Effects of dietary guanidinoacetic acid and betaine supplementation on performance, blood biochemical parameters and antioxidant status of broilers subjected to cold stress

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ABSTRACT
This study was conducted to determine the effects of dietary guanidinoacetic acid (GAA) and betaine supplementation on performance, antioxidant status and biochemical parameters of broilers subjected to cold stress. Based on a 2 × 2 factorial arrangement, 384-day-old male broiler chicks (Cobb) were randomly distributed between four experimental diets (with 8 replicates and 12 birds per replicate) included basal diet (as control) and the basal diet supplemented with 1200 mg/kg GAA; 600 mg/kg betaine and 1200 mg/kg GAA + 600 mg/kg betaine. No significant dietary effects were seen on performance, haematological and blood biochemical parameters including plasma glucose, uric acid, total antioxidant status, lactate, lactate dehydrogenase, red blood cell, haemoglobin, haematocrit and heterophil-to-lymphocyte ratios. However, malondialdehyde (MDA), and the activity of creatine kinase (CK), superoxide dismutase (SOD) and glutathione peroxidase (GPx) were affected by the experimental diets. Compared with the other groups, betaine supplementation decreased liver MDA level and SOD activity while increased activity of liver GPx and serum CK and decreased serum level of MDA were observed in birds fed the GAA-included diet. Overall, based on the results, it seems that dietary GAA and betaine could have beneficial effects on antioxidant status of broilers subjected to cold stress.

Introduction
Animals face a variety of environmental stressors every day, such as cold stress, which commonly exists in cold regions. The optimal temperature range for efficient broiler production is 18–21°C. Cold stress occurs when the ambient temperature decreases below 18°C. In cold environments, the energy requirement for maintenance increases to maintain the core body temperature, and the remaining small proportion of energy is further decreased, which dramatically affects bird growth (Sakomura 2004). Low ambient temperatures also cause increased feed intake (FI) and decreased nutrient digestibility, which in turn negatively influences the broiler performance. Also, cold stress causes oxidative stress in tissues such as heart, kidney, small intestine, thymus, adrenal glands and lungs (Venditti et al. 2010). Cold temperature and rapid growth enhance the metabolic rate, which is followed by creating a high oxygen requirement, and causes an imbalance between the respiration system and the high oxygen requirement, leading to systemic hypoxia (Wideman & Kirby 1995), which increases production of reactive oxygen species (ROS) in mitochondria (Lu et al. 2010). When the rate of ROS production exceeds the protective capacity of antioxidant, the excess ROS oxidizes proteins and DNA within the cell, damages the polyunsaturated fatty acids on the bio-membrane and initiates lipid peroxidation chain reaction (Bottje & Wideman 1995). Due to susceptibility of tissues to oxidative stress, some antioxidant systems are present in living organisms. Antioxidant system is diverse and responsible for scavenging the ROS and protecting cells against the free radicals. The major antioxidant enzymes are superoxide dismutase (SOD), GPx and Catalase (Cadenas & Davies 2000) and non-enzymatic antioxidant, including natural fat-soluble compounds (vitamins A, E, carotenoids, ubiquinones, etc.) and water-soluble compounds (ascorbic acid, uric acid, taurine, carnitine, etc.) (Surai 2002). The levels of malondialdehyde (MDA), as a biomarker of oxidative stress, in heart, liver and abdominal fat of control group in the cold-exposed broilers, were found higher than groups fed the diet supplemented with antioxidants (Seven et al. 2009).

Haematological parameters such as red blood cells (RBCs), white blood cells, mean corpuscular volume, mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) are valuable in monitoring the health status of farm animals (Oyawoye & Ogunkunle 2004). A reduced RBC count implies a decline in the level of oxygen that would be carried to the tissues and the level of carbon dioxide returned to the lungs, and haemoglobin is the iron-containing oxygen-transport metalloprotein in the RBC with physiological function of transporting oxygen to tissues and carbon dioxide out of the body (Ugwuene 2011).
The elevation of the erythrocyte counts is induced by low temperature exposure in birds (Blahová et al. 2007). Maekawa et al. 2013 observed erythrocyte counts enhanced by low temperature to increase oxygen supply to peripheral tissues for heat production in endothermic animals. Maekawa et al. (2013) described a significantly relationship between erythropoiesis, haematological parameters and low temperature in mice.

CreAmino®, a granulated product of guanidinoacetic acid (GAA) and precursor of creatine, is a compound synthesized from glycine and arginine by L-arginine: glycine amidinotransferase in avian kidney and liver. Subsequently, GAA is methylated by S-adenosylmethionine to creatine, and finally, ATP donates a phosphorus moiety to form the high-energy compound, phosphocreatine (Dilger et al. 2013). GAA has a potential to be a feed additive for chicks not only to replace dietary arginine, the fifth limiting amino acid in corn-soybean diets for being a feed additive for poultry, but also to support overall energy homeostasis of the bird. On the other hand, GAA is a more suitable feed additive compared with creatine and arginine due to lower price than both of arginine and creatine and is more chemically stable than creatine (Dilger et al. 2013).

Betaine is a common term for trimethylglycine, a substrate for betaine-homocysteine methyltransferase in liver and kidney (Attia et al. 2009). Betaine has two primary metabolic roles, including a methyl group donor and an osmolyte that assists in cellular water homeostasis. The antioxidant mechanism of betaine has been demonstrated to enhance non-enzymatic antioxidant defences via the methionine–homocysteine cycle and form a protective membrane by hydrophobicity and hydrophilicity properties of the three methyl groups and the carboxyl of betaine, respectively (Zhang et al. 2016).

The addition of GAA for creatine synthesis imposes a methyl-group demand on the body because creatine synthesis is considered to be the major user of methyl groups from SAM (S-adenosyl methionine). This process utilizes more SAM than all of the other physiological methyltransferases combined, accounting for about 75% of homocysteine formation (Mudd & Poole 1975). Thus, methyl group of betaine can be used in transmethylation reactions for synthesis of creatine and may reduce the requirement for other methyl group donors such as methionine and choline (Siljander-Rasi et al. 2003).

However, we presumed that betaine with its methionine sparing effect can help energy homeostasis in broilers during cold stress so that more methionine can be used for expansion of body protein mass. The ergogenic potential of betaine was first proposed by Borsook et al. (1952) after poliomyelitis patients supplemented with betaine–guanidinoacetate experienced improvements in general strength and endurance. The goal of the present study was to evaluate the effects of dietary supplemental betaine and GAA on performance, blood parameters and oxidative status in broiler chickens subjected to cold stress.

### Materials and methods

All experimental protocols adhered and were approved by the guidelines of the Animal Ethics Committee of Razi University (Kermanshah, Iran). A total of 384-day-old Cobb male broiler chicks were randomly distributed in 32 replicate cages. Based on a $2 \times 2$ factorial arrangement in a completely randomized design, birds in every eight replicates $(n = 12)$ were assigned to feed one of the four experimental diets, including basal diet and basal diet supplemented with 1200 mg/kg GAA; 600 mg/kg betaine; and 1200 mg/kg GAA + 600 mg/kg betaine. GAA and betaine were added in the form of CreAmino® and betaine hydrochloride, respectively. From day 1 of age, all chickens were kept under a 24-h light regimen and provided ad libitum access to water. The starter (2985 Kcal metabolizable energy, ME/kg and 22.12% crude protein, CP), the grower (3058 Kcal ME/kg and 21.57% CP) and the finisher diets (3130 Kcal ME/kg, 19.50% CP) were formulated and fed chicks during 1–10, 11–22 and 23–42 days of age, respectively (Table 1). All birds were brooded in 32–35°C from 1 to 7 days of age, then 30°C from 8 to 11 days. Thereafter, all birds were subjected to a step-down temperature programme of 1–2°C per day to 12–15°C on 21 days by set the thermostat as low as earlier day, and it remained constant until the end of the experiment (Igbal et al. 2001).

### Table 1. Ingredients and composition of experimental diets supplemented with guanidinoacetic acid (GAA) and betaine (g/kg, as-fed basis).

| Ingredient (%): | Age, days | Starter (1–10 days) | Grower (11–22 days) | Finisher (23–42 days) |
|----------------|-----------|---------------------|---------------------|----------------------|
| Corn grain (7.8% CP) | 47.665 | 53.348 | 53.197 |
| Corn Gluten | 2.996 | 1.888 | – |
| Soybean meal, 470 g of CP/kg | 40.280 | 35.696 | 36.623 |
| Soybean oil | 3.958 | 4.406 | 5.967 |
| Limestone (CaO3) (38% Ca) | 1.213 | 1.002 | 0.954 |
| Dicalcium phosphate | 2.301 | 2.039 | 1.855 |
| Common salt | 0.283 | 0.289 | 0.315 |
| Sodium bicarbonate (Na HCO3) | 0.119 | 0.095 | 0.043 |
| Mineral premix | 0.250 | 0.250 | 0.250 |
| Multivitamin premix | 0.250 | 0.250 | 0.250 |
| DL-Met, 990 g/kg | 0.307 | 0.284 | 0.268 |
| L-Lys-HCl, 780 g/kg | 0.164 | 0.140 | 0.062 |
| L-Threonine, 985 g/kg | 0.034 | 0.133 | 0.033 |
| Filler | 0.180 | 0.180 | 0.180 |
| Nutrients, % (as-fed basis): | | | |
| Metabolisable energy (Kcal/kg) | 2985 | 3058 | 3130 |
| Crude protein (%) | 22.12 | 21.57 | 19.50 |
| Calcium (%) | 1.043 | 0.899 | 0.845 |
| Available phosphorus (%) | 0.497 | 0.449 | 0.418 |
| Sodium (%) | 0.169 | 0.164 | 0.160 |
| Crude fibre (%) | 3.800 | 3.560 | 3.574 |
| Choline, ppm | 1.695 | 1.608 | 1.590 |
| Lys (TFD) % | 1.290 | 1.154 | 1.100 |
| Met (TFD)% | 0.645 | 0.589 | 0.552 |
| M + C (TFD)% | 0.970 | 0.885 | 0.834 |
| THR (TFD)% | 0.824 | 0.642 | 0.722 |
| TRP (TFD)% | 0.257 | 0.231 | 0.232 |
| Arg (TFD)% | 1.500 | 1.351 | 1.344 |
| Leu (TFD)% | 1.986 | 1.768 | 1.595 |
| Val (TFD) % | 1.019 | 0.917 | 0.883 |

*Contains 18.5% P and 21% Ca.

*Mineral premix provided per kilogram of diet: Mn (from MnSO4·H2O), 65 mg; Zn (from ZnO), 55 mg; Fe (from FeSO4·H2O), 50 mg; Cu (from CuSO4·5H2O), 8 mg; I (from Ca(IO3)2·H2O), 1.8 mg; Se, 0.30 mg; Co (from CoCl2), 0.20 mg; Mo, 0.16 mg.

*Vitamin premix provided per kilogram of diet: vitamin A (from vitamin A acetate), 11,500 IU; cholecalciferol, 2100 IU; vitamin E (from dl-tocopheryl acetate), 22 IU; vitamin B12, 0.60 mg; riboflavin, 4.4 mg; nicotinamide, 40 mg; calcium pantothenate, 35 mg; menadione (from menadione dimethyl-pyrimidinol), 1.50 mg; folic acid, 0.80 mg.

*Supplement Guanidinoacetic acid (GAA) and betaine were added at the expense of filler (sand).

*True Fecal Digestible Amino acid of the ingredients were obtained from Evonik Degussa Gmbh, Hanau, Germany.
At the end of the experiment, body weight gain (BWG) and FCR of broiler chickens were measured and feed conversion ratio (FCR) was calculated in each replicate cage. Also, one chick from each replicate cage was randomly selected and after 4 h starvation, blood samples from the wing vein were taken and then the bled bird were killed and tissue samples from liver and breast muscle were taken to evaluate antioxidant status. To measure total RBC, haemoglobin (HB), haematocrit (HCT), MCH, MCHC and plasma levels of glucose, albumin, LDL, HDL, lactate, MDA, uric acid, total antioxidant status (TAS), lactate dehydrogenase (LDH), CK and the heterophil-to-lymphocyte ratio, blood samples were collected into K2 EDTA tubes. Plasma was separated by centrifugation (3500 rpm for 15 min) of blood samples, transferred into 2 mL eppendorf tubes, and stored at −80°C until analysis. Plasma samples were thawed, and glucose, lactate, uric acid, CK and the LDH enzyme activities were determined using an autoanalyser (Abbott alcyon 300, USA). TAS was measured by TAS kit from Randox (NX 2332). To measure cytosolic enzymes activity and MDA level, the liver and breast samples were taken and then the bled bird were killed and tissue samples from liver and breast muscle were taken to evaluate antioxidant activity of liver in 42 days of age was detected, so that lower SOD activity and MDA were observed in birds fed the GAA-supplemented diet. Supplementation of betaine had no significant effect on tissue and plasma level of MDA, but adding 1200 mg/kg GAA to diet resulted in decreased level of serum MDA (P < .05).

**Statistical analysis**

Data were subjected to analysis of variance, using the general linear model procedure of SAS (2009). Duncan’s multiple range test was used to compare the means when treatment effects were statistically significant (P ≤ .05). The following model was used:

\[ Y_{ijk} = \mu + A_i + B_j + AB_{ij} + e_{ijk} \]

where \( Y_{ijk} \) is the individual observation, \( \mu \) is the overall mean, \( A_i \) is the GAA effect, \( B_j \) is the betaine effect, \( AB_{ij} \) is the GAA by betaine interaction and \( e_{ijk} \) is the random error.

**Results**

Effects of dietary betaine and GAA supplementation on daily BWG, FI and FCR are presented in Table 2. There were no significant effects of dietary treatments on daily BWG, FI and FCR of broilers. A significant interaction between dietary treatments and FCR was detected. The FCR was lower in broilers fed GAA+ betaine supplementation similar to control group.

Based on the results shown in Table 3, no significant effects of dietary treatment on the blood levels of uric acid, lactate, TAS and LDH during cold stress were detected. A significant interaction between dietary inclusion GAA and betaine on the plasma level of CK was observed during cold stress. Since the interaction between dietary supplemental GAA and betaine on the plasma level of CK was significant, the activity of CK was higher in cold-stressed birds fed the GAA and GAA + betaine-supplemented diets.

Blood levels of glucose, albumin and LDL were not influenced by supplementation of GAA and betaine, but a significant effect of GAA supplementation on HDL was detected (Figure 1). Haematological parameters are reported in Table 4. There was no diet effect on total RBCs, HB, HCT, MCH, MCHC and the heterophil-to-lymphocyte ratio.

As shown in Table 5, a significant effect of dietary treatment on antioxidant activity of liver in 42 days of age was detected, so that lower SOD activity and MDA were observed in birds fed the betaine-included diet and higher GPx activity was found in birds fed the GAA-supplemented diet. Supplementation of betaine had no significant effect on tissue and plasma level of MDA, but adding 1200 mg/kg GAA to diet resulted in decreased level of serum MDA (P < .05).

**Discussion**

**Performance parameters**

In the presented study, dietary inclusion GAA and betaine had no significant effect on performance of broilers. Control group and

Table 2. Effects of dietary supplemental guanidinoacetic acid and betaine on performance of the broiler chickens subjected to cold stress.

| Treatments (mg/kg) | FI (gr/day) | BWG (gr/chick/day) | FCR |
|--------------------|-------------|--------------------|-----|
| 0                  | 93.46       | 42.57              | 2.04|
| 1200               | 95.89       | 41.11              | 2.10|
| Betaine            |             |                    |
| 0                  | 94.91       | 41.53              | 2.06|
| 600                | 94.45       | 42.16              | 2.09|
| SEM                | 0.980       | 0.148              | 0.022|
| GAA                | .234        | .536               | .181|
| Betaine            | .820        | .789               | .421|
| GAA × betaine      | .628        | .236               | .011|
| Interaction means  | 93.20       | 43.67              | 1.97a|
| Control            | 93.72       | 41.47              | 2.12b|
| Control + GAA      | 96.61       | 39.38              | 2.14b|
| Control + betaine  | 95.17       | 42.84              | 2.06b|

Note: Values in column with no common superscript differ significantly (P < .05). GAA, guanidinoacetic acid.

Table 3. Effects of dietary supplemental guanidinoacetic acid and betaine on blood parameters and oxidative status of the broiler chickens subjected to cold stress.

| Treatments (mg/kg) | Lactate (mmol/lit) | Uric acid (mg/dl) | TAS (mmol/lit) | CK (U/lit) | LDH (U/lit) |
|--------------------|--------------------|-------------------|---------------|------------|-------------|
| GAA                | 64.41              | 3.55              | 1.15          | 4582b      | 1663        |
| 1200               | 66.61              | 3.12              | 0.99          | 9090b      | 2086        |
| Betaine            |                    |                   |               |            |             |
| 0                  | 55.41              | 3.36              | 1.05          | 7290       | 1666        |
| 600                | 69.61              | 3.31              | 1.09          | 6382       | 2083        |
| SEM                | 3.957              | 0.398             | 0.073         | 679        | 199.824     |
| P-value            | .062               | .517              | .325          | <.0001**   | .311        |
| GAA × betaine      | .068               | .938              | .785          | .119       | .311        |
| Interaction means  | .147               | .938              | .738          | .008**     | .398        |
| Control            | 62.80              | 3.55              | 1.10          | 4180f      | 1630        |
| Control + betaine  | 66.02              | 3.55              | 1.20          | 4985c      | 1697        |
| Control + GAA      | 48.02              | 3.17              | 0.99          | 10,400a    | 1702        |
| Control + betaine+ | 73.21              | 3.07              | 0.98          | 7780b      | 2470        |

Note: Values in column with no common superscript differ significantly (P < .05). GAA, guanidinoacetic acid level; Mmol/ lit, milmole/litre; mg/dl, milligram/decilitre; U/lit, unit/litre.
the birds fed GAA + betaine-included diet had lower FCR. There is no report on the effect of GAA on cold-stressed birds, and reports on the effect of dietary GAA supplementation on broiler performance under normal temperature are limited. In earlier studies, supplementation of GAA improved FCR and with no significant effect on BW gain in broilers (Mousavi et al. 2013). Also, supplemental GAA significantly improved FCR and breast meat yield, which might be related to increase muscle levels of creatine (Heger et al. 2014). Creatine is necessary for energy haemostasis of cells, especially in birds fed the creatine-deficient diets exclusively based on vegetable ingredients such as corn and soybean meal (Michiels et al. 2012). The GAA might be important in poultry nutrition in order to support overall energy homeostasis of the bird; an impact which is beyond the arginine sparing effect of GAA (Dilger et al. 2013).

The addition of 600 mg/kg betaine did not influence BWG and FCR compared to the control group, but improved FCR was observed in the birds fed diet supplemented with 1200 mg/kg betaine (Hamidi et al. 2010). In other study, the addition of betaine to diet improved BWG and FCR in broilers (El-Husseiny et al. 2007).

In the present study, numerically higher BWG was seen in control group and chicks fed GAA + betaine-included diet. Thus, when the combination of GAA and betaine were added, an improvement in FCR was obtained. The observed responses in FCR may have resulted from effects of adding GAA and betaine to the diet.

**Figure 1.** Effects of dietary supplemental guanidinoacetic acid and betaine on serum biochemical parameters of the broiler chickens subjected to cold stress. Values in columns with no common superscript differ significantly ($P < .05$).

**Table 4.** Effects of dietary supplemental guanidinoacetic acid and betaine on haematological parameters of the broiler chickens subjected to cold stress.

| Treatments (mg/kg) | RBC ($\times 10^6/\mu l$) | HB (g/dL) | HCT (%) | MCH (pg) | MCHC (g/dL) |
|---------------------|--------------------------|-----------|---------|----------|--------------|
| GAA                 |                          |           |         |          |              |
| 0                   | 2.795                    | 12.468    | 40.725  | 0.216    | 44.668       | 30.675        |
| 1200                | 2.820                    | 12.531    | 41.431  | 0.200    | 44.368       | 30.237        |
| Betaine             |                          |           |         |          |              |
| 0                   | 2.792                    | 12.462    | 41.181  | 0.192    | 44.587       | 30.281        |
| 600                 | 2.823                    | 12.537    | 40.975  | 0.223    | 44.450       | 30.631        |
| SEM                 | 0.083                    | 0.375     | 1.277   | 0.019    | 0.412        | 0.225         |
| P-value             |                          |           |         |          |              |
| GAA                 | .828                     | .906      | .698    | .690     | .610         | .179          |
| Betaine             | .795                     | .888      | .909    | .445     | .815         | .280          |
| GAA x Betaine       | .903                     | .591      | .563    | .926     | .194         | .876          |

Note: Values in column with no common superscript differ significantly ($P < .05$). GAA, guanidinoacetic acid level; pg, pico gram.

**Table 5.** Effects of dietary supplemental guanidinoacetic acid and betaine on the oxidative stability of the broiler chickens subjected to cold stress.

| Treatments (mg/kg) | Liver SOD (unit/mg protein) | Liver GPX (unit/mg protein) | Liver MDA (nmol/mg protein) | Breath MDA (nmol/mg protein) | Serum MDA (nmol/ml) |
|---------------------|----------------------------|-----------------------------|-----------------------------|------------------------------|---------------------|
| GAA                 |                            |                             |                             |                              |                     |
| 0                   | 4.77                       | 0.35$^b$                    | 0.35                        | 0.015                        | 1.31$^a$            |
| 1200                | 4.78                       | 0.40$^a$                    | 0.42                        | 0.009                        | 1.03$^b$            |
| Betaine             |                            |                             |                             |                              |                     |
| 0                   | 5.29$^a$                   | 0.39                        | 0.45$^a$                    | 0.015                        | 1.22                |
| 600                 | 4.26$^a$                   | 0.36                        | 0.32$^a$                    | 0.010                        | 1.12                |
| SEM                 | 0.185                      | 0.012                       | 0.165                       | 0.001                        | 0.064               |
| P-value             |                            |                             |                             |                              |                     |
| GAA                 |                            |                             |                             |                              |                     |
| Betaine             | .975                       | .047$^*$                    | .264                        | .109                         | .032$^*$            |
| GAA x Betaine       | .659                       | .849                        | .507                        | .277                         | .523                |

Note: Values in column with no common superscript differ significantly ($P < .05$). GAA, guanidinoacetic acid level; nmol/mg, nano mol/milligram; nmol/ml, nano mol/millilitre.
betaine on creatine synthesis metabolism and arginine and methionine that could be more available for other functions, including protein anabolism (Bertolo & McBrearty 2013; Dilger et al. 2013).

**Blood biochemical and haematological parameters**

Various studies have been conducted to evaluate the effect of cold stress on blood biochemical or haematological parameters of broiler chickens, but only the present study assessed the effect of dietary supplementation GAA and betaine on the mentioned parameters in broiler chicks reared in cold condition. Low ambient temperature caused a significant increase in the RBC count, HB level (MCH and MCHC) (Olanrewaju et al. 2010; Yang et al. 2014), that is a reflection of physiological oxygen-transport capacity. A high value for haematocrit in broilers with a high metabolic rate and under cold stress is an adaptive advantage because the blood’s oxygen-carrying capacity is enhanced (Ipek & Sahan 2006). Also, Glucose, lactate, LDH and CK enzymes are as markers that show energy status especially at the stress condition.

In the present study, no significant effect of GAA and betaine supplementation on haematological and serum biochemical parameters was observed, but numerically high levels of lactate were detected in birds fed the diet included 600 mg/kg betaine, and HDL level was significantly lower in broilers fed GAA-included diet. In hypoxia, serum cholesterol and LDL levels in mice fed creatine supplementation were lower compared to the control birds fed normal diet (Savransky et al. 2007). High basal cholesterol levels and reduced blood total cholesterol have been observed in human males and females fed supplemental creatine (Earnest et al. 1996).

In the current study, addition of 1200 mg/kg GAA yielded increases in the activity of CK. The phosphocreatine/creatine system buffers ATP/ADP ratio for all energy consuming functions of the cell. CK catalyses the conversion of creatine and utilizes ATP to create phosphocreatine and ADP. The rise in CK activity and serum creatinine could be either due to reduced clearance or due to overproduction or both (Hekimsoy & Oktem 2005). GAA might be able to support creatine production and increase CK activity. In the present study, the increased level of circulating CK indicates the previously reported potential role in maintaining high ATP turnover at increased level of circulating CK indicates the previously reported potential role in maintaining high ATP turnover at increased level of circulating CK indicates the previously reported potential role in maintaining high ATP turnover at increased level of circulating CK indicates the previously reported potential role in maintaining high ATP turnover at high temperature (Jayasundara et al. 2015). Similar to this study, a significant increase in CK activity was observed in the group supplemented with creatine (Dobgenski et al. 2016). Also, a significant increase in CK activity is observed in the male CFY rats kept in induced hypothermia condition, and it is concluded that CK played a role in energy supply of muscle tissue during the hypothermia condition due in part to the increased plasma level of CK (Buris & Debreczeni 1982).

**Antioxidant capacity and enzyme activity**

Antioxidant enzymes are the first defence level in antioxidant system of animal cells. A significant increase in free radical production was reported along with an increase in the expression of antioxidant enzymes during a period of non-damaging exercise (McArdle & Jackson 2000). These increases in antioxidant enzyme activities have been considered as protective responses to oxidative stress (Altan et al. 2003). Also, the condition of oxidative stress results in enhanced production of ROS that induce lipid peroxidation reactions, which are in turn manifested by an increased level of MDA in plasma and tissues (Sahin et al. 2002).

In the present study, the increased activity of the liver GPx and the lower level of serum MDA observed in birds fed the GAA-supplemented diet and lower activity of the liver SOD and MDA was seen in birds fed the betaine-supplemented diet. There is little information about the effects of exogenous GAA on human and animal antioxidant–antioxidant system, but since GAA has a guanidinium ion of conjugate base, which easily donates an electron, it may affect on oxidoantioxidant system of animals. Thus, the higher concentrations of GAA may generate a hydroxyl radical, and impede antioxidant capacity (Hiramatsu 2003).

GAA induces oxidative stress after experimental intrastratial infusion (Zugno et al. 2008), or excessive accumulation in pathological conditions (Mori et al. 1996), while dietary GAA improves antioxidant status by elevating total antioxidant capacity and the activities of several antioxidant enzymes (Wang et al. 2012). Contrasting results of the previous studies are accompanied with different levels of GAA achieved after GAA administration/accumulation. Pro-oxidant effects have been seen after intracerebral accumulation and highly elevated brain GAA concentrations (~100 µmol/L), while antioxidant effects seem to appear after GAA ingestion and relatively low post-administration serum levels of GAA (~5 µmol/L) (Ostojic 2015).

On the other hand, GAA-related metabolites (creatine and arginine) might be able to quench free radicals after GAA ingestion (Wang et al. 2012), suggesting indirect antioxidant effect of GAA utilization. Theoretically, if GAA metabolism remains undisturbed and/or low-to-modest quantities of exogenous GAA have been provided, a cumulative effect of the intervention might be neutral or antioxidant protection through creatine-and arginine-driven medium. Creatine, the end product of GAA utilization, is thought to have antioxidant capacity in some studies (Sestili et al. 2006; Sestili et al. 2009), but reduce antioxidant status in others (Percário et al. 2012). According to Fathi and Tanha (2015), arginine supplementation increased plasma GPx activity and decreased MDA level in the broiler with cold-induced acites. Thus, the more information is needed to know how GAA affects oxidant–antioxidant system to help its use as a nutritional compound under stress condition.

Zhang et al. (2016) reported that betaine exerts its antioxidant activity via two mechanisms. One mechanism involves scavenging ROS in cells via up-regulation of endogenous non-enzymatic antioxidant defences. Betaine was able to increase the levels of S-adenosylmethionine and methionine via the methionine–homocysteine cycle and improve the ROS-scavenging ability of the methionine sulfoxide reductase system. The other mechanism inhibits ROS generation by isolating cells from the oxidative stress inducer. Betaine is an amphiphilic molecule with a hydrophilic group, carboxyl terminal, and the three hydrophobic methyl groups at the N terminal, and may form a protective membrane around cells to prevent the contact of free radicals with the cytomerbrane. The structure of the protective membrane is such that the three hydrophobic methyl
groups at the N terminus are close to the lipid bilayer, and the carboxyl terminus is close to the water. Betaine is a zwitterion, and its carboxyl terminus is electronegative, which means that the outside surface of the protective membrane is also electronegative. Free radicals are molecules with one or more unpaired electrons. Therefore, free radicals are electronegative. The electronegative outside surface of the protective membrane may repel free radicals, preventing them from damaging the cytomembrane (Zhang et al. 2016).

Conclusions

Overall, the results of the present study showed that dietary inclusion GAA and betaine improved FCR. Adding GAA at 1200 mg/kg decreased plasma level of MDA, and increased antioxidant enzyme and creatine kinase activity in the broilers subjected to cold stress. In addition, the low level of liver MDA and SOD activity was observed in the birds fed diets supplemented with 600 mg/kg betaine. In conclusion, it seems that dietary GAA and betaine can alleviate the negative effect of cold stress via reducing lipid peroxidation and increasing activity of antioxidant enzymes, although more research is needed in this regard.

Geolocation information

Kermanshah (34°18′N 47°4′E) is located in the middle of the western part of Iran.

Note

1. Evonik Degussa GmbH, Hanau-Wolfgang, Germany.

Disclosure statement

No potential conflict of interest was reported by the authors.

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