimental factors on blood cation- anion balance (CAB) of broilers under heat stress are shown in Table 4. Feed restriction had no effect on Na^+ and Cl^- levels in the broilers' blood at days 28 and 42, but it significantly reduced blood K^+level at day 42 (P<0.05), so that, the blood CAB also significantly decreased (P<0.05). Using betaine caused a significant reduction in Na^+ and K^+ and an increase in Cl^- levels at day 28 (P<0.01), and a significant decrease in Na^+ and an elevation in Cl^- levels at day 42 (P<0.01). Therefore, blood CAB significantly decreased at days 28 and 42 (P<0.01). In addition, the interaction between feed restriction and betaine supplementation had a significant effect on blood CAB. At day 28, treatment groups fed by betaine had lower levels of Na^+ and K^+ and higher level of Cl^- (P<0.01), and so they had lower blood CAB in comparison with treatments without betaine in diet (P<0.01). At day 42, the treatment containing ad libitum feed without betaine had higher blood CAB than the treatment containing feed restriction with betaine in diet (P<0.01).

Body temperature

Table 5 shows the effects of experimental treatments on cloacal temperature of broilers before and under heat stress. Feed restriction only on day 14 of age and under stress caused a significant reduction in cloacal temperature (P<0.01), but there was no significant difference at days 21 and 28. On the other hand, the effect of betaine supplementation significantly reduced the cloacal temperature under heat stress at days 14 and 21 (P<0.01) and day 28 (P<0.05), but no significant difference was observed before heat stress at these days. Also, the effects of interaction between experimental factors were significant. At days 14 and 21 under heat stress, the treatment group with feed restricted diet containing betaine had the least cloacal temperature (P<0.01), but before the heat stress no significant differences were observed among treatments.

Table 4: Effect of feed restriction and betaine supplementation on blood cation-anion balance (mEq/L) of broilers under heat stress

Treatment	Day 28					Day 42			
	Na^+	K^+	Cl^-	CAB[1]		Na^+	K^+	Cl^-	CAB
Feed Restriction									
Ad libitum Diet	141.33	23.05	0.36	164.02		141.00	18.63[a]	0.40	159.23[a]
Feed restricted Diet	140.16	22.10	0.37	161.89		140.33	17.68[b]	0.41	157.61[b]
SEM	0.763	0347	0.002	0.973		0.311	0.230	0.003	0.425
P-value	NS	NS	NS	NS		NS	*	NS	*
Betaine Supplementation									
Without Betaine	143.83[a]	24.90[a]	0.34[b]	168.38[a]		140.83	20.15[a]	0.39[b]	160.59[a]
1 g/Kg Betaine in diet	137.66[b]	20.24[b]	0.38[a]	157.53[b]		140.50	16.16[b]	0.41[a]	156.25[b]
SEM	0.763	0.347	0.002	0.973		0.311	0.230	0.003	0.425
P-value	**	**	**	**		NS	**	**	**
Interactions									
Ad libitum Diet × Without Betaine	145.33[a]	25.56[a]	0.34[c]	170.55[a]		141.33	20.98[a]	0.38[b]	161.93[a]
Ad libitum Diet ×1 g/Kg Betaine in diet	137.33[b]	20.53[b]	0.37[b]	157.49[b]		140.66	16.28[c]	0.41[a]	156.54[c]
Feed restricted Diet × Without Betaine	142.33[a]	24.23[a]	0.35[c]	166.21[a]		140.33	19.32[b]	0.39[b]	159.26[b]
Feed restricted Diet ×1 g/Kg Betaine in diet	138.00[b]	19.96[b]	0.38[a]	157.58[b]		140.33	16.05[c]	0.42[a]	155.96[c]
SEM	1.080	0.491	0.004	1.376		0.440	0.325	0.005	0.602
P-value	**	**	**	**		NS	**	**	**

[1]Cation Anion Balance; [a,b,c] Within the same column, means with different superscripts differ significantly; NS: Not Significant; *P < 0.05; ** P < 0.01.

Table 5: Effect of feed restriction and betaine supplementation on cloacal temperature (˚C) of broilers before and under heat stress

Treatment	Day 14		Day 21		Day28	
	Before HS[1]	Under HS	Before HS	Under HS	Before HS	Under HS
Feed Restriction						
Ad libitum Diet	39.52	41.13[a]	39.70	40.63	39.80	40.38
Feed restricted Diet	39.66	40.15[b]	39.50	40.29	39.63	40.17
SEM	0.221	0.155	0.182	0.111	0.080	0.103
P-value	NS	**	NS	NS	NS	NS
Betaine Supplementation						
Without Betaine	39.64	41.14[a]	39.51	40.98[a]	39.67	40.50[a]
1 g/Kg Betaine in diet	39.54	40.13[b]	39.69	39.94[b]	39.76	40.05[b]
SEM	0.221	0.155	0.182	0.111	0.080	0.103
P-value	NS	**	NS	**	NS	*
Interactions						
Ad libitum Diet × Without Betaine	39.72	41.73[a]	39.60	41.15[a]	39.80	40.65
Ad libitum Diet ×1 g/Kg Betaine in diet	39.32	40.53[b]	39.81	40.11[b]	39.80	40.12
Feed restricted Diet × Without Betaine	39.57	40.56[b]	39.43	40.81[a]	39.54	40.36
Feed restricted Diet ×1 g/Kg Betaine in diet	39.76	39.74[c]	39.56	39.76[b]	39.73	39.98
SEM	0.313	0.219	0.257	0.157	0.113	0.146
P-value	NS	**	NS	**	NS	NS

[1] Heat Stress; [a,b,c] Within the same column, means with different superscripts differ Significantly; NS: Not Significant; * P < 0.05; ** P < 0.01.

Bone characteristics

Tibia characteristics are shown in Table 6. According to the table, feed restriction caused tibia length of broilers to decrease significantly at days 28 (P<0.01) and 42 (P<0.05), but tibia ash percentage had no significant difference among treatments. Betaine supplementation significantly increased tibia length (P<0.01) and ash (P<0.05). Also, interaction results showed that the treatment group with ad libitum diet containing betaine had maximum, and the one with feed restricted diet without betaine had minimum tibia length at day 28 (P<0.01). At day 42, the group with ad libitum feed without betaine had the lowest tibia length and its difference with other treatment groups was significant (P<0.05). The highest percentage of tibia ash at day 28 was in the group with ad libitum feed containing betaine and it was significantly different from the feed restricted without betaine treatment group ($P<0.05$). At day 42, groups supplemented with betaine had the highest percentage of tibia ash and their difference with ad libitum fed without betaine group were significant ($P<0.05$).

Table 6: Effect of feed restriction and betaine supplementation on bone characteristics of broilers under heat stress

Treatment	Day 28		Day 42	
	Tibia length (mm)	% Tibia ash	Tibia length (mm)	% Tibia ash
Feed Restriction				
Ad libitum Diet	6.75[a]	47.18	8.48[b]	45.10
Feed restricted Diet	6.42[b]	45.63	8.67[a]	46.28
SEM	0.043	0.545	0.044	0.483
P-value	**	NS	*	NS
Betaine Supplementation				
Without Betaine	6.44[b]	45.26[b]	8.44[b]	44.69
1 g/Kg Betaine in diet	6.73[a]	47.54[a]	8.71[a]	46.69
SEM	0.043	0.545	0.044	0.483
P-value	**	*	**	*
Interactions				
Ad libitum Diet × Without Betaine	6.58[b]	46.08[ab]	8.31[b]	43.98[b]
Ad libitum Diet ×1 g/Kg Betaine in diet	6.92[a]	48.28[a]	8.66[a]	46.23[a]
Feed restricted Diet × Without Betaine	6.31[c]	44.45[b]	8.58[a]	45.41[ab]
Feed restricted Diet ×1 g/Kg Betaine in diet	6.54[b]	46.80[ab]	8.76[a]	47.15[a]
SEM	0.061	0.771	0.062	0.683
P-value	**	*	*	*

[a,b,c] Within the same column, means with different superscripts differ significantly; NS: Not Significant; * $P < 0.05$; ** $P < 0.01$

Discussion

Stress in birds is followed by an increase in glucocorticoids which in turn decreases immune responses (Munck et al. 1984). Zulkifli et al. (2004) stated that humoral immunity of the birds under heat stress is weakend and causes a reduction in antibody synthesis. According to Mashaly et al. (2004), T-helper cytokines, which are important for antibody production, are depressed by heat stress.

In this study betaine supplementation had no effect on antibody titer against NDV, but it caused an increase in bursa and spleen weights, as well as the antibody titer against IBD, and led to improvement of the humoral immune response of broilers under heat stress. Our findings are in agreement with Alahgholi et al. (2014) who reported using betaine does not affect antibody titer against NDV and SRBC, and also spleen weight, but significantly increases bursa weight and titer against Influenza. Bursa of fabricius and spleen, which are respectively primary and secondary lymphatic organs, are directly related to immune system and support cellular and humoral immunity (Esmailzadeh et al. 2013). Zhan (2001) reported that adding betaine to broiler diet improves immune system and therefor preserves the bird from heat stress damages. Yan et al. (2000) attributed the positive effects of betaine on the poultry immune system which lead to producing antibodies and phagocytes under heat stress and protecting poultry from critical conditions such as diseases and various stresses.

In this study feed restriction had no harmful effect on immune responses of chickens exposed to heat stress. This result was compatible with Fassbinder-Orth and Karasov (2006) findings. Likely, Fanooci and Torki (2010) observed no reduction in antibody titers against NDV with 90% feed restriction, but Jahanpour et al. (2012) reported that a 75% feed restriction in the second week of age, reduced antibody titer against NDV. These differences may be due to the severity of the applied restrictions.

Brees *et al.* (1989) reported that chickens' blood pH increased in high temperature conditions, which is because of panting that leads to CO_2 loss. Teeter *et al.* (1999) observed that panting results in additional respiratory alkalosis which causes changes in blood flow patterns, distribution of water in the body and mineral and ionic balance (Smith and Teeter, 1993). Osmotic pressure and acid-base balance can be maintained by Na^+, K^+ and Cl^-, among essential elements (Honarbakhsh *et al.* 2007).

In this study, reduction of blood CAB as a result of betaine supplementation showed the positive effects of betaine in dealing with heat stress. According to Panda *et al.* (2008), betaine caused cytoplasmic osmotic pressure to increase under heat stress. Adding betaine in feed or water can prevent dehydration of birds that suffer from heat stress and maintain optimal performance in the poultry. Our findings were in agreement with Khattak *et al.* (2012) who reported betaine supplementation reduced concentration of serum Na^+ and K^+, but did not affect serum Cl^-, which led to CAB reduction in serum. Alahgholi *et al.* (2014) also reported Na^+, K^+ and Cl^- concentration of serum did not changed by just betaine supplementation, but interaction of water TDS and betaine supplementation significantly influenced plasma Na^+, K^+ and Cl^-, which reflects the beneficial effects of betaine under stress.

It seems that reduction in K^+ concentration in broilers under feed restriction condition is due to secretion of Aldestrone. Aldosterone's activity is increased through stress occurrence which increases K^+ excretion from kidneys (Roberts and Balnave, 1992).
According to our results supplementing betaine was quite useful in decreasing broilers body temperature under heat stress. Zhan et al. (2006) reported that betaine feeding decreased rectal temperature from 43.2ºC to 41.9ºC. These results disagree with those obtained by Gudevi et al. (2011) who found rectal temperature did not changed by betaine supplementation or enviromental temperature fluctuation. Egbuniwe et al. (2015) reported that betaine had no effect on reducing the cloacal temperature of chickens under heat stress; while in this study, the betaine fed and feed restricted treatment group had the lowest cloacal temperature. It seems feed restrictions is effective on beneficial impact of betaine on reducing the cloacal temperature.

According to Abu-Dieyeh (2006) heat generation and cloacal temperature is reduced as a result of less feed intake, thereby heat tolerance increases, so in our study the reduction of cloacal temperature at day 14 may be due to feed restriction, and also discontinuing of the process at day 21 can be because of increased feed intake after dietary restriction.

In this study, feed restriction under heat stress condition had a negative impact on the tibia length of chickens, which indicated that the retention and storage of minerals in the bones of broilers under heat stress was affected by feed restriction; but tibia ash was not affected by feed restriction. Bruno *et al.* (2000) reported that in broiler chickens under heat stress, dietary restriction leads to a reduction in the width and length of tibia and femur. But in another experiment, length and width of tibia and femur were not affected by feeding programs. Also growth of tibia and femur bones were not affected by environmental temperature and birds reared in cold temperatures always had less bone mass in comparison with birds reared in warm temperatures or thermal equilibrium. Moreover, no significant interaction between nutrition program and environmental temperature or the nutrition program and age was found (Bruno *et al.* 2007). Evidently, the effect of feed restriction and heat stress on bone characteristics is dependent on their duration and severity.

The positive effects of betaine on tibia characteristics is probably due to its impact on reducing the effects of heat stress (Zhan *et al.* 2006) and lengthening the intestinal villi (Alahgholi *et al.* 2014), thus improving the absorption of nutrients.

Conclusion The results of this experiment reflect the positive effects of betaine on immune responses, blood electrolyte balance, body temperature and bone characteristics under heat stress in broilers. Also, feed restriction in this situation is partly effective in improving these factors except tibia length. Interaction between these factors indicated that supplementing betaine and applying feed restriction together can be used as management strategy against heat stress.

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