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Application of Trimethylglycine Supplement in Broiler Chicken's Diets with a High Dose of Dietary Guanidinoacetic Acid: Influence on Growth Performance and Physiological Variables

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Abstract

The present experiment studied the effect of a high dose of guanidinoacetic acid (GAA; 2.25 g/kg), with or without trimethylglycine (TMG) on growth performance and physiological variables of broiler chickens. A total of 300 day-old males Cobb 500 were randomly assigned to three dietary treatments with 5 replicates of 10 birds in each. Treatments were including a basal diet (control treatment), the basal diet supplemented with 2.25 g/kg GAA, and the GAA diet with 1 g/kg TMG. All birds received feed and water *ad libitum* during a 40-days rearing period. Results indicated that weight gain during days 31-40 of the rearing period was impaired by feeding the GAA diet. Feed conversion ratio was impaired in all feeding stages when the high dose of GAA was included in the diet. However, the inclusion of TMG in the GAA diet restored these responses to a comparable level to the control group. The GAA diet caused a significant increase in malondialdehyde concentration in serum compared to the control treatment. Moreover, the use of GAA decreased the heterophil to lymphocyte ratio than other treatments. A high dose of GAA caused higher serum levels of creatine and homocysteine; however, TMG supplementation re-established those responses. In addition, TMG supplement significantly up-regulated hepatic adenosyl homocysteinase and methionine adenosyltransferase II beta genes. In conclusion, feeding a high dose of GAA could impact broiler growth performance, but this effect could be ameliorated by dietary inclusion of TMG supplement, suggesting the negative effects of high doses of GAA were linked to the methyl donor deficiency. The practical implication is to include TMG in diets when a high dose of GAA is going to feed.

Introduction

Guanidinoacetic acid (GAA) is the key substrate for the biosynthesis of creatine. Creatine, in the phosphorylated form, is a labile energy reserve in the skeletal muscle to regenerate cellular energy status (Khajali *et al*., 2020). While creatine as a feed supplement has poor stability when exposed to thermal treatment during poultry feed processing (Vranes *et al*., 2017), GAA has durable thermal stability and safely withstands the feed pelleting process. In addition, GAA is highly bioavailable and cost-effective. Dietary levels of GAA in poultry feeds that are associated with improved energy utilization and growth performance are in the range of 0.6 to 1.2 g/kg (Khajali *et al*., 2020). The optimal level of GAA for broiler chickens reared at high altitudes has been reported to be greater than the recommended range (Ahmadipour *et al*., 2018).

Supplementing poultry diets with more than 2 g/kg GAA could deteriorate the broiler response (Ahmadipour *et al*., 2018; Faraji *et al*., 2019). This is thought to be associated with the metabolic burden of methyl donor deficiency (McBreairty *et al*., 2015). GAA undergoes the S-adenosylmethionine (SAM) dependent methylation to convert to creatine. This conversion is not regulated by a feedback mechanism. A recent study reported that administration of GAA with methionine (a methyl donor) was even more

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effective than creatine alone in improving poultry performance (Ibrahim *et al*., 2019). The *S*-adenosylhomocysteine hydrolase enzyme converts *S*-adenosylhomocysteine to homocysteine. Moreover, methionine adenosyl transferase II beta catalyzes the biosynthesis of S-adenosylmethionine from methionine and ATP. Both genes are responsible in the interrelationships between GAA and homocystenine (Jose *et al*., 2002). Since the conversion of GAA to creatine needs a methyl donor, we hypothesized that concomitant use of GAA and a methyl donor may counterbalance the impact of a high dose of GAA. Therefore, the present study investigated the simultaneous use of a high dose of GAA (2.25 g/kg) and TMG as a methyl donor on live performance, physiological response, and *S*-adenosylhomocysteine hydrolase and methionine adenosyl transferase II beta genes expression of broiler chickens.

Materials and Methods

Birds and housing

The study was carried out at the Poultry Research Center of Shahrekord University, Shahrekord, Iran, (2100 m above sea level). All procedures were observed by the Directive 2010/63/ EU in Europe and approved by the Ethical Committee of Shahrekord University Research Council.

A total of 300 one-day-old male broiler chicks (Cobb 500 strain) were randomly distributed across 15-floor pens (1.5 m^2 each). Initial bird weight was similar among all pens (48.5 g ± 1.3). The house temperature was set at 32±1 °C on day 1 and declined to 25±1 °C on day 7, 20 ± 1 °C on day 14 and 15 ± 1 °C on day 14 onward, as previously described (Moghaddam *et al*., 2017). The lighting program was set in compliance with the Cobb 500 Management Guide (2018). Birds had free access to feed and water.

Treatments

Experimental diets were formulated and prepared to meet the Cobb 500 recommendations for broiler chickens. A basal diet (Table 1) was regarded as the control. The basal diet was supplemented with a high dose of GAA (2.25 g/kg; CreAMINO® by Evonik Industries, Germany) to make the second dietary treatment. The third dietary treatment was prepared by supplementing TMG (1 g/kg; Betafin S1, Marlborough, UK) to the GAA diet.

Table 1. The composition of basal diets of broiler chickens at different feeding stages.

Item (% unless stated otherwise)	Starter (1-8 d)	Grower (9-18 d)	Finisher 1 (19-28 d)	Finisher 2 (28-40 d)
Corn (8.5% CP)	56.4	59.3	61.3	63.5
Soybean meal (44% CP)	35.5	32	30	27.5
Wheat bran* (15% CP)	0.3-0.8	1-1.5	0.5-1	0.5-1
Soy oil	3	3.2	4	4.3
Dicalcium phosphate	1.8	1.7	1.5	1.5
Oyster shell	1.3	1.2	1.1	1.1
Salt	0.3	0.3	0.3	0.3
DL-methionine	0.25	0.2	0.2	0.2
L-lysine	0.15	0.1	0.1	0.1
Vitamin premix[1]	0.25	0.25	0.25	0.25
Trace mineral premix[2]	0.25	0.25	0.25	0.25
Total*	99.5	99.5	99.5	99.5
Metabolizable energy (Kcal/kg)	2980	3025	3100	3150
Crude protein	21.1	19.8	19	18
Methionine+Cystine	0.95	0.90	0.85	0.80
Lysine	1.25	1.15	1.07	1.02
Calcium	0.9	0.85	0.76	0.76
Available phosphorus	0.45	0.42	0.36	0.36

[1]Provided the following per kilogram of diet: vitamin A (*trans* retinyl acetate), 3,600 IU; vitamin D_3 (cholecalciferol), 800 IU; vitamin E (dl-α-tocopheryl acetate), 7.2 mg; vitamin K_3, 1.6 mg; thiamine, 0.72 mg; riboflavin, 3.3 mg; niacin, 0.4 mg; pyridoxin, 1.2 mg; cobalamine, 0.6 mg; folic acid, 0.5 mg; choline chloride, 200 mg.

[2]Provided the following per kilogram of diet: Mn (from MnSO4·H2O), 40 mg; Zn (from ZnO), 40 mg; Fe (from FeSO4·7H2O), 20 mg; Cu (from CuSO4·5H2O), 4 mg; I [from Ca (IO3)2·H2O], 0.64 mg; Se (from sodium selenite), 0.08 mg.

* 0.5% used to substitute wheat bran for feed additives (guanidinoacetic acid trimethylglycine)

Measurements

Body weight and feed consumption were measured and presented at 10-day intervals. Feed conversion ratio (FCR) was then calculated by taking into account the mortality body weights. At the end of the experiment, 10 birds from each treatment were selected for blood collection (~3 mL) from the brachial vein. Blood samples were then centrifuged at 2500 g for 10 min to collect sera to determine malondialdehyde (MDA) and creatinine. Serum MDA was measured

according to the procedure described by Behrooj *et al*. (2012). Serum creatinine was measured colorimetrically using a commercial kit (Chronolab® Barcelona, Spain). Serum homocysteine levels were measured using an enzymatic assay kit (HY4036, Randox Laboratories, County Antrim, UK).

A smear of blood was also prepared on glass slides for the May–Grunwald and Giemsa staining to determine differential leukocyte count (Lucas and Jamroz, 1961). At the end of the experiment, two birds from each pen (10 birds per treatment) with a weight close to the mean pen weight were selected for carcass processing. Livers were collected and stored in liquid nitrogen for quantitative real-time PCR analysis.

Quantitative real-time PCR analysis

Livers were homogenized in a digestion buffer and then exposed to RNX-Plus reagent (Sinaclon Bioscience, Tehran, Iran). An amount of 200 μL chloroform was added and mixed with homogenates, followed by centrifugation at 15,000 g at 4°C for 15 min. Total RNA (the supernatant) was deposited with isopropanol, treated with 75% ethanol, and again suspended in diethyl dicarbonate (DEPC) treated water. DNAase (Sinaclon Bioscience) was added to remove residual DNA and the RNA was quantified by a spectrophotometer. RNA with A260/A280 greater than 1.9 was used to reverse-transcribe into cDNA using the PrimeScript™ RT Reagent Kit (Takara Bio Inc., Japan). The temperature of the mixture reached 85°C for 5 s to inactivate reverse transcriptase (RT) and to denature the RNA and stored at −20°C. The levels of adenosyl homocysteinase (AHCY), methionine adenosyl transferase II beta (MAT2B), and tyrosine 3-mono-oxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ) transcripts were quantified by real-time RT-PCR using SYBR® Premix Ex Taq™ II (TliRnase H Plus; Takara Bio Inc.). YWHAZ was considered as an endogenous standard to normalize the input load of cDNA across samples. Table 2 demonstrates specific primers of the transcripts, which determined by Primer-Blast (www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). PCR samples were run in a real-time PCR cycler (Rotor Gene Q 6000; Qiagen, USA) in three replicates per liver sample. A total of 1 μL cDNA was added to 10 µl of SYBR® Premix Ex Taq II Mix as well as 1 µM of each specific primer (total volume of 20 µl). The settings of the thermocycler were 95°C for 30 s, 40 cycles of 94°C for 40 s, 64°C for 35 s, and 72°C for 30 s. At the end of each phase, the fluorescence intensity was measured. Gene regulation results were then normalized to YWHAZ. Data were analyzed using LinRegPCR software version 2012.0 (Amsterdam, the Netherlands) to determine the threshold cycle number and reaction efficiency (Ruijter *et al*., 2009). Relative transcript levels and fold changes in transcript abundance were determined using the efficiency-adjusted Paffl methodology (Dorak, 2006).

Table 2. Primers used for quantitative real-time PCR analysis of chicken mRNAs

Accession No.	PCR Product	Primers	Target
NM_001031343.1	84 bp	F:5′-AGGAGCCGAGCTGTCCAATG-3′ R:5′-CTCCAAGATGACCTACGGGCTC-3′	β-Actin
XM_417331.5	197 bp	F:5′-CTGCCTCCATATGACCGTGC-3′ R:5′-GGGTCTGCTCAATGCACCAC-3	AHYC
XM_015293669.1	81 bp	F:5′-CTTCCCAGCAGCCACTTGAG-3′ R:5′-GCAGTCAAGCTGAGCGTTCC-3′	MAT2B

AHCY: adenosyl homocysteinase; MAT2B: methionine adenosyltransferase II beta; bp: base pair

The housekeeping gene YWHAZ was used as an endogenous standard to normalize the input load of cDNA across samples. Table 2 depicts specific primers of the transcripts that were designed with Primer-Blast (www.ncbi.nlm.nih. gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). PCRs were run in a real-time PCR cycler (Rotor Gene Q 6000; Qiagen, USA) in three replicates per liver sample. An amount of 1 μL cDNA was added to 10 µl of SYBR® Premix Ex Taq II Mix with 1 µM of each specific primer (total volume of 20 μL). The thermocycler was set at 95°C for 30 s, 40 cycles of 94°C for 40 s, 64°C for 35 s, and 72°C for 30 s. Fluorescence intensity was measured at the end of each phase. Gene expression data were normalized to YWHAZ. Data were analyzed using LinRegPCR software version 2012.0 (Amsterdam, the Netherlands) to obtain the threshold cycle number and reaction efficiency (Ruijter *et al*., 2009). Relative transcript levels and fold changes in transcript abundance were calculated using the efficiency-adjusted Paffl methodology (Dorak, 2006).

Statistical analysis

Data were subjected to the ANOVA procedure of the SAS software (version 9.2; 2008) to analyze in a completely randomized design. Treatment means were then separated by Duncan's multiple range test. Effects were considered significant at P <0.05.

Results

Table 3 shows the weight gain and FCR of broilers as affected by GAA and TMG. Body weight gain was significantly reduced by feeding a high dose of GAA

31-40 d. In addition, FCR deteriorated following feeding a high GAA diet in all feeding stages. Supplementing the GAA diet with TMG restored the responses to a level similar to the control group.

Table 3. Effect of dietary supplementation of trimethylglycine (TMG) and a high level of guanidinoacetic acid (GAA) on growth performance of broiler chickens

Weight gain (g/b)	Control	GAA	GAA+ TMG	*SEM*	*P*-vaue
1-10d	150	146	153	4.79	0.520
11-20d	381[ab]	362[b]	392[a]	7.75	0.635
21-30d	644	615	618	9.82	0.741
31-40d	665[a]	628[b]	664[ab]	9.04	0.036
1-40d	1840	1750	1827	21.04	0.810
Feed conversion ratio					
1-10d	1.37[b]	1.46[a]	1.36[b]	0.014	0.022
11-20d	1.61[b]	1.86[a]	1.67[b]	0.020	0.015
21-30d	1.76[b]	1.89[a]	1.74[b]	0.015	0.031
31-40d	2.10[b]	2.23[a]	2.12[b]	0.017	0.015
1-40d	1.82[b]	1.97[a]	1.83[b]	0.018	0.026

[a,b] Means within each row with different superscripts have significant difference

Table 4 presents carcass and circulatory variables of broiler chickens that received dietary supplements. The high level of dietary GAA caused a significant decrease in breast meat yield with no impact on carcass yield. The serum concentration of MDA was increased in the GAA group compared to the control. However, adding TMG to the GAA group brought back the response to a comparable level to the control. A high dose of GAA caused a higher level of serum creatinine. Nevertheless, the use of GAA and TMG mixture re-established the response.

Table 4. Effect of dietary supplementation of trimethylglycine (TMG) and high level of guanidinoacetic acid (GAA) on carcass and blood variables of broiler chickens

	Control	GAA	GAA+ TMG	*SEM*	*P*-value
Carcass yield (%)	71.2	70.6	71.4	0.62	0.580
Breast yield (%)	26.2[a]	24.0[b]	27.0[a]	0.71	0.040
Serum malondialdehyde (µMol/L)	2.91[b]	4.11[a]	2.73[b]	0.35	0.013
Serum creatinine (mg/dL)	0.32[b]	0.40[a]	0.30[b]	0.01	0.024
Serum homocysteine (µMol/L)	32[b]	40[a]	30[b]	1.74	0.006
H/L[*]	0.59[b]	0.86[a]	0.51[b]	0.043	0.005

[a,b] Means within each row with different superscripts have significant differences
[*]Heterophils to lymphocytes ratio
Number of observations in each group=10

When GAA was included at 2.25 g/kg, the heterophils to lymphocytes ratio (H/L) significantly increased relative to the control. In the interim, adding TMG significantly restored the situation.

Table 5 indicates the effects of GAA and TMG on hepatic gene expression of broiler chickens. AHCY and MAT2B were significantly overexpressed by TMG supplementation.

Table 5. Effect of dietary supplementation of trimethylglycine (TMG) and high level of guanidinoacetic acid (GAA) on hepatic gene expression of broiler chickens

	Control	GAA	GAA + TMG	*SEM*	*P*-value
AHCY	0.019[b]	0.030[b]	0.283[a]	0.140	0.002
MAT2B	0.0017[b]	0.0081[b]	0.055[a]	0.041	0003

[a,b] Means within each row with different superscripts have significant difference
AHCY: adenosyl homocysteinase; MAT2B: methionine adenosyltransferase II, beta

Discussion

In general, broiler performance was substantially lower compared to the performance objectives presented in the Cobb-Vantress manual (2018). The poor performance might be due to the high altitude (2100 m) that birds raised. For instance, weight gain and FCR for Hubbard broilers during 7 to 35 days of age were 250 g/b and 4.36, respectively, when raised at an altitude of 3500 m, which was significantly lower than that of the Hubbard manual for the same period (2058 g/b and 1.48) (Kalia *et al*., 2017).

Significantly impaired weight gain and FCR of chickens that received GAA at 2.25 g/kg were aligned with the finding of Ahmadipour *et al*. (2018). They observed a curvilinear response to different levels of GAA in broiler chickens. However, these researchers failed to explain how high doses of GAA could impact growth performance. Improved growth performance by feeding TMG suggests that the methyl donor deficiency played a causal role in decreasing performance when a high dose of GAA was being fed (McBreairty *et al*., 2015). In fact, homocysteine produced from the metabolism of GAA must be converted to methionine through remethylation reactions that are catalyzed by means of the enzyme betaine-homocysteine methyltransferase (Olthof and Verhoef, 2005; Elango, 2020) and TMG serves as a substrate to regenerate methionine from homocysteine (Ueland *et al*., 2005). In line with our findings, Ibrahim *et al*. (2019) indicated that feeding GAA with a 0.4% methionine supplement (a methyl donor) enhanced the growth performance of poultry through up-regulating hepatic insulin-like growth factor-1, growth hormone, and muscle myogenin. In the study of Sharma *et al.* (2021), however, the researchers failed to observe a difference between GAA alone or combined with TMG when reduced-protein diets were fed to broilers.

Increased serum concentration of homocysteine could be expected by feeding a high dose of GAA. In view of the GAA metabolism (Khajali *et al*., 2020), it is anticipated that supplementary GAA uses available methionine in the form of SAM to be converted to creatine. This reaction generates S-adenosyl homocysteine, which is further metabolized to convert to homocysteine. In experimental studies with animal models, GAA has sometimes been used to induce hyperhomocysteinemia (Setoue *et al*., 2008; Liu *et al*., 2014). TMG is a helpful substrate to restore the situation through the betaine-homocysteine methyltransferase as mentioned earlier. A high level of homocysteine is regarded as stress, which is known to have a link with several diseases (Olthof and Verhoef, 2005). This is likely the reason for a higher H/L ratio in the GAA group in the present study. In fact, the H/L ratio is a well-known indicator of stress in birds. This physiological response could be anticipated as high doses of GAA have been shown to intensify the generation of reactive oxygen species and predisposes chickens to oxidative stress (Mori *et al*., 1996; Faraji *et al*., 2019). The oxidative stress *per se* is reflected as a higher level of MDA in the GAA group. In this regard, there is some evidence that dietary TMG supplement reduced liver MDA levels and SOD activity in broiler chickens (Nasiroleslami *et al*., 2018). It has been reported that the simultaneous inclusion of antioxidants such as coenzyme Q_{10} in broiler diets with a high dose of GAA could reduce serum MDA concentration (Faraji *et al*., 2019).

Supplementing broiler diets with TMG resulted in a significant up-regulation of hepatic adenosyl homocysteinase (AHCY) and methionine adenosyl transferase II beta (MAT2B). AHCY is the enzyme that converts *S*-adenosyl homocysteine to homocysteine and MAT2B is a hepatic enzyme that forms S-adenosylmethionine, a key biological methyl donor. Both AHCY and MAT2B are associated with the methyl donor function and over-expression of these genes suggests the role of TMG as a key methyl donor.

In conclusion, feeding a high dose of GAA could impact broiler growth performance. The situation was restored when TMG was included in the diet, suggesting the negative effects of high doses of GAA, at least in part, were associated with the methyl donor deficiency. In practical nutrition, a source of TMG should be included in broiler diets when high doses of GAA are going to feed.

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