Influence of dietary supplemental guanidinoacetic acid on performance, haematological parameters, carcass characteristics and enzyme activities in male broilers with cold-induced ascites

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ABSTRACT
This study aimed to evaluate the effects of dietary guanidinoacetic acid (GAA) on performance, enzyme activity and biochemical variables of broilers with cold-induced ascites. A total of 640 day-old male broiler chicks (Cobb 500) were randomly assigned to four treatments (with 8 replicates of 20 birds) including a control and diets supplemented with 0.6, 1.2 and 1.8 g of GAA per kg of feed. On day 14, the temperature was reduced to 17°C during the daytime and 14°C at night. Dietary inclusion of 0.6 g/kg GAA decreased BWG in compared to those fed other levels in the grower period (P < 0.05). The ratio of right to total ventricle, mortality and relative weights of liver were lower in birds fed the diet with 1.2 g/kg GAA compared to bids fed other levels (P < 0.05). The plasma activity of creatine kinase (CK) increased in the birds fed with 1.2 and 1.8 g/kg GAA compared to chicks fed the control diet. It can be concluded that dietary supplemental 1.2 g/kg GAA would be a beneficial way to decrease the mortality rate of broiler reared under cold stress conditions. In addition, the blood levels of HDL-C increased in broilers fed the 1.2 g/kg GAA diet.

Introduction

During the past several years, the main objectives were to improve feed conversion ratio (FCR), mortality rate and carcass yield in fast-growing meat-type chicken (broiler) industry. Moreover, providing welfare is considered an essential factor in order to increase the animal production systems. Metabolic diseases increase compromised welfare and financial losses because of deterioration of production value on account of increased mortality rate, less efficient utilization of nutrients and condemnation of carcasses; also, both intensive, quantitative selection and improved diet and management have caused to improve the overall performance of broilers over the past 50 years (Kalmar et al. 2010). Ascites syndrome (AS) or pulmonary hypertension syndrome (PHs) is a metabolic disorder of meat-type lines/hybrids broilers which have caused to improve the overall performance of broilers with cold-induced ascites. A total of 640 day-old male broiler chicks (Cobb 500) were randomly assigned to four treatments (with 8 replicates of 20 birds) including a control and diets supplemented with 0.6, 1.2 and 1.8 g of GAA per kg of feed. On day 14, the temperature was reduced to 17°C during the daytime and 14°C at night. Dietary inclusion of 0.6 g/kg GAA decreased BWG in compared to those fed other levels in the grower period (P < 0.05). The ratio of right to total ventricle, mortality and relative weights of liver were lower in birds fed the diet with 1.2 g/kg GAA compared to bids fed other levels (P < 0.05). The plasma activity of creatine kinase (CK) increased in the birds fed with 1.2 and 1.8 g/kg GAA compared to chicks fed the control diet. It can be concluded that dietary supplemental 1.2 g/kg GAA would be a beneficial way to decrease the mortality rate of broiler reared under cold stress conditions. In addition, the blood levels of HDL-C increased in broilers fed the 1.2 g/kg GAA diet.

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deficient diets and also improve growth performance in Arg-adequate diets (Michiels et al. 2012). Also, Khajali et al. 2020, reported the effect of GAA on growth performance, energy utilization and muscle development (protein accretion). If the cost of GAA is less expensive than, or equal to, commercially available Arg, then GAA supplementation would be more advantageous to supplement because of the improvement observed in Arg-adequate diets. Therefore, the poultry industry may use GAA as an Arg replacement, relieving the necessity for Arg supplementation in current poultry diets.

Based on the available literature, there is no study investigating the effect of dietary supplemental GAA on the performance of broiler chickens with induced ascites. It was hypothesized that supplemental GAA when included in Arg-adequate practical broiler diets would elicit a positive influence on alleviating the negative effects of ascites in broiler reared under cold-temperature conditions.

Materials and methods

The experiment was conducted at the animal husbandry station, Razi University, Kermanshah, western region of Iran positioned between 34°18' N and 47°03' E at a height of 1350 m from sea level.

Bird source and management

A total of 640 newly hatched Cobb male chicks were purchased from a commercial hatchery (male chicks were selected by vent sexing from a population of 1600, according to body weight; those with extreme weights were discarded). Chicks were randomly assigned into 4 groups, with eight replicates per treatment (20 birds per battery replicate cage). Birds were reared in battery cages (2.4 × 0.6 × 0.6 m) with a screen-wired floor. Each cage was equipped with a feeder to be manually filled daily. The temperature in the rearing chamber was 28–30°C in the first week and daily decreased by 1°C till 30°C on d 10. From d 11, all birds were exposed to a temperature cycle of 17°C during the daytime and 14°C at night in order to increase ascites susceptibility until the end of the experiment (Fig. 1) (Shlosberg et al. 1992; Korte et al. 1999; Buyse et al. 2001). Except for the applied temperature schedule, the chickens were kept under conditions that closely resembled commercial practice. Continuous light was provided for 24 h for the first 3 days, and then, 23 L: 1D light was adopted for the rest of the trial period.

Feeding and experimental diets

The basal diet was free of animal by-products in order to avoid the contribution of creatine from an additional source. Prior to the experiments, the experimental diets were analysed for dry matter (DM), crude protein (CP) and amino acids (AAs) using near-infrared spectroscopy. Metabolizable energy (ME) contents of corn and soybean meal were estimated by using the regression models (NRC 1994). All dietary nutrients met or exceeded (Cobb 2008) recommendations (Table 1). The basal diet was supplemented with 0.0, 0.6, 1.2 or 1.8 g GAA per kg of feed. GAA was added in the form of CreAMINO® and supplied at the expense of corn. The birds had ad libitum access to feed and water throughout the trial period. All birds received a pre-starter diet from 1 to 10 d. Grower and finisher diets were provided from 11 to 21 d and 22 to 42 d of age, respectively. The ME and CP contents of starter, grower and finisher diets were 2850, 3058 and 3130 Kcal/kg; 250, 225 and 215 g/kg, respectively. No type of medication was administered during the entire experimental period.

Growth performance

Feed intake (FI) and body weight (BW) were recorded at 1, 10, 24, and 42 days of age, whereas mortality was recorded daily throughout the study. FI was corrected for mortality. From these data, average FI and body weight gain (BWG) and FCR were calculated per cage for different rearing periods.

Blood parameters

At 42 d of age, 3 ml of blood was collected from a brachial vein (eight birds per treatment). Sera were separated by centrifugation at 3000 × g for 10 min. Triglyceride (TG), cholesterol (CHOL), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) in serum samples were determined using a corresponding reagent kit (Pars Azmoon Co., Tehran, Iran) and an automatic biochemical analyser (Clima, Ral. Co, Spain).

Thyroid hormones

The measurement of thyroid hormones was done by gama kit (Bio-source Europe Com.). In order to separate the serum from the samples, the clotted blood was centrifuged twice at 5000 rpm for two minutes. Then, 25 microliters of the sample was isolated for the 3, 5, 3′-triiodothyronine (T3) test, and 200 microliters of I125 iodine was added. After incubation for one hour in the shaker unit, it was perused by the gama kit. In order to evaluate the thyroxine (T4), 500 microliters of I125 iodine was added (Georgiou and Christofidis 1996). Then, the mixture was incubated in the shaker unit for one hour and finally read by the gama kit.

Enzyme activity

The activities of cytosolic creatine kinase (CK; EC 2.7.3.2) and lactate dehydrogenase (LDH; EC 1.1.1.27) enzymes were measured in heart tissue obtained from the mid-portion of the left ventricle free wall. Briefly, aliquots of 300 mg of frozen samples were homogenized in phosphate buffer (50 mM, pH 7.4) in the ratio of 100 mg tissue per 1 ml buffer in test tubes pre-cooled with ice. The homogenate was centrifuged at 2500 g for 10 min at 4 °C, and the suspension was further centrifuged at 12,000 g for 10 min. The supernatant was used for activity measurements of CK and LDH using a CK kit (Roche Diagnostics, Indianapolis, IN, USA) and LD-L10 (Sigma Diagnostics Inc., St. Louis, MO, USA), respectively. The
final activity measurements were performed during the linear phase of responses pre-established during the validation phase. These measurements were performed in a single assay for each enzyme in order to avoid inter-assay variability (Nain 2008).

**Organ index and ascites-related parameters**

On day 42 of the trial, six chicks, close to the mean of herd weight, were selected and sequentially slaughtered with intervals of approximately 3 min between the slaughters of individuals. When the head, shanks and feathers were removed, the carcass was eviscerated by cutting around the vent to remove all of the viscera. Once eviscerated, the carcass without giblets was weighed and expressed as a percentage of live weight and considered as the carcass yield. Breasts were weighed and expressed as percentages of the live body weight. The weights of the abdominal fat, liver, heart and gastrointestinal sections (after removal of digesta) were also measured to the nearest 0.01 g and expressed as a percentage of the live BW. Moreover, after removing the intestinal contents, the lengths of duodenum (pancreatic loop), jejunum (from the pancreatic loop to Meckel’s diverticulum), ileum (from Meckel’s diverticulum to ileocecal junction), and caeca were recorded. Postmortem examinations were performed on all dead chickens during the experimental period in order to diagnose ascites. Moreover, on day 42, a total of 40 birds per each treatment (5 birds per each cage) were randomly selected, sacrificed, and used for the weight of right ventricular (RV) to total ventricular (TV) (RV/TV) determination. Ascites-related mortality was diagnosed when an accumulation of abdominal or pericardial fluid was present, and the RV/TV was higher than 0.29 (Julian 1987). In order to calculate the RV/TV, hearts were collected, and the pericardium, peripheral adipose tissues, and atria were removed. The left ventricle and RV were separated, and their individual weights were measured on an analytical balance (Scaltec SBA41, Germany; precision 10-13 g), and the RV/TV was calculated (Julian 2005).

**Statistical analysis**

The data were analysed based on a completely randomized design using the GLM procedure of SAS® (SAS 2008). The results are reported as means. The data were tested for linear and quadratic contrasts using the incremental dietary GAA treatments (0 or control diet, 0.6, 1.2 or 1.8 g GAA per kg of feed). Data were tested for distribution normality and homogeneity of variance. Variables with significant F-tests ($P < 0.05$) were compared using Duncan’s multiple range test. Results were considered as significant when $P$-values were less than 0.05. Mortality data were subjected to Chi-square analysis.

**Results**

**Growth performance**

The effects of diet inclusion of GAA on the growth performance of broilers are summarized in Table 2. During the starter period (0–10 days), FI was significantly higher ($P < 0.05$) in birds fed the control diet (138.55 g) compared to those fed the diets included with 0.6 g/kg GAA (96.62 g). It was not observed significant difference between the control group and those fed with FI in birds fed the diets included 1.2 (121.56 g) or 1.8 (106.92 g) g/kg GAA in the starter period. FI was significantly higher in 1.2 GAA in comparison to the 0.6 GAA included diets in comparison to the 0.6 GAA group ($P < 0.05$) in grower and finisher periods. The birds in the control diet showed significantly higher BWG in comparison to the 0.6 GAA group ($P < 0.05$) in grower and finisher periods ($P < 0.05$). No significant differences were observed ($P > 0.05$) in FCR among all dietary treatments during the starter period (0–10 d). At both, 11–24 and 25–42 days of age, chicks receiving the control diet showed lower ($P < 0.05$) FCR compared to the chicks receiving the 0.6 and 1.8 GAA-included diets. The ratio of right to total ventricle, mortality and relative
weights of liver were significantly lower in birds fed the diet with 1.2 g/kg GAA compared to those in birds fed other levels (P < 0.05).

As indicated in Table 2, RV/TV ratio was significantly lower in the birds fed diet included 1.2 g/kg GAA compared to other groups (P < 0.05). Also, quadratic responses to GAA levels were observed in terms of RV/TV ratio.

### Blood parameters

As it is shown in Table 3, the serum concentrations of TG, total CHOL and LDL-C were not significantly affected by dietary treatments (P > 0.05). Birds fed the diet supplemented with 1.2 g/kg GAA showed a significantly higher level of blood HDL-C (P < 0.05) in comparison to other groups.

### Enzyme activity and Thyroid hormones

No significant effect of dietary treatments was found on serum levels of T3 and T4 (Table 4). In terms of enzyme activity, the activity of LDH was decreased in the birds fed the diet of 1.2 g/kg GAA in comparison to that of 1.2 g/kg GAA (Table 4). The activity of CK was lower in chickens fed control and 0.6 g/kg GAA diets compared to 1.2 and 1.8 g/kg GAA.

### Organ index and asces-related parameters

Effects of dietary treatments on carcass traits are represented in Table 5. No significant impacts of supplemental GAA on breast meat, abdominal fat, heart, and relative length of duodenum and ileum were observed. The relative length of jejunum was significantly lower (P < 0.05) in the birds fed 0.6 and 1.2 g/kg GAA compared to birds fed the control diet (P < 0.05). The relative weight of the liver was significantly lower in the birds fed 1.2 g/kg GAA compared to other groups.

### Discussion

**Growth performance**

Based on our knowledge, there are no other reports evaluating the effect of in-feed supplementation of GAA on the performance of broiler chicks with induced ascites. Our findings for growth performance were conflicting. Significant decreased FI in the birds fed the diet included 0.6 g/kg GAA were observed in the starter period, which is in line with the previous reports in which 0.6 g/kg GAA and higher concentrations caused a significant decrease in FI and BWG (European Food Safety Authority 2009). However, other levels did show a significant difference for FI in the starter period. In contrast, broiler chicks fed with the level of 1.2 GAA consumed more FI in comparison to those fed level of 0.6 GAA. It cannot be certainly stated that decreased FI was attributed to higher levels of GAA because it was not observed significant between levels of 0.6 and 1.2 g/kg GAA compared to the control birds. Another study did not show a significant effect of dietary supplemental GAA on FI in broilers (Ringel et al. 2007 and Khodambashi Emami et al. 2017). Guanidinoacetic acid not only spares Arg (Dilger et al. 2013) but it is also a precursor for the synthesis of nitric oxide (NO). Wang et al. (2014) showed that dietary
Arg may regulate appetite in ducks by conversion to NO. It can be argued that GAA may not increase NO, and thus, it does not improve NO. In the present study, increased FCR was detected in chicks receiving 0.6 and 1.8 g/kg GAA in the grower period and 1.2 g/kg supplemental GAA during the finishing period which is consistent with results reported by Michiels et al. (2012). However, several studies have reported positive effects of GAA on FCR (Lemme et al. 2007; Ringel et al. 2008). Dilger et al. (2013) reported that supplemental GAA to Arg-deficient diet improved in BWG and FCR. Tossenberger et al. (2016) did not observe a significant effect of diet supplementation of 0.6 g/kg GAA on BWG, FCR. Tossenberger et al. (2016) did not observe a significant effect of diet supplementation of 0.6 g/kg GAA on BWG, FCR.

Table 2. Growth performance at different rearing periods, right ventricle to total ventricle ratio at day 42 of age, and mortality from 1 to 42 days of age in male broilers given guanidinoacetic acid (GAA)-supplemented diets.

| Item                  | Control$^1$ | GAA0.6 | GAA1.2 | GAA1.8 | SEM$^1$ | Orthogonal polynomials contrasts$^2$ |
|-----------------------|------------|--------|--------|--------|---------|----------------------------------|
| Feed intake (g/bird)  | 3870.55$^b$ | 3672.48$^b$ | 4054.31$^a$ | 3790.85$^b$ | 71.17   | 0.657 0.649                      |
| Body weight gain (g/bird) | 2100.32$^a$ | 1925.46$^b$ | 2045.87$^a$ | 2038.63$^b$ | 41.55   | 0.730 0.053                      |
| Feed conversion ratio (g/g) | 1.49$^b$ | 1.56$^b$ | 1.62$^b$ | 1.51$^b$ | 0.03    | 0.424 0.026                      |
| RV/TV$^5$             | 0.37$^a$ | 0.38$^a$ | 0.28$^b$ | 0.33$^a$ | 0.00    | 0.015 0.194                      |
| PHs mortality (%)     | 28.25$^a$ | 27.50$^a$ | 23.75$^b$ | 27.25$^a$ | 0.85    | 0.01 0.103                       |

$^1$Values in the same row not sharing a common roman letter differ significantly (P < 0.05).
$^2$SEM = Standard error of mean.
$^3$Linear (L) and quadratic (Q) orthogonal contrasts were tested using the incremental dietary GAA treatments (0 or control, 0.6, 1.2 and 1.8 g/kg).
$^4$RV/TV = right ventricle to total ventricle weight ratio.

Table 3. Biochemical parameters in serum 42 d in male broilers given guanidinoacetic acid (GAA)-supplemented diets.

| Item                  | Control$^1$ | GAA0.6 | GAA1.2 | GAA1.8 | SEM$^1$ | Orthogonal polynomials contrasts$^2$ |
|-----------------------|------------|--------|--------|--------|---------|----------------------------------|
| TG (mg/dL)$^6$        | 52.75      | 36.00  | 52.33  | 64.00  | 0.92    | 0.048 0.368                      |
| CHOL (mg/dL)          | 121.75     | 112.00 | 119.00 | 115.33 | 8.15    | 0.087 0.712                      |
| HDL-C (mg/dL)         | 32.23$^b$  | 28.73$^b$ | 109.33$^a$ | 26.00$^b$ | 0.92    | 0.232 0.373                      |
| LDL-C (mg/dL)         | 5.75       | 4.00   | 6.00   | 7.00   | 1.16    | 0.290 0.259                      |
| Feed intake (g/bird)  | 3870.55$^b$ | 3672.48$^b$ | 4054.31$^a$ | 3790.85$^b$ | 71.17   | 0.657 0.649                      |
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$^2$SEM = Standard error of mean.
$^3$Linear (L) and quadratic (Q) orthogonal contrasts were tested using the incremental dietary GAA treatments (0 or control, 0.6, 1.2 and 1.8 g/kg).
$^4$TG = Triglycerides; CHOL = Cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol

Table 4. Thyroid hormones, lactate dehydrogenase and creatine kinase activity in male broilers given guanidinoacetic acid (GAA)-supplemented diets.

| Item                  | Control$^1$ | GAA0.6 | GAA1.2 | GAA1.8 | SEM$^1$ | Orthogonal polynomials contrasts$^2$ |
|-----------------------|------------|--------|--------|--------|---------|----------------------------------|
| Thyroid hormones (ng/ml)$^5$ |            |        |        |        |         |                                    |
| T3 (ng mL$^{-1}$)     | 2.35       | 2.70   | 3.27   | 2.45   | 0.438   | 0.663 0.205                      |
| T4 (µg dL$^{-1}$)     | 3.750      | 3.975  | 4.875  | 3.625  | 0.377   | 0.761 0.074                      |
| Enzyme activity (U/L) |            |        |        |        |         |                                    |
| Lactate dehydrogenase | 163.00$^{ab}$ | 119.00$^{ab}$ | 85.67$^{b}$ | 208.67$^{a}$ | 0.93    | 0.971 0.494                      |
| Creatine kinase       | 418$^{b}$  | 464$^{b}$ | 801$^{a}$ | 747$^{a}$ | 0.94    | 0.526 0.890                      |

$^1$Values in the same row not sharing a common roman letter differ significantly (P < 0.05).
$^2$SEM = Standard error of mean.
$^3$Linear (L) and quadratic (Q) orthogonal contrasts were tested using the incremental dietary GAA treatments (0 or control, 0.6, 1.2 and 1.8 g/kg).
$^4$T3 = 3,5,3’-triiodothyronine; T4 = thyroxine.
suggested that birds cannot compensate for reduced BWG caused by low temperature until market age. It can be stated that GAA in the different levels showed conflicting results which were not in agreement with most previous studies. The differences between our findings and others can be attributed to levels of GAA, type of birds and especially rearing conditions (stress vs normal condition).

RV/TV ratio is criteria in order to show ascites incidence (Balog et al. 2000; Julian 2005; Daneshyar et al. 2009; Izadi-nia et al. 2010). In broiler chicks, a RV/TV ratio higher than 0.25–0.30 is considered ascites (Julian 1987). Walton et al. (2001) observed higher RV/TV value and increased ascites morbidity in the broilers with cold-induced ascetic. All the groups except group 1.2 g/kg GAA, other groups showed higher values for RV/TV. In addition, 1.2 g/kg GAA showed lower mortality. Supplemening GAA in vegetable-based diets could be an efficacious replacement for dietary Arg in young birds (Dilger et al. 2013). Regarding arginine, studies have reported that Arg supplementation in broiler diets significantly alleviated the adverse effect of cold stress on RV/TV (Tan et al. 2007; Khajali et al. 2014). Our findings showed that a level of 1.2 g/kg GAA showed better efficiency, while a level of 1.8 g/kg did not show such effects. The reason for conflicting results is unknown.

**Blood parameters**

In terms of serum lipid profiles, there were no significant effects of dietary treatments on serum TG, total CHOL and LDL-C. Conversely, other studies have shown that dietary supplement of GAA improves lipid metabolism in poultry (Daneshyar et al. 2009; Mohammadalipour et al. 2017). HDL-C was increased in group 1.2 g/kg GAA which is parallel with findings for mortality. Increased HDL-C can be considered a health index. More studies would be needed to evaluate the GAA on blood parameters.

**Thyroid hormones**

Thyroid hormones (T3 and T4) are key metabolic hormones that are closely correlated with growth performance and energy metabolism in animals (Zhan et al. 2007). In the current study, although there were no significant differences among treatments, the birds fed diets with supplemental GAA showed a trend to increase the higher level of T3 in sera (Table 4), which is an indicator of increased metabolic rate and oxygen demand. Under these conditions, it seems that erythropoiesis enhances a physiological response to increase the oxygen transport capacity, increased red blood cell (RBC) counts and haemoglobin (Hb), and provide sufficient oxygen for the high metabolic rate. Luger et al. (2001) reported increased plasma concentrations of T3 in response to cold-temperature exposure, accompanied by reduced plasma T4 one week before death in induced-ascetic broilers. The plasma concentration of thyroid hormones plays important role in increased metabolism in the birds and ascites incidence (Hassanzadeh et al. 2000; Scheele et al. 2003). It was found conflicting results for growth performance; thus, it is natural that the data for thyroid hormones are not significant.

**Enzyme activity**

Significant increase in activity of plasma CK and LDH enzymes in 1.8 g/kg GAA in the current study is suggesting the occurrence of necrotic damage to the myocardial membrane. Biopsy samples were taken from patients suffering from congestive heart failure also revealed higher activity of LDH (York et al. 1976; Schultheiss et al. 1980; Fathi et al. 2016). It was reported that LDH is released in the blood from the damaged heart, liver and pulmonary system (Jaffe et al. 1996). LDH activities were decreased in-feed GAA under cold-temperature conditions. In this situation, it is feasible that GAA prevents heart, liver and pulmonary system damage, probably due to their antioxidant property.

**Organ index and ascites-related parameters**

The relative length of the jejunum was increased by the inclusion of 0.6 and 1.2 g/kg GAA in the diets. The increased length of the jejunum in 0.6 and 1.2 g/kg GAA in the diets can be explained by the findings that dietary inclusion of Arg in the culture medium stimulates the growth of chicken intestinal epithelial cells (Yuan et al. 2015). Increased breast meat yield with an increasing tendency with GAA level in the diet.
5. Conclusion

Based on the results of the present investigation, it can be concluded that dietary supplemental 1.2 g/kg GAA would be a beneficial way to decrease the mortality rate of broiler reared under cold stress conditions. In addition, the increased blood level of HDL-C and the decreased relative weight of the liver were seen in broilers fed the 1.2 g/kg GAA-included diet. This level can be advised in order to alleviate the adverse effects of ascites. It will be needed in future studies with higher levels and/or compared with arginine in order to clear the exact mechanism.

Disclosure statement
No potential conflict of interest was reported by the author(s).

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