PROCESSING AND PRODUCTS

Effect of the level and source of supplementary dietary zinc on egg production, quality, and zinc content and on serum antioxidant parameters and zinc concentration in laying hens

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ABSTRACT Zinc is vital for proper functioning of an animal. Two sources of zinc are commonly supplemented in animal feed, organic and inorganic zinc, and there are reports that the former is absorbed to a greater extent than the latter. We hypothesized that supplementary zinc would increase zinc content in eggs of laying hens and that organic zinc would be more effective than inorganic zinc. To test these hypotheses, we examined the effect of levels and sources of supplemental dietary zinc on average daily feed intake (**ADFI**), egg production, and zinc content in eggs and on serum antioxidant capacity and zinc concentration in laying hens. A total of 720 Roman laying hens (21-week-old) were randomly assigned to 5 treatment groups with 6 replicates, with 24 hens in each replicate. Two sources of zinc, organic (zinc amino acid complex) and inorganic (zinc sulfate), each with 2 levels, low (35 mg/kg) and high (70 mg/kg), comprised 4 treatment groups, and a control group without supplementary zinc was the fifth group. Seven days were allowed for adjustment to the conditions, and then measurements were taken over 42 D. There was no difference in ADFI, average egg weight (**EW**), ADFI-to-EW ratio, and egg quality ($P > 0.05$) among the 5 treatment groups; supplemental zinc increased serum concentrations of Zn^{2+} and Cu-Zn superoxide dismutase and tended to increase superoxide dismutase content ($P = 0.065$). Zinc content in eggs increased linearly with supplementary organic zinc ($N = 18$, $R^2 = 0.363$, $P = 0.008$) and with supplementary inorganic zinc ($N = 18$, $R^2 = 0.366$, $P = 0.008$) treatment, but there was no difference between the source treatments of zinc. Therefore, our first hypothesis was supported, but our second one was not supported. We concluded that zinc supplementation is effective in enhancing zinc content in eggs and in improving antioxidant capacity in laying hens.

Key words: zinc-rich egg, laying hen, performance, egg quality

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INTRODUCTION

Zinc (**Zn**) is one of the pivotal microelements necessary for normal animal function, including physical growth and muscle development (Liu et al. 2018), immunity (Maywald et al., 2018), reproduction, milk and egg production, and eggshell quality (Hoang et al., 2016). The World Health Organization (WHO, 2008) reported that Zn deficiency ranks 11th among the 20 most major risk factors contributing to disease worldwide and fifth among the 10 major important factors in developing countries. Hence, strategies to increase Zn intake in people could be crucial in many parts of the world.

Zinc is a major additive in the feed industry, with both organic and inorganic forms used. Inorganic Zn is relatively cheap; however, organic sources are more easily absorbed than inorganic sources, which would allow lower concentrations to be added to feed (Li et al., 2019). According to the National Research Council, a supplemental dose of 35 mg of Zn per kg is recommended for laying hens (NRC. 1994), although Cufadar et al. (2020) reported that 20 mg/kg of supplemental Zn was

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adequate for sustaining performance, good eggshell quality, and bone status, while also reducing Zn excretion and soil pollution. Furthermore, Zn is a component of the metalloenzyme carbonic anhydrase that is necessary for eggshell formation (Rodríguez-Navarro et al., 2015; Supuran et al., 2018).

Eggs are eaten widely by most people in the world and, consequently, would be an ideal food to be a source of Zn intake and alleviate Zn deficiencies in humans. Despite its potential importance, we are aware of only 2 studies that measured Zn content in eggs (Plaimast et al., 2008; Abedini et al., 2018). The aim of this study was to examine the effect of Zn intake on Zn content in eggs and to test whether a linear relationship exists between Zn content in eggs and Zn intake. We hypothesized that Zn content in eggs could be increased by feeding supplemental Zn to laying hens and that the increase would be dose dependent and also that an organic source would be more effective than an inorganic source. To test these hypotheses, we examined the effect of 2 levels and 2 sources (one organic and one inorganic) of supplemental dietary Zn on egg production, quality, and Zn content in eggs and on serum antioxidant factors and Zn concentration in laying hens.

MATERIALS AND METHODS

Animals and Management

This experiment was conducted in a commercial poultry farm (Yiyang city, Hunan province, China). All procedures on chickens were approved by the Committee of Laboratory Animal Management and Animal Welfare of Hunan Agricultural University (Changsha city, Hunan province, China) and Ethical Committee of Hunan Agricultural University (ethics approval number: 201607-6).

Seven hundred twenty Roman laying hens, of 21 wk of age and of similar body size and laying rates, were selected. A completely randomized design was used in which 5 treatment groups were allocated, each consisting of 6 replicates with 24 hens per replicate. The 5 dietary treatments included the following: control group with no supplementary Zn; group supplemented with an organic Zn source, zinc amino acid complex (Availa Zn; Zinpro Corporation, Eden Prairie, MN), at a dose of 35 mg/kg (low level [AvZn-L]) and 70 mg/kg (high level [AvZn-H]); and group supplemented with an inorganic Zn source, zinc sulfate (**$ZnSO_4$**), at a dose of 35 mg/kg (low level [$ZnSO_4$-L]) and 70 mg/kg (high level [$ZnSO_4$-H]). All diets were formulated to meet nutrient requirements (Table 1), except for Zn, as suggested by the National Research Council. (1994). The hens were housed in a caged layer house with 8 hens per cage (50 cm × 50 cm × 60 cm) equipped with 2 nipple drinkers and one feeder. The house was well ventilated and was under a lighting regime of 16 h per day. The chickens were fed 2 times per day, at 05:00 h and 13:00 h, and water and feed were provided ad libitum. The measured Zn content of the concentrate feed was 20.56 mg/kg of dry matter (**DM**), and therefore, the concentration of Zn in the low-concentration treatments was 55.56 mg/kg of DM and in the high-concentration treatment was 90.56 mg/kg of DM. Seven days were allowed for adjustment to the conditions, and then, measurements were taken over 42 D. Feeding and vaccinations were carried out following the management practices of the company.

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

Ingredients	Content	Nutrition level	Content
		ME, MJ/kg[2]	11.12
Corn, %	61.0	CP, %	15.90
Soybean meal, %	23.0	Ca, %	3.50
Limestone, %	8.00	AP, %	0.34
Rapeseed meal, %	3.00	Lys, %	0.84
Soybean oil, %	1.00	Met, %	0.33
Premix,[1] %	4.00	Zn, mg/kg	20.56

Abbreviations: AP, available phosphorus; Ca, calcium; CP, crude protein; Lys, lysine; Met, methionine; ME, metabolic energy.

[1]The premix contained 0 mg of Zn but provided the following per kg of diet: vitamin A, 7,715 IU; vitamin D_3, 2,755 IU; vitamin E, 8.8 IU; vitamin K, 2.2 mg; vitamin B_{12}, 0.01 mg; vitamin B_2, 4.41 mg; vitamin B_3, 5.51 mg; vitamin B, 0.55 mg; nicotinic acid, 19.8 mg; folic acid, 0.28 mg; Mn, 50 mg; Fe, 25 mg; Cu, 2.5 mg; Se, 0.15 mg; I, 1.0 mg.

[2]Calculated according to NRC. (1994).

Sample Collection and Analysis

Feed and egg samples Daily feed intake was calculated by the difference between feed offered and orts. Feed samples were collected on day 1, 21, and 42, dried at 65°C in a forced-air oven for 72 h, air equilibrated for 12 h, ground to pass through a 1-mm screen, then packed in self-locked plastic bags, and stored at −20°C. Nitrogen content was measured using the Kjeldahl method, and CP was calculated as 6.25 × N content. For Ca analysis, 1 g of the sample was ashed at 600°C for 8 h in a muffle furnace. The resulting ash was digested and diluted in a 100-mL volumetric flask with deionized water, and then, Ca was measured using an inductively coupled plasma optical emission spectrometer (Varian ICP, VISTA MPX, CCD Simultaneous, Lancashire, UK). Lysine concentration was determined using an amino acid analyzer (Hitachi L-8900; High-Technologies Corporation, Tokyo, Japan) after the samples were hydrolyzed with 6 *N* HCl at 110°C for 24 h. Methionine was determined as methionine sulfone and cysteic acid after cold performic acid oxidation overnight and hydrolyzing with 7.5 *N* HCl at 110°C for 24 h using an amino acid analyzer (Hitachi L-8900; Tokyo, Japan). All samples were analyzed in duplicate following AOAC. (1999).

Daily egg production, total weight per replicate, and eggshell quality (broken or soft) were recorded daily. Feed conversion ratio was calculated as average daily feed intake (**ADFI**; g) per replicated weight of eggs produced (g). On day 42, 6 eggs per replicate were selected randomly, and Zn concentration and egg quality were determined. Shell thickness (mm), as a mean of 3 sites, and albumen height (mm) were measured using a

Table 2. Effects of dietary supplementation of different levels and sources of zinc on performance of laying hens.

Parameters	CON, 0 mg/kg	AvZn-L, 35 mg/kg	AvZn-H, 70 mg/kg	$ZnSO_4$-L, 35 mg/kg	$ZnSO_4$-H, 70 mg/kg	*P*-values		
						L	S	L × S
ADFI, g/day	115 ± 1.97	113 ± 1.21	115 ± 1.93	116 ± 12.82	114 ± 0.15	0.931	0.185	0.039
EW, g	54.8 ± 0.48	55.1 ± 0.27	55.1 ± 0.42	54.6 ± 0.51	54.8 ± 0.63	0.612	0.008	0.040
ADFI:EW	2.22 ± 0.01	2.25 ± 0.04	2.24 ± 0.08	2.31 ± 0.07	2.19 ± 0.05	0.012	0.823	0.094
Laying rate, %	94.0 ± 0.94	90.4 ± 1.94	93.2 ± 2.66	91.7 ± 1.83	95.0 ± 1.55	<0.001	0.091	0.432
Broken or soft shell rate, %	0.85 ± 0.41	1.18 ± 0.74	1.16 ± 0.74	0.46 ± 0.25	0.26 ± 0.18	0.786	0.005	0.098

Abbreviations: AvZn, Availa Zn, control group; L, zinc levels; S, zinc sources; S × L, the interaction of zinc sources and levels; $ZnSO_4$, zinc sulfateCON. Total dietary Zn per kg of dry matter: CON, 20.56 mg/kg; AvZn-L, 55.56 mg/kg; AvZn-H = 90.56 mg/kg; $ZnSO_4$-L, 55.56 mg/kg; $ZnSO_4$-H, 90.56 mg/kg. Values under treatment denote supplementary dietary zinc.

micrometer (ETG-1061A; Robotmation, Co., Ltd., Tokyo, Japan). Egg shape index (long axis:short axis, mm:mm) and egg yolk index (yolk height:yolk diameter, mm:mm) were determined according to the method of Romanoff and Romanoff (1949). Haugh units were calculated using the following formula: Haugh unit = 100 × log ($H + 7.57 - 1.7 \times W^{0.37}$), where H is the albumen height (mm) and W is the egg weight (**EW**; g) (Caudill et al., 2010). Egg yolks and eggshells were weighed using an electronic balance (Liaoning Instrument Co., Ltd., Shenyang, China). Eggs were dried at 65°C in a forced-air oven for 72 h, air equilibrated for 12 h, and then ground to pass through a 1-mm screen. Egg and feed samples were analyzed in duplicate for Zn content (AOAC 1999). Two grams of the dried sample was placed in a crucible, and 9 mL of nitric acid, 1 mL of perchloric acid, and 2 mL of hydrogen peroxide were added to the sample. The sample was heated at 180°C in a microwave oven (MARS 5; CEM Corp., Matthews, NC) at a pressure of 1.207 kPa and then diluted to 25 mL with distilled water. Zinc concentration was determined by inductively coupled plasma optical emission spectrometry (ICP-ASE; Agilent Technologies, Santa Clara, CA).

Blood collection Using a vacuum tube, 5 mL of blood samples were collected from a wing vein from 2 random hens in each replicate on day 42. The samples were allowed to settle at room temperature for 30 min and then centrifuged at 3,000 rpm for 10 min. The serum was stored in a 1.5-mL tube at −20°C. Serum concentrations of Zn^{2+}, Cu-Zn superoxide dismutase, and superoxide dismutase (**SOD**) were determined using assay kits according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Statistical Analysis

Two-way analysis of variance was used to test the effect of Zn sources and levels on the dependent variables using SPSS software (SPSS 21; SPSS Inc., Chicago, IL). Data are presented as means ± SD, and statistical significance was accepted at $P \leq 0.05$.

RESULTS AND DISCUSSION

ADFI and Egg Production

There was no difference among treatments in ADFI, but the interaction between levels and sources was significant ($P = 0.04$) (Table 2). Average EW was affected by the source of Zn, with the organic Zn treatment producing heavier eggs than inorganic Zn treatment ($P = 0.008$), and the level × source interaction was significant ($P = 0.04$). Hens receiving the higher level of dietary Zn required more ADFI per EW produced ($P = 0.012$) but laid eggs at a higher rate than hens receiving the lower level of dietary Zn. The laying rate of soft-shelled eggs was affected by the Zn source ($P = 0.005$), with the organic Zn treatment producing more soft-shelled eggs than the inorganic Zn treatment. In the present study, the level and source of dietary Zn did not affect ADFI. However, the higher level of supplementary dietary Zn, 70 mg/kg of DM, decreased ADFI per EW and increased the laying rate than the lower level of supplementary dietary Zn, 35 mg/kg of DM; while dietary organic Zn increased EW and also increased the laying rate of soft-shelled eggs when compared with dietary inorganic Zn. The results have been equivocal on

Table 3. Effects of dietary supplementation of different levels and sources of zinc on egg quality of laying hens.

Parameters	CON, 0 mg/kg	AvZn-L, 35 mg/kg	AvZn-H, 70 mg/kg	$ZnSO_4$-L, 35 mg/kg	$ZnSO_4$-H, 70 mg/kg	*P*-values		
						L	S	S × L
Egg shape index	1.28 ± 0.04	1.28 ± 0.04	1.30 ± 0.04	1.28 ± 0.02	1.28 ± 0.04	0.542	0.244	0.189
Eggshell thickness, mm	0.49 ± 0.02	0.50 ± 0.03	0.50 ± 0.04	0.51 ± 0.02	0.49 ± 0.03	0.321	0.929	0.612
Eggshell weight, g	6.24 ± 0.22	6.40 ± 0.30	6.38 ± 0.61	6.14 ± 0.47	6.32 ± 0.37	0.552	0.278	0.671
Eggshell strength, kg/m^2	5.07 ± 0.60	5.11 ± 0.49	5.33 ± 0.63	5.58 ± 0.64	5.09 ± 0.67	0.491	0.658	0.237
Yolk weight, g	15.8 ± 0.78	15.8 ± 1.30	16.1 ± 0.97	15.1 ± 1.14	15.7 ± 0.73	0.306	0.040	0.229
Yolk index	0.42 ± 0.03	0.43 ± 0.04	0.44 ± 0.03	0.42 ± 0.03	0.42 ± 0.02	0.974	0.015	0.059
Albumen height, mm	3.52 ± 1.26	3.49 ± 1.50	3.50 ± 1.32	3.31 ± 1.34	4.39 ± 1.47	0.275	0.494	0.375
Haugh unit	54.1 ± 8.67	60.5 ± 5.28	61.7 ± 8.67	55.5 ± 6.21	60.3 ± 11.7	0.304	0.125	0.442

Abbreviations: AvZn, Availa Zn; CON, control group; L, zinc levels; S, zinc sources; S × L, the interaction of zinc sources and levels; $ZnSO_4$, zinc sulfate. Total dietary Zn per kg dry matter; CON, 20.56 mg/kg; AvZn-L, 55.56 mg/kg; AvZn-H = 90.56 mg/kg; $ZnSO_4$-L, 55.56 mg/kg; $ZnSO_4$-H, 90.56 mg/kg. Values under treatment denote supplementary dietary zinc.

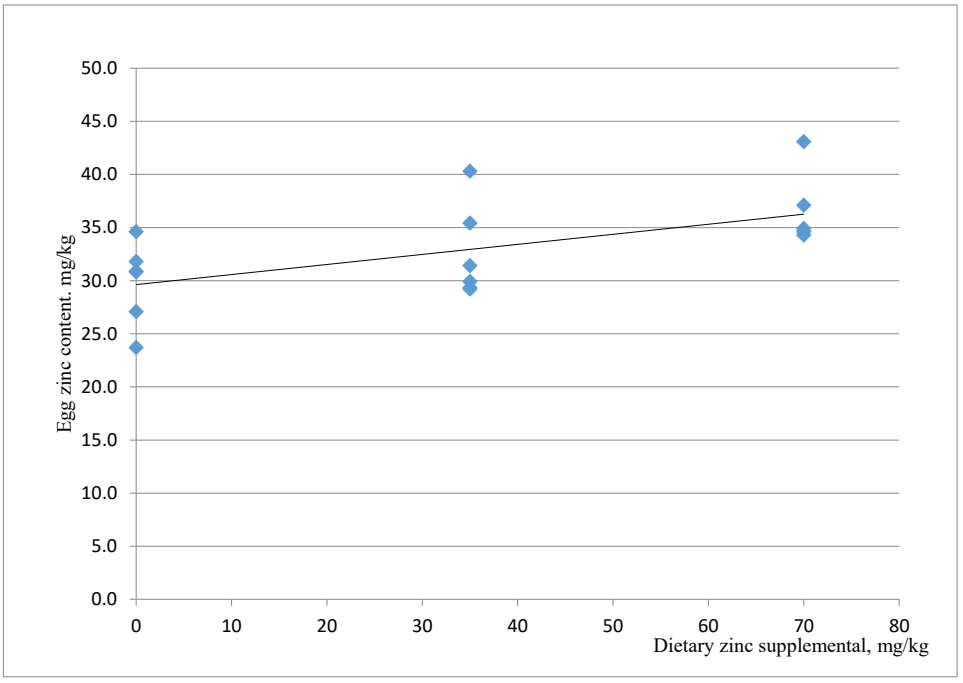

Figure 1. Linear regression of zinc content (mg/kg) in eggs of laying hens on supplemental different levels of AvZn (mg/kg). Abbreviation: AvZn, Availa Zn.

the effect of the level and source of supplementary dietary Zn on the performance of laying hens. Cufadar et al. (2020) reported that different levels of added dietary Zn, namely, 20, 40, 60, 80, and 100 mg/kg of DM, and different sources of Zn, namely, inorganic zinc oxide, organic zinc proteinate, and zinc oxide nanoparticles (**NP**), did not affect feed intake, egg production, EW, and ADFI-to-EW ratio. There was some support for these findings as Mao and Lien (2017) reported that egg production, EW, and ADFI-to-EW ratio of laying hens were not affected by a dietary supplement of 80 mg/kg of DM of Zn whether it was in the form of inorganic, organic, or nano Zn. In addition, Olgum and Yildiz (2017) reported that egg production in laying hens was not affected by supplementary nano Zn, inorganic Zn, or organic Zn. In contrast, it was reported that 20 mg/kg of supplemental Zn was adequate to sustain high egg production, good eggshell quality, and proper bone development, while also reducing Zn excretion and soil pollution (Cufadar et al., 2020), and Li et al. (2019) found that a supplementary dose of 80 mg/kg via Zn methionine decreased feed intake and ADFI-to-EW ratio to a greater extent than via $ZnSO_4$.

Egg Quality and Egg Zn Content

There was no difference among treatments in egg shape index, eggshell thickness, eggshell weight, eggshell strength, albumen height, and Haugh units ($P > 0.05$, Table 3). Yolk weight was more ($P = 0.040$) and yolk index ($P = 0.015$) was higher in hens receiving the higher level of Zn than the lower level of Zn.

We expected eggshell quality to improve with supplementary dietary Zn as Zn is a component of the carbonic anhydrase enzyme, which is essential for supplying carbonate ions for eggshell formation (Gutowska, 1945). Tsai et al. (2016) reported that eggshell thickness was increased by supplementary dietary organic Zn and nano Zn, and Abedini et al. (2018) reported that eggshell thickness increased with all sources of supplementary Zn, but to a greater extent with nano Zn than with inorganic Zn. However, in the present study, the level and source of supplementary dietary Zn did not affect egg shape index, eggshell thickness, eggshell weight, eggshell strength, albumen height, and Haugh unit. These results were supported by Cufadar et al. (2020), who also reported that eggshell weight and eggshell strength were not affected by the Zn level and sources, which was also supported by other studies that reported different sources of Zn did not affect eggshell thickness in laying hens (Tabatabaie et al., 2007; Idowu et al., 2011). Shell strength was strengthened by supplementary nano and organic Zn, but not by supplementary inorganic Zn (Abedini et al., 2018).

Zinc content of the eggs increased linearly with an increase in supplementary dietary Zn, but there was no difference between the organic and inorganic source treatments of Zn. A linear regression of supplemental AvZn on Zn content in eggs took the form: $Y = 0.0946X + 29.638$ ($N = 18$, $R^2 = 0.363$, $P = 0.008$; Y: the content of egg Zn deposition; X: the dietary Zn supplementary; Figure 1); whereas the linear regressions of supplemental $ZnSO_4$ on Zn content in eggs took the form: $Y = 0.0764X + 29.058$ ($N = 18$, $R^2 = 0.366$, $P = 0.008$; Y: the content of egg Zn deposition; X: the dietary Zn supplementary; Figure 2). Therefore, our first hypothesis was supported, but our second one was not supported. Similarly, Zn content in eggs

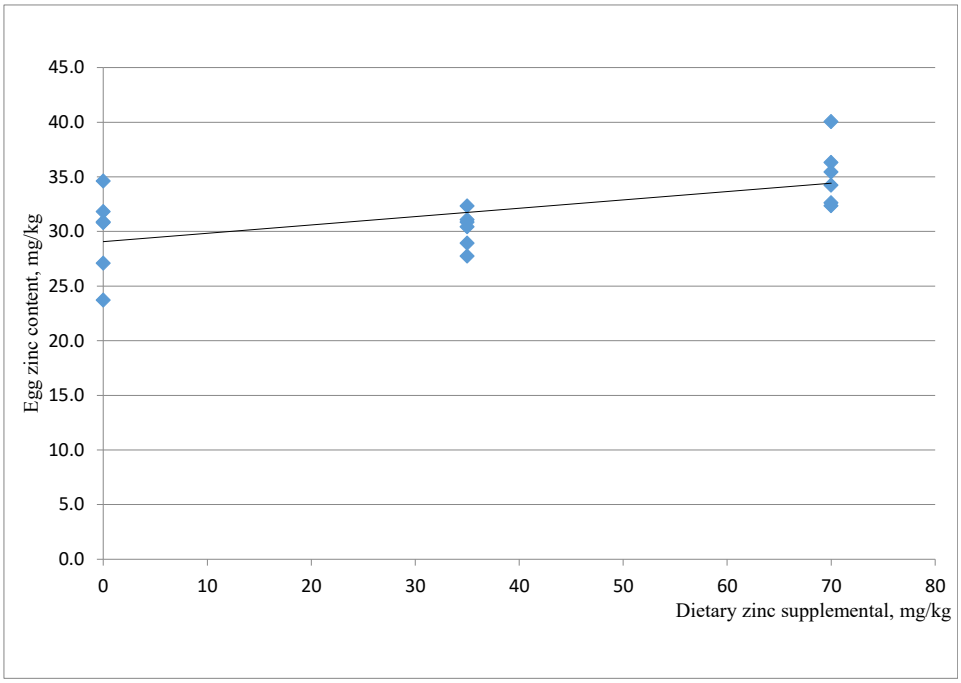

Figure 2. Linear regression of zinc content (mg/kg) in laying hens on supplemental levels of $ZnSO_4$ (mg/kg). Abbreviation: $ZnSO_4$,zinc sulfate.

increased when chicken feed was supplemented with 80 mg/kg of Zn oxide, Zn methionine, and ZnO NP; however, Zn content in eggs from chickens fed with the organic Zn and Zn NP were higher than in eggs from chickens fed with inorganic Zn (11.6 and 12.0 vs 9.9 mg/kg, respectively) (Abedini et al., 2018). Plaimast et al. (2008) reported that in chickens that received either supplementary organic or inorganic Zn so that the total Zn intake was 300 or 600 mg/kg, Zn content in the egg yolk did not differ between the 2 levels of Zn or the 2 sources. However, the chickens that received 600 mg/kg of Zn had higher Zn content in eggs than the controls (60 mg/kg), but the eggs of chickens that received 300 mg/kg did not have a higher Zn content.

Serum Antioxidant Factors

Much effort is being taken to improve the antioxidant capability of laying hens to decrease stress and, consequently, help maintain high egg production. In the present study, hens supplemented with the 70 mg/kg of DM of Zn had higher Zn^{2+} and Cu-ZnSOD ($P < 0.05$; Table 4) content and tended to have higher serum concentrations of SOD ($P = 0.065$) than hens supplemented with the 35 mg/kg of DM of Zn, but the source of Zn did not have an effect. Zinc is an essential component of SOD, which has an important function in the detoxification of superoxide free radicals and protection of cells against oxidative stress by catalyzing the conversion of superoxide anion (O_2^-) to H_2O_2. Superoxide anion (O_2^-) has been linked to the pathogenesis of cardiovascular diseases, including hypertension and atherosclerosis. Cu-ZnSOD is the major superoxide scavenger in the cytoplasm, nucleus, lysosomes, and intermembrane space of mitochondria (Elchuri et al., 2005; Fukai et al., 2011). Therefore, in general, supplementary Zn improved antioxidant capacity in laying hens in the present study, which in agreement with previous studies (Yuan et al., 2011; Yalcinkaya et al., 2012; Li et al., 2019). Abedini et al. (2018) reported similar results when 80 mg/kg of Zn was added: supplementary Zn increased SOD content, but the content was not affected by the source of the Zn. In the study of Li et al. (2019), serum Cu-ZnSOD concentration was not affected in laying hens offered 20 to 100 mg/kg of organic Zn, but concentration in the liver increased at the 60 mg/kg level.

Table 4. Effects of dietary supplementation of different levels and sources of zinc on serum antioxidant factors and zinc concentration in laying hens.

Parameters	CON, 0 mg/kg	AvZn-L, 35 mg/kg	AvZn-H, 70 mg/kg	$ZnSO_4$-L, 35 mg/kg	$ZnSO_4$-H, 70 mg/kg	*P*-values		
						L	S	L × S
Zn^{2+}, μmol/L	21.5 ± 3.44	26.8 ± 3.25	29.1 ± 6.09	23.6 ± 4.82	28.6 ± 6.56	0.007	0.456	0.676
SOD, μ/ml	157 ± 6.71	164 ± 5.95	167 ± 5.68	158 ± 12.12	164 ± 4.59	0.065	0.174	0.540
Cu-ZnSOD, μ/ml	165 ±35.44	170 ±22.69	182 ±24.96	169 ± 24.67	181 ± 20.29	0.009	0.902	0.996

Abbreviations: AvZn, Availa Zn; CON, control group; L, zinc levels; S, zinc sources; S × L, the interaction of zinc sources and levels; $ZnSO_4$, zinc sulfate. Total dietary Zn per kg dry matter; CON, 20.56 mg/kg; AvZn-L, 55.56 mg/kg; AvZn-H = 90.56 mg/kg; $ZnSO_4$-L, 55.56 mg/kg; $ZnSO_4$-H, 90.56 mg/kg. Values under treatment denote supplementary dietary zinc.

CONCLUSIONS

Supplementary Zn increased Zn content in eggs linearly, but the source of Zn did not have an effect, and consequently, our first hypothesis was supported, but our second hypothesis was not supported. ADFI was not affected by supplementary Zn, but egg production was higher in the group supplemented with high level of Zn than in the group supplemented with low level of Zn and tended to be higher in the inorganic Zn–supplemented hens than in organic Zn–supplemented hens. Egg quality was not affected by supplementary Zn, except for the yolk index, which was higher in the organic Zn–sumpplemented than in the inorganic Zn–supplemented hens. Serum concentrations of Zn^{2+} and Cu-ZnSOD were higher, and SOD content tended to be higher in the group supplemented with high level of Zn than in the group supplemented with low level of Zn, but there was no difference between the source treatments of Zn. We concluded that supplementation of Zn is effective in enhancing Zn content in eggs and improving antioxidant capability in laying hens.

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