Effects of dietary supplementation of guanidinoacetic acid on physiological response of broiler chicken exposed to repeated lactic acid injection

Zeinab Boroumandnia, Heshmatollah Khosravinia, Babak Masouri and Bahman Parizadian Kavan
Department of Animal Sciences, Lorestan University, Khorramabad, Iran

ABSTRACT
Increased blood lactic acid is likely to be involved in the incidence of sudden death syndrome (SDS) in broiler chicken. Guanidinoacetic acid (GAA) with direct or indirect influence on the cardiovascular system may provide, to some degree, protection against lactic acidemia in birds. A total number of 144 male broiler chicks (Ross 308) were used in a complete randomised block design to investigate the effects of dietary supplementation of GAA at 0.6, 1.2, 1.8, 2.4 and 3 g/kg on the physiological response of broiler chicks to an acute lactic acidosis in an 8-days experiment. Birds receiving 1.8 and 3 g/kg GAA exhibited lesser mortality percentage compared with the control birds and those received dietary GAA at 0.6 and 1.2 g/kg \((p < .03)\). Dietary inclusion of 1.2 g/kg GAA to broiler chicks increased serum concentration of uric acid compared to the control birds and those received 2.4 g/kg \((p < .02)\). The mean serum concentration of creatinine was greater in the birds receiving different GAA levels compared with the control birds \((p < .0001)\). The serum concentration of nitric oxide decreased in the birds receiving 1.2 g/kg GAA by 21.05 and 15.57% compared with the control birds and those received 1.8 g/kg of GAA, respectively \((p < .04)\). A lesser frequency for liver colour score 5 was observed in the birds fed on the diet with 0.6 g/kg GAA \((p < .0001)\). It was concluded that dietary administration of GAA deteriorates the adaptive physiological responses to an acute lactic acidosis in broiler chicken possibly through liver and kidney dysfunction.

HIGHLIGHTS
- Dietary guanidinoacetic acid decreased mortality but showed an adverse effect on growth performance in chickens exposed to acute lactic acidosis.
- The adverse effects of guanidinoacetic acid may happen through liver and kidney dysfunction.
- No firm evidence was found to suggest a significant effect of extra nutritional guanidinoacetic acid on the incidence of sudden death syndrome in chicken.

Introduction
Lactic acidosis results from an acid-base imbalance in the body due to an excess of lactic acid, which may realise through its increased rate of production or decreased rate of removal (Ahmed et al. 2019; Kamel et al. 2020). Many reports suggest that increased blood lactic acid is likely to be involved in the incidence of sudden death syndrome (SDS) in broiler chicken (Summer et al. 1987; Jacob et al. 1990; Imaeda 2000). It was shown that the cardiac system is easily damaged by an increased circulatory concentration of lactic acid in birds (Olkowski et al. 2008; Meshram and Bijoy 2017). Previously, in research for SDS prevention in broiler chicken, we suggested that providing extra nutritional levels of certain nutrients with direct or indirect influence on the cardiovascular system may provide to some degree protection against hyperlactic acidemia in the same birds. Such speculation was based on results from many reports (Hulan et al. 1980; Whitehead and Randall 1982; Campbell and Classen 1989; Cherian 2007). Guanidinoacetic acid can be considered as a candidate nutrient for the abovementioned purpose since it is synthesised in the body from arginine, an essential amino acid for poultry that serves as the precursor for nitric oxide (NO), and modulates blood pressure (Wideman et al. 2013). Nitric
oxide known as a potent pulmonary vasodilator and also plays an important role in glucose transport into the muscle during muscular contraction (Balon and Nadler 1997; Roberts et al. 1999). Recent research has shown that GAA can be effectively replaced the arginine in the diet of broiler chickens (Dilger et al. 2013). It has been reported that the addition of GAA to the diet is important not only for saving energy but also for reducing arginine requirements and helping to maintain overall energy homeostasis in the poultry body (Wyss and Kaddurah-Daouk 2000). It was also shown that dietary GAA can increase the performance of broiler chickens by increasing muscle creatine reserves (cellular energy source) and providing arginine for protein synthesis and cell proliferation (Michiels et al. 2012). Moreover, Raei et al. (2020) reported that GAA supplementation up to 1.2 g/kg of the diet increased serum creatine level, an active entity that increases the antioxidant capacity in serum through its reactive oxygen scavenger property.

These results support our idea that GAA may improve cardiovascular function and attenuate the adverse effects of lactic acidosis. Therefore, this study aimed to investigate the effects of dietary inclusion of GAA in a wide range of 0 to 3 g/kg on physiological response of broiler chickens challenged with an acute lactic acidosis.

### Material and methods

#### Birds and diets

One-hundred and forty-four 32-day-old healthy male broiler chickens with an average body weight of 1473 ± 50 g were used in this experiment. The birds were chosen from a commercial flock of Ross 308 male broiler chicks, which were obtained from a local hatchery and raised in a force ventilated grow out-house where they received a pre-starter (1–10 days), starter (11–20 days), grower (21–31 days) and a finisher diet (32–40 days) for *ad libitum* consumption (Table 1). The initial brooding temperature was held at 32°C for the first 3 days and then gradually lowered to 25°C by the end of the experiment. Photoperiods were maintained at 24 h during the first day and decreased to 23 h a day for the remainder of the trial. The experimental design was a randomised complete block design comprising of six treatments with four replicates of six birds each. The treatments consisted of a pelleted finisher diet supplemented with GAA (company Evonik Degussa) at 0 (control), 0.6, 1.2, 1.8, 2.4 and 3 g/kg. The GAA was blended with warm distilled water with pH-neutral (Vranes et al. 2017) and sprayed on the pelleted feed (with 4 and 10 mm for diameter and length, respectively) prior to feeding twice a day at 6 a.m. and 6 p.m.

#### Table 1. Ingredients and nutrient composition of basal diets.

|                              | Pre-starter (1–10 days) | Starter (11–20 days) | Grower (21–31 days) | Finisher (32–40 days) |
|------------------------------|-------------------------|----------------------|---------------------|-----------------------|
| Ingredients, % (as fed basis)|                         |                      |                     |                       |
| Yellow maize                 | 55.77                   | 60.97                | 64.97               | 69.12                 |
| Soybean meal                 | 39.00                   | 34.00                | 30.00               | 26.00                 |
| Oyster shell                 | 1.20                    | 1.10                 | 1.10                | 1.00                  |
| Calcium phosphate            | 1.32                    | 1.09                 | 0.94                | 0.78                  |
| Vegetable oil               | 0.80                    | 1.00                 | 1.20                | 1.40                  |
| Salt                         | 0.13                    | 0.15                 | 0.13                | 0.08                  |
| Vitamin mix                  | 0.50                    | 0.50                 | 0.50                | 0.50                  |
| Mineral mix                  | 0.50                    | 0.50                 | 0.50                | 0.50                  |
| Sodium bicarbonate          | 0.10                    | 0.10                 | 0.10                | 0.10                  |
| DL-Methionine                | 0.21                    | 0.19                 | 0.17                | 0.16                  |
| L-Lysine HCl                 | 0.18                    | 0.16                 | 0.15                | 0.14                  |
| Methionine + Cystine         | 0.22                    | 0.20                 | 0.18                | 0.17                  |
| L-Threonine                  | 0.07                    | 0.06                 | 0.06                | 0.05                  |
| Nutrient composition (calculated) |                       |                      |                     |                       |
| Metabolizable energy, Kcal/kg| 2910.00                 | 2975.00              | 3030.00             | 3090.00               |
| Crude protein, %             | 22.45                   | 20.60                | 19.15               | 17.75                 |
| Crude fat, %                 | 3.54                    | 4.09                 | 4.20                | 4.28                  |
| Crude fibre, %               | 2.78                    | 2.59                 | 2.56                | 2.56                  |
| Calcium, %                   | 0.96                    | 0.87                 | 0.78                | 0.75                  |
| Available phosphorus, %      | 0.48                    | 0.44                 | 0.39                | 0.38                  |
| Sodium, %                    | 0.19                    | 0.19                 | 0.18                | 0.18                  |
| Potassium, %                 | 0.70                    | 0.65                 | 0.65                | 0.65                  |
| Chlorine, %                  | 0.19                    | 0.19                 | 0.19                | 0.19                  |
| Methionine, %                | 0.56                    | 0.51                 | 0.47                | 0.44                  |
| Methionine + Cystine, %      | 1.08                    | 0.99                 | 0.90                | 0.85                  |
| Threonine, %                 | 1.44                    | 1.29                 | 1.15                | 1.08                  |
| Lysine, %                    | 0.97                    | 0.88                 | 0.78                | 0.73                  |

*Vitamin A = 440,000 international units; Vitamin D3 = 160,000 international units; Vitamin E = 1500 international units; Vitamin K3 = 128 mg; Vitamin B1 = 74 mg; Vitamin B2 = 260 mg; Vitamin B3 = 490 mg; Vitamin B5 = 1600 mg; Vitamin B6 = 120 mg; Vitamin B9 = 60 mg; Vitamin B12 = 0.6 mg; Vitamin H2 = 4 mg; Anti-oxidant = 250 mg; Choline chloride = 20,000 mg.*

*Manganese = 4800 mg; Zinc = 4400 mg; Copper = 650 mg; Selenium = 12 mg; Iodine = 48 mg; Iron = 2000 mg.*
Acute lactic acidosis induced through the repeated intravenous administration of 0.3, 0.3 and 0.4 mL lactic acid 40% solution (100366, Merck Co. Germany) in 48 h intervals when initiated at 48 h after starting to feed the GAA-supplemented diet at day 34 of age. The administrated lactic acid doses were equivalent to 195, 195 and 260 mL/kg body weight, respectively. The selected doses were determined based on the results from a preliminary study (Table 2) which was performed based on the ideas received from the reports of Huang et al. (1994) and Schwedhelm et al. (2013).

In the preliminary test, lactic acid solution (40%) was injected into the brachial vein at increasing doses from 0 to 0.6 mL. Each dose of the lactic acid was injected into five broiler chicks with a bodyweight close to the experimental chicks. The chicks were monitored for SDS-like death over 24 h post-injection.

**Performance data**

Individual BW and pen wise FI were recorded on days 32 and 40 of age, and the data were used to calculate daily weight gain (DWG), daily feed intake (DFI) and feed conversion ratio (FCR) for the same period. Mortality was recorded upon occurrence.

**Carcase characteristics**

At the close of day 40, all the birds were killed by puncturing jugular vein and carotid arteries, scalded, de-feathered mechanically, eviscerated manually. Then, they evaluated for heart, liver, lungs, bursa of Fabricius, thymus, pancreas and spleen percentage of live body weight.

**Blood biochemistry**

At day 40 of age, all treated birds that survived were killed and a 10 mL blood sample was collected and kept on slush-ice pending serum extraction. Samples of coagulated whole blood were centrifuged at 1800 ×g for 15 min and the collected sera were deposited at −20 °C pending analysis. The sera samples were assayed for the liver activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) enzymes. Likewise, the serum concentration of glucose (GLU), total triglycerides (TG), total cholesterol (TC), high-density lipoproteins (HDL), low-density lipoproteins (LDL), total protein (TP), albumin (ALB), uric acid (UA), urea, creatinine, lactate and certain electrolytes including Ca, Cl and Mg were also determined using an autoanalyser (Selects E Autoanalyzer, Sr. No. 8-7140, Vital Pharma BV, Maarheeze, The Netherlands). This analyser employs enzymatic procedures using Diagnostic Kits (Pars Azmun Co., Iran). Very low-density lipoprotein cholesterol was estimated as [Triglycerides/5] (Friedewald et al. 1972).

Serum sodium and potassium concentrations were measured using a flame photometer (480, Corning, USA). Total antioxidant capacity (TAC) using Randox kit (Randox Laboratories Ltd, UK), blood superoxide dismutase (SOD) activity by Ransod spectrophotometric kit (Ransod, Randox Laboratories Ltd. UK) and blood glutathione peroxidase (GPX) activity by Ransel spectrophotometric kit (Ransel, Randox Laboratories Ltd. UK) were determined. Lipid peroxidation was assayed by the content of thiobarbituric acid reactive substances (TBARS) in the serum with malondialdehyde (MDA) assay kit. Nitric oxide (NO) concentration in sera samples was determined by commercially available NO kit (Novin Navand Salamat Pishtaz Co. Urmia, Iran) immunologically using an Eliza Reader.

**Liver measurement**

Intact livers were collected from all the slaughtered birds and weighed. The livers were then subjected to a visual appraisal and scored on a 5-point scale for their colour appearance. The normal colour was given a score of 1, and the most yellowish was given a score of 5 (Choi et al. 2012). All livers were again appraised based on morphological changes. In a 6-point scale, scores were assigned to each category as follows; livers with normal morphological appearance were given score 0; 1–10 for subcapsular petechial haemorrhages; 2 for more than 10 subcapsular petechial

| Age, days | Administration route | Criterion a | Lactic acid levels, mL |
|-----------|----------------------|-------------|------------------------|
| 40        | Oral gavageing        | SDS-like death 0 | 0 0 0 0 0 0 0 0 0 20% |
| 40        | Oral gavageing        | SDS-like death 0 | 0 0 0 0 0 0 0 0 0 40% |
| 33        | Intravenous injection | SDS-like death 0 | 0 0 0 1 5 5 5 5 5 40% |
| 40        | Intravenous injection | SDS-like death 0 | 0 0 0 1 5 5 5 5 5 40% |

aSDS-like death described by Newberry et al. (1987).
haemorrhages; and 3–5 for large and massive haemorrhages (Diaz et al. 1999; Rozenboim et al. 2016). Then, the liver samples were kept at −18°C pending fat extraction. Extraction of total lipids from liver tissue was conducted using the Folch et al. (1957) method with slight modification. Briefly, approximately 1 g of liver tissue were weighed, added to chloroform/methanol (2/1) to a final volume of 20-times the volume of the tissue sample vortexed for 1 min, allowed to stand with agitation for 2 h. The separated liquid was filtered through Whatman number 1 filter paper into a 100-mL 54 graduated cylinder, and 5 mL of 7.5% Potassium chloride solution was added and blended. After phase separation, the top layer was entirely drained off. Total lipids were measured gravimetrically after evaporating the solvent. The samples were then dried and weighed, and the total lipid weight was expressed as the percentage of liver fat against the total liver weight. Liver dry matter percentage was determined gravimetrically by oven drying at 105°C to a constant mass (AOAC 2005, 930.15). Total ash content was determined by combustion of each sample at 550°C for 8 h. (AOAC 2005,923.03).

Heart measurements

The thickness of the left ventricular wall (LVWT), right ventricle wall (RVWT) and interventricular septum (IVST) were measured at five different locations by a digital calliper (HB-101-111, Guanglu, China) and averaged (Harash et al. 2019). Fat, ash and dry matter of the heart tissue were measured as described for the liver.

White blood cell differential count

For differential leukocyte counts (lymphocytes, monocytes, eosinophils, basophils and heterophiles), blood smears were stained with Wright-Giemsa staining. Slides were examined by light microscope (Leica Galeni III. USA) equipped with a camera to obtain counts of each category per 100 leukocytes (Krams et al. 2012). The heterophile to lymphocyte ratio was determined by the method of Grass and Siegel (1983).

Statistical analysis

A complete randomised block design with six treatments and four replicates of six birds each was used to evaluate the response of the broiler chickens to dietary GAA (0, 0.6, 1.2, 1.8, 2.4 and 3 g/kg diet) in considered variables. All data were analysed using PROC Mixed in Statistical Analysis System, version 9.1 (SAS Institute 2003). The Tukey test was used for multiple treatment comparisons (Kramer 1956). Liver colour and health scores were subjected to frequency analysis using PROC FREQ in the same statistical analysis software (SAS Institute 2003). For all tests, the maximum likelihood for type-II error was set at 5% (p < .05). Specific orthogonal contrasts (linear and quadratic) were applied to determine the effects of incremental inclusion levels (0, 0.6, 1.2, 1.8, 2.4 and 3 g/kg diet) of GAA. A mortality data with a lack of normality was analysed using the Kruskal-Wallis test.

Results

The results of the preliminary experiment intended to adjust the lactic acid dose for induction of lactic acidosis showed that oral gavageing of lactic acid solution (20% and 40%) was not able to create acute SDS-like death in birds. However, intravenous injection of lactic acid at doses above 0.3 and 0.4 mL per bird caused acute SDS-like death due to lactic acidosis in days 33 and 40 of age, respectively (Table 2). Doses beyond 0.3 and 0.4 mL per bird resulted in death in all the treated birds in days 33 and 40 of age. Dietary GAA in a range of 0.6 to 3.0 g/kg showed no influence on ADG, ADFI and FCR of the birds during days 32 to 40 of age (Table 3), but it modified the mortality rate. The birds receiving 1.8 and 3 g/kg GAA exhibited lesser mortality percentage compared with the control birds and those received dietary GAA at 0.6 and 1.2 g/kg (p < .03; Table 3).

No change in the relative weight of bursa of Fabricius, spleen, thymus, lungs, liver, heart and liver fat, dry matter and ash percentage as well as LVVWT,

Table 3. Means of average daily gain (g), average daily feed intake (g), feed conversion ratio (g:g) and mortality (%) in the broiler chicks exposed to lactic acidosis and received different levels of GAA in days 32 to 40 of age.

| GAA, g/kg | ADG  | ADFI  | FCR   | Mortality† (number/total) |
|----------|------|-------|-------|--------------------------|
| 0        | 48.88| 135.67| 2.79  | 41.67a (10/24)           |
| 0.60     | 48.85| 128.14| 2.63  | 58.33a (14/24)           |
| 1.20     | 48.19| 119.73| 2.53  | 54.17a (13/24)           |
| 1.80     | 50.83| 128.02| 2.53  | 37.50a (9/24)            |
| 2.40     | 50.02| 124.84| 2.51  | 45.83ab (11/24)          |
| 3        | 47.38| 127.70| 2.73  | 37.50a (9/24)            |
| SEM      | 4.71 | 9.29  | 0.13  | 4.39                     |
| p-Value  | GAA  | .99   | .87   | .50                      |
| Linear   | .97  | .60   | .53   | .05                      |
| Quadratic| .75  | .40   | .06   | .07                      |

ADG: average daily gain; ADFI: average daily feed intake; FCR: feed conversion ratio; GAA: guanidinoacetic acid; SEM: standard error of mean. 

†Data were subjected to analysis of variance using the nonparametric Kruskal-Wallis test.
Table 4. Means of relative internal organs weight (% of live body weight), liver and heart fat, dry matter, ash percent and cardiovascular parameters (mm) in broiler chicks exposed to lactic acidosis and received different levels of GAA in day 40 of age.

| Variables      | GAA, g/kg | SEM GAA | Linear | Quadratic |
|----------------|-----------|---------|--------|-----------|
|                | 0         | 0.6     | 1.2    | 1.8       | 2.4      | 3        |
| Bursa of Fabricius, % | 0.16      | 0.16    | 0.14   | 0.14      | 0.12     | 0.15     | 0.01     | .16 | .07 | .23 |
| Spleen, %      | 0.13      | 0.14    | 0.14   | 0.13      | 0.15     | 0.15     | 0.01     | .33 | .07 | .97 |
| Thymus, %      | 0.23      | 0.21    | 0.24   | 0.22      | 0.22     | 0.22     | 0.02     | .82 | .56 | .78 |
| Pancreas, %    | 0.25ab    | 0.23b   | 0.23b  | 0.28a     | 0.27ab   | 0.26b    | 0.01     | .04 | .01 | .92 |
| Lung, %        | 0.50      | 0.52    | 0.50   | 0.47      | 0.52     | 0.52     | 0.02     | .39 | .58 | .42 |
| Liver, %       | 2.82      | 2.66    | 2.90   | 2.59      | 2.65     | 2.90     | 0.10     | .08 | .85 | .13 |
| Fat, %         | 8.55      | 8.00    | 8.08   | 8.45      | 8.42     | 8.05     | 0.58     | .96 | .87 | .90 |
| DM, %          | 28.86     | 29.21   | 29.04  | 29.76     | 29.53    | 29.80    | 0.57     | .93 | .79 | .58 |
| Ash, %         | 4.37      | 4.50    | 4.00   | 4.30      | 4.48     | 4.37     | 0.18     | .29 | .83 | .27 |
| Heart, %       | 0.46      | 0.49    | 0.50   | 0.46      | 0.49     | 0.51     | 0.02     | .19 | .12 | .96 |
| LWWT, mm       | 6.12      | 5.85    | 5.33   | 5.70      | 5.89     | 5.81     | 0.23     | .46 | .52 | .07 |
| RVWT, mm       | 1.50      | 1.92    | 1.45   | 1.50      | 1.52     | 1.83     | 0.14     | .06 | .65 | .27 |
| IVST, mm       | 5.32      | 4.94    | 4.80   | 5.13      | 4.56     | 4.87     | 0.24     | .20 | .10 | .43 |
| Fat, %         | 5.89      | 5.83    | 5.96   | 5.92      | 5.58     | 5.81     | 0.25     | .88 | .40 | .66 |
| DM, %          | 23.24     | 22.15   | 22.25  | 22.87     | 22.67    | 23.40    | 0.37     | .14 | .33 | .02 |
| Ash, %         | 4.56      | 4.45    | 4.58   | 4.55      | 4.76     | 4.68     | 0.18     | .87 | .30 | .76 |

GAA: guanidinoacetic acid; SEM: standard error of mean; DM: dry matter; LVWT: left ventricular wall thickness; RVWT: right ventricular wall thickness; IVST: interventricular septum thickness.

abMeans in the same row without same superscript differ significantly (p < .05).

RWVT, IVST, heart fat, dry matter and ash percentage was observed in the broiler chickens treated with different levels of GAA compared with the control birds at day 40 of age (p > .05; Table 4). The relative weight of the pancreas was greater by 15.05% and 14.76% in the birds that received 1.8 g/kg GAA compared with those that received 0.6 and 1.2 g/kg, respectively (p < .04). Administration of GAA modified serum concentration of TG, LDL and VLDL in a linear trend (p < .0009). Dietary GAA levels decreased serum concentration of TG, LDL and VLDL compared with the control birds (p < .0008 and p < .04, respectively). Administration of GAA showed a non-linear pattern of changes in pancreas percentage (p < .01; Table 4).

The serum concentration of TC, HDL, lactate, Na, K, Mg, Ca, Cl⁻, MDA, LDH and AST did not alter in the birds receiving supplementary GAA compared with the control birds during 40 days of age (Table 4). The relative weight of the pancreas was greater by 15.05% and 14.76% in the birds that received 1.8 g/kg GAA compared with those that received 0.6 and 1.2 g/kg, respectively (p < .04). Administration of GAA modified serum concentration of TG, LDL and VLDL compared with the control birds in day 40 of age (Table 5). Dietary GAA levels decreased serum concentration of TG, LDL and VLDL compared with the control birds in the same age (p < .04, p < .008 and p < .04, respectively). Administration of GAA showed a non-linear pattern of changes in LDL (p < .001) where birds receiving 1.2, 1.8 and 2.4 g/kg GAA exhibited 21.89, 27.29 and 31.56% lesser serum concentration of LDL compared with the control birds, respectively (p < .008; Table 5). Dietary inclusion of GAA modified serum concentration of TG and VLDL in a linear trend (p < .02) so that the birds receiving 0.6, 1.8, 2.4 and 3 g/kg GAA showed lesser serum TG and VLDL concentration with 20.22, 25.44, 19.34 and 21.24% compared with the control birds, respectively (p < .04; Table 5).

The serum concentration of TP and ALB in the birds receiving 2.4 g/kg GAA was lesser than all other experimental groups (p < .01 and p < .04, respectively). Mean serum concentration of GLU in the birds grown on the diets containing 1.2 g/kg GAA was greater by 33.97, 18.78 and 33.24% than those receiving 0.6, 2.4 and 3 g/kg of GAA, respectively (p < .0009). Dietary GAA modified serum concentration of GLU, UA and creatinine in a quadratic trend (p < .005, p < .04 and p < .001, respectively). Inclusion of GAA into the diet at 1.2 g/kg increased serum concentration of UA than control birds and those received 2.4 g/kg (p < .02; Table 5). The birds receiving 3 g/kg GAA exhibited 20.12, 20.29 and 21.88% greater serum concentration of urea compared with the control birds and those provided 1.2 and 2.4 g/kg GAA, respectively (p < .007). Mean serum concentration of creatinine in the birds receiving different GAA levels was also greater compared with the control birds (p < .0001). Dietary GAA caused a quadratic pattern of changes in serum concentration of nitric oxide and GPX activity (p < .04 and p < .0007, respectively) where serum nitric oxide level was declined in the birds receiving 1.2 g/kg GAA by 21.05 and 15.57% compared with the control birds and those received 1.8 g/kg of GAA, respectively (p < .04; Table 5). Serum GPX activity in the birds receiving 3 g/kg GAA was greater by 37.59, 28.10 and 22.73% than those received 2.4, 1.8 and 0.6 g/kg, respectively (p < .001). Serum SOD activity in the birds receiving 0.6 and 1.2 g/kg GAA was lesser by 25.00 and 24.05%, respectively, compared with the control birds (p < .03). The birds receiving 1.2 g/kg GAA showed greater serum TAC activity than the control birds and those received 2.4, and 3 g/kg of GAA (p < .005; Table 5). Dietary GAA levels modified serum concentration of TAC in a non-linear trend (p < .01). Serum ALP activity changed linearly by dietary administration of GAA where it was increased in birds receiving 0.6 g/kg GAA in the birds fed on diets containing greater levels of supplementary GAA (p < .01). Serum ALT activity was greater in the birds receiving 1.8 g/kg.
The frequency of liver colour scores 2 and 3 did not differ in the birds that received dietary GAA compared with the control birds (Table 7). The relative frequency of score 4 was greater (30.43%) in the control birds and was lesser (8.70%) in those that received 1.2 g/kg GAA. On the contrary, a lesser frequency for score 5 was observed in the birds receiving 0.6 g/kg GAA by 26.86, 30.37 and 32.96% compared with the control birds and those receiving 1.2 and 2.4 g/kg GAA, respectively (p < .002; Table 5).

Dietary GAA showed no effect on lymphocyte, monocyte and basophil percentage in broiler chickens in day 40 of age (Table 6). However, addition of GAA into the diet at 1.2 g/kg increased heterophile count compared with the control birds and those received 2.4 and 3 g/kg dietary GAA (p < .008). The birds receiving 3 g/kg GAA exhibited greater eosinophil count than the control and other birds (p < .0002). Moreover, dietary GAA resulted in a non-linear pattern of changes in heterophile and H/L ratio (p < .0007 and p < .0008, respectively) where the same ratio was greater the birds receiving 1.2 g/kg GAA compared with the control birds and those received 2.4 and 3 g/kg GAA (p < .009; Table 6).

Table 5. Means of blood biochemical parameters, electrolytes, antioxidant indices concentration and hepatic enzymes activity in broiler chicks exposed to lactic acidosis and received different levels of GAA in day 40 of age.

| Variable          | GAA, g/kg | SEM | p-Value |
|-------------------|-----------|-----|---------|
| TG, mg/dL         | 63.23±    | 0.6 | 1.2 | 1.8 | 2.4 | 3 | SEM | GAA | Linear | Quadratic |
| TC, mg/dL         | 117.54±   | 0.6 | 1.2 | 1.8 | 2.4 | 3 | SEM | GAA | Linear | Quadratic |
| LDL, mg/dL        | 54.56±    | 0.6 | 1.2 | 1.8 | 2.4 | 3 | SEM | GAA | Linear | Quadratic |
| HDL, mg/dL        | 50.05±    | 0.6 | 1.2 | 1.8 | 2.4 | 3 | SEM | GAA | Linear | Quadratic |
| VLDL, mg/dL       | 12.65±    | 0.6 | 1.2 | 1.8 | 2.4 | 3 | SEM | GAA | Linear | Quadratic |
| TG, mg/dL         | 63.23±    | 0.6 | 1.2 | 1.8 | 2.4 | 3 | SEM | GAA | Linear | Quadratic |
| TC, mg/dL         | 117.54±   | 0.6 | 1.2 | 1.8 | 2.4 | 3 | SEM | GAA | Linear | Quadratic |
| LDL, mg/dL        | 54.56±    | 0.6 | 1.2 | 1.8 | 2.4 | 3 | SEM | GAA | Linear | Quadratic |
| HDL, mg/dL        | 50.05±    | 0.6 | 1.2 | 1.8 | 2.4 | 3 | SEM | GAA | Linear | Quadratic |
| VLDL, mg/dL       | 12.65±    | 0.6 | 1.2 | 1.8 | 2.4 | 3 | SEM | GAA | Linear | Quadratic |

GAA: guanidinoacetic acid; SEM: standard error of mean; TG: total triglycerides; TC: total cholesterol; LDL: low density lipoproteins; HDL: high density lipoproteins; VLDL: very low density lipoproteins; TC: total protein; ALB: albumin; GLU: glucose; UA: uric acid; NO: nitric oxide; Na: sodium; K: potassium; Mg: magnesium; Ca: calcium; Cl: chlorine; GPX: glutathione peroxidase; SOD: superoxide dismutase; TAC: total antioxidant capacity; MDA: malondialdehyde; LDH: lactate dehydrogenase; ALP: alkaline phosphatase; AST: aspartate aminotransferase; ALT: alanine aminotransferase.

Means in the same row without same superscript differ significantly (p < .05).
was found in the birds receiving 1.2 g/kg GAA compared with all other experimental bird groups (\(p < .0001\)). The frequency of score 4 was also greater in the control birds as well as those provided with the diet containing 3 g/kg GAA (\(p < .0001;\) Table 7).

### Discussion

Intravenous infusion of lactic acid has been shown to cause SDS-like death in broilers through an increased blood concentration of lactate (Imaeda 2000). Such elevated serum lactate results in myalgia, failure of balance, strong muscular contractions, ultimately violent flapping and overturning. The same phenomenon may frequently happen in well-nourished commercial broilers where inadequate oxygen supply owing to insufficient growth of visceral organs (heart, lung, etc.) induces hypoxia and lack of aerobic metabolism, an event which led to lactate generation from pyruvate by lactate dehydrogenase (Saki and Hemati 2011). Therefore, it can be announced that the parenteral administration of lactic acid in an adjusted dosage can be used as an effective method to induce SDS-like death in broilers. This result confirms the previous works reported by Imaeda (2000), Meshram and Bijoy (2017) and Kamel et al. (2020) who all revealed that acidemia caused by increased blood lactic acid levels is able to induce SDS-like death in broiler chicken.

DeGroot et al. (2018) observed that dietary GAA in a balanced diet and irrespective of Arginine level increased muscle phosphocreatine (PCr), a finding which indicates that GAA improves energy homeostasis in muscle cells in broiler chicken. These results were announced in corroboration with the observations by Michiels et al. (2012), where improvements in creatine and phosphocreatine/ATP concentrations in breast meat were reported as a result of graded GAA supplementation of Arginine-adequate diets. Although, muscle creatine and ATP have not been evaluated in the current study, however, our results showed that dietary GAA increased serum concentration of creatinine, urea and uric acid. It was shown that GAA is metabolically converted to creatine in the liver and then enters the muscles where it changed through an irreversible process to creatinine (Khajali et al. 2020). Therefore, from these data can be speculated that GAA has to some extent been converted to creatine in the experimental birds. However, creatinine is transported to the kidney and excreted as it has no nutritional value (Tossenberger et al. 2016; DeGroot et al. 2018).

Obviously, the elevated serum creatinine, urea and uric acid concentrations, as indicators of renal function, may also be considered in a different aspect where they may indicate a certain degree of kidney damage by supplementary GAA at different doses. Moreover, increased serum activity of ALT in the GAA-receiving birds and increased liver fat beyond 8% may also suggest predisposing of the birds to the fatty liver by dietary GAA. These results agree with Ale Saheb Fosoul et al.’s (2019) findings who reported Energy retention as fat and total energy retention were increased when birds received diets supplemented with 1.2 g/kg GAA. Therefore, supplementary GAA may provide some positive influences in muscle energy metabolism but it is suspicious for imposing certain

### Table 7. Frequency of apparent liver health and liver colour scores in broiler chicks exposed to repeated lactic acidosis and received different levels of GAA in day 40 of age.

| GAA, g/kg | Liver health score<sup>a</sup> | Liver colour score<sup>b</sup> |
| --- | --- | --- |
| 0 | 0.00 | 0.00 | 7.69 | 11.11 | 23.91 | 0.00 | 0.00 | 33.33 | 30.43 | 12.00 |
| 0.6 | 0.00 | 0.00 | 30.77 | 5.56 | 10.87 | 0.00 | 0.00 | 50.00 | 33.33 | 13.04 | 10.00 |
| 1.2 | 0.00 | 0.00 | 7.69 | 27.78 | 10.87 | 0.00 | 0.00 | 50.00 | 33.33 | 8.70 | 14.00 |
| 1.8 | 0.00 | 0.00 | 30.77 | 16.67 | 17.39 | 0.00 | 0.00 | 0.00 | 0.00 | 13.04 | 24.00 |
| 2.4 | 0.00 | 0.00 | 15.38 | 22.22 | 13.04 | 0.00 | 0.00 | 50.00 | 33.33 | 8.70 | 14.00 |
| 3 | 0.00 | 0.00 | 7.69 | 16.67 | 23.91 | 0.00 | 0.00 | 0.00 | 0.00 | 21.74 | 20.00 |

<sup>a</sup>Liver health score: 0 indicating normal liver, 1 up to 10 sub capsular petechial haemorrhages, 2 for more than 10 sub capsular petechial haemorrhages, and 3 to 5 for large and massive haemorrhages.

<sup>b</sup>Liver colour score: 1 indicating normal liver, score from 2 to 5 from dark red to light yellowish red.

GAA: guanidinoacetic acid.

DeGroot et al. (2018) observed that dietary GAA in a balanced diet and irrespective of Arginine level increased muscle phosphocreatine (PCr), a finding which indicates that GAA improves energy homeostasis in muscle cells in broiler chicken. These results were announced in corroboration with the observations by Michiels et al. (2012), where improvements in creatine and phosphocreatine/ATP concentrations in breast meat were reported as a result of graded GAA supplementation of Arginine-adequate diets. Although, muscle creatine and ATP have not been evaluated in the current study, however, our results showed that dietary GAA increased serum concentration of creatinine, urea and uric acid. It was shown that GAA is metabolically converted to creatine in the liver and then enters the muscles where it changed through an irreversible process to creatinine (Khajali et al. 2020). Therefore, from these data can be speculated that GAA has to some extent been converted to creatine in the experimental birds. However, creatinine is transported to the kidney and excreted as it has no nutritional value (Tossenberger et al. 2016; DeGroot et al. 2018).

Obviously, the elevated serum creatinine, urea and uric acid concentrations, as indicators of renal function, may also be considered in a different aspect where they may indicate a certain degree of kidney damage by supplementary GAA at different doses. Moreover, increased serum activity of ALT in the GAA-receiving birds and increased liver fat beyond 8% may also suggest predisposing of the birds to the fatty liver by dietary GAA. These results agree with Ale Saheb Fosoul et al.’s (2019) findings who reported Energy retention as fat and total energy retention were increased when birds received diets supplemented with 1.2 g/kg GAA. Therefore, supplementary GAA may provide some positive influences in muscle energy metabolism but it is suspicious for imposing certain...
adverse effects on the liver as well as kidney function, the topics which both need to be characterised in detail. In the present experiment, the birds receiving different GAA levels exhibited greater serum concentration of creatinine compared with the control birds, the results, which agree with those of Raei et al. (2020), report who observed that the increasing dietary GAA levels from 0.6 to 1.8 g/kg enhanced serum concentration of creatinine compared to the birds in the control group.

Results of the current study concerning blood biochemistry, enzymes activity and liver parameters which are commonly considered as surrogates of stress response in chicken (Puvadolpirod and Thaxton 2000a, 2000b; Post et al. 2003) demonstrated no consistent evidence for the influence of supplementary GAA on metabolism capability of broiler chickens to attenuate acute lactic acidosis stress. Increased serum GLU, CHO, HDL, TG, UA and decreased ALB, TP concentrations as well as greater liver weight and fat content have previously been indicated by Puvadolpirod and Thaxton (2000a) and later confirmed by many researchers (Khosravinia 2015; Khosravinia and Manafi 2016) as elicited adaptive responses to stressors in broiler chicks. In the current study, acute lactic acidosis induced through repeated injection of lactic acid over a week, as physiological stress caused inconsistent alterations in almost all these variables in the control chicks. Nevertheless, dietary GAA exerted no effect on the pattern of changes that occurred in almost all variables and provided no support of stress relief in the treated birds.

Our results demonstrated that serum antioxidant capacity was improved by the inclusion of the greater GAA levels in the broiler diets evidenced by elevated GPX activity in the birds receiving GAA at 3 g/kg of diet. It was shown that creatine, the end product of GAA utilisation, is thought to have the antioxidant capacity (Sestili et al. 2006; Sestili et al. 2009). According to Fathi and Tanha (2015), arginine supplementation increased plasma GPX activity and decreased MDA level in the broiler with cold-induced ascites. Thus, more information is needed to know how GAA affects oxidant–antioxidant system to help its use as a nutritional compound under stressful conditions. Results of the current study confirm Nasiroleslami et al. (2018) report who showed an increase in liver GPX activity and a reduction in serum MDA content as the addition level of GAA increased to 1.2 g/kg in broiler diet as well as Wang et al. (2012) findings who observed a quadratic increase in antioxidant enzymes activity by 0.8, 1.2 and 2 g/kg of GAA supplementation in pig diet.

In the present study, surprisingly serum concentration of nitric oxide declined in the birds receiving dietary GAA at 1.2 g/kg compared with the control birds. Contrary to our results, Raei et al. (2020) showed with increasing GAA supplementation, serum nitric oxide concentrations increased linearly. Our results also showed that supplementary GAA affected the percentage of heterophile and the ratio of heterophile to lymphocyte in broiler chickens exposed to lactic acid injection. The birds receiving 2.4 and 3 g/kg supplementary GAA showed lesser heterophile percentage and H/L ratio compared with other birds. Although changes in blood cell components cannot be easily interpreted, however, Attia et al. (2011) reported that arginine supplementation improved the immunity of chicken and tolerance to stress in birds exposed to extreme ambient temperatures. We suggest further examination of the GAA short and long-term effect in the contact of lactic acidemia in broiler chicken considering the evaluation of performance criteria for the whole course of the experiment with day-old chicks.

Conclusions
It is concluded that the dietary administration of GAA at 2.4 and 3.0 g/kg decreased mortality in broiler chickens exposed to acute lactic acidosis. Dietary GAA did not modify surrogates of adaptive physiological stress responses towards a certain degree of lactic acidosis stress relief in the treated birds. Elevated serum creatinine, urea and uric acid concentrations may suggest to a certain degree kidney damage by supplementary GAA at different doses. Moreover, increased serum activity of ALT and increased liver fat beyond 8% in the GAA-receiving birds may also provide disputed clues for predisposing of the birds to the fatty liver by dietary GAA. This evidences collectively suggest that dietary GAA at levels greater than 0.6 g/kg must be closely monitored and interpreted since it possibly exceeds the metabolic capacity of the bird’s liver for its conversion to arginine and then exerts deleterious effects on broilers metabolism.

Acknowledgement
The authors would like to thank the Research Directorate, Lorestan University for partial financial support.
Ethical approval

All procedures carried out in this experiment were reviewed and approved by the Animal Care and Use Committee of Lorestan University, Khorramabad, Iran.

Disclosure statement

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

ORCID

Heshmatollah Khosravinia http://orcid.org/0000-0001-6752-4237

References

Ahmed HH, De Bels D, Attou R, Honore PM, Redant S. 2019. Elevated lactic acid during ketoacidosis: pathophysiology and management. J Transl Int Med. 7(3):115–117.

Ale Saheb Fosoul SS, Azarfar A, Gheisari A, Khosravinia H. 2019. Performance and physiological responses of broiler chicks to supplemental guanidinoacetic acid in arginine-deficient diets. British Poult Sci. 60(2):161–168.

AOAC. 2005. Official methods of analysis, 17th ed. Arlington (VA): AOAC International.

Attia YA, Hassan RA, Tag El-Din AE, Abou-Shehema BM. 2011. Effect of ascorbic acid or increasing metabolizable energy level with or without supplementation of some essential amino acids on productive and physiological traits of slow-growing chicks exposed to chronic heat stress. J Anim Physiol Anim Nutr. 95(6):744–755.

Balon TW, Nadler JL. 1997. Evidence that nitric oxide increases glucose transport in skeletal muscle. J Appl Physiol. 82(1):359–363.

Campbell GL, Classen HL. 1989. Effect of dietary taurine supplementation on sudden death syndrome in broiler chickens. Can J Anim Sci. 69(2):509–512.

Cherian G. 2007. Metabolic and cardiovascular diseases in poultry: role of dietary lipids. Poult Sci. 86(5):1012–1016.

Choi YI, Ahn HJ, Lee BK, Oh ST, An BK, Kang CW. 2012. Nutritional and hormonal induction of fatty liver syndrome and effects of dietary lipotropic factors in egg-type male chicks. Asian Australas J Anim Sci. 25(8):1145–1152.

DeGroot AA, Braun U, Dilger RN. 2018. Efficacy of guanidinoacetic acid on growth and muscle energy metabolism in broiler chicks receiving arginine-deficient diets. Poult Sci. 97(3):890–900.

Díaz GJ, Squires EJ, Julian RJ. 1999. The use of selected plasma enzyme activities for the diagnosis of fatty liver hemorrhagic syndrome in laying hens. Avian Dis. 43(4):768–773.

Dilger RN, Bryant-Angeloni K, Payne RL, Lemme A, Parsons CM. 2013. Dietary guanidino acetic acid is an efficacious replacement for arginine for young chicks. Poult Sci. 92(1):171–177.

Fathi M, Tanha T. 2015. Effects of L-arginine supplementation on liver & plasma antioxidant status and growth performance in broiler with cold induced ascites. Anim Sci J. 108:83–94.

Folch J, Lees M, Sloane Stanley GH. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem. 226(1):497–509.

Friedewald WT, Levy RI, Fredrickson DS. 1972. Estimation of concentration of low-density lipoprotein cholesterol in plasma without use of the ultra-centrifuge. Clin Chem. 18:449–502.

Grass WB, Siegel HS. 1983. Evaluation of the heterophile/lymphocyte ratio as a measure of stress in chickens. Avian Dis. 27:927–979.

Harash GK, Richardson C, Alshamy Z, Hunigen H, Hafez HM, Plendl J, Al Masri S. 2019. Heart ventricular histology and microvasculature together with aortic histology and elastic lamellar structure: a comparison of a novel dual-purpose to a broiler chicken line. PLoS One. 14(3):e0214158.

Huang Y, Yee JB, Yip WH, Wong KC. 1994. Lactic acidosis and pH on the cardiovascular system. Adv Pharmacol. 31:551–564.

Hulan HW, Proudfoot FG, McRae KB. 1980. Effect of vitamins on the incidence of mortality and acute death syndrome (“flip-over”) in broiler chickens. Poult Sci. 59(4):927–931.

Imaeda N. 2000. Characterization of lactic acid formation and adenosine triphosphate consumption in calcium-loaded erythrocytes of broiler chickens. Poult Sci. 79(11):1543–1547.

Jacob JP, Blair R, Gardiner EE. 1990. Effect of dietary lactate and glucose on the incidence of sudden death syndrome in male broiler chickens. Poult Sci. 69(9):1529–1532.

Kamel KS, Oh MS, Halperin ML. 2020. L-lactic acidosis: pathophysiology, classification, and causes; emphasis on biochemical and metabolic basis. Kidney Int. 97(1):75–88.

Khajali F, Lemme A, Rademacher-Heilshorn M. 2020. Guanidinoacetic acid as a feed supplement for poultry. World’s Poult Sci J. 76(2):270–291.

Khosravinia H. 2015. Effects of intrayolk sac inoculation of olive oil on physiological adaptiveresponses in newly hatched broiler chicks subjected to neonatal fasting. Jpn Poult Sci. 52(4):304–311.

Khosravinia H, Manafi M. 2016. Broiler chicks with slow-feathering (K) or rapid-feathering (k+) genes: effects of environmental stressors on physiological adaptive indicators up to 56th posthatch. Poult Sci. 95(8):1719–1725.

Krams I, Vrublevska J, Cirule D, Kivleniece I, Krama T, Rantala MJ, Sild E, Hõrak P. 2012. Heterophil/lymphocyte ratios predict the magnitude of humoral immune response to a novel antigen in great tits (Parus major). Comp Biochem Physiol A Mol Integr Physiol. 161(4):422–428.

Kramer CY. 1956. Extension of multiple range tests to group means with unequal number of replications. Biometrics. 12(3):307–310.

Meshram PV, Bijoy F. 2017. Managemental and nutritional disease-sudden death syndrome in broilers. Int J Sci Environ Technol. 6:260–266.

Michiels J, Maertens L, Buyse J, Lemme A, Rademacher M, Dierick NA, de Smet S. 2012. Supplementation of guanidinoacetic acid to broiler diets: effects on performance, carcass characteristics, meat quality, and energy metabolism. Poult Sci. 91(2):402–412.

Nasiroleslami M, Torki M, Saki AA, Abdolmohammadi AR. 2018. Effects of dietary guanidinoacetic acid and betaine supplementation on performance, blood biochemical
parameters and antioxidant status of broilers subjected to cold stress. J Appl Anim Res. 46(1):1016–1022.
Newberry RC, Gardiner EE, Hunt JR. 1987. Behavior of chickens prior to death from sudden death syndrome. Poult Sci. 66(9):1446–1450.
Olkowski AA, Wojnarowicz C, Nain S, Ling B, Alcorn JM, Laarveld B. 2008. A study on pathogenesis of sudden death syndrome in broiler chickens. Res Vet Sci. 85(1):131–140.
Post J, Rebel JMJ, ter Huurne AAHM. 2003. Physiological effects of elevated plasma corticosterone concentrations in broiler chickens. An alternative means by which to assess the physiological effects of stress. Poult Sci. 82(8):1313–1318.
Puvadolpirod S, Thaxton JP. 2000a. Model of physiological stress in chickens 1. Response parameters. Poult Sci. 79(3):363–369.
Puvadolpirod S, Thaxton JP. 2000b. Model of physiological stress in chickens 2. Dosimetry of adrenocorticotropic. Poult Sci. 79(3):370–376.
Raei A, Karimi A, Sadeghi A. 2020. Performance, antioxidant status, nutrient retention and serum profile responses of laying Japanese quails to increasing addition levels of dietary guanidinoacetic acid. Ital J Anim Sci. 19(1):75–85.
Roberts CK, Barnard RJ, Jasman A, Balon TW. 1999. Acute exercise increases nitric oxide synthase activity in skeletal muscle. Am J Physiol. 277:390–394.
Rozenboim I, Mahato J, Cohen NA, Tirosh O. 2016. Low protein and high-energy diet: a possible natural cause of fatty liver hemorrhagic syndrome in caged White Leghorn laying hens. Poult Sci. 95(3):612–621.
Saki AA, Hemati M. 2011. Does nutrition help to alleviate sudden death syndrome in broiler chicken? Glob Vet. 6:262–268.
SAS Institute. 2003. SAS users guide: statistics. Ver. 6. Cary (NC): SAS.