Effects of organic zinc on tibia quality, mineral deposit, and metallothionein expression level of aged hens

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ABSTRACT The study aimed to determine the effects of methionine hydroxy analog chelate zinc on the tibia quality, mineral deposit, apparent retention of nutrients, and liver metallothionein (MT) expression level of aged laying hens. A total of 960 layers (Hy-Line Grey, 57 wk old) were randomly assigned into 4 groups, and each group had 8 replicates of 30 hens. During the first 2 wk, groups were fed a basal diet without extra zinc (Zn: 35.08 mg/kg). During the ensuing 14 wk, 4 levels of Zn (inorganic Zn: 80 mg/kg; organic Zn: 20, 40, 80 mg/kg) were added to the diet. The results indicated that both the Zn source and level did influence tibia strength and calcium (Ca) and Zn concentrations of tibia ($P < 0.05$), whereas there were no differences in the copper (Cu) and phosphorus (P) concentrations of the tibia and the tibia length ($P > 0.05$). Moreover, dietary supplementation with 40 or 80 mg/kg of organic Zn showed higher Zn and Ca concentrations in the tibia and higher tibia strength. The Cu concentration in the liver showed no difference among the 4 treatments, whereas the Zn concentration in the liver increased with the increasing Zn level. The apparent retention of P, iron (Fe), and manganese (Mn) was not affected by the Zn level or source ($P > 0.05$). However, the organic Zn group increased the apparent retention of Cu, Zn, Ca, crude protein (CP), and energy, and the group supplemented with 40 or 80 mg/kg of organic Zn obtained significant effects ($P < 0.05$). Moreover, dietary supplementation with 40 or 80 mg/kg organic Zn increased the MT mRNA expression of the liver at week 72, whereas 20 mg/kg of organic Zn decreased it ($P < 0.05$). In conclusion, this study suggested that an optimum dietary (40 mg/kg) organic Zn level plays a key role in promoting the apparent retention of minerals and nutrients, trace element deposit, and MT mRNA expression.

Key words: organic Zn, tibia quality, mineral deposit, metallothionein, aged laying hen

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INTRODUCTION

Zinc **(Zn)**, an essential trace element for laying hens, plays a significant role in many biological processes, such as growth, tissue growth and repair, skeletal development, and immune competence (Salim et al., 2008; Abdallah et al., 2009; Richards et al., 2010; Gheisari et al., 2011). Generally, inorganic Zn (Zn sulfate, Zn oxides, etc.) is used in poultry production. However, various disadvantages of the inorganic form, such as high excretion, low bioavailability, high hydrophobicity, and oxidation and destruction of nutrients in the diet, were found in previous studies (Wedekind et al., 1992; Cao et al., 2000). Currently, organic Zn products are used as an alternative source.

A series of experiments have been conducted to estimate the effect of organic Zn sources relative to inorganic Zn based on performance, tissue Zn concentration, and relative metallothionein **(MT)** expressions in chicken (Wedekind and Baker, 1990; Mohanna and Nys, 1999; Jahanian and Rasouli, 2015). Some studies demonstrated that organic Zn sources were more available than inorganic Zn sources (Wedekind et al., 1992; Cao et al., 2000, 2002), whereas others showed no differences in bioavailability between the 2 types of Zn sources (Mohanna and Nys, 1999; Huang et al., 2009, 2013; Liu et al., 2013). Such discrepancies might be explained by the types and quality characteristics of tested organic Zn sources or the criteria used in bioavailability assays. Numerous studies on the relative bioavailability of Zn-methionine complex for chicks have been reported (Mohanna and Nys, 1999; Cao et al., 2002; Jahanian and Rasouli, 2015). However, studies have rarely been conducted to estimate the effects of methionine hydroxy analog chelate zinc **(MHA-Zn)** on aged laying hens. Chemically, MHA-Zn is more stable than other organic Zn forms and has

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higher bioavailability in broilers (Yan and Waldroup, 2006; Yi et al., 2007) and laying hens from 39 to 52 wk of age (Sun et al., 2012).

Laying performance and egg quality are often used as parameters to estimate dietary Zn requirements for laying hens (Batal et al., 2001; Berg et al., 2004). However, laying performance and egg quality are usually not sensitive when hens are fed with a conventional corn-soybean meal diet containing higher Zn levels (Tabatabaie et al., 2007). More recently, tissue Zn concentrations have been considered to be sensitive criteria for estimating the Zn requirements of chicks (Huang et al., 2007; Sunder et al., 2008; Liao et al., 2013). Previous studies suggested that tibia Zn concentration might be a more suitable and reliable parameter for the Zn requirement estimation of laying hens (Qin et al., 2017). In addition, the Zn concentrations of liver tissue and liver MT mRNA level were also regarded as potential indices to estimate the Zn requirement of birds (Dewar et al., 1983; Bao et al., 2007).

Therefore, the objective of this study was to investigate the effects of MHA-Zn compared with inorganic Zn on Zn concentrations in the tibia and liver tissue and liver MT mRNA level for aged laying hens (from 57 to 72 wk) to estimate the optimal dietary Zn level of aged laying hens fed a corn-soybean meal diet from 57 to 72 wk of age.

MATERIALS AND METHODS

Experimental Design and Diets

The experiment was performed in a layer unit. Housing, management and care of birds were conformed to standard feeding guidelines of Animal Care and Use Committee of the Northwest A&F University, Yangling, China. A total of 960 layers (Hy-line Grey) aged 59 wk were fed a basal diet without extral zinc (Zn = 35.08 mg/kg) for 2 wk to exhaust Zn accumulated in the early phase. The basal diet was composed mainly of corn and soybean meal and was formulated to supply adequate levels of all nutrients (except for Zn) in accordance with nutrient recommendations from the layer nutrition demand (China layer feeding standard: NY/T33—2004), combined with the recommendation of Hy-Line International. Both feed and water were provided ad libitum throughout the study. After 2 wk, the birds were randomly divided into 4 groups according to their body weight and laying rate. Each group had 8 replicates of 30 layers, and 2 hens in a cage (45 cm × 45 cm × 45 cm). During the ensuing 14 wk, 4 levels of Zn ($ZnSO_4$: 80 mg/kg; MHA-Zn: 20, 40, 80 mg/kg) were supplemented to the basal diet. Research on living animals met the guidelines approved by the Institutional Animal Care and Use Committee.

The basal diet was formulated to provide all the other nutrients, except Zn, The dietary composition and nutrient levels are listed in Table 1. The actual concentrations of the total Zn analyzed in the 4 treatment diets are shown in Table 2. The inorganic trace elements in the diet were $ZnSO_4$ (Zn ≥ 34.5%), $CuSO_4$ (Cu ≥ 25.0%), $FeSO_4$ (Fe ≥ 30.0%), $MnSO_4$ (Mn ≥ 32.0%), Na_2SeO_3 (S ≥ 1.0%), and Ca $(IO_3)_2$ (I ≥ 1.0%). The organic Zn was MHA-Zn (MINTREX®), purchased from Novus International Trading (Shanghai) Company.

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

Ingredients	%	Nutrients	%
Corn	58.50	Metabolic energy (MJ/kg)	12.29
Soybean meal	23.00	Crude protein[2]	15.47
Ground limestone	8.74	Calcium[2]	3.60
$CaHPO_4$	1.10	Total phosphorus	0.62
Wheat bran	7.50	Available phosphorus	0.32
Soybean oil	0.50	Lysine	0.94
NaCl	0.24	Methionine	0.44
Methionine	0.16	Threonine	0.70
Threonine	0.03	Zinc[2] (mg/kg)	35.08
Lysine	0.05		
Premix[1]	0.18		
Total	100.00		

[1]Supplied the following per kilogram of diet: vitamin A 10,000 IU, VD3 31,800 IU, VE 10 IU, VK 10 mg, VB 125 μg, thiamine l 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 (as manganese sulfate) 60 mg, I (as calcium iodate) 0.4 mg, Fe (as ferrous sulfate) 60 mg, Cu (as copper sulfate) 8 mg, Se (sodium selenite) 0.30 mg.
[2]Analyzed values, each value based on triplicate determinations.

Table 2. Concentrations of zinc in experimental treatment diets (air-dry basis).[1]

Dietary zinc supplementation	Calculated (mg/kg)	Analyzed[1] (mg/kg)
80 mg/kg $ZnSO_4$[2]	115.08	115.53
20 mg/kg MHA-Zn[3]	55.08	55.38
40 mg/kg MHA-Zn	75.08	75.97
80 mg/kg MHA-Zn	115.08	114.18

[1]Value based on triplicate determinations.
[2]$ZnSO_4$: Zinc Sulphate.
[3]MHA-Zn: methionine hydroxy analog chelate zinc, methionine yielded 80%.

Sample Collection and Preparations

Feed samples from 4 treatments (8 replicates/treatment) were taken for dry matter **(DM)**, crude protein **(CP)**, calcium **(Ca)**, phosphorus **(P),** and Zn analyses. At the end of 62, 66, 70, and 72 wk of age, after fasting for 12 h, 1 bird from each replicate was chosen according to body weight. Those birds were slaughtered. The liver was taken and frozen in liquid nitrogen for the Zn and copper **(Cu)** analysis. The left tibia was taken and stored at 4°C for tibia length and strength analysis. The right tibia was also taken and frozen at –20°C for the analysis of Ca, P, Zn, and Cu concentrations. Right tibia bones were boiled in deionized water for 5 min, and all soft tissues were removed. Then, bones were dried for 12 h at 105°C and incinerated in a muffle furnace at 550°C for 16 h. At the end of 72 wk, another liver sample was frozen

Table 3. Nucleotide primer sequences of PCR primers of metallothionein.

Gene	Sequence no.	Product size/bp	Primer sequence (5′→3′)
β-actin	NM_205518	113	F: ATTGTCCACCGCAAATGCTTC R: AAATAAAGCCATGCCAATCTCGTC
Metallothionein	NM_205275	103	F: TCTTGGGTATGGAGTCCTG R: TAGAAGCATTTGCGGTGG

in liquid nitrogen and stored at −80°C for the MT mRNA level assay. At the end of the trial (72 wk), 3 layers with similar body weights, within 8 replications of each treatment, were placed into a single cage. After 3 d of adaptation, all layers were followed for 3 d to collect feces. All feces were mixed together, dried at 65°C, and preserved for the analysis of minerals and nutrients.

Sample Analysis

The Zn and Cu concentrations in the tibia and Cu, iron **(Fe)**, manganese **(Mn),** and Zn concentrations in the feed and feces were measured by flame atomic absorption spectrophotometry (AOAC, 2005 Proc.No. 968.08) after dry digestions with HNO_3 and HCl. The Zn concentration in the liver was measured by flame atomic absorption spectrophotometry (AOAC, 2005 Proc. No. 968.08) after wet digestions with HNO_3 and $HClO_4$. The nutrient components in the feed and feces were determined using standard procedures from AOAC procedures (AOAC, 2005) for DM (No.934.01), CP (No.976.05), Ca (927.02), and P (No.965.17). All feed and feces samples were analyzed for nutrients and minerals, which were used to calculate the apparent retention: apparent retention of mineral (%) = (mineral intake- mineral output in feces)/mineral intake × 100%; apparent retention of nutrient (%) = (nutrient intake-nutrient output in feces)/nutrient intake × 100%.

The tibia length was measured by Vernier calipers, and the tibia strength was measured by a microcomputer controlled electronic test machine (Shenzhen Xinsansi Material Detection Co Ltd, China). A 3-point bending test of the tibia was carried out. The tibia surface was placed horizontally on the scaffold, and the distance of the 2 fulcrum was adjusted to 20 mm, so that the center of the fulcrum and the loading point of the midpoint of tibia coincided with each other. The upper compression head was uniformly loaded to the specimen at 10 mm/min to record the fracture strength of the tibia. Bone strength was expressed in Newtonian (Wang, 2010).

The MT mRNA level of the liver was determined using a reverse transcription polymerase reaction (**RT-PCR**). Total RNA was isolated from the liver using TRIzol reagent (TaKaRa, Japan) according to the manufacturer's instructions. The total RNA concentration was determined by a NanoDrop 2000 Spectrophotometer (Thermo Scientific), and the RNA quality was analyzed in agarose gels stained with ethidium bromide. RNA was reverse transcribed. The resultant cDNA was synthesized using a PrimeScript^{TM} RT reagent kit with gDNA Eraser (TaKaRa). The reverse transcription reaction (20 μL) was conducted in a mixture containing 2 μL of 5 × DNA Eraser Buffer, 1 μL of gDNA Eraser, 1 μL of total RNA, and 6 μL of RNase-free double distilled water (ddH_2O), and it was then incubated for 2 min in a 42°C environment. Next, 4 μL of 5 × Prime Script Buffer 2, 1 μL of Prime Script RT Enzyme Mix I, 1 μL of RT Primer Mix, and 5 μL of RNase-free ddH_2O were added to the reaction solution, and the reaction was run at 37°C for 15 min, 85°C for 5 s and 4°C for 10 min. Reverse transcription products (cDNA) were stored at −20°C for real-time PCR. Metallothionein primers and β-actin were designed using Primer Premier Software (Premier Biosoft International, CA). The primer sequences used in PCR and their gene bank accession numbers are listed in Table 3. Gene expression levels were assessed by a real-time quantitative PCR using ABI 7900HT (Bio-Rad) and the following profiles: stage 1, 1 cycle at 95.0°C 30 s; and stage 2, 40 cycles at 95.0°C 5 s, 60.0°C 30 s. At the end of the PCR reactions, a melt curve analysis was performed for all genes. The relative gene expression level was normalized according to the expression of β-actin. The results were expressed as $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001).

Statistical Analysis

Data were analyzed by a 1-way ANOVA of SPSS 21.0 (SPSS Inc., Chicago, IL), the Duncan's multiple comparisons was used to test the significance of the differences between treatment means, significance was declared at $P < 0.05$.

RESULTS

Tibia Quality and Mineral Concentration

As shown in Table 4, the tibia length and P and Cu concentrations in the tibia were not influenced by the dietary Zn supplemental level or source from week 59 to 62, week 63 to 66, week 67 to 70, or week 71 to 72 ($P > 0.05$). Tibia strength did not differ among the 4 treatments from week 59 to 62. However, compared to the inorganic group, dietary supplementation with 40 or 80 mg/kg MHA-Zn increased the tibia strength from week 63 to 66, week 67 to 70, and week 71 to 72 ($P < 0.05$). Significant differences among treatments were noted in the tibia Ca concentration from week 59 to 62,

Table 4. Effects of MHA-Zn on tibia quality and mineral concentration in tibia of laying hens.[1]

Items	Experimental treatment				*P*-Value
	80 mg/kg $ZnSO_4$	20 mg/kg MHA-Zn[2]	40 mg/kg MHA-Zn	80 mg/kg MHA-Zn	
Tibia length (mm)					
Week 59 to 62	111.48 ± 4.32	111.49 ± 2.59	111.44 ± 2.70	111.56 ± 3.78	1.000
Week 63 to 66	111.83 ± 2.60	111.01 ± 3.02	111.99 ± 1.86	111.66 ± 3.75	0.913
Week 67 to 70	111.84 ± 2.24	111.90 ± 2.76	111.28 ± 2.98	111.41 ± 4.16	0.971
Week 71 to 72	111.67 ± 2.09	111.50 ± 2.11	111.35 ± 2.55	111.40 ± 2.83	0.994
Tibia strength (N)					
Week 59 to 62	166.35 ± 7.65	165.25 ± 12.11	168.51 ± 6.25	165.82 ± 9.65	0.904
Week 63 to 66	150.56 ± 7.09^{b}	149.42 ± 8.74^{b}	158.38 ± 6.10^{a}	161.78 ± 5.32^{a}	0.004
Week 67 to 70	148.43 ± 6.64^{b}	140.08 ± 9.56^{c}	156.29 ± 6.67^{a}	160.40 ± 7.24^{a}	<0.001
Week 71 to 72	145.27 ± 5.97^{b}	138.77 ± 10.24^{b}	155.37 ± 8.30^{a}	158.29 ± 7.20^{a}	<0.001
Ca content (%)					
Week 59 to 62	21.82 ± 0.46^{b}	21.02 ± 2.22^{b}	22.02 ± 1.42^{b}	23.69 ± 1.02^{a}	0.006
Week 63 to 66	20.87 ± 1.55^{b}	20.81 ± 1.31^{b}	22.19 ± 1.46^{a}	23.55 ± 1.04^{a}	<0.001
Week 67 to 70	20.84 ± 0.98^{b,c}	20.37 ± 1.46^{c}	21.90 ± 1.24^{a,b}	22.47 ± 1.58^{a}	0.009
Week 71 to 72	20.82 ± 1.12^{b,c}	20.27 ± 1.18^{c}	21.79 ± 1.16^{a,b}	22.33 ± 0.87^{a}	0.010
P content (%)					
Week 59 to 62	8.83 ± 0.34	8.67 ± 0.64	8.74 ± 0.64	8.76 ± 0.69	0.997
Week 63 to 66	8.76 ± 0.60	8.39 ± 0.42	8.48 ± 0.42	8.23 ± 0.40	0.177
Week 67 to 70	8.63 ± 0.32	8.52 ± 0.24	8.56 ± 0.69	8.56 ± 0.68	0.982
Week 71 to 72	8.25 ± 0.49	8.76 ± 0.55	8.65 ± 0.53	8.60 ± 0.99	0.393
Zn content (mg/kg)					
Week 59 to 62	504.73 ± 64.50	494.38 ± 61.01	503.1 ± 58.14	507.41 ± 49.67	0.978
Week 63 to 66	443.05 ± 49.89^{b}	427.72 ± 49.05^{b}	462.64 ± 47.97^{a,b}	499.14 ± 34.48^{a}	0.030
Week 67 to 70	420.78 ± 45.07^{b}	410.62 ± 41.34^{b}	448.36 ± 40.02^{a,b}	486.21 ± 23.99^{a}	0.008
Week 71 to 72	377.21 ± 36.41^{b}	376.78 ± 71.39^{b}	438.36 ± 40.02^{a}	476.21 ± 23.99^{a}	0.001

[1]Data represent the means from 8 replicates per treatment. Values are the means ± SD.
[2]MHA-Zn: methionine hydroxy analog chelate zinc.
[a–c]Means with different superscripts within the same row are significantly different ($P < 0.05$).

week 63 to 66, week 67 to 70, and week 71 to 72 ($P < 0.05$). In different phases, tibia Ca concentration in the group that added 80 mg/kg MHA-Zn was the highest, whereas the group with 20 mg/kg MHA-Zn had the lowest Ca concentration in tibia, but it did not differ from the inorganic group. The Zn concentration in the tibia did not differ among the experimental treatments during the starter phase of the study (week 59 to 62, $P > 0.05$). The higher Zn concentration in the tibia was observed in the organic groups with the added 40 or 80 mg/kg MHA-Zn compared to the inorganic group from week 63 to 66, week 67 to 70, and week 71 to 72 ($P < 0.05$), but no difference was found between the 2 organic groups.

Mineral Deposit and Metallothionein Expression Level of Liver

The Cu concentration in the liver was not significantly influenced by dietary Zn level or source ($P > 0.05$, data not shown) from week 59 to 62, week 63 to 66, week 67 to 70, and week 71 to 72. As presented in Table 5, the Zn concentrations in the liver were statistically affected from week 59 to 62, week 63 to 66, week 67 to 70, and week 71 to 72 ($P < 0.05$). The Zn concentrations for the group fed with 40 or 80 mg/kg MHA-Zn were dramatically higher ($P < 0.05$) than those for the inorganic group, whereas there was no difference found between the 2 groups.

Compared to the inorganic group, dietary supplementation with 20 mg/kg MHA-Zn markedly decreased the MT-mRNA expression in the liver. However, dietary supplementation with 40 or 80 mg/kg MHA-Zn significantly increased the MT-mRNA expression ($P < 0.05$). Moreover, the expression of MT-mRNA increased with the increase in the Zn level.

Apparent Retention of Minerals and Nutrients

Table 6 indicates that no significant differences in the apparent retention of Fe and Mn were observed among the 4 groups ($P > 0.05$). The apparent retention of Cu in the organic groups was significantly higher than that in the inorganic group ($P < 0.05$), and the diet with 40 mg/kg MHA-Zn had the highest Cu utilization. Compared to the control group, groups with organic Zn could improve the apparent retention of Zn, but no significant difference was found ($P > 0.05$). Besides, the highest Zn utilization was found in the group with 40 mg/kg MHA-Zn.

Moreover, Table 6 shows that both the Zn level and source had no effects on the apparent retention of P ($P > 0.05$). The dietary supplementation with organic Zn significantly improved the apparent retention of energy

Table 5. Effects of MHA-Zn on mineral concentration and MT mRNA expression of liver of aged laying hens.[1]

Items	Experimental treatment				*P*-Value
	80 mg/kg $ZnSO_4$	20 mg/kg MHA-Zn[2]	40 mg/kg MHA-Zn	80 mg/kg MHA-Zn	
Zn concentration of liver (mg/kg)					
Week 59 to 62	81.27 ± 4.31^{a,b}	78.94 ± 5.19^{b}	86.27 ± 5.92^{a}	86.76 ± 6.72^{a}	0.032
Week 63 to 66	78.59 ± 5.56^{b,c}	75.94 ± 6.56^{c}	83.16 ± 3.26^{a,b}	84.74 ± 6.13^{a}	0.015
Week 67 to 70	76.27 ± 4.31^{a,b}	74.08 ± 4.20^{b}	81.28 ± 6.54^{a}	82.11 ± 7.13^{a}	0.035
Week 71 to 72	74.09 ± 4.34^{b}	73.94 ± 6.56^{b}	80.92 ± 3.75^{a}	81.29 ± 4.65^{a}	0.004
MT mRNA expression of liver					
Week 72	1.00 ± 0.03^{c}	0.62 ± 0.07^{d}	2.45 ± 0.07^{b}	2.67 ± 0.04^{a}	<0.001

[1]Data represent the means from 8 replicates per treatment. Values are the means ± SD.
[2]MHA-Zn: methionine hydroxy analog chelate zinc.
[a–d]Means with different superscripts within the same row are significantly different ($P < 0.05$). MT: metallothionein.

Table 6. Effects of MHA-Zn on apparent retention of trace element and nutrients of aged laying hens.[1]

Items	Experimental treatment				*P*-Value
	80 mg/kg $ZnSO_4$	20 mg/kg MHA-Zn[2]	40 mg/kg MHA-Zn	80 mg/kg MHA-Zn	
Apparent retention of nutrients (%)					
Energy	69.89 ± 0.95^{c}	71.89 ± 0.72^{b}	74.24 ± 1.26^{a}	74.57 ± 0.46^{a}	<0.001
CP	69.70 ± 0.98^{b}	69.37 ± 0.94^{b}	70.11 ± 1.90^{a}	72.03 ± 1.19^{a}	0.002
Ca	50.95 ± 1.20^{b}	51.65 ± 2.15^{b}	53.70 ± 3.44^{a,b}	54.56 ± 2.84^{a}	0.041
P	51.31 ± 2.78	51.03 ± 2.27	51.93 ± 2.70	51.66 ± 3.60	0.928
Apparent retention of trace elements (%)					
Cu	32.21 ± 7.11^{b}	41.31 ± 7.29^{a}	44.97 ± 8.27^{a}	42.32 ± 5.65^{a}	0.014
Fe	20.63 ± 3.46	22.21 ± 2.00	21.80 ± 4.92	22.16 ± 4.52	0.865
Mn	15.40 ± 2.55	16.91 ± 4.76	17.21 ± 4.89	16.73 ± 4.24	0.884
Zn	11.14 ± 5.09^{b}	14.80 ± 2.92^{a,b}	19.21 ± 5.40^{a}	16.43 ± 6.27^{a,b}	0.086

[1]Data represent the means from 8 replicates per treatment. Values are the means ± SD.
[2]MHA-Zn: methionine hydroxy analog chelate zinc.
[a–c]Means with different superscripts within the same row are significantly different ($P < 0.05$).

($P < 0.05$). Groups with 40 or 80 mg/kg MHA-Zn significantly promoted the apparent retention of CP ($P < 0.05$). Dietary supplementation with 80 mg/kg MHA-Zn significantly improved the apparent retention of Ca ($P < 0.05$).

DISCUSSION

In the current study, both the Zn source and level affected the mineral retention of the tibia. Zn plays a crucial role in bone calcification (Rossi et al., 2001; Huang et al., 2007; Star et al., 2012). Previous studies reported that Zn concentration in the tibia is a sensitive indicator of Zn requirement for broilers (Ao et al., 2006; Sunder et al., 2008; Liao et al., 2013). Moreover, Qin et al. (2017) demonstrated that the tibia Zn concentration responded quadratically to the dietary supplemental Zn level in laying hens. In the present study, the Zn concentration in the tibia increased as dietary Zn increased during the late phase (63 to 72 wk) and with the same Zn level. Organic Zn increased Zn deposition in the tibia compared to inorganic Zn, especially organic Zn with 80 mg/kg dose, which indicated that the parameter is a sensitive and suitable criterion for the Zn requirement estimation for laying hens. Additionally, the current study revealed that the Ca concentration in the tibia and tibia strength improved with increasing dietary Zn supplementation. These results suggested that organic Zn is better absorbed than inorganic Zn, especially 80 mg/kg dose. In addition, Ao et al. (2009) also reported that organic Zn caused more Zn accumulation in the tibia of chicks than the inorganic Zn source, and the mechanism for this might be explained by the antagonism occurring between Zn and other minerals (such as Cu and Ca) when the inorganic forms, but not organic forms, were included in the diet. We concluded that the increase of Zn in the tibia improved carbonic anhydrase activity, which increased carbonic acid hydrolysis. Furthermore, the above process promoted the deposition of calcium carbonate and tibia quality.

The liver is the metabolism center of nutrients. The Zn concentration in the liver can be used as an indicator of the organism level of Zn (Jahanian and Rasouli, 2015). In our study, the Zn concentration in the liver was increased with the increasing of Zn supplementation. The effect of organic Zn was better than that of inorganic Zn, which was consistent with Wang et al. (2012) and Sandoval et al. (1998) but inconsistent with Ao et al. (2006) and Huang et al. (2009), who reported that there was no significant difference in liver Zn concentrations between different Zn sources. These

discrepancies might be related to the degree of chelation or complexation of the Zn ion to organic ligands.

Metallothionein is a low molecular weight protein with high cysteine concentration, and it has a high Zn-binding capacity (Fernando et al., 1989). Tissue MT expression levels are often proportional to Zn status and respond to dietary Zn (Cao et al., 2000, 2002; Huang et al., 2007). Metallothionein might potentially be a useful biomarker to assess the bioavailability of different Zn sources under normal physiological conditions. Studies in chicks have demonstrated that there was a significant change in hepatic or pancreas MT mRNA level with dietary supplementation with different concentrations of Zn (Huang et al., 2007, 2009, 2013). In the present study, the MT mRNA level in the liver increased as the Zn level increased.

Trace elements play a vital role in poultry production, and the current trace elements used are mainly from inorganic sources. However, inorganic trace elements are not easily absorbed and easily form complexes with other ingredients, such as phytic acid and tannin, in the diets and other anti-antagonists to form insoluble compounds (Cao et al., 2000). Compared to Zn sulfate, MHA-Zn would be more stable and better resistant to interferences from pH, phytic acid, or other ligands in the digestive tract and then, it could arrive at the absorptive site in the small intestine as an intact structure and might have more Zn absorption and a higher Zn bioavailability (Suo et al., 2015). Excessive use of inorganic trace elements not only seriously affects animal gastrointestinal health but also causes environmental pollution. Some experiments showed that a low dose of organic trace elements is well absorbed and can reduce environmental pollution (Creech et al., 2004; Ao et al., 2009). Trace elements utilization reflects the body's absorption and utilization of trace elements. In this study, the organic Zn group improved the apparent utilization of Zn and Cu and reduced the waste of trace elements to protect the environment. Hydroxy methionine chelate minerals, transporting trace elements for easier absorption and utilization, also have methionine activity, which can minimize the antagonism of trace elements and achieve higher bioavailability. The apparent utilization of nutrients can be an intuitive reaction of the body nutrient utilization.

In our study, compared with the inorganic group, dietary supplementation with organic Zn significantly improved the energy efficiency. The 80 mg/kg organic Zn supplementation significantly promoted the utilization of CP and Ca, which agreed with Abdallah et al. (2009) and Huang et al. (2013). Organic Zn in vivo can improve the activity of digestive enzymes, thereby improving the apparent nutrient digestibility of feed. It is also possible that Zn is effectively protected by zinc methionine, so the absorption and utilization of Zn are higher than those of $ZnSO_4$.

In conclusion, dietary organic Zn supplementation could influence Zn and Ca deposits in the tibia and liver, tibia quality, the apparent retention of nutrients, mineral utilization, and the MT expression level in layers. On the contrary, both 40 and 80 mg/kg of organic Zn had a better effect. In view of economic costs and environmental pollution, the optimal supplementation of MHA-Zn is 40 mg/kg.

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