Effects of Zinc Hydroxy Methionine on Production Performance, Egg Quality, and Immune-Related Gene Expression in Late-Phase Laying Hens: Postprint

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Abstract

This study aimed to investigate the effects of zinc methionine hydroxy analogue (MHA-Zn) on production performance, egg quality, and immune-related gene expression in laying hens during the late laying period. A total of 960 57-week-old Hy-Line Gray laying hens with similar body weight and laying rate were randomly allocated to 4 groups, each consisting of 8 replicates of 30 hens. A corn-soybean meal basal diet (containing 35.08 mg/kg zinc) was formulated according to the NRC (1994) standards for laying hens and the nutrient recommendations of Hy-Line Company. The control group received the basal diet supplemented with 80 mg/kg zinc sulfate (as zinc), while the experimental groups received the basal diet supplemented with 20, 40, or 80 mg/kg MHA-Zn (as zinc), respectively. The experiment included a 4-week pre-trial period followed by a 12-week formal trial period. The results showed: 1) No significant differences were observed among groups in laying rate, average egg weight, average daily feed intake, feed-to-egg ratio, or daily egg mass ($P > 0.05$). 2) No significant differences were found among groups in albumen height, Haugh unit, or yolk color ($P > 0.05$). However, eggshell thickness and eggshell strength in the 40 and 80 mg/kg MHA-Zn groups were significantly higher than those in the control group and the 20 mg/kg MHA-Zn group ($P < 0.05$), while the broken egg rate was significantly lower ($P < 0.05$). 3) No significant differences were detected among groups in the relative mRNA expression levels of the spleen immune-related genes nuclear factor- B1 (NF- B1), tumor necrosis factor- (TNF-), and interleukin-10 (IL-10) ($P > 0.05$). The relative mRNA expression level of spleen interleukin-8 (IL-8) in the 80 mg/kg MHA-Zn group was significantly lower than that in the 40 mg/kg MHA-Zn group ($P < 0.05$), which in turn was significantly lower than that in the 20 mg/kg MHA-Zn group ($P < 0.05$). In conclusion, dietary supplementation with 40 or 80 mg/kg MHA-Zn significantly improved eggshell thickness and strength, reduced the broken egg rate, and decreased the relative mRNA expression level of the pro-inflammatory factor IL-8. The recommended optimal supplementation level of MHA-Zn in diets for laying hens during the late laying period is 40 mg/kg.

Full Text

Effects of Methionine Hydroxy Analog Chelate Zinc on Performance, Eggshell Quality and Immune-Related Gene Expression of Laying Hens during Later Period of Laying

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Abstract

This experiment was conducted to investigate the effects of methionine hydroxy analog chelate zinc (MHA-Zn) on performance, eggshell quality, and immune-related gene expression in laying hens during the later period of production. A total of 960 Hy-Line Grey laying hens aged 57 weeks, with similar body weight and laying rate, were randomly allocated into four groups, each consisting of eight replicates of 30 hens. A corn-soybean meal basal diet containing 35.08 mg/kg zinc was formulated according to the NRC (1994) standards for laying hens and Hy-Line Company' s recommended nutrient levels. The control group received the basal diet supplemented with 80 mg/kg zinc sulfate (as zinc), while the experimental groups received the basal diet supplemented with 20, 40, or 80 mg/kg MHA-Zn (as zinc), respectively. The pre-experimental period lasted 4 weeks, followed by a 12-week experimental period.

The results showed: (1) No significant differences were observed among all groups in laying rate, average egg weight, average daily feed intake, feed-to-egg ratio, or daily egg mass ($P > 0.05$). (2) No significant differences were found in albumen height, Haugh units, or yolk color among groups ($P > 0.05$). However, eggshell thickness and eggshell strength in the 40 and 80 mg/kg MHA-Zn groups were significantly higher than those in the control and 20 mg/kg MHA-Zn groups ($P < 0.05$), while broken egg rate was significantly lower ($P < 0.05$). (3) No significant differences were detected among groups in the mRNA relative expression levels of splenic immune-related genes nuclear factor- B1 (NF- B1), tumor necrosis factor- (TNF-), and interleukin-10 (IL-10) ($P > 0.05$). The splenic IL-8 mRNA relative expression level in the 80 mg/kg MHA-Zn group was significantly lower than that in the 40 mg/kg MHA-Zn group ($P < 0.05$), which in turn was significantly lower than that in the 20 mg/kg MHA-Zn group ($P < 0.05$).

In conclusion, dietary supplementation with 40 or 80 mg/kg MHA-Zn significantly improved eggshell thickness and strength, reduced broken egg rate, and decreased the mRNA relative expression level of the pro-inflammatory factor IL-8. The recommended optimal dietary MHA-Zn supplementation level for laying hens during the later production period is 40 mg/kg.

Keywords: laying hens; MHA-Zn; performance; eggshell quality; immune-related gene

Zinc is present in all living organisms and regulates various metabolic pathways, participating in growth, development, reproduction, and immune responses [1]. It plays a crucial role in protein synthesis [2], protein deposition, and deposition in the isthmus of the eggshell membrane [3]. Zinc also modulates innate and adaptive immune responses, with zinc deficiency causing cell-mediated immune dysfunction [4] and affecting epithelial cell quality. In rats fed a zinc-deficient diet for 30 days, plasma leptin content, metabolic rate, and enzyme activity were significantly reduced [5].

In commercial production, inorganic zinc is typically added in its free divalent metal state, which readily chelates with phytates or lignin, reducing zinc utilization efficiency. Methionine hydroxy analog chelate zinc (MHA-Zn) is an organic zinc chelated with methionine hydroxy analog, offering advantages in animal production including low toxicity, stable chemical properties, good palatability, and non-irritating effects [6]. Research indicates that organic zinc (such as zinc amino acid complexes) is absorbed via amino acids or small peptides, resulting in higher bioavailability than inorganic zinc [7], with lower excretion and reduced environmental pollution. Therefore, replacing inorganic zinc with MHA-Zn can reduce the required zinc supplementation level.

Zinc affects egg and eggshell quality. Dietary supplementation with 80 mg/kg zinc can increase eggshell strength [8], while excessive supplementation reduces average egg weight [9]. Zinc regulates eggshell structure, influencing deposition and ultrastructural crystal arrangement [10]. Low doses of organic zinc can meet production requirements [11], and inorganic zinc at 70 mg/kg shows better production effects [12]. However, few studies have investigated MHA-Zn as a replacement for inorganic zinc in laying hen diets, and the molecular mechanisms of immune regulation by different zinc sources remain unexplored. Partial or complete replacement of conventional inorganic zinc with MHA-Zn may achieve comparable effects on production performance, egg quality, and immune function. Based on this, the present study further explored the optimal dietary MHA-Zn supplementation level and its effects on immune-related gene expression to elucidate the molecular mechanisms of immune regulation in laying hens and provide a theoretical basis for improving performance and egg quality during the later laying period.

1.1 Experimental Materials

The zinc sources used were zinc sulfate (zinc content 34.5%) and MHA-Zn (methionine hydroxy analog mass fraction 80%, zinc content 12.1%, chelation rate 90%), provided by Novus International Shanghai.

1.2 Experimental Design

A total of 960 healthy Hy-Line Grey laying hens aged 57 weeks with consistent laying performance were randomly assigned using a single-factor completely randomized design into four groups, each comprising eight replicates of 30 hens. Based on NRC (1994) standards and Hy-Line Company's recommended nutrient levels, a corn-soybean meal basal diet containing 35.08 mg/kg zinc was formulated. The composition and nutrient levels of the basal diet are presented in Table 1 . During the pre-experimental period, hens were fed the basal diet without zinc supplementation. During the experimental period, the control group received the basal diet supplemented with 80 mg/kg zinc sulfate (as zinc), while the experimental groups received the basal diet supplemented with 20, 40, or 80 mg/kg MHA-Zn (as zinc). The total trial lasted 16 weeks, including a 4-week pre-experimental period (57–60 weeks of age) and a 12-week experimental period (61–72 weeks of age).

Table 1 Composition and nutrient levels of the basal diet (air-dry basis)

Note: The premix provided the following per kg of diet: VA 10,000 IU, VD3 1,800 IU, VE 10 IU, VK 10 mg, VB12 5 g, thiamine 1 mg, riboflavin 4.5 mg, calcium pantothenate 50 mg, niacin 24.5 mg, pyridoxine 5 mg, biotin 1 mg, folic acid 1 mg, choline 500 mg, Mn 60 mg, I 0.4 mg, Fe 80 mg, Cu 8 mg, Se 0.3 mg. CP, Ca, TP and Zn were measured values, while the others were calculated values.

1.3 Management

The feeding trial was conducted at the experimental poultry farm of Northwest A&F University in Yangling, Shaanxi. Hens were housed in three-tier step cages with two hens per cage in a windowed poultry house. Hens from the same group were evenly distributed throughout different spaces in the house. They had free access to feed and water, with mechanical manure removal and 16 hours of daily lighting. House temperature ranged from 18 to 29 °C, with relative humidity of 50–70%. Feed was provided three times daily (08:00, 11:00, 16:00), and eggs were collected at 17:00. Daily health observations and mortality records were maintained, with routine immunization, disinfection, and management practices.

1.4.1 Performance Metrics

During the experimental period, daily egg number, egg weight, feed amount, hen inventory, and broken eggs were recorded per replicate. Remaining feed was weighed weekly. Performance metrics including laying rate, daily egg mass, average egg weight, average daily feed intake, and feed-to-egg ratio were calculated for the early (61–64 weeks), middle (65–68 weeks), and late (69–72 weeks) experimental phases.

1.4.2 Egg Quality

At the ends of weeks 4, 8, and 12 of the experimental period, three normal eggs were randomly selected from each replicate for two consecutive days to determine egg quality parameters, including egg weight, albumen height, eggshell strength, eggshell thickness, egg shape index, yolk color, Haugh unit, and broken egg rate. Eggshell thickness was measured at three points (top, middle, bottom) using an eggshell thickness gauge (ETG-1601A, Robotmation, Japan) and averaged. Eggshell strength was measured using an eggshell force gauge (EFG-0503, Robotmation, Japan). Albumen height, yolk color, and Haugh unit were determined using an automatic egg quality analyzer (EMT-5200, Robotmation, Japan).

1.4.3 Expression of Inflammatory and Immune Cytokine-Related Genes in Spleen

At the end of the trial (72 weeks of age), one healthy hen with body weight close to the replicate average was randomly selected from each replicate, slaughtered after blood collection, and approximately 2 g of spleen tissue was excised, quickly cut into 30–50 mg pieces, placed in cryovials, snap-frozen in liquid nitrogen, and stored at -80 °C for analysis.

Total RNA was extracted from samples using the Trizol reagent kit (TaKaRa). Nucleic acid concentration analyzer confirmed absorbance ratios at 260 and 280 nm of 1.80–2.10, with concentrations automatically recorded. RNA quality was assessed by agarose gel electrophoresis. One microgram of RNA sample was reverse-transcribed to cDNA using a TaKaRa reverse transcription kit according to the manufacturer' s instructions. Primers were designed using Primer 6.0 software, with PCR primer sequences shown in Table 2 . Using cDNA as template, real-time fluorescent quantitative PCR was employed to detect mRNA relative expression levels of inflammatory and immune-related genes in spleen [nuclear factor- B1 (NF- B1), interleukin-10 (IL-10), interleukin-8 (IL-8), tumor necrosis factor- (TNF-)] on an Applied Biosystems 7300 Real Time PCR System. The 20 L reaction mixture contained: 10 L 2×SYBR Green PCR Master Mix, 2 L cDNA, 0.8 L forward primer, 0.8 L reverse primer, 0.4 L ROX Reference Dye, and 6 L ddH2O. Cycling conditions were: 95 °C pre-denaturation for 30 s; 40 cycles of 95 °C denaturation for 0.05 s, 60 °C annealing for 34 s. mRNA relative expression levels were calculated using the 2^(-ΔΔCT) method based on threshold cycle (CT) values of target and reference genes.

Table 2 Primer sequences for PCR

1.5 Data Processing

Experimental data were initially processed using Excel 2013. One-way ANOVA was performed using SPSS 21.0, with inter-group means compared using Duncan' s multiple range test. Data are expressed as "mean ± standard deviation," with $P < 0.05$ considered statistically significant.

2.1 Effects of MHA-Zn on Performance of Laying Hens during Later Period of Laying

As shown in Table 3 , no significant differences were observed among groups in average daily feed intake, laying rate, feed-to-egg ratio, daily egg mass, or average egg weight during the early, middle, or late experimental phases ($P > 0.05$).

Table 3 Effects of MHA-Zn on performance of laying hens during later period of laying

Note: In the same row, values with different small letter superscripts indicate significant difference (P < 0.05), while values with the same or no letter superscripts indicate no significant difference (P > 0.05). The same applies below.

2.2 Effects of MHA-Zn on Egg Quality of Laying Hens during Later Period of Laying

As shown in Table 4 , no significant differences were found among groups in yolk color, Haugh units, or albumen height during any phase ($P > 0.05$). However, eggshell thickness and eggshell strength in the 40 and 80 mg/kg MHA-Zn groups were significantly higher than those in the control and 20 mg/kg MHA-Zn groups during the early, middle, and late phases ($P < 0.05$). Broken egg rate in the 40 and 80 mg/kg MHA-Zn groups was significantly lower than that in the control and 20 mg/kg MHA-Zn groups during all phases ($P < 0.05$).

Table 4 Effects of MHA-Zn on egg quality of laying hens during later period of laying

2.3 Effects of MHA-Zn on mRNA Relative Expression Level of Immune-Related Genes in Spleen of Laying Hens during Later Period of Laying

As shown in Table 5 , the mRNA relative expression level of splenic IL-8 in the 80 mg/kg MHA-Zn group was significantly lower than that in the 40 mg/kg MHA-Zn group ($P < 0.05$), which in turn was significantly lower than that in the 20 mg/kg MHA-Zn group ($P < 0.05$). No significant differences were observed among groups in the mRNA relative expression levels of TNF- , IL-10, or NF- B1 ($P > 0.05$).

Table 5 Effects of MHA-Zn on mRNA relative expression level of immune-related genes in spleen of laying hens during later period of laying

3.1 Effects of MHA-Zn on Performance of Laying Hens during Later Period of Laying

Zinc is an essential trace element involved in the synthesis of numerous enzymes. Studies have shown that dietary zinc supplementation at various levels can improve broiler performance and meat quality [13], likely because zinc is crucial for maintaining metalloprotein structure, and deficiency affects protein metabolism [14]. Organic zinc demonstrates higher bioavailability than inorganic zinc, and dietary zinc methionine supplementation can significantly improve laying hen performance, egg quality, and immune function [15]. Xue et al. [16] reported that supplementing trace elements in organic form significantly increased laying rate and reduced feed-to-egg ratio. Supplementing basal diets with 70 or 140 mg/kg zinc methionine significantly increased daily egg mass and laying rate while decreasing feed-to-egg ratio [17].

In contrast, the present study found that dietary MHA-Zn supplementation at different levels had no significant effects on average daily feed intake, feed-to-egg ratio, average egg weight, laying rate, or daily egg mass in late-phase laying hens. This discrepancy with previous research may be attributed to the basal diet zinc content of 35.08 mg/kg in our study, which already met the requirements for growth and production in laying hens. Additional zinc supplementation beyond this level may not further improve certain performance metrics. Furthermore, fixed levels of copper, manganese, and iron in the diet may have created antagonistic interactions with high zinc levels, affecting absorption and utilization.

3.2 Effects of MHA-Zn on Egg Quality of Laying Hens during Later Period of Laying

Eggshell breakage is a primary cause of egg rejection in production. Trace elements participate in eggshell membrane and shell formation as critical enzyme cofactors. Zinc is an essential component of carbonic anhydrase, which provides crucial carbonate ions (HCO3-) during eggshell formation, and carbonate deposition increases eggshell weight. Zinc deficiency reduces HCO3- availability, substantially decreasing eggshell thickness and affecting eggshell weight [10]. Some studies have reported significant differences in eggshell strength without corresponding differences in eggshell thickness at various dietary zinc levels [18], suggesting that eggshell strength may be more closely related to shell structure. Zinc influences protein synthesis and may affect eggshell membrane structure during shell formation [3]. Trace elements also significantly impact ultrastructural crystal arrangement during shell deposition, with high dietary zinc levels increasing eggshell strength and thickness while reducing broken egg rate [19]. Other research indicates that organic trace elements have higher absorption rates than inorganic forms, with organic zinc and manganese supplementation significantly increasing eggshell thickness and strength while decreasing broken egg rate compared to inorganic controls [18].

Our results align with these findings, demonstrating that dietary MHA-Zn supplementation at 40 or 80 mg/kg significantly increased eggshell weight and strength and reduced broken egg rate compared to 80 mg/kg zinc sulfate. These metrics did not change significantly with increasing organic zinc levels, and no significant differences existed between the 40 and 80 mg/kg MHA-Zn groups, consistent with previous research showing that beyond a certain zinc level, continued supplementation does not progressively improve eggshell parameters. Dietary supplementation with 40 mg/kg MHA-Zn achieved effects comparable to 80 mg/kg zinc sulfate in commercial production.

3.3 Effects of MHA-Zn on Expression of Immune-Related Genes in Spleen of Laying Hens during Later Period of Laying

Zinc deficiency impairs immune function [5]. Accumulating evidence indicates that zinc acts as a negative regulator of the nuclear factor- B (NF- B) inflammatory signaling pathway, influencing inflammatory cytokine expression [20]. Zinc deficiency upregulates pro-inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), and TNF- [21]. NF- B1 is a highly conserved nuclear transcription factor mediating pro-inflammatory responses, closely associated with apoptosis and involved in transcriptional regulation of multiple apoptosis-related genes, exhibiting dual roles in inhibiting and promoting cell death. IL-8 is an immune chemokine that regulates proliferation and differentiation of various cells including hematopoietic cells, B lymphocytes, T lymphocytes, endothelial cells, and nerve cells, and can induce local inflammatory responses [22]. IL-10 acts on multiple immune cell types to inhibit pro-inflammatory cytokines including IL-1, IL-6, IL-12, and TNF- [23].

In broiler studies, providing breeder hens and their offspring with high-dose organic zinc significantly reduced mRNA expression of pro-inflammatory factors IL-1 , TNF- , and IL-8, whereas low-dose organic zinc produced opposite results. Additionally, offspring from breeder hens fed high-dose inorganic zinc showed significantly lower jejunal mRNA expression of IL-6, IL-1 , and IL-8 compared to those from hens fed normal-dose organic zinc [24]. In humans, zinc supplementation reduces IL-1 and TNF- mRNA expression by binding to zinc finger protein A20 and inhibiting NF- B activation, thereby downregulating inflammatory cytokines [16]. Dietary organic zinc supplementation enhances animal antioxidant capacity, and previous research has shown that MHA-Zn significantly increases total antioxidant capacity in serum and liver of laying hens [11].

In the present study, the 80 mg/kg MHA-Zn group showed significantly lower mRNA expression of the pro-inflammatory cytokine IL-8 compared to the 20 mg/kg MHA-Zn group, consistent with Li [23] who reported that low dietary zinc levels significantly upregulated pro-inflammatory factors IL-1 , TNF- , and IL-8. However, dietary MHA-Zn supplementation at different levels did not significantly affect mRNA expression of splenic NF- B1, TNF- , or IL-10. This may be due to differences in animal age and physiological status, as late-phase laying hens have diminished physiological functions. Additionally, interactions among trace elements may have influenced absorption, as short-term changes in zinc nutrition can affect other trace element uptake.

4 Conclusion

Dietary supplementation with 40 or 80 mg/kg MHA-Zn significantly improved eggshell thickness and strength, reduced broken egg rate, and decreased the mRNA relative expression level of the pro-inflammatory factor IL-8 in spleen. The recommended optimal dietary MHA-Zn supplementation level for laying hens during the later production period is 40 mg/kg.

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