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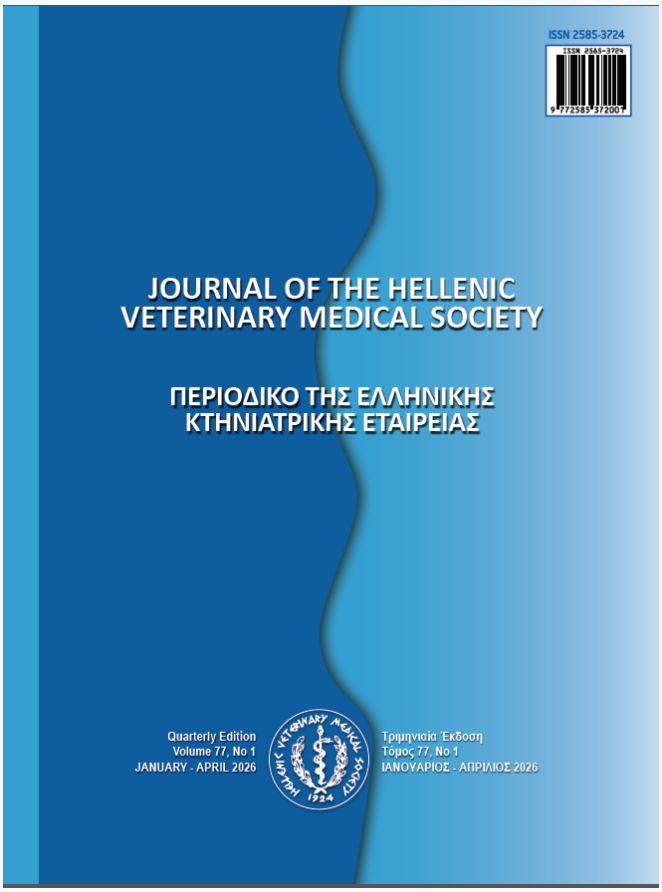

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Research article
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Effect of dietary guanidinoacetic acid supplementation on antioxidant status, biochemical parameters, inflammation cytokines and growth performance in broiler chickens

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ABSTRACT: This study examined the effects of dietary guanidinoacetic acid (GAA) on biochemical parameters, antioxidant status and inflammatory responses of chicks. Three hundred 1-d-old male broiler chicks (Ross 308) were randomly assigned to fifteen pens containing 20 chicks each, which was subjected to one of three dietary treatments supplemented with different levels of guanidinoacetic acid (GAA) at levels of (control, 0, 6 and 9 g/kg). On day 42, 2 birds per cage were weighed and euthanized, and samples were collected. Dietary GAA in 6 and 9 g/kg supplementation reduced feed intake (FI), body weight gain (BWG) and increased feed conversion ratio (FCR) than broilers in the control group. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), Gamma glutamyltransferase (GGT), Alkaline Phosphatase (ALP) activity, and creatinine & urea levels showed a marked significant ($P<0.05$) increase in 6 and 9 g/kg groups supplemented groups compared to the control group. Also, high levels of GAA significantly reduced serum Nitric oxide levels without affecting cholesterol and triglyceride. Malondialdehyde (MDA) markedly increased along with a significant decrease of glutathione peroxidase (GPx), superoxide dismutase (SOD) and in serum and liver, and catalase (CAT) in serum in both 6 and 9g/kg groups compared to control group. None of the serum and liver inflammatory parameters were affected by high levels of GAA. In conclusion, high levels of dietary GAA supplementation induced oxidative stress in broiler chickens, as evidenced by increased lipid peroxidation and decreased antioxidant function, while no significant inflammatory responses were observed. Therefore, the reduction in growth performance associated with high GAA levels is likely attributed to oxidative stress rather than inflammation.

Keyword: Antioxidant status; broiler; enzyme activity; guanidinoacetic acid; performance; inflammation response

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INTRODUCTION

Genetic selection in poultry has progressed continuously since the early 1960s, resulting in faster growth rates and higher meat production (Hartchera and Lum, 2019). Over the past 60 years, body weight gain has increased fourfold from 1957 to 2005 with a simultaneous 50% reduction in feed conversion ratio (Havenstein et al. 2003; Zuidhof et al. 2014). At the same time, the growth, development, structure, and overall metabolism have been modified by such selection, which has probably resulted in modifications affecting biochemical (Petracci et al. 2019).

Moreover, several studies have documented that in intensive large-scale breeding conditions, fast-growing broilers are susceptible to oxidative stress and inflammatory response due to their fast growth and high feeding density (Fathi et al., 2023). It has been reported that oxidative stress can decrease production, growth rate, and feed efficiency of poultry, impair meat quality and immune defense. Therefore, it is important to reduce the oxidative stress in the broiler industry (Bailey et al. 2015; Chen et al. 2019; Fathi et al. et al. 2022; Wei et al. 2021).

Energy is the main limiting nutrient for fast growing chickens with enormous muscle growth and increase the body's ability to cope with adverse environmental conditions and diseases. In cellular metabolism, energy transfers from adenosine-tri-phosphate (ATP) to various metabolic processes (Tossenberger et al., 2016). In this context, a pool of phosphocreatine and creatine kinase are located in skeletal muscle keeping adenosine-diphosphate (ADP) and ATP levels constant as a kind of buffering system, which is important for proper functioning of cellular energy metabolism (Ahmadipour et al., 2018). GAA is the biochemical precursor of creatine, which, in its phosphorylated form, plays an important role as a high-energy carrier in the muscle. GAA is formed from the amino acids glycine and arginine in the kidney or absorbed from the gut (Oviedo-Rondón and Córdova-Noboa, 2020). It is transformed to creatine in the liver. Phosphocreatine serves as a dynamic reservoir of high energy phosphate. The phosphocreatine/ creatine system buffers ATP/ADP ratio for all energy consuming functions of the cell (Bonilla et al., 2021). The degradation of creatine results in creatinine, which is excreted in the urine. Therefore, creatine must be continually replaced from dietary sources or synthesized de novo from GAA. In contrast to animal proteins, creatine is not found in plant feedstuffs and may be thus deficient in all-vegetable diets. GAA as a creatine source is more stable and less expensive than creatine itself (Ostogic et al. 2015; Baker, 2009). However, pathological accumulation of GAA can induce oxidative stress, as shown by decreased total radical-trapping antioxidant potential, total thiol content and antioxidant enzymes activity following intrastriatal infusion in rats (Zugno et al. 2008)

The objective of the present experiment was to determine the effects of high level of GAA (trade name CreAMINO®, Evonik Industries) on biochemical parameters, antioxidant status and inflammatory responses in chicks.

MATERIALS AND METHODS

Animal ethics statement

All efforts were made to minimize animal suffering. Animal handling and experimental procedures of the study were performed based on following the general ethical guideline of the Animal Ethics Committee of Payame Noor University, Tehran, Iran (Number: 3831.03.31.1396).

Birds, diets and experimental design

Total of 300, day-old male Ross 308 broilers were randomly allocated into three dietary treatments with 5 replication pens, each pen had 20 chicks. Birds received basal diet supplemented with (control, 0; GAA1, 6 g/kg and GAA3, 9 g/kg GAA). Diets (Table 1) and fresh water were provided ad libitum. GAA was added in the form of CreAMINO (Evonik Degussa GmbH, Hanau-Wolfgang, Germany) and supplied at the expense of corn. Each cage was equipped with bell-drinker and a feeder. All management procedures were conducted under standard commercial conditions. Broilers were housed in flooring pens. The experimental pens measured 1.0 × 2.0 m. Broilers were provided with continuous 24-h lighting. Water and feed were provided ad libitum throughout the experimental period. Average housing temperatures were maintained at 25–27◦C, with temperatures during the first week set at 32–35◦C to promote optimal broiler growth. Additionally, relative humidity was kept at 75–80 % (Fathi et al., 2023)

The use GAA in the suggested way (Vranes et al. 2017; Boroumandnia et al., 2021) was as follows The GAA was blended with warm distilled water with pH-neutral and sprayed on the pelleted feed prior to feeding twice a day at 6 a.m. and 6 p.m.

Table 1. The ingredients and composition of the basal diet

	Starter (0-10 d)	Grower (11-24 d)	Finisher (25-42 d)
Ingredients (%)			
Maize, 8% CP	47.53	51.63	57.56
Soybean meal, 44%CP	42.35	37.99	32.35
Soybean oil, 9000 kcal/kg	5.54	6.24	6.29
Limestone, 38% Ca	1.20	1.12	1.05
Di-calcium phosphate, 21%Ca	1.79	1.56	1.34
Vitamin premix	0.25	0.25	0.25
Mineral premix	0.25	0.25	0.25
NaCl	0.40	0.40	0.40
DL-Methionine, 99%	0.37	0.32	0.28
Lysine, 78%	0.28	0.22	0.22
Threonine, 98.5%	0.05	0.02	0.00
Calculated values			
Metabolizable energy, kCal/kg	2990	3082	3218
Crude protein, %	23	21.3	19.3
Calcium (Ca), %	0.96	0.87	0.79
Available phosphorus, %	0.456	0.409	0.361
Sodium (Na), %	0.16	0.16	0.16
Methionine, %	0.71	0.64	0.58
Methionine+cysteine, %	1.07	0.89	0.89
Lysine, %	1.46	1.30	1.17
Arginine, %	1.56	1.45	1.30
Threonine, %	0.96	0.87	0.78
Tryptophan, %	0.35	0.32	0.29

Vitamin concentrations per kilogram of diet: retinol, 13.50 mg; cholecalciferol, 4.15 mg; tocopherol acetate, 32.00 mg; vitamin K3, 2 mg; thiamin, 2 mg; riboflavin, 6.00 mg; biotin, 0.1 mg; cobalamin, 0.015 mg; pyroxidine, 3 mg; niacin, 11.00 mg; d-pantothenic acid, 25.0; menadione sodium bisulphate, 1.10; folic acid, 1.02; choline chloride, 250 mg; nicotinamide, 5 mg;

Mineral concentrations per kilogram of diet:calcium pantothenate, 25 mg; Fe (from ferrous sulphate), 35 mg; Cu (from copper sulphate), 3.5 mg; Mn (from manganese sulphate), 60 mg; Zn (from zinc sulphate), 35 mg; I (from calcium iodate), 0.6 mg; Se (from sodium selenite), 0.3 mg.

Sample preparation

On day 42, after 12-hour fasting, 6 broilers per treatment (two chicks per from each replication pen) randomly selected, blood samples (2.5 mL) were collected from the brachial and kept on ice until serum was separated by centrifugation for 10 min at 2,500 rpm. Serum samples were stored at -20oC until assayed. BWG and FI were measured weekly, and average FCR was calculated.

Serum biochemical parameters

At the age of 42 d, two chicks per replicate were randomly selected and then 5 ml of blood was collected from wing vein or jugular vein using sterile needles. Whole clotted blood was centrifuged to separate serum for determining the biochemical and antioxidant parameters. Blood samples (2.5 mL) were taken via wing vein and kept on ice until serum was separated by centrifugation for 10 min at 2,500 rpm. Serum samples were stored at 80oC until assayed. Cholesterol, triglyceride, nitric oxide, urea and creatinine were determined using an autoanalyser (Abbott alcyon 300, USA) by laboratory kits (Pars Azmoon, Tehran, Iran). AST, ALT, ALP and GGT were measured using appropriate laboratory kits (Pars Azmoon, Tehran, Iran).

Antioxidant Capacity

Blood was collected by cardiac puncture using an anticoagulant-free vacutainer tube, later centrifuged

at 3,000 × g for 10 min to obtain serum. GPx activity was determined using a commercially available enzyme kit (Ransel, RANDOX/RS-504 supplied by Randox Laboratories, Crumlin, UK), CAT and SOD activity was determined using the commercially available enzyme kit (Ransod, RANDOX/SD-125 supplied by Randox Laboratories) and autoanalyzer (Alcyon 300, USA) according to the manufacturers' protocols. The level of MDA in serum was measured with the thiobarbituric-acid reaction by the method of Fathi et al (2022). This method evaluates oxidative stress by measuring MDA, the last product of lipid breakdown caused by oxidative stress.

Liver parameters

Approximately 2 g of chicken liver homogenized with 9 mL of 0.9% sodium chloride buffer (w/v, 1:9) on ice, and then centrifuged at 1,000 g at 4°C for 10 min to obtain the supernatant. The supernatant was stored at -80°C until further analysis. The SOD, GPx, CAT activities and MDA concentration in the supernatant of the liver homogenate was determined using commercially available assay kits (Pars Azmoon, Tehran, Iran) via an automated spectrophotometric analyzer (Alcyon 300, USA). All procedures were performed per the manufacturer's instruction.

Inflammatory tests

Inflammatory parameters such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin- 10 (IL-10) concentrations were determined by using ELISA kits (Pars Azmoon, Tehran, Iran) according to the manufacturer's instructions.

Statistical Analysis

Data from all response variables were subjected to one-way analysis of variance by applying the SAS program (SAS, 2005) based on a completely randomized design (CRD) with four treatments and five replicates per treatment using a general liner model (GLM). Significant differences among treatment means were separated using Tukey's test at 5 % probability.

RESULTS AND DISCUSSION

Growth performance

As shown in Table 2, In this work, a significant decline in performance parameters, so that, dietary inclusion of high levels of GAA reduced FI, BWG, and FCR compared to the control birds ($P < 0.05$). In the present study, the use of high levels of GAA significantly caused a drop in growth indices in broiler chickens. One of the possible explanations for the negative effect of high levels of GAA on the growth performance of broilers is that high levels of GAA induce oxidative stress, thereby reducing feed intake and weight gain in broilers.

Previous studies have demonstrated that oxidative stress can significantly reduce feed intake and body weight gain in broiler chickens (Arab et al., 2006; Fathi et al., 2022). Oxidative stress impairs growth performance by disrupting cellular homeostasis and causing damage to vital organs, particularly the liver, which plays a key role in metabolism and nutrient utilization. Elevated levels of reactive oxygen species (ROS) may impair mitochondrial function, reduce ATP production, and interfere with appetite regulation mechanisms, ultimately leading to decreased feed intake and inefficient nutrient absorption. This cascade of physiological disturbances can result in markedly reduced growth rates and overall performance (Arab et al., 2006; Surai, 2020).

Biochemical parameters

Based on the results shown in Table 3, a significant increasing in uric acid and creatinine by dietary inclusion of high levels of GAA compared to the control birds ($P<0.05$). It is also, a significant decreasing in nitric oxide (NO) by dietary inclusion of high levels of GAA compared to the control birds ($P<0.05$). There was no significant difference

Table 2. Growth performance of broilers fed diets containing guanidinoacetic acid (GAA)

Parameters	Treatments (GAA levels)				
	Control (0 g/kg)	6 g/kg	9g/kg	SEM	P Value
FI (g)	3878[a]	3493[b]	3455[b]	56	0.01>
BWG (g)	2486[a]	2130[b]	2120[b]	42	0.01>
Average FCR	1.56[b]	1.64[a]	1.63[a]	0.03	0.01>

[a, b, c] Mean values in the same row with different superscript letters were significantly different ($P<0.05$). BWG, body weight gain; FI, feed intake; FCR, feed conversion ratio.

Table 3. Biochemical parameters of broilers fed diets containing guanidinoacetic acid (GAA)

Parameters	Treatments (GAA levels)				
	Control (0 g/kg)	6 g/kg	9g/kg	SEM	P Value
Triglyceride (mg/dL)	63.5	62.5	61.5		0.19
Cholesterol (mg/dL)	252.78	262.78	270.38		0.17
Nitric oxide (µM/L)	9.25[a]	7.02[b]	5.20[c]		0.01>
Urea (mg/dL)	3.55[c]	4.20[b]	4.89[a]		0.01>
Creatinine(mg/dL)	0.09[c]	0.12[b]	0.25[a]		0.01>

[a, b, c] Mean values in the same row with different superscript letters were significantly different (P<0.05).

in serum triglyceride and cholesterol levels of fed birds with high levels of GAA and control birds (P>0.05). GAA appeared to elevate serum creatinine, perhaps due to increased creatine utilization after administration (Ostojic et al. 2013). These results were announced in corroboration with the observations Khajali et al. 2020 where 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. 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; De Groot 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 high 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. (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 characterized 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.

In the present study, surprisingly serum concentration of nitric oxide declined in the birds receiving high dietary GAA compared with the control birds. creatine synthesis from GAA might affect depletion of arginine, a precursor of creatine and a key source of nitric oxide (NO) and creatine overload may induce disturbances in NO bioavailability (Karamat et al. 2015; Naseem.2005).

Antioxidant capacity in serum and liver

As shown in Table 4, a significant effect of dietary treatment on antioxidant activity of serum and liver in 42 days of age was detected, so that higher MDA and lower GPx and SOD activity in serum and liver were observed in birds fed the high levels of GAA diet. Also, a lower CAT activity in serum was found in birds fed the high levels of GAA-supplemented diet compared to control group birds (P<0.05).

There is little information about the effects of exogenous GAA on animal oxidant–antioxidant system, but Since GAA has a guanidinium ion of conjugate base, which easily donates an electron, higher concentrations of GAA may generate a hydroxyl radical, strong free radical and impede antioxidant capacity (Hiramatsu 2003). This might be rather detrimental to individuals supplemented with GAA. Moreover, there are report that show GAA induces oxidative stress after experimental intrastriatal infusion (Zugno et al. 2008). Pro-oxidant effects have seen after intracerebral accumulation and highly elevated brain GAA concentrations (~100 µmol/L), while antioxidant effects seem to appear after GAA ingestion and relatively low post-administration serum levels of GAA (~5 µmol/L) (Ostojic, 2015). Creatine, the

Table 4. Serum and liver antioxidant status of broilers fed diets containing guanidinoacetic acid (GAA)

		Treatments (GAA levels)				
	Parameters	**Control (0 g/kg)**	**6 g/kg**	**9g/kg**	**SEM**	**P Value**
Serum	GPx (Mu/mL)	1395.9[a]	1199.9[b]	1152.9[c]	18.10	<0.01
	SOD (U /mL)	302.50[a]	272.50[b]	271.50[b]	10.50	<0.01
	CAT (N mol/min/mL)	71.64[a]	29.25[b]	26.25[b]	4.50	<0.01
	MDA (n mol/ml)	10.01[b]	14.32[a]	14.45[a]	0.12	<0.01
Liver	GPx (Mu/mL)	14.0[a]	12.2[b]	11.1[b]	1.10	<0.01
	SOD (U /mL)	695[a]	609[a]	520[b]	14.90	<0.01
	CAT (N mol/min/mL)	33.2	30.5	30.7	5.5	0.31
	MDA (n mol/ml)	0.55[b]	0.78[a]	0.80[a]	0.01	<0.01

[a, b, c] Mean values in the same row with different superscript letters were significantly different (P<0.05). GPx, glutathione peroxidase; SOD, Superoxide dismutase; CAT, catalase; MDA, Malondialdehyde

end product of GAA utilization, is thought to have Pro-oxidant effects in athletes (Percário et al., 2012).

One of the theories about the negative effect of high levels of GAA on the body's antioxidant capacity is that the transformation of GAA to creatine requires a donation of a methyl group (-CH3) from σ-adenosyl-L-methionine, an excessive GAA intake can hypothetically drain the stores of methyl donors in the human body (e.g., methionine, choline, folic acid, B vitamins). The metabolic burden of methyl donor deficiency can perturb many cellular functions, including DNA methylation, neurotransmission, antioxidant defense, and protein synthesis (Ostojic, 2022).

Inflammatory cytokines in the serum and liver

The content of interleukins including; IL-1*β*, TNF-*α*, and IL-10 in the serum and liver are presented in Table 5. No significant difference was observed between serum and liver interleukins between different treatments (P > 0.05). Our results align with recent findings by Peng et al. (2023), who reported that GAA supplementation in broiler chickens only downregulated the expression of IL-1β among several inflammatory markers, while other indicators remained unaffected. This supports the notion that the anti-inflammatory effects of GAA may be limited, underscoring the need for further studies to better understand its role in inflammation modulation. Similarly, another study investigating the ileum gene expression in broilers found that dietary GAA had no significant impact on inflammatory responses in broilers exposed to heat stress (Barekatain et al., 2025).

In line with our findings, Ostojic et al. (2018) reported that supplemental GAA had no significant effects on biomarkers of cardiometabolic risk and

Table 5. Serum and liver cytokines of broilers fed diets containing guanidinoacetic acid (GAA)

		Treatments (GAA levels)				
	Parameters	**Control**	**6 g/kg**	**9g/kg**	**SEM**	**P Value**
Serum	IL-10 (ug/mL)	32.20	35.20	36.20	3.95	0.21
	IL-1*β* (ug/mL)	10.10	11.10	12.90	2.16	0.17
	TNF-*α* (ug/mL)	11.20	13.30	14.20	4.87	0.24
Liver	IL-10 (ug/mL)	16.20	14.95	14.20	3.18	0.31
	IL-1*β* (ug/mL)	0.90	1.01	1.10	0.17	0.26
	TNF-*α* (ug/mL)	16.20	18.60	18.30	5.10	0.19

[a, b, c] Mean values in the same row with different superscript letters were significantly different (p<0.05).

IL-10, interleukin-10; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor- α

inflammation in healthy human volunteers. After 10 weeks of GAA administration at a dosage of 3 grams per day, neither serum triglycerides nor HDL cholesterol levels were affected. Similarly, Osna et al. (2016) demonstrated that prolonged administration of high doses of GAA in mice did not alter inflammatory parameters, although it significantly increased liver damage. These studies collectively support the notion that GAA supplementation may have limited or no anti-inflammatory effects under certain physiological conditions

Enzyme activity

Based on the results shown in Table 6, the use of high levels of GAA significantly increased the serum levels of enzymes (AST, ALT, GGT and ALP) compared to the control group. There are reports that show GAA significantly increased the serum activity of ALP and CK without affecting the serum activity of ALT, AST and GGT enzymes (Ostojic, 2015). It is also reported that the use of high levels of GAA increased ALT activity in broilers (Boroumandniaet al. 2021). There are many reports that GAA has a positive effect on increasing the serum activity of GGT, ALP and CK in laying Japanese quails (Raei et al., 2020) and broilers chickens (Nasiroleslamia et al. 2018).

Probably one of the reasons for the increase in serum enzyme activity is the induction of oxidative stress due to the use of high levels of GAA in this study (Table 3). There have already been reports that during oxidative stress and the production of high levels of free radicals in fast-growing broilers, liver tissue destruction occurs, followed by an increase in the activity of liver enzymes in the serum (Arab et al., 2006). 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 (Boroumandniaet al. 2021).

In the current study, addition of 6 and 9 g/kg GAA yielded increases in the activity of CK. The phosphocreatine/creatine system buffers ATP/ADP ratio for all energy consuming functions of the cell. CK catalyzes the conversion of creatine and utilizes ATP to create phosphocreatine and ADP. The rise in CK activity and serum creatinine could be either due to reduced clearance or due to overproduction or both (Hekimsoy & Oktem 2005). GAA might be able to support creatine production and increase CK activity. In the present study, the increased level of serum CK indicates the previously reported potential role in maintaining high ATP turnover at low temperature (Jayasundara et al. 2015). Similar to this study, a significant increase in CK activity was seen formerly in the group supplemented with creatine (Dobgenski et al. 2016).

CONCLUSIONS

In the present study, dietary supplementation of guanidinoacetic acid (GAA) at 6 and 9 g/kg negatively affected growth performance, as evidenced by reduced feed intake and body weight gain and increased feed conversion ratio. Biochemical analysis indicated that high levels of GAA significantly increased liver enzymes (AST, ALT, GGT, ALP) and markers of renal function (creatinine and urea), suggesting possible hepatic and renal stress. Additionally, GAA supplementation decreased antioxidant enzyme activities (GPx, SOD, CAT) and increased lipid peroxidation (MDA), indicating oxidative stress. Importantly, no significant effects of GAA were observed on inflammatory markers in serum or liver. These findings suggest that excessive levels of dietary GAA may impair performance and oxidative status in broilers without exerting measurable effects on inflammatory responses. Further research is warranted to evaluate the long-term impact of lower GAA doses and their potential interaction with environmental stressors such as heat or immune challenge.

Table 6. Serum enzymes activity of broilers fed diets containing guanidinoacetic acid (GAA)

Treatments (GAA levels)	ALT (U/L)	AST (U/L)	GGT (U/L)	ALP (U/L)
Control (0 g/kg)	4.80[b]	152.98[b]	15.0[b]	359.2[b]
6 g/kg	8.50[a]	196.08[a]	21.2[a]	630.6[a]
9 g/kg	9.00[a]	183.98[a]	22.5[a]	660.2[a]
SEM	1.55	23.70	1.50	650
P Value	<0.01	<0.01	<0.01	<0.01

[a, b, c] Mean values in the same row with different superscript letters were significantly different (P<0.05). Alanine aminotransferase, ALT; aspartate aminotransferase, AST; Gamma-glutamyltransferase, GGT; ALP, Alkaline Phosphatase

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