# Postprint: Evaluation of Tolerance to Guanidinoacetic Acid in Arbor Acres Broiler Chickens

**Authors:** Zhang Defu, Juntao Li, Tian Yaoyao, Yang Libin, Zhang Liying

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## Abstract

This study aimed to investigate the effects of dietary guanidinoacetic acid (GAA) supplementation on growth performance, hematological indices, organ indices, tissue homocysteine content, and tissue morphology in Arbor Acres (AA) broiler chickens, and to systematically evaluate the tolerance of AA broiler chickens to GAA. A total of 540 one-day-old AA male broiler chicks were randomly assigned to five groups with six replicates per group and 18 birds per replicate. The five groups were fed basal diets supplemented with 0, 800, 1,600, 4,000, and 8,000 mg/kg GAA, respectively. The 42-day experimental period was divided into two phases: starter phase (days 1-21) and grower phase (days 22-42). The results showed that compared with the control group, dietary supplementation with 800-4,000 mg/kg GAA significantly improved average daily gain (ADG) during the starter phase, grower phase, and overall period ($P<0.05$), and significantly decreased feed conversion ratio (FCR) during the grower phase and overall period ($P<0.05$), but had no significant effect on average daily feed intake (ADFI) in any phase ($P>0.05$). The ADG, ADFI, and FCR of the 8,000 mg/kg GAA group did not differ significantly from the control group ($P>0.05$). Dietary supplementation with 800-8,000 mg/kg GAA had no significant effects on routine blood parameters, serum biochemical parameters, organ indices, or tissue homocysteine content in broiler chickens at 21 and 42 days of age ($P>0.05$). Dietary supplementation with 4,000 and 8,000 mg/kg GAA had no adverse effects on liver and kidney tissue morphology. These results indicate that dietary GAA supplementation had no adverse effects on growth performance, hematological indices, organ indices, tissue homocysteine content, or tissue morphology in broiler chickens, and that broiler chickens can tolerate GAA up to 8,000 mg/kg.

## Full Text

# Evaluation of Tolerance to Guanidinoacetic Acid in Arbor Acres Broilers

**Authors:** ZHANG Defu[1],[2], LI Juntao[1], TIAN Yaoyao[2], YANG Libin[2], *ZHANG Liying*[1]

**Affiliations:**

[1]State Key Laboratory of Animal Nutrition, Ministry of Agriculture Key Laboratory of Feed Safety and Biological Efficacy, China Agricultural University, Beijing 100193, China

[2]Beijing Gendone Agricultural Technology Co., Ltd, Beijing 100085, China

### Abstract

This experiment was conducted to investigate the effects of dietary guanidinoacetic acid (GAA) supplementation on growth performance, hematological indices, organ indices, tissue homocysteine content, and histomorphology in Arbor Acres (AA) broilers, thereby providing a systematic evaluation of AA broiler tolerance to GAA. A total of 540 one-day-old AA male broiler chicks were randomly allocated to five groups with six replicates per group and eighteen birds per replicate. The five groups received a basal diet supplemented with 0, 800, 1,600, 4,000, and 8,000 mg/kg GAA, respectively. The 42-day experimental period was divided into two phases: the starter phase (days 1–21) and the grower phase (days 22–42).

The results demonstrated that compared with the control group, dietary GAA supplementation at 800–4,000 mg/kg significantly increased average daily gain (ADG) during the starter, grower, and overall periods ($P<0.05$) and significantly decreased feed-to-gain ratio (F/G) during the grower and overall periods ($P<0.05$), while having no significant effect on average daily feed intake (ADFI) during any period ($P>0.05$). The 8,000 mg/kg GAA group showed no significant differences from the control group in ADG, ADFI, or F/G ($P>0.05$). Dietary GAA supplementation at 800–8,000 mg/kg had no significant effects on hematological indices, serum biochemical parameters, organ indices, or tissue homocysteine content in broilers at 21 and 42 days of age ($P>0.05$). Supplementation at 4,000 and 8,000 mg/kg GAA produced no adverse effects on liver or kidney histomorphology.

In conclusion, dietary GAA supplementation showed no adverse effects on growth performance, hematological indices, organ indices, tissue homocysteine content, or histomorphology in broilers. Broilers can tolerate GAA at dietary levels up to 8,000 mg/kg.

**Keywords:** guanidinoacetic acid; broilers; tolerance; growth performance; hematological indices; organ indices; homocysteine

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## Introduction

Depletion of fish stocks has led to fishmeal shortages, high prices, and severe adulteration, while meat-and-bone meal faces restrictions in animal feed due to food safety concerns. Consequently, animal-derived protein feedstuffs are generally lacking in livestock and poultry diets in China. Animals fed purely plant-based diets exhibit reduced performance, likely because animal-derived feedstuffs contain creatine whereas plant-derived feedstuffs do not. Creatine is an essential nutrient for rapidly growing young animals, and its phosphorylated form, phosphocreatine, together with ATP constitutes the phosphagen energy system. This system provides immediate energy for muscle tissue development without oxygen requirement. Animals can synthesize creatine endogenously from arginine, glycine, and methionine, with approximately 75% of total creatine requirements met through de novo synthesis. However, about 1.7% of creatine is non-enzymatically excreted daily as creatinine, and modern breeding practices have accelerated muscle growth, thereby increasing creatine demands. Thus, additional creatine supplementation is necessary.

Exogenous creatine supplementation is limited by high cost and instability. Moreover, supplemental creatine downregulates L-arginine:glycine amidinotransferase gene expression, inhibiting endogenous creatine synthesis. Research has demonstrated that exogenous guanidinoacetic acid (GAA) is more effective than creatine for increasing tissue creatine stores. Previous studies have confirmed that supplementation of 600 mg/kg GAA in plant-based diets improves broiler growth performance and breast muscle weight, achieving performance comparable to fishmeal supplementation. Additionally, GAA has been shown to improve performance in weaned piglets, growth performance and carcass and meat quality in finishing pigs, reproductive performance in broiler breeders and quail, and growth performance and energy metabolism in Jian carp. As the sole precursor of creatine, GAA has attracted considerable attention from animal nutritionists. However, research on high-dose GAA supplementation and its tolerance remains scarce. Therefore, this study aimed to systematically evaluate the tolerance of AA broilers to GAA by examining its effects on growth performance, hematological indices, organ indices, tissue homocysteine (HCY) content, and histomorphology, thereby providing a theoretical basis for safe GAA application.

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## Materials and Methods

### 1.1 Experimental Material

The GAA product used in this experiment was Gendone® provided by Beijing Gendone Agricultural Technology Co., Ltd, containing $ $98% GAA.

### 1.2 Experimental Animals and Design

A total of 540 one-day-old AA male broiler chicks with an average initial body weight of 45.13 g were randomly divided into five groups with six replicates per group and eighteen birds per replicate. The five groups received a basal diet supplemented with 0, 800, 1,600, 4,000, and 8,000 mg/kg GAA, respectively. The 42-day experimental period was divided into two phases: days 1–21 and days 22–42.

### 1.3 Basal Diet

A corn-soybean meal basal diet was formulated according to the *Feeding Standard of Chicken* (NY/T 33–2004). The composition and nutrient levels of the basal diet are presented in Table 1 . During diet preparation, GAA was progressively diluted and amplified with corn meal before mixing with other ingredients.

**Table 1** Basal diet composition and nutrient levels (as-fed basis)

### 1.4 Management

The experiment was conducted in the metabolism room of the College of Animal Science and Technology at China Agricultural University for 42 days. Broilers were housed in three-tier cages with ad libitum access to feed and water. Lighting was provided 24 hours daily. Temperature was maintained at 34–35°C during the first week and reduced by 2°C weekly until reaching 20–26°C; relative humidity was maintained at 45–55%. Conventional vaccination and management procedures for broilers were followed, and flock health and behavior were observed daily.

### 1.5 Measurements

**1.5.1 Growth Performance** Initial body weight was recorded at the start of the experiment. At 08:30 on days 21 and 42 (end of each feeding phase), broilers were weighed after fasting and feed consumption was recorded. Average daily gain (ADG), average daily feed intake (ADFI), and feed-to-gain ratio (F/G) were calculated for days 1–21, 22–42, and 1–42.

**1.5.2 Hematological Indices** On days 21 and 42, six birds per group (one per replicate) were randomly selected. Three milliliters of blood were collected via cardiac puncture into EDTA-$Na_2$ anticoagulant tubes, gently mixed, and used for hematological analysis. Parameters included white blood cell count (WBC), red blood cell count (RBC), hemoglobin (HGB), mean corpuscular volume (MCV), platelet count (PLT), and hematocrit (HCT), measured using an automatic analyzer (Sysmex Microcell Counter CL-180). Additionally, 5 mL of non-anticoagulated blood was collected, allowed to clot at room temperature for 0.5 h, centrifuged at 3,000 rpm for 10 min, and serum was stored at -20°C for biochemical analysis. Serum parameters included alanine aminotransferase

(ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein (TP), albumin (ALB), urea nitrogen (UN), creatinine (CRE), glucose (GLU), and total bilirubin (TBILI), measured using commercial kits (Zhongsheng Beikong Biotechnology Co., Ltd, Beijing) on an automatic biochemical analyzer (Hitachi 7160, Hitachi Group, Japan).

**1.5.3 Organ Indices** On day 42, six birds per group (one per replicate) were randomly selected and slaughtered. Heart, liver, spleen, and kidney were weighed to calculate organ indices.

Organ index = organ fresh weight (g) / live body weight before slaughter (kg).

**1.5.4 Tissue HCY Content** On day 42, six birds per group (one per replicate) were slaughtered, and liver, kidney, and breast muscle samples were collected. Tissue homogenates were prepared and HCY content was measured using a commercial kit (Axis-Shield Diagnostics Ltd, Norway).

**1.5.5 Histomorphology** On day 42, six birds from the control group and the 4,000 and 8,000 mg/kg GAA groups (one per replicate) were randomly selected. Heart, liver, kidney, and spleen tissues were fixed in 10% buffered formalin for 48 h, dehydrated overnight in a Sakura automatic dehydrator, embedded, sectioned at 5  m thickness, stained with hematoxylin-eosin, and manually coverslipped. Images were captured using true-color image analysis software to observe histomorphological changes.

### 1.6 Statistical Analysis

Data were analyzed using one-way ANOVA in SAS 8.0 software. Differences among groups were evaluated using Duncan' s multiple comparison test, with $P<0.05$ considered statistically significant.

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## Results

### 2.1 Effects of GAA on Broiler Growth Performance

As shown in Table 2 , no significant differences were observed in ADFI among groups during any phase ($P>0.05$). During the starter phase (days 1-21), ADG in the 800-4,000 mg/kg GAA groups was significantly higher than in the control and 8,000 mg/kg GAA groups ($P<0.05$), and F/G in the 800 mg/kg GAA group was significantly lower than the control ($P<0.05$). During the grower phase (days 22-42) and overall period (days 1-42), ADG was significantly higher and F/G was significantly lower in the 800-4,000 mg/kg GAA groups compared with the control and 8,000 mg/kg GAA groups ($P<0.05$). No significant differences were found between the control and 8,000 mg/kg GAA groups in ADG, ADFI,

or F/G during any phase (P>0.05). Compared with the control group, 21-day body weight increased by 15.25%, 11.86%, and 8.47% (P<0.05), and 42-day body weight increased by 9.50%, 8.00%, and 7.50% (P<0.05) in broilers fed diets supplemented with 800–4,000 mg/kg GAA. These results indicate that dietary GAA supplementation at 800–4,000 mg/kg effectively improved growth rate and feed efficiency, thereby increasing market weight. However, supplementation at 8,000 mg/kg did not improve growth performance but also caused no significant adverse effects.

**Table 2** Effects of dietary GAA supplementation on growth performance of broilers

### 2.2 Effects of GAA on Broiler Hematological Indices

As shown in Table 3 , no significant differences were observed in any hematological parameters (WBC, RBC, HCT, HGB, MCV, PLT) among groups at 21 or 42 days of age (P>0.05). These results indicate that dietary GAA supplementation at 800–8,000 mg/kg had no significant adverse effects on hematological indices in broilers.

**Table 3** Effects of dietary GAA supplementation on blood routine indices of broilers

### 2.3 Effects of GAA on Broiler Serum Biochemical Parameters

As shown in Table 4 , no significant differences were observed in serum ALP, ALT, or AST activities, or in UN, TBILI, GLU, TP, ALB, or CRE concentrations among groups at 21 or 42 days of age (P>0.05). These results indicate that dietary GAA supplementation at 800–8,000 mg/kg had no significant adverse effects on serum biochemical parameters in broilers.

**Table 4** Effects of dietary GAA supplementation on serum biochemical parameters of broilers

### 2.4 Effects of GAA on Broiler Organ Indices

As shown in Table 5 , no significant differences were observed in heart, liver, spleen, or kidney indices among groups on day 42 (P>0.05). These results indicate that dietary GAA supplementation at up to 8,000 mg/kg had no significant effects on organ indices in broilers.

**Table 5** Effects of dietary GAA supplementation on organ indices of broilers

### 2.5 Effects of GAA on Tissue HCY Content in Broilers

As shown in Table 6 , no significant differences were observed in HCY content in breast muscle, kidney, or liver among groups on day 42 (P>0.05). These results indicate that dietary GAA supplementation at 800–8,000 mg/kg had no significant adverse effects on tissue HCY content in broilers.

**Table 6** Effects of dietary GAA supplementation on tissue HCY content of broilers

### 2.6 Effects of GAA on Broiler Histomorphology

Histomorphological observations of liver and kidney tissues are presented in Figures 1 [Figure 1: see original paper] and 2 [Figure 2: see original paper], respectively. Tissue sections from all groups appeared normal with no significant histological changes among groups.

**Figure 1** Kidney morphological structure (200×)
**Figure 2** Liver morphological structure (200×)

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## Discussion

### 3.1 Effects of GAA on Broiler Growth Performance

Previous research has demonstrated that dietary GAA supplementation increases muscle creatine content and the phosphocreatine/ATP ratio in broilers and finishing pigs. The phosphocreatine/ATP ratio reflects cellular energy metabolism status, with higher values indicating improved energy metabolism that provides more immediate ATP for muscle contraction, cell movement, anabolism, and ion balance. Furthermore, 600 mg/kg GAA has been shown to spare 209.3 kJ of metabolizable energy and significantly reduce F/G in broilers. These findings suggest that GAA may improve broiler growth performance by enhancing muscle energy metabolism and promoting muscle fiber development. Animals can synthesize GAA from arginine and glycine, and dietary GAA supplementation can spare these essential amino acids. It has been confirmed that GAA supplementation effectively spares arginine in arginine-deficient broiler diets. Additionally, GAA can promote secretion of insulin-like growth factor-I and insulin, thereby stimulating growth. The present study found that dietary GAA supplementation at 800–4,000 mg/kg improved ADG and reduced F/G in AA broilers, thereby enhancing growth performance. However, the 8,000 mg/kg GAA group showed numerically lower ADFI and ADG compared with the control group, without further improving growth performance. Tossenberger et al. reported that dietary GAA supplementation at 6,000 mg/kg significantly reduced body weight gain due to decreased feed intake, and that true GAA utilization efficiency dropped sharply from 76% to 46% when supplementation increased from 600 to 6,000 mg/kg. In summary, appropriate GAA supplementation improves broiler growth performance likely by enhancing energy utilization efficiency, sparing essential amino acids such as arginine and glycine, and promoting secretion of hormones favorable for protein synthesis. However, excessive GAA supplementation may reduce feed intake and consequently decrease daily gain.

### 3.2 Effects of GAA on Broiler Hematological Indices

Hematological indices are fundamental indicators of blood status closely related to metabolism and health. EFSA previously reported that dietary GAA supplementation at 1,500 mg/kg increased MCV and RBC, possibly due to insufficient methyl donors (vitamin $B_{12}$ 7.5  g/kg, folic acid 0.5 mg/kg, choline chloride 100 mg/kg) in the diet, and also decreased WBC. However, a recent dose-response study with the same GAA level found no significant effects on mean corpuscular volume, hemoglobin, or WBC when adequate methyl donors were provided (vitamin $B_{12}$ 20  g/kg, folic acid 1.0 mg/kg, choline chloride 460 mg/kg). The present study found no significant effects of GAA on hematological indices, likely because the basal diet contained adequate methyl donors (vitamin $B_{12}$ 0.1 mg/kg, folic acid 1.0 mg/kg, choline chloride 500 mg/kg).

### 3.3 Effects of GAA on Broiler Serum Biochemical Parameters

Serum ALT, AST, and ALP activities reflect liver function, with elevated activities indicating liver damage. Serum TP, ALB, and TBILI concentrations can also detect liver metabolic status. Blood UN and CRE concentrations reflect kidney function, with serum UN indicating amino acid utilization. Creatinine, as a metabolite of muscle creatine breakdown, is excreted in urine in proportion to muscle mass. Tossenberger et al. found that dietary GAA supplementation at 600 and 6,000 mg/kg had no significant effects on serum TP, ALB, GLU, UN, or uric acid, but increased serum CRE at 6,000 mg/kg. The present study found no significant effects of GAA on serum biochemical parameters. The lack of increased serum CRE in this study may be because GAA more substantially increased creatine content in muscle tissue and urine. Research has confirmed that high-dose GAA supplementation increases creatine content in muscle tissue and urine to a greater extent than in serum.

### 3.4 Effects of GAA on Broiler Organ Indices

Organ indices are relatively stable in healthy animals, and changes can reflect organ congestion, hyperplasia, atrophy, or degenerative changes, and may corroborate histomorphological alterations. This study found that dietary GAA supplementation at up to 8,000 mg/kg had no significant effects on heart, liver, spleen, or kidney indices in AA broilers, consistent with the lack of significant changes in hematological and serum biochemical parameters.

### 3.5 Effects of GAA on Tissue HCY Content in Broilers

Dietary GAA supplementation increases demand for methyl donors, and insufficient methyl donors can elevate tissue HCY content. Blood HCY is a risk indicator for coronary atherosclerosis and myocardial infarction. HCY content in edible broiler tissues may affect human blood HCY levels and is therefore a concern in tolerance studies. This study found that dietary GAA supplementation at up to 8,000 mg/kg did not alter HCY content in breast muscle, kidney,

or liver, likely due to adequate methyl donors in the basal diet. Tossenberger et al. found that 6,000 mg/kg GAA increased plasma HCY using a basal diet with lower methyl donor levels (vitamin $B_{12}$ 7.5 g/kg, folic acid 0.5 mg/kg, choline chloride 100 mg/kg) than those used in the present study. Current research indicates that methyl donors such as choline, betaine, folic acid, and vitamin $B_{12}$ can prevent hyperhomocysteinemia caused by GAA-induced elevation of blood HCY.

### 3.6 Effects of GAA on Broiler Histomorphology

Kidney and liver tissues are closely involved in GAA metabolism. This study found that high-dose GAA (4,000 and 8,000 mg/kg) caused no obvious lesions in kidney or liver tissues, consistent with the lack of significant changes in hematological indices and organ indices.

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## Conclusions

1. Dietary GAA supplementation at 800–4,000 mg/kg effectively improved growth rate and feed efficiency, thereby increasing market weight in broilers. Supplementation at 8,000 mg/kg did not improve growth performance but caused no significant adverse effects.
2. Dietary GAA supplementation at 800–8,000 mg/kg had no adverse effects on hematological indices, serum biochemical parameters, organ indices, tissue HCY content, or histomorphology in broilers.
3. Broilers can tolerate dietary GAA at levels up to 8,000 mg/kg.

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*Note: Figure translations are in progress. See original paper for figures.*