

Tolerance of Laying Hens to Dietary Zinc Methionine (Postprint)

Authors: Nana Chen, He Junna, Guo Yang, Bing Liu, Xiong Pingwen, Yu Dongyou

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Abstract

This study aimed to investigate the effects of high-dose dietary zinc methionine supplementation on production performance, hematological indices, and histopathology in laying hens, in order to determine the tolerance dose of feed-grade zinc methionine for laying hens. The experiment adopted a single-factor completely randomized design. A total of 540 healthy 20-week-old Hy-Line White laying hens with similar body weight and laying rate were selected and randomly divided into 6 groups, with 6 replicates per group and 15 hens per replicate. Each group was fed experimental diets with zinc methionine supplementation levels of 0 (control), 70 (effective dose), 140 (2×), 350 (5×), 700 (10×), and 1,400 mg/kg (20×). The experiment lasted for 8 weeks. The results showed: 1) Compared with the control group, the 70 mg/kg and 140 mg/kg groups significantly increased daily egg weight and laying rate ($P < 0.05$), and significantly decreased feed-to-egg ratio ($P < 0.05$); the above-mentioned indices in the 350 mg/kg and 700 mg/kg groups showed no significant differences from the control group ($P > 0.05$); compared with the 0-700 mg/kg groups, the 1,400 mg/kg group extremely significantly decreased daily egg weight and laying rate ($P < 0.01$), and extremely significantly increased feed-to-egg ratio ($P < 0.01$). 2) Dietary zinc methionine supplementation had no significant effect on routine blood indices in laying hens ($P > 0.05$). 3) Compared with the control group, dietary supplementation with 70-700 mg/kg zinc methionine had no significant effect on serum biochemical indices in laying hens ($P > 0.05$), but the 1,400 mg/kg group extremely significantly increased serum glucose, uric acid, creatinine, and total bilirubin contents, as well as aspartate aminotransferase and alkaline phosphatase activities ($P < 0.01$). 4) Supplementation with 20× the recommended effective dose (1,400 mg/kg) of zinc methionine caused damage and lesions in major organ tissues. These results suggest that the tolerance dose of zinc methionine in laying hen diets is 700 mg/kg.

Full Text

Tolerance of Laying Hens to Zinc-Methionine in Diet

CHEN Nana, HE Junna, GUO Yang, LIU Bing, XIONG Pingwen, YU Dongyou*

Key Laboratory of Animal Nutrition and Feed in East China of MOA, College of Animal Science, Zhejiang University, Hangzhou 310058, China

Abstract

This experiment was conducted to determine the tolerant dosage of zinc-methionine supplementation in diet through evaluating its effects on performance, hematology indices and histopathological changes of laying hens. Five hundred and forty healthy 20-week-old Hy-Line white laying hens with similar body weight and laying rate were randomly allocated into 6 groups with 6 replicates per group and 15 birds per replicate. Birds were fed basal diets supplemented with 0 (control), 70 (the recommended available dose, RAD), 140 (two folds), 350 (five folds), 700 (ten folds) and 1 400 mg/kg (twenty folds) zinc-methionine, respectively. The trial lasted for 8 weeks. The results showed as follows: 1) compared with the control group, daily egg mass and laying rate were significantly increased and the ratio of feed to egg was remarkably reduced in 70 mg/kg and 140 mg/kg groups ($P < 0.05$); there was no significant difference among the groups of 350 mg/kg, 700 mg/kg or the control in the indices above ($P > 0.05$); compared with the groups of 0 to 700 mg/kg, 1 400 mg/kg group significantly reduced daily egg mass and laying rate ($P < 0.01$), and increased the ratio of feed to egg ($P < 0.01$). 2) Dietary zinc-methionine supplementation had no significant effects on blood routine parameters ($P > 0.05$). 3) Compared with the control group, dietary supplementation with 70 to 700 mg/kg zinc-methionine had no remarkably effects on serum biochemical indices ($P > 0.05$), however, supplementation with 1 400 mg/kg zinc-methionine significantly increased the contents of glucose, uric acid, creatinine and total bilirubin and improved the activities of glutamic-oxalacetic transaminase and alkaline phosphatase ($P < 0.01$). 4) Dietary supplementation with twenty folds of RAD (1 400 mg/kg) zinc-methionine damaged major organ tissues. It is indicated that the tolerant dosage of zinc-methionine in laying hens diet is 700 mg/kg.

Key words: laying hens; zinc-methionine; tolerance

Introduction

Zinc is one of the essential trace elements in animals. As a necessary component of over 300 enzymes and proteins in animal bodies, zinc regulates material metabolism, growth and development, immune function, and reproductive performance through enzymatic catalytic mechanisms, exerting extensive biological functions and earning the designation of a “life element” for the organism [1].

Zinc-methionine is a metal chelate with a unique cyclic structure formed by methionine and inorganic zinc under specific conditions [2]. Studies have shown that appropriate supplementation of zinc-methionine in laying hen diets can not only improve laying rate and egg quality, but also enhance fertility and hatchability in breeding hens, while strengthening antioxidant capacity, immunity, and stress resistance [3-6]. As an efficient novel organic trace mineral additive, zinc-methionine is approved for use as a feed additive in the Ministry of Agriculture's "Announcement No. 1224" [7]; however, its safety limit in laying hen diets has not been established. Therefore, this experiment aimed to determine the tolerance dose of dietary zinc-methionine in laying hens by investigating the effects of high-level supplementation on production performance, hematological indices, and organ histopathology, thereby providing reference and theoretical basis for scientifically guiding the application of zinc-methionine in production practice.

Materials and Methods

1.1 Experimental Materials

Zinc-methionine was provided by Shandong Jining Heshi Biotechnology Co., Ltd. (product batch number: 2014080701), with a zinc content of 17.72% and a methionine content of 79.34%.

1.2 Animal Management

A total of 540 healthy 20-week-old Hy-Line white laying hens with normal feed intake [$(89.17 \pm 1.27) g/d$], similar body weight [$(1.36 \pm 0.03) kg$], and consistent laying rate [$(80.23 \pm 0.98)\%$] were selected and housed in a closed chicken house with three-tier step cages ($45\text{ cm} \times 30\text{ cm} \times 30\text{ cm}$), with 5 cages per tier and 3 hens per cage. The experimental hens in each group were equally distributed across the upper, middle, and lower tiers. Conventional feeding management practices of the farm were followed. Hens had ad libitum access to feed, which was provided at 08:00 and 14:30 daily.

1.3 Experimental Design

A single-factor experimental design was employed, with 540 hens randomly divided into 6 groups, each consisting of 6 replicates of 15 hens. Based on previous studies, supplementation of 40-70 mg/kg zinc-methionine is considered beneficial for improving production performance and egg quality in laying hens [8-11]. According to the relevant provisions in the "Guidelines for Tolerance Evaluation of Target Animals to Diets and Feed Additives (Trial)" issued by the Ministry of Agriculture, the highest effective supplementation level of zinc-methionine (70 mg/kg) was adopted as the recommended available dose (RAD) for tolerance evaluation in laying hen diets. Six dietary treatment groups were established: a control group without zinc-methionine supplementation, and groups supplemented with RAD, $2 \times \text{RAD}$, $5 \times \text{RAD}$, $10 \times \text{RAD}$, and $20 \times \text{RAD}$, corresponding

to dietary zinc-methionine levels of 0, 70, 140, 350, 700, and 1 400 mg/kg (as zinc), respectively. The experimental period lasted for 8 weeks. The basal diet was formulated according to NRC (1994) and the “Feeding Standard of Chickens” (NY/T 33–2004), combined with the Hy-Line laying hen management manual. The composition and nutrient levels of the basal diet are presented in Table 1 .

1.4 Sample Collection and Preparation

At the end of weeks 2, 4, 6, and 8, one hen per replicate was randomly selected and 2 mL and 5 mL of blood were collected from the wing vein using an anti-coagulant tube and a coagulant-promoting tube, respectively. The 2 mL whole blood sample was immediately submitted for blood routine parameter analysis. The 5 mL blood sample was centrifuged at 3 000 r/min for 10 min to prepare serum, and the supernatant was aliquoted into 1.5 mL Eppendorf tubes and stored at -80°C. At the end of week 8 (27 weeks of age), one healthy hen with similar body weight per replicate was slaughtered, and the same portions of heart, liver, spleen, lung, and kidney were collected and fixed in 4% neutral formaldehyde.

1.5 Analytical Methods

1.5.1 Production Performance On a replicate basis, daily egg number (including soft-shelled and broken eggs) and egg weight were recorded. Feed consumption was calculated weekly using the reduction method. Production performance indices including daily egg mass, average egg weight, average daily feed intake, laying rate, and feed-to-egg ratio were then calculated.

1.5.2 Blood Routine Parameters Whole blood white blood cell count, red blood cell count, and platelet count were determined using a hemocytometer. Hematocrit was measured by high-speed centrifugation, and hemoglobin content was determined using a UV spectrophotometer.

1.5.3 Serum Biochemical Indices Alkaline phosphatase, glutamic-oxalacetic transaminase, and glutamic-pyruvic transaminase activities, as well as total protein, albumin, uric acid, urea nitrogen, creatinine, glucose, and total bilirubin contents were measured using assay kits from Nanjing Jiancheng Bioengineering Institute and a SpectraMax M5 multi-functional microplate reader.

1.5.4 Histopathological Examination Fixed specimens were processed through water washing, gradient ethanol dehydration, xylene clearing, paraffin infiltration, and embedding. Six-micrometer sections were prepared using a Leica RM2255 paraffin microtome (Germany). After hematoxylin-eosin staining, histopathological morphological changes were observed under an

Eclipse 80i microscope, and corresponding tissue section images were captured using a DigiLab -C image acquisition system.

1.5.5 Determination of Zinc Content in Basal Diet Approximately 0.5 g of basal diet was placed in a digestion tube, and 6 mL of concentrated nitric acid was added. The sample was digested in a CEM-MARS5 microwave digestion system. After digestion, acid was evaporated in a DV4 000 precision temperature-controlled electric digester. The cooled sample was diluted to 100 mL with deionized water. Zinc content in the sample was determined using an ICE 3 000 atomic absorption spectrometer.

1.6 Statistical Analysis

Data were processed using Excel 2007 and subjected to statistical analysis using one-way ANOVA procedure of SPSS 18.0 software. Bonferroni' s method was used for significance testing. Results were expressed as mean values, with $P < 0.05$ considered statistically significant and $P < 0.01$ considered highly significant.

Results

2.1 Effects of Zinc-Methionine on Production Performance of Laying Hens

As shown in Table 2 , dietary zinc-methionine supplementation had no significant effect on average daily feed intake or average egg weight ($P > 0.05$). Compared with the control group, the 70 mg/kg and 140 mg/kg groups showed significantly increased daily egg mass and laying rate ($P < 0.05$) and significantly reduced feed-to-egg ratio ($P < 0.05$). The 350 mg/kg and 700 mg/kg groups showed no significant differences from the control group ($P > 0.05$). Compared with the 0-700 mg/kg groups, dietary supplementation with 1 400 mg/kg zinc-methionine highly significantly reduced daily egg mass and laying rate ($P < 0.01$) and highly significantly increased feed-to-egg ratio ($P < 0.01$).

2.2 Effects of Zinc-Methionine on Blood Routine Parameters of Laying Hens

As shown in Table 3 , dietary zinc-methionine supplementation had no significant effect on blood routine parameters including white blood cell count, red blood cell count, platelet count, hemoglobin, and hematocrit ($P > 0.05$).

2.3 Effects of Zinc-Methionine on Serum Biochemical Parameters of Laying Hens

As shown in Table 4 , compared with the control group, dietary supplementation with 70-700 mg/kg zinc-methionine had no significant effect on serum

indices of laying hens ($P > 0.05$). However, the 1 400 mg/kg group highly significantly increased serum glucose, uric acid, creatinine, and total bilirubin contents, as well as glutamic-oxalacetic transaminase and alkaline phosphatase activities ($P < 0.01$).

2.4 Effects of Zinc-Methionine on Histopathological Changes of Major Organs

Histopathological observations from Figure 1 [Figure 1: see original paper] revealed the following:

Heart: Groups A, B, C, D, and E exhibited normal cardiac tissue structure with neatly arranged myocardial fibers. Myocardial cells were short cylindrical with centrally located oval or rectangular nuclei. However, in group F, 2 cases showed disorganized and fractured myocardial arrangement, necrotic muscle cells with disappeared nuclei, and local inflammatory cell infiltration.

Liver: Groups A, B, C, D, and E showed normal hepatic architecture with hepatocytes radially arranged around central veins forming hepatic cords, with bile canaliculi in the center and hepatic sinusoids in the interspaces. Hepatocytes were conical with large round nuclei. In contrast, group F exhibited mild hepatocyte swelling in 3 cases, occasional fatty degeneration, massive hepatocyte necrosis near portal areas, and aggregated inflammatory cell infiltration.

Spleen: All groups displayed clear and normal splenic structure, composed of white pulp and red pulp. Small arteries were surrounded by periarterial lymphatic sheaths, with visible splenic nodules, cord-like splenic cords, and interconnected splenic sinusoids.

Lung: Group A showed normal pulmonary structure, with parenchyma consisting of primary, secondary, and tertiary bronchi, pulmonary atria, and pulmonary capillaries. Tertiary bronchi and surrounding atria formed pulmonary lobules. Groups B, C, D, and E were essentially consistent with group A. However, group F showed interstitial hyperplasia in 2 cases, serous exudate in pulmonary atria, and substantial inflammatory cell infiltration.

Kidney: Groups A, B, C, D, and E demonstrated normal renal architecture, with parenchyma composed of cortical and medullary lobules. Renal corpuscles consisted of glomeruli encapsulated by Bowman's capsule, with normal renal tubules and collecting ducts. In group F, 3 cases exhibited glomerular swelling with local inflammatory cell infiltration.

These results indicate that dietary supplementation with 20 times the recommended dose (1 400 mg/kg) of zinc-methionine caused varying degrees of damage and lesions in major organ tissues.

Discussion

3.1 Effects of Zinc-Methionine on Production Performance of Laying Hens

Studies have shown that appropriate zinc supplementation in laying hen diets can prevent zinc deficiency and improve production performance [12], while excessive supplementation can cause toxic effects and negatively impact laying performance [13-15]. The present results demonstrated that dietary supplementation with 70 and 140 mg/kg zinc-methionine significantly increased daily egg mass and laying rate while reducing feed-to-egg ratio. Supplementation with 350 and 700 mg/kg had no significant effect on production performance. However, when dietary zinc-methionine reached 1 400 mg/kg, daily egg mass and laying rate were highly significantly reduced, while feed-to-egg ratio was highly significantly increased. Yang et al. [13] reported that dietary supplementation with 4 000 mg/kg zinc sulfate significantly reduced laying rate and increased feed-to-egg ratio in laying hens. Wang and Shan [14] found that 3 000 mg/kg zinc sulfate significantly decreased laying rate and feed conversion efficiency. The zinc dose causing negative effects in this study was substantially lower than those reported previously, likely because zinc-methionine has higher biological availability than inorganic zinc, thus eliciting toxic effects at lower levels. Furthermore, excessive zinc supplementation not only antagonizes the absorption and utilization of other nutrients in the diet [16-17], but also causes conformational abnormalities in zinc-dependent enzymes involved in nutrient metabolism, leading to reduced or lost enzyme activity and decreased utilization of carbohydrates, proteins, and lipids, ultimately impairing production performance [18-19]. Additionally, Schutte et al. [20] and Jiang [21] reported that excessive dietary methionine reduced feed conversion and egg production in laying hens. In the highest dose group (1 400 mg/kg) of this study, dietary methionine level reached 1.02%, which may have caused amino acid imbalance and antagonism among amino acids [22], thereby reducing nutrient utilization and causing production performance decline. Therefore, the reduction in production performance may have resulted from the combined effects of high-dose zinc and high-dose methionine.

3.2 Effects of Zinc-Methionine on Hematological Indices of Laying Hens

Studies have demonstrated that zinc plays an important role in animal blood circulation and material metabolism. Although animals have high tolerance to zinc, exceeding certain limits can cause zinc toxicity, disrupt homeostasis, and negatively affect blood circulation and metabolism [23-24]. This experiment showed that dietary zinc-methionine supplementation had no significant effect on blood routine parameters of laying hens, indicating that under the experimental conditions, supplementation up to 1 400 mg/kg did not adversely affect these parameters. Serum biochemical indices are important indicators for evaluating metabolic status and health condition. Cui et al. [25] reported that 1 400 mg/kg zinc sulfate significantly increased serum glutamic-pyruvic transaminase

activity in broilers. Wang et al. [26] found that 5 000 mg/kg nano-zinc significantly elevated serum alkaline phosphatase and glutamic-pyruvic transaminase activities in mice. The present results showed that dietary supplementation with 70-700 mg/kg zinc-methionine had no significant effect on serum biochemical indices of laying hens, while 1 400 mg/kg zinc-methionine highly significantly increased serum glucose, uric acid, creatinine, and total bilirubin contents, as well as alkaline phosphatase and glutamic-pyruvic transaminase activities. These results indicate that dietary supplementation with 1 400 mg/kg zinc-methionine negatively impacted liver and kidney function and glucose metabolism in laying hens. The underlying mechanisms may include: 1) excessive zinc deposition in the liver causing hepatocyte necrosis, leading to massive release of transaminases and alkaline phosphatase (normally present in hepatocyte cytoplasm) into serum, while the damaged liver could not timely process bilirubin, resulting in elevated serum bilirubin [27]; 2) excessive zinc deposition in the kidneys causing renal lesions, and the impaired kidneys could not efficiently filter and clear uric acid and creatinine from serum, leading to increased serum levels [28]; and 3) excessive zinc may cause conformational changes and abnormal molecular folding of insulin, preventing it from performing its glucose-lowering function and thus causing hyperglycemia in laying hens [29-30].

3.3 Effects of Zinc-Methionine on Histopathological Changes of Major Organs

Histopathological examination is an auxiliary tool for evaluating animal health status and diagnosing diseases, and is currently used as an important indicator for safety evaluation of feed additives. Excessive dietary zinc can cause poisoning in poultry, resulting in varying degrees of organ damage and lesions [16-17]. Gasaway et al. [31] found that 3 000 mg/kg zinc carbonate caused focal necrotic lesions and sandy-yellow discoloration in mallard duck livers. Yang et al. [13] reported that 4 000 mg/kg zinc sulfate caused hepatic congestion and renal necrosis in laying hens. Zhao et al. [32] observed that 2 400 mg/kg zinc sulfate induced cardiac and hepatic damage in Avian broilers. This experiment demonstrated that dietary supplementation with 70-700 mg/kg zinc-methionine had no significant effect on major organ tissues of laying hens, while 1 400 mg/kg zinc-methionine caused varying degrees of lesions and damage to heart, liver, lung, and kidney tissues, consistent with the results of production performance and serum biochemical indices. The variation in zinc toxicity levels is mainly related to differences in zinc preparation type, formulation, animal species, and physiological stage. The mechanism of high-dose zinc toxicity remains unclear. Some scholars propose that direct toxic effects of zinc alter the stability of lipid bilayers and glycoprotein content in membrane structures, change the conformation of zinc-containing enzymes, and destroy mitochondrial cristae, thereby causing cell damage or necrosis [16-17]. Others suggest that high zinc levels antagonize the absorption and utilization of other mineral elements, leading to indirect toxic effects [33-34].

Conclusion

Dietary supplementation with 70-700 mg/kg zinc-methionine had no negative effects on production performance, hematological indices, or major organ tissues of laying hens. However, when the supplementation level reached 20 times the recommended available dose (1 400 mg/kg), it affected certain indices such as production performance and serum biochemistry, and caused damage and lesions in major visceral organs. In conclusion, under the conditions of this experiment, the tolerance dose of zinc-methionine in corn-soybean meal diets for laying hens is 700 mg/kg.

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