Acta Veterinaria Hungarica 66 (1), pp. 116–124 (2018)
DOI: 10.1556/004.2018.011

PULMONARY HYPERTENSIVE RESPONSE OF BROILER CHICKENS TO ARGININE AND GUANIDINOACETIC ACID UNDER HIGH-ALTITUDE HYPOXIA

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(Received 1 September 2017; accepted 6 November 2017)

This study assessed the preventive effects of arginine (ARG) and guanidinoacetic acid (GAA) on the incidence of pulmonary hypertension syndrome (PHS) in broiler chickens. Four isoenergetic and isonitrogenous diets were prepared, including: (i) the control, (ii) the control supplemented with 1 g/kg ARG, (iii) the control supplemented with 1 g/kg GAA, and (iv) the control supplemented with 1.5 g/kg GAA. These diets were fed to broilers (Ross 308) from day 1 to 42 post-hatch. Criteria evaluated in the experiment were growth performance, carcass characteristics, serum and blood variables, lead-II electrocardiogram, and ET-1 and iNOS gene expression in heart and lungs. Mortality from PHS was recorded daily. The results showed that ARG and GAA supplements improved the feed conversion ratio (FCR) compared to the control ($P < 0.05$). Supplementation of ARG and GAA significantly ($P < 0.05$) increased serum nitric oxide (NO) concentration. ARG and GAA supplementation significantly reduced the haematocrit value and the heterophil to lymphocyte ratio in the blood. A significant ($P < 0.05$) decline in S-wave amplitude of the lead-II electrocardiogram, right to total ventricular weight ratio (RV:TV) and ascites mortality was observed by supplementing ARG or 1.5 g/kg GAA. Addition of ARG and GAA supplements did not significantly change ET-1 and iNOS gene expression in the heart and lung relative to the control. In conclusion, GAA supplementation at 1.5 g/kg had a potential to improve growth performance and could prevent PHS.

Key words: Ascites, arginine, chicken, guanidinoacetic acid, pulmonary hypertension

Pulmonary hypertension syndrome (PHS) in broilers is associated with hypoxia, which prevails at high altitude. Arginine (ARG) is a key factor in the prevention of PHS because it serves as a substrate for the synthesis of nitric oxide (NO), a potent vasodilator molecule. Arginine is an indispensable amino acid for birds because of the lack of a functional urea cycle (Khajali and Wideman, 2010). Research has shown that ARG requirements for maximising growth performance of broiler chickens raised at high altitude remarkably exceed the NRC

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(1994) recommendation (1.32 *vs.* 1.1%; Basoo et al., 2012). It is suggested that ARG requirements advocated by the NRC (1994) are not adequate to support maximal growth and immune function at high altitudes. In fact, the NRC recommendations provide only minimal requirements established under thermoneutral conditions in low-altitude areas (Khajali and Wideman, 2016).

The issue with ARG supplementation is that this amino acid is not available in the market as feed-grade supplement. The limited availability and high cost of ARG have propelled researchers to find commercially available and competitive alternatives. Guanidinoacetic acid (GAA) is of high interest since it acts as an immediate precursor of creatine and its phosphorylated derivative, phosphocreatine. The latter serves as a rapidly mobilisable reserve of high-energy phosphates in the body of birds. Guanidinoacetic acid has also been reported to spare ARG requirements of broiler chickens (Michiels et al., 2012; Dilger et al., 2013). In a recent study, GAA supplementation at 1.2 g/kg in the diet improved jejunal villus surface area in broilers without any impact on growth performance (Kodambashi Emami et al., 2017). Nevertheless, the role of GAA in the prevention of PHS in broilers has not been studied. The current study was designed to investigate the effects of different doses of GAA (1 and 1.5 g/kg) for the prevention of PHS in broiler chickens reared at high altitude. To compare the efficacy of GAA with ARG, an experimental treatment was considered to provide 1 g/kg ARG.

Materials and methods

Birds and experimental facility

The experiment was carried out at the Poultry Research Center of Shahrekord University, Shahrekord, Iran (altitude of 2,100 m above sea level). The experimental animals were treated according to the Institutional Animal Care and Use Committee of Shahrekord University.

A total of 240 day-old male broilers (Ross 308) were obtained from Behjoojeh Co., Shahrekord, Iran. Chicks were randomised across 16 floor pens measuring 1.8 m^2 (15 birds per pen with initial body weights of 42 g ± 0.7 g on average). Each pen was equipped with a bell drinker and a feed trough. The temperature of the experimental house was set at 32 °C upon chick arrival and reduced to 25 °C on day 7, 20 °C on day 14, and 15 °C on day 21 and thereafter. All chicks had free access to feed and water and provided with 23 h light and 1 h dark per day, throughout the trial.

Treatments

A commercial broiler diet was prepared according to the NRC (1994) recommendations for the starter/grower (1 to 21 days of age) and finisher (21 to 42 days of age) stages and regarded as control (Table 1). Three additional diets were

prepared by supplementing 1 g/kg ARG or 1 and 1.5 g/kg GAA to the control diet. ARG and GAA were provided by Evonik Degussa, Tehran, Iran. All diets had similar metabolisable energy and protein content and were offered in mash form.

Table 1

Composition of the control diet for broiler chickens during the starter and grower stages

Item (% unless noted)	Starter/Grower (1–21 days)	Finisher (21–42 days)
Corn	51.10	60.5
Soybean meal (44% CP)	39.85	31.9
Soy oil	5.00	4.00
Dicalcium phosphate	1.50	1.30
Oyster shell	1.50	1.40
Salt	0.35	0.30
DL-Methionine	0.20	0.1
Mineral supplement[a]	0.25	0.25
Vitamin supplement[b]	0.25	0.25
Calculated composition		
Metabolisable energy (kcal/kg)	3050	3100
Crude protein	21.95	19.20
Methionine + Cysteine	0.95	0.72
Lysine	1.20	1.03
Threonine	0.90	0.88
Arginine	1.30	1.20
Ca	0.95	0.85
Available P	0.43	0.35

[a]Provided the following per kg of diet: vitamin A (trans retinyl acetate), 3600 IU; vitamin D3 (cholecalciferol), 800 IU; vitamin E (dl-α-tocopheryl acetate), 7.2 mg; vitamin K3, 1.6 mg; thiamine, 0.72 mg; riboflavin, 3.3 mg; niacin, 0.4 mg; pyridoxine, 1.2 mg; cobalamin, 0.6 mg; folic acid, 0.5 mg; choline chloride, 200 mg; [b]Provided the following per kg of diet: Mn (from $MnSO_4 \times H_2O$), 40 mg; Zn (from ZnO), 40 mg; Fe (from $FeSO_4 \times 7H_2O$), 20 mg; Cu (from $CuSO_4 \times 5H_2O$), 4 mg; I [from Ca $(IO_3)_2 \times H_2O$], 0.64 mg; Se (from sodium selenite),0.08 mg; Supplement of arginine (1 g/kg) and guanidinoacetic acid (1 or 1.5 g/kg) was added to the basal diet to prepare experimental groups

Measurements

Daily feed intake and weight gain were recorded during the 1–42 d period. Feed conversion ratio (FCR) was also calculated and corrected for mortality body weights. At 42 days of age, eight birds per treatment were selected for blood sampling. Blood (3 ml) was collected from the brachial vein and centrifuged at 2500 *g* for 10 min to obtain sera. Serum samples were used for the determination of nitric oxide (NO) (nitrate + nitrite) according to the method described by Behrooj et al. (2012).

Samples of blood were collected in microhaematocrit tubes for measuring haematocrit. An aliquot of blood was spread on glass slides to obtain a blood

smear for the determination of different forms of leukocytes. The May-Grünwald and Giemsa stains were used for staining the smears 3 h after methyl alcohol fixation (Lucas and Jamroz, 1961). One hundred leukocytes, including granular (heterophils) and non-granular forms (lymphocytes), were enumerated and the heterophil to lymphocyte ratio (H:L) was calculated. All chemical reagents were obtained from Sigma-Aldrich Co. (Sigma-Aldrich Co., St. Louis, MO, USA).

Electrocardiographic recording

Eight birds from each treatment were randomly selected at day 40 and leads II of electrocardiograms (ECG) were recorded by an automatic instrument (Cardiomax FX-2111, Fukuda, Japan) while standardised at 10 mm = 1 mV with a chart speed of 50 mm/s. The amplitudes of the T, R and S waves were measured and analysed.

Quantitative real-time PCR analysis

At 42 days of age, 8 chickens from each treatment group were randomly selected, weighed and killed by CO_2 euthanasia. Carcass characteristics including liver, abdominal fat and heart were obtained. The hearts were further dissected to obtain the right-to-total ventricular weight ratio (RV:TV). The hearts (right ventricles) and the lungs were immediately frozen in liquid nitrogen and stored at –70 °C for subsequent RNA analysis. Total tissue RNA was extracted using RNXPlus reagent (Sinaclon Bioscience, Tehran, Iran). An amount of 100 mg tissue was homogenised in digestion buffer. The homogenate was mixed with chloroform. After centrifuging the mixture, total RNA settled in the upper aqueous phase. Following precipitation with isopropanol, the RNA pellet was rinsed with 75% ethanol. The samples of RNA were resuspended in DEPC-treated water. To remove residual DNA, the RNA was treated by DNase (Sinaclon Bioscience, Tehran, Iran). The RNA was measured and qualified spectrophotometrically. Only RNA with an absorbance ratio (A260/A280) greater than 1.9 was used for synthesis of cDNA. Total RNA was reverse transcribed into cDNA using PrimeScript™ RT Reagent Kit (Takara Bio Inc., Japan). The reverse transcription mix was heated to 85 °C for 5 sec to inactivate reverse transcriptase and denature the RNA and then stored at –20 °C. The levels of inducible nitric oxide synthase (iNOS), endothelin-1 (ET-1) and β-actin transcripts were determined by real-time PCR using SYBR® Premix Ex Taq™ II (Takara Bio Inc., Japan). In order to normalise the input load of cDNA among samples, β-actin was used as an endogenous standard. Details of the specific primer pairs are listed in Table 2. The PCRs were done in a real-time thermocycler (Rotor Gene Q 6000, Qiagen, USA) in three replicates for each sample of ventricles. One microlitre of cDNA was added to the 10 µl of SYBR® Premix Ex Taq II Mix and 0.5 µM of each specific primer in a total volume of 20 µl. The thermal profile was 95 °C for 30 sec, 40

cycles of 94 °C for 40 sec, 64 °C for 35 sec and 72 °C for 30 sec. At the end of each phase, the measurement of fluorescence was done and used for quantitative objectives. Gene expression data were normalised to β-actin. Data were analysed using RotorGene software, version 2.0.2 (build 4) (Qiagen, Hilden, Germany) and LinRegPCR software version 2012.0 (Amsterdam, Netherlands) to obtain the threshold cycle number and reaction efficiency (Ruijter et al., 2009). Relative transcript levels were calculated using efficiency adjusted Pfaffl methodology (Dorak, 2006).

Table 2

Primers used for quantitative real-time PCR analysis of chicken mRNAs

Target	Primers	PCR product	Accession No.
β-Actin	139 bp	5'-AGCGAACGCCCCCAAAGTTCT-3' 5'-AGCTGGGCTGTTGCCTTCACA-3'	NM-205518.1
iNOS	371 bp	5'-AGGCCAAACATCCTGGAGGTC-3' 5'-TCATAGAGACGCTGCTGCCAG-3'	U46 504
ET-1	141 bp	5'-GGACGAGGAGTGCGTGTATT-3' 5'-GCTCCAGCAAGCATCTCTG-3'	XM418943

Mortality from ascites was checked daily and whenever the RV:TV was greater than 0.25, it was regarded as pulmonary hypertension (Saedi and Khajali, 2010; Ahmadipour et al., 2015).

Statistical analysis

Data were analysed by the ANOVA procedure of SAS software (SAS Institute Inc., 2007) in a completely randomised design and the means were separated by Duncan's multiple range test.

Results

While feed intake was unaffected by treatment, weight gain, final body weight and FCR were significantly ($P < 0.05$) improved at 1 and 1.5 g/kg GAA compared to the control (Table 3).

Table 4 shows blood and serum variables of broiler chickens fed different levels of GAA and ARG. Although dietary supplementation with GAA (1 and 1.5 g/kg) and ARG (1 g/kg) significantly ($P < 0.05$) increased serum NO concentration, it reduced the haematocrit level and the heterophils to lymphocytes ratio compared to the control group.

As indicated in Table 5, there was a significant decrease in S-wave amplitude of birds fed a diet supplemented with ARG and GAA at 1.5 g/kg compared to the control when measured at 40 days of age. R-wave amplitude in bids fed a

diet supplemented with ARG and GAA at 1.5 g/kg was significantly decreased when compared to the control.

Table 3

Effect of ARG and GAA supplementation on the growth performance of broiler chickens (1 to 42 days)

Variable	Control	ARG (1 g/kg)	GAA (1 g/kg)	GAA (1.5 g/kg)
Weight gain (g/d)	$47.8^{b} \pm 2.09$	$53.1^{ab} \pm 0.55$	$52.6^{ab} \pm 1.44$	$53.8^{a} \pm 2.05$
Body weight (g/bird)	$2050^{b} \pm 32$	$2272^{a} \pm 29$	$2251^{a} \pm 31$	$2301^{a} \pm 28$
Feed intake (g/d)	93 ± 3.1	95 ± 5.4	93 ± 7.6	95 ± 4.7
Feed conversion ratio	$1.91^{a} \pm 0.043$	$1.87^{ab} \pm 0.036$	$1.77^{b} \pm 0.006$	$1.76^{b} \pm 0.016$

ARG: arginine, GAA: guanidinoacetic acid. Means in the same row with different letters are significantly different ($P < 0.05$). Each mean represents values from four replicates

Table 4

Effect of ARG and GAA supplementation on serum and blood variables in broiler chickens measured at 42 days of age

Variable	Control	ARG (1 g/kg)	GAA (1 g/kg)	GAA (1.5 g/kg)
Serum nitric oxide (μmol/l)	$10.2^{b} \pm 1.55$	$18.7^{a} \pm 1.44$	$17.9^{a} \pm 2.60$	$19.5^{a} \pm 1.80$
Hematocrit (%)	$43.1^{a} \pm 2.07$	$34.7^{b} \pm 0.91$	$35.6^{b} \pm 0.80$	$33.8^{b} \pm 0.92$
H:L	$1.30^{a} \pm 0.113$	$0.63^{b} \pm 0.063$	$0.72^{b} \pm 0.043$	$0.62^{b} \pm 0.070$

ARG: arginine, GAA: guanidinoacetic acid, H:L: heterophil to lymphocyte ratio. Means in the same row with different letters are significantly different ($P < 0.05$). Each mean represents values from 8 replicates

Supplements of ARG and GAA significantly reduced right ventricular weight ratio (RV:TV) compared to the control. However, ascites mortality was significantly prevented by ARG or GAA when added at 1.5 g/kg.

Real-time PCR results showed that the relative gene expressions of ET-1 and iNOS in the heart (right ventricle) and lung of chickens fed GAA and ARG were not significantly different from the control (Table 6).

Discussion

The growth performance of birds was lower compared to the expected criteria set by the strain performance objectives (ROSS 308). This is, in fact, the result of raising birds at high altitude (2100 m). High altitude imposes severe hypobaric hypoxia to birds. There is a highly-correlated relationship between growth performance and oxygen concentration in broiler chickens (Beker et al., 2001).

Supplementation of GAA at 1 and 1.5 g/kg significantly improved the FCR, the body weight gain and the final body weight. This is in accordance with the results of previous studies where GAA supplementation improved feed conversion efficiency in broiler chickens (Michiels et al., 2012; Dilger et al., 2013; Mousavi et al., 2013). This finding suggests that GAA improves energy status in birds, which can be mediated through creatine synthesis. In this regard, Stahl et al. (2003) found a significant improvement in feed conversion by creatine supplementation. Moreover, Zhao et al. (2017) reported that *in ovo* feeding of creatine could improve energy status and resulted in enhanced breast muscle weight of broiler chickens.

Table 5

Effect of ARG and GAA supplementation on lead II electrocardiogram and ascites mortality in broilers

Variable	Control	ARG (1 g/kg)	GAA (1 g/kg)	GAA (1.5 g/kg)
R wave(mV)	$0.230^{a} \pm 0.015$	$0.183^{b} \pm 0.017$	$0.196^{ab} \pm 0.014$	$0.187^{b} \pm 0.013$
S wave(mV)	$-0.351^{a} \pm 0.011$	$-0.301^{b} \pm 0.019$	$-0.318^{ab} \pm 0.010$	$-0.303^{b} \pm 0.012$
T wave(mV)	0.153 ± 0.015	0.123 ± 0.014	0.131 ± 0.012	0.133 ± 0.012
RV: TV ratio	$0.33^{a} \pm 0.013$	$0.23^{b} \pm 0.022$	$0.24^{b} \pm 0.018$	$0.22^{b} \pm 0.036$
Ascites mortality* (%)	$27.5^{a} \pm 3.66$	$15.5^{b} \pm 1.97$	$20.0^{ab} \pm 3.80$	$17.0^{b} \pm 2.09$

ARG: arginine, GAA: guanidinoacetic acid. Means in the same row with different letters are significantly different ($P < 0.05$). Each mean represents values from 8 replicates. *As percent of the whole group

Table 6

Effect of ARG and GAA supplementation on gene expression in broiler chickens

Variable	Control	ARG (1 g/kg)	GAA (1 g/kg)	GAA (1.5 g/kg)
Heart (right ventricle)				
iNOS	0.337 ± 0.059	0.412 ± 0.074	0.415 ± 0.088	0.488 ± 0.068
ET-1	0.370 ± 0.053	0.207 ± 0.078	0.228 ± 0.053	0.239 ± 0.087
Lung				
iNOS	0.213 ± 0.050	0.307 ± 0.059	0.339 ± 0.054	0.338 ± 0.073
ET-1	0.474 ± 0.118	0.381 ± 0.118	0.382 ± 0.064	0.340 ± 0.037

ARG: arginine, GAA: guanidinoacetic acid. Each mean represents values from 8 replicates

Serum NO levels in the blood increased as a consequence of ARG and GAA supplementation. This observation suggests that extra ARG became for the formation of NO due to the fact that ARG is a precursor of NO synthesis (Khajali and Wideman, 2010). Elevation of circulatory NO by GAA supplementation to the extent induced by ARG suggests that GAA could effectively replace ARG in broiler diets. This observation confirmed previous studies that reported sparing activity

between ARG and GAA in broiler chickens (Michiels et al., 2012; Dilger et al., 2013). Supplying both ARG and GAA significantly decreased haematocrit level compared to the control. This finding indicates less burden on the heart to pump less viscous blood through the pulmonary vasculature, which is reflected in the significantly lower RV:TV ratio. The above-mentioned observations explained the decrease in ascites mortality by supplementing ARG and GAA to broiler diets. The ratio of H:L is an index of stress in the chicken (Khajali et al., 2008). This ratio was much lower for the birds fed ARG and GAA than for the control, which means birds fed diets supplemented with ARG or GAA adapted to stress conditions more efficiently than those fed the control diet.

Electrocardiographic results indicated that the amplitudes of the R and S waves were decreased by ARG and GAA supplemented at 1.5 g/kg. Increased negative S wave amplitude is the most prominent ECG manifestation (lead II) in broilers subjected to ascites (Kirby et al., 1999). These authors reported a relatively high correlation ($R^2 = 0.79$) between S-wave amplitude and RV:TV. Significant reductions in S-wave amplitude and RV:TV ratio in birds receiving ARG and GAA at 1.5 g/kg supplementation level suggest a lower rate of right ventricular hypertrophy and dilation. Elevation of the RV:TV ratio and S-wave amplitude reflects right ventricular hypertrophy that can be directly related to pulmonary hypertension and ascites (Yousefi et al., 2013). Ascites mortality data reported herein are in good accordance with S-wave amplitude and RV:TV. In fact, supplements of ARG and GAA at 1.5 g/kg could effectively prevent right ventricular hypertrophy and resulted in a lower rate of ascites mortality in chickens kept at high altitude.

The addition of ARG and GAA at levels used in broiler diets did not significantly change the gene expression of ET-1 and iNOS. Apparently, the regulation of these genes is multifactorial and it is not influenced by nutrition alone.

In conclusion, guanidinoacetic acid (GAA) is a suitable substitute for arginine (ARG) to improve broiler performance and prevent right ventricular hypertrophy in broiler chickens reared at high altitude. Like ARG, GAA supplementation has a direct effect on elevating circulatory nitric oxide, although this effect is not mediated by the overexpression of iNOS in the heart and lungs.

Acknowledgement

This study was supported by Shahrekord University (Grant# 1395).

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