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Impact of Dietary Replacement of Inorganic Zinc by Organic or Nano Sources on Productive Performance, Immune Response and Some Blood Biochemical Constituents of Laying Hens

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ABSTRACT

Two hundred, 55 weeks old of *Isa brown* laying hens were used to investigate the effects of replacement of inorganic zinc with organic or nano zinc on egg production performance, immune response, some blood biochemical parameters. Hens were randomly allotted into 5 groups for 10 weeks experimental period. Zinc sources were added to the basal diet as following: Group1 fed the basal diet supplemented with 60 ppm inorganic zinc oxide, G2-G3: supplemented with 60 and 30 ppm organic zinc and G4-G5 received the same levels but from zinc oxide nanoparticles (ZnO-NPs). Results showed that replacement of inorganic zinc by 60 ppm of organic zinc or 30ppm of zinc nanoparticles significantly reduced body weight losses. Moreover, addition of organic or nano zinc significantly increased egg production % and average yolk weight, non-significantly ($P \geq 0.05$) increased average shell weight while lower level of nano zinc (30ppm) significantly ($P<0.05$) reduced average albumen weight. Higher supplementation level of organic zinc (60 ppm) significantly reduced cracked shell % ($P<0.05$) while nano zinc significantly reduced average albumen index. Inclusion of zinc polysaccharide or nanoparticles had no significant effect on antibody production against avian influenza while improved it against Newcastle disease at 63rd and 65th weeks of layer age. Significant improvement of phagocytic activity and index, increased serum SOD with improved MDA and GPx activities and non-significant increased serum total protein and albumin concentrations were found in laying hens supplemented with organic or nano zinc forms. Serum liver enzymes (GOT and GPT) activities were reduced in laying hens supplemented with organic zinc and 30mg of nano zinc/kg diet. Moreover, organic or nano zinc oxide supplementation non-significantly reduced serum triglycerides while, increased ($P \geq 0.05$) serum phosphorus, zinc, total cholesterol and high density lipoprotein concentrations except lower level of nano zinc reduced serum cholesterol. In conclusion, organic or nano forms of zinc at 60 or 30 ppm/kg diet could be used as safe alternative sources of inorganic zinc in laying hen diet without any detrimental effect on their productive performance.

1. INTRODUCTION

Zinc (Zn) is an essential trace element for growth, bone development, enzyme structure and function, and eggshell formation in poultry. Moreover, a cofactor and/or structural component of carbonic anhydrase enzyme which is very important for supplying the carbonate ions needed during eggshell formation (Nys *et al.*, 2004). Zn content of the diet is very low compared to the Zn requirement of poultry (NRC, 1994). In practice, chicken diets are generally supplemented with higher levels of Zn than the recommended by NRC. This results in high levels of Zn residue in the excreta of chickens, leading to environmental pollution. Therefore, enhancing Zn absorption can help alleviate both these issues.

Zinc bioavailability is 6–11% in monogastric animals (Brody 1997).This bioavailability and tissue accumulation of Zn depend upon various factors such as its chemical form, feed composition, age and physiological state of hens, and interactions with other minerals (Leeson and Summers, 2005). Diets are commonly supplemented with inorganic sources such as oxide, carbonate, chloride, or sulfate salts due to its cost and commercial availability. However, due to the pH changes that naturally occur in the digestive tract of poultry, there may be antagonism and interactions among trace minerals, as well as with other compounds in diet forming insoluble compounds, preventing their absorption in body (Aksu *et al.*, 2012) and causing its deficiency. Consequently, poultry producers added these inorganic salts to the diets at levels higher than recommended by NRC to meet bird requirements, avoiding mineral deficiency and achieve maximum performance, (Aksu *et al.*, 2012), which in turn increases mineral excretion resulting in environmental pollution (Mohanna and Nys, 1998). Furthermore, higher inclusion levels of Zn may affect the balance of other trace elements in the body, reduce the stability of vitamins and other nutrients, and increase its accumulation inside the animal body (Zhao *et al.*, 2014) .

The reduced bioavailability of the inorganic mineral sources and environmental issues increased the interest in finding more available alternative sources such as organic or nano sources. Chelated or organic trace minerals do not suffer ionic dissociation in the acidic gastric pH like the inorganic sources, remaining stable and protected from chemical reactions with other molecules in the intestinal lumen and consequently optimizes their absorption and bioavailability relative to inorganic sources (Światkiewicz *et al.*, 2014). So, chelated minerals can be included in diet at lower concentrations than inorganic source without adverse effect on production performance. Earlier studies documented that egg quality and performance improvement were achieved with the addition of organic trace minerals in the diet of laying hens (Fernandes *et al.*, 2008; Figueiredo Júnior *et al.*, 2013; Saldanha *et al.*, 2009). Wedekind *et al.* (1992) reported that Zn-methionine was more bioavailable than Zn-sulphate or Zn-oxide. The bioavailability of organic zinc is higher than that of inorganic zinc, but the application of organic zinc in animal diets is limited due to its cost (Zhao *et al.*, 2014).

The development of nanotechnology has brought new trends and integrated to many fields. As one of its applications in the food industry, nanotechnology has been successfully used to produce food additives and to improve the utilization efficiency of trace elements in diets. Furthermore, nanoparticles show new characteristics of transport and uptake and exhibit high absorption efficiency (Davda and Labhasetwar, 2002). As a rule, the smaller the particles, the higher and the more effective their absorption, especially if the size below 100 nm (Hett, 2004). Zinc Oxide nanoparticles (ZnO-NPs) have attracted a great attention because they show novel characteristics such as size, shape, large surface area, high surface activity, high catalytic efficiency, and strong adsorbing ability (Wijnhoven *et al.*, 2009). Compared with ZnO, nano-ZnO has a stronger chemical activity and participates in oxidation reactions with a variety of organic compounds. In addition, the permeability of nano-ZnO can also help prevent adverse gastrointestinal reactions and improve the absorption (Zhao *et al.*, 2014).

Therefore, the objectives of this study were to investigate the comparative effects of replacement of inorganic zinc with organic or nano zinc on egg production performance, eggshell quality, immune response and some blood biochemical parameters of laying hens.

2. MATERIALS AND METHODS

This experiment was done at the Faculty of veterinary Medicine, Alexandria University, Egypt.

2.1. Birds management, Experimental design and feeding program

Bird management procedures were approved and followed the requirements of the local ethical committee of Animal use from Faculty of Veterinary Medicine, Alexandria University, Egypt. Two hundred, 55 weeks old of *Isa brown* laying hens were obtained from local trade Company. Hens were weighted separately and randomly allotted into 5 separate groups (40 hens per group). They were housed in a clean well-ventilated room partitioned into 5 equal compartments. Each compartment was provided by suitable feeder and waterer allowing free access to feed and water for 10 weeks experimental period. Birds were fed the basal diet (BD) containing 17% crude protein and other nutrients according the requirements (NRC, 1994). Basal diet was formulated with zinc sources (inorganic zinc, organic zinc, nano zinc) and two concentrations (100% and 50%) for the organic and nano sources according to the *Isa brown* management guide as outlined in the experimental design presented in table 1. The ingredients composition and chemical analysis of the basal diet used in the experiment are shown in table 2 and 3. Proximate chemical analysis of experimental diet samples was done according to (AOAC, 1985). Hens were weighed individually at the beginning and ending of the experiment and changes in live body weight change was recorded. Feed intake (FI) and produced egg mass were taken and the feed conversion ratio (FCR) was calculated accordingly.

2.2. Laying performance

Egg production performance: The eggs produced from various groups were collected daily (during 14 days' period for five successive periods) for calculating the following parameters: Average egg weight (g) = Total egg weight per period/Number of produced eggs per period. Average egg production (%) = Number of produced eggs per period/Number of laying hens.

Egg mass = Grams of egg produced/hen/day

Egg quality measurements: At the end of each laying periods (57th, 59th, 61th, 63th and 65th weeks of hens age), a sample of 20 eggs from each group was collected to estimate the following parameters according to (Card and Nesheim, 1972) .

Egg shape index = Egg width / Egg length.

Yolk weight and relative weight to total egg weight.

Yolk index = Yolk height / Yolk width.

Albumin weight and its relative weight.

Albumen index = Albumen height/ (Albumen length +Albumen width).

Shell thickness and weight as well as its relative weight.

The percent of cracked shell was recorded daily.

2.3. Evaluation of immune response: was done by a group of parameters including phagocytic activity (PA), phagocytic index (PI), differential leukocytic count and haemagglutination inhibition test for detection of antibodies against Newcastle and avian influenza diseases. At the end of the experiment (65 week of hen's age), Five blood samples were collected from each group in clean dry vials containing anticoagulant (0.1ml sodium citrate 3.8%) for determination of PA, PI, and differential leukocytes count.

Table 1. The experimental design

Groups No.	Experimental diet	Inorganic zinc concentration*	Organic zinc concentration**	Nano-zinc concentration***
1	Basal diet	60 ppm	--	--
2	""""	--	60 ppm	--
3	""""	--	30 ppm	--
4	""""	--	--	60 ppm
5	""""	--	--	30 ppm

*Inorganic zinc oxide: Using zinc oxide (ZnO) as fed basis produced by El-Gomhoria Co., Egypt with guaranteed minimum of 80% Zn).**Organic zinc: Used zinc polysaccharide complex (Quali Tech, Chaska, MN) with guaranteed minimum of 30% Zn.***Nano zinc: Used zinc oxide nano particles produced by Mknano Co. " M K Impex Corp, Canada" with 30nm.

Table 2. Ingredients composition of basal diet.

Ingredients	**%**
Yellow corn ground	57.88
Soybean meal (44% CP)	22.0
Corn gluten meal (60% CP)	4.0
Wheat bran	3.0
Vegetable oils (sunflower oil)	1.25
Ground lime stone[1]	9.80
Monocalcium phosphate[2]	1.30
Common salt	0.25
Vitamin premix (0.15%)[3]	0.15
Mineral premix (0.1%)[4]	0.10
Lysine[5]	0.02
Methionine[6]	0.10
Choline[7]	0.10
Mycotoxin adsorbent	0.05

[1]Lime stone contains 37% calcium & locally produced. [2]Mono calcium phosphate: contain 21% phosphorus and 17% calcium. [3]Vitamin premix: Each 1.5kg contains: Vit A (12000000Iu), vit D (2000000Iu), vit E(10gm), vit K3 (2gm), vit B1 (1gm), vit B2 (5gm), vitB6 (1.5gm), vit B12 (10gm), nicotinic acid (30gm), pantothinic acid (10gm), folic acid (1gm), biotin (50mg). produced by Archar Daniels method company De Caur LL. Made in U. S. A. [4]Mineral Premix was formulated and composed of (1 kg): 70000 mg Mn, 60000mg Zn (Using zinc oxide (ZnO) and replaced by zinc polysaccharide complex or nano zinc particles), 8000mg Cu,50000 Fe, 1000mg I, 250mg Se and 150mg Co and calcium carbonate up to 1 kg.
[5]Lysine: 98% lysine hydrochloride, Produced by Shandyoung Longue Co. China.
[6]DL-methionine produced by Evoink Co. Guranted analysis 99.5% DL- methionine.
[7]Choline: choline chloride 60% with vegetable carrier (corn powder) produced by Shandyuong pharmaceutical Co. Chin. 7- Beta-2-x. [8]Nitrogen free extract calculated by difference.

Table 3. Chemical composition of the used basal diet.

Chemical composition (%)	
Moisture	11.65
Crude protein	17.07
Ether extract	4.06
Ash	12.76
Crude fiber	4.43
NFE8	50.03
Calcium	3.72
Phosphorus	0.63
ME Kcal/kg*	2746.88

* ME were calculated according to (NRC, 1994) .

2.3.a. Phagocytic activity (PA) and phagocytic index (PI): were determined according to (Kawahara *et al.*, 1991). They were calculated according to the following equations: PA = macrophages containing yeast/total number of macrophages*100 while, PI= number of cells phagocytized/number of phagocytic cells. Blood film was prepared according to the method described by (Lucky, 1977) for differential leukocytic count. The percentage and absolute value for each type of cells were calculated according to (Schalm, 1986) **.**

2.3.b. Haemagglutination Inhibition test for detection of Newcastle and avian influenza antibodies: Blood samples were collected at end of laying periods (55th, 59th, 61th, 63th and 65th weeks of hens age) from five chickens of each group. Serum was separated by centrifugation at 3000 rpm for 10 minutes. Micro-technique of haemagglutination inhibition test was done according to (Takatsy, 1955). Geometric mean titer (GMT) was calculated according to (Brugh, 1978).

2.4. Blood biochemical Parameters

Another blood sample was obtained from the same birds and left to coagulate at room temperature then separation of serum was done by centrifugation of coagulated blood at 3000 rpm for 10 minutes. The clear serum samples were kept in freezer at 20 ^{C} for analysis of blood biochemical parameters. Serum samples were thawed and used for analysis of some biochemical constituents including glucose, total protein, albumin, triglycerides, total cholesterol, Low density lipoprotein (LDL), High density lipoprotein (HDL), GOT (Glutamic-oxaloacetic transaminase), GPT (glutamic--pyruvic transaminase), uric acid and creatinine, superoxide dismutase (SOD), glutathione peroxide (GPx) and lipid peroxidase (Malondialdehyde "MDA") and some minerals as calcium, phosphorus and zinc by spectrophotometer using commercial kits from Biodiagnostic (Diagnostic and Research reagents) and Vitro Scient companies , Egypt.

2.5. Statistical analysis: The obtained results were analyzed using analysis of variance (one way anova) using (SAS, 2004) to measure the significant differences between the means of different variables. All statistical tests were considered significant at $P \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Productive performance of laying hens.

3.1.1. Body weight changes

As presented in table (4), replacement of inorganic zinc by 60mg of organic zinc (polysaccharide zinc) or by 30mg of ZnO-NPs/kg diet significantly reduced body weight losses during production periods compared with laying hens fed the BD supplemented with 60 ppm inorganic zinc. However, replacement by 30mg of organic zinc or by 60mg of ZnO-NPs had no significant effect on body weight changes of laying hens. The obtained data are in accordance with (Olgun and Yildiz, 2017) who reported that different forms and dosages of Zn (inorganic, organic or nano) had no significant differences in body weight change. While, disagree with (Mohammadi *et al.*, 2015) who stated that the dietary supplementation of nano zinc decreased body weight when compared to other Zn sources used.

3.1.2 Hen day egg production (HDEP %)

Replacement of inorganic zinc by 60 or 30mg of polysaccharide zinc non-significantly ($P \geq 0.05$) increased hen day egg production (HDEP%) during 1st and 2nd periods while, significantly ($P<0.05$) increased it during 3rd, 4th and 5th periods (table 5). Moreover, supplementation of organic zinc significantly increased average HDEP% throughout the whole experimental period. The obtained data are in accordance with (Abedini *et al.*, 2018) who stated that laying hens supplemented with Zn-Methionine had greater HDEP % compared to the control. Moreover, Abd El-Hack *et al.* (2017) reported that 50 mg of ZnO and 100 mg of Zn-Met. resulted in significantly higher egg production (EP) rate. This result may be attributed to increased bioavailability of organic Zn. Other theory postulated that dietary Zn possibly improves egg production by interacting with the endocrine system. Hence, it is essential for progesterone synthesis and its deficiency can induce an excessive secretion of prolactin hormone which initiates the broodiness and stops egg production (Park *et al.*, 2004). On the contrary, Tabatabaie *et al.* (2007) pointed out that zinc source did not affect egg production in laying hens. Also, Olgun and Yildiz (2017) concluded that supplementation of the glycine form of Zn to the diet decreased egg production rate of laying hens compared to those fed inorganic forms of Zn.

Nano zinc supplementation (60 or 30 ppm/kg diet) non-significantly ($P \geq 0.05$) decreased HDEP% during 1st and 2nd periods while, increased during 3rd, 4th and 5th periods compared with those received inorganic form. However, Zn-NPs significantly ($P<0.05$) increased average HDEP % throughout whole experimental periods. These findings are in line with (Abedini *et al.*, 2018) who observed the positive effect of dietary supplementation of nano Zn on egg production in laying hens. Higher bioavailability of Zn in the form ZnO-NPs could explain the improved egg production. Olgun and Yildiz (2017), reported a contrast result as supplementation of Zn in nano form did not affect egg production rate of laying hens. On the other hand, organic zinc supplementation increased egg production % compared with laying hens fed on the same level of supplemented zinc from nano source.

Table 4. Effect of dietary replacement of inorganic zinc with lower levels of organic or nano zinc on body weight changes of laying hens.

Age/weeks	Zinc source and supplementation levels (mg/Kg diet)				
	Inorganic	**Organic**		**Nano**	
	60	**60**	**30**	**60**	**30**
55 weeks	2092.30 ±28.0	2060.70 ±24.0	2080.60 ±25.0	2120.60 ±30.0	2042.00 ±24.0
65 weeks	2025.60 ±27.0	2020.6 ±26.0	2020.7 ±27.0	2055.10 ±25.0	2009.50 ±28.0
Body weight change (65 – 55)	-67.00 ±5.6^{b}	-40.10 ±6.6^{a}	-59.8 ±4.6^{b}	-65.50 ±6.5^{b}	-32.50 ±10.5^{a}

Values are means ± standard error. Means within the same row of different letters are significantly different at ($P<0.05$).

Table 5. Effect of dietary replacement of inorganic zinc by lower levels of organic or nano zinc on Hen day egg production (HDEP %) of laying hens.

Periods/week	Zinc source and supplementation levels (mg/Kg diet)				
	Inorganic	**Organic**		**Nano**	
	60	**60**	**30**	**60**	**30**
55 – 57	82.32±1.40	83.92±1.50^{a}	83.6±1.80	80.17±1.20	81.96±1.60
57 – 59	80.35±1.10^{bc}	82.85±1.20^{ab}	85.00±1.40^{a}	78.62±0.90^{c}	81.42±0.60^{bc}
59 – 61	76.96±1.60^{b}	81.96±1.10^{a}	83.03±1.00^{a}	79.82±0.85^{ab}	83.39±1.00^{a}
61 – 63	74.28±1.60^{b}	79.82±0.90^{a}	79.82±1.20^{a}	79.82±0.60^{a}	78.92±1.00^{a}
63 – 65	72.32±1.00^{b}	78.39±1.30^{b}	78.21±0.70^{a}	78.21±0.70^{a}	74.82±0.70^{a}
55 – 65 (Average)	77.25±0.75^{c}	81.39±0.60^{a}	81.91±0.65^{a}	79.33±0.40^{b}	80.10±0.60^{ab}

Values are means ± standard error. Means within the same row of different letters are significantly different at ($P<0.05$).

Table 6. Effect of dietary replacement of inorganic zinc by lower levels of organic or nano zinc on Feed conversion ratio (FCR) values of laying hens

Periods/week	Zinc source and supplementation levels (mg/Kg diet)				
	Inorganic	**Organic**		**Nano**	
	60	**60**	**30**	**60**	**30**
55 – 57	2.10±0.03	2.02±0.04	2.05±0.05	2.12±0.03	2.14±0.04
57 – 59	2.15±0.03	2.09±0.03	2.08±0.04	2.18±0.03	2.12±0.02
59 – 61	2.24±0.04^{a}	2.10±0.03^{b}	2.10±0.03^{b}	2.17±0.03^{ab}	2.09±0.04^{b}
61 – 63	2.41±0.06^{a}	2.24±0.03^{b}	2.24±0.03^{b}	2.27±0.03^{b}	2.26±0.03^{b}

63 – 65	2.43±0.05[a]	2.28±0.04[c]	2.28±0.03[c]	2.31±0.03[bc]	2.41±0.02[ab]
55 – 65	2.26±0.03[a]	2.15±0.02[b]	2.15±0.02[b]	2.21±0.02[ab]	2.20±0.02[b]

Values are means ± standard error. Means within the same row of different letters are significantly different at (P<0.05).

3.1.3. Feed conversion ratio (FCR)

Inclusion of organic or nano zinc sources as alternatives to inorganic form had no significant effect on FCR of laying hens at 1st and 2nd periods of the experiment while improvement was observed during 3rd, 4th and 5th periods. Furthermore, organic zinc at 60 or 30mg and 30mg of nano zinc/kg diet significantly (P<0.05) improved average FCR, while high supplementation level of zinc oxide nano particles (60ppm) non-significantly (P≥0.05) improved average FCR (table 6).

Present data are supported by (Abd El-Hack *et al.*, 2017) who indicated that 50mg of organic zinc improved FCR of laying hens while in contrary with Olgun and Yildiz (2017) as organic zinc (zinc-glycine complex) deteriorated FCR in laying hens compared with inorganic zinc supplemented group and nano zinc source had no significant effect. Also, Tabatabaie *et al.* (2007) recorded that the source or level (25 and 50 ppm) of zinc supplements did not affect feed intake or feed conversion ratio in laying hens.

3.1.4. Egg mass (EM), external egg quality (shell quality) and internal egg quality

As presented in table 7, Dietary inclusion of organic zinc at 60 or 30mg/kg diet instead of inorganic zinc oxide significantly (P<0.05) increased average egg mass (EM), while nano zinc supplementation at the same levels non-significantly (P≥0.05) increased average EM throughout the whole experimental periods. These results are supported by (Bahakaim *et al.*, 2014) who indicated that the EM increased with the addition of organic Zn (Zn-methionine) compared to Zn-sulfate in laying hens. Also, Abd El-Hack *et al.* (2017) reported that replacement of inorganic zinc by zinc-methionine significantly increased EM of laying hens. Moreover, organic zinc increased average EM compared with birds received supplemental zinc from nano source. Regarding external egg quality based on egg index, shell weight, shell thickness and cracked shell%, results presented in table 7, Organic or nano zinc supplementation had no significant effect on average egg index compared with laying hens supplemented with inorganic zinc. Additionally, replacement of inorganic Zn with organic or nano zinc non-significantly (P≥0.05) increased average shell weight, had no significant effect on average shell thickness.

Zinc plays an important role in the isthmus, where eggshell membranes are produced. Dietary zinc improved eggshell quality because it is a component of the carbonic anhydrase enzyme, which is necessary for the formation of eggshells (Innocenti *et al.*, 2004) **.** On the other hand, eggshell thickness was non-significantly decreased when birds were supplemented with organic form of Zn, but this effect was not observed between nano and inorganic sources of Zn. Similar to the current study results, (Idowu *et al.*, 2011; Tabatabaie *et al.*, 2007) stated that the addition of different sources of Zn had no effect on eggshell thickness. However, Bahakaim *et al.* (2014) found that the eggshell thickness improved as a result of using organic Zn in diets. Also, Abd El-Hack *et al.* (2017) stated that organic Zn supplementation had no significant effect on egg shell quality of laying hens. On contrary, Olgun and Yildiz (2017) reported that the lowest eggshell thickness (P<0.05) was obtained in nano Zn-oxide*100 eggs, and the highest eggshell thickness was obtained in Zn-glycine*100 eggs.

Table 7. Effect of dietary replacement of inorganic zinc by lower levels of organic or nano zinc on egg mass, shell quality and internal egg quality of laying hens

	Zinc source and supplementation levels (mg/Kg diet)				
	Inorganic	**Organic**		**Nano**	
	60	**60**	**30**	**60**	**30**
Egg mass(g)	48.91±0.50[b]	51.38±0.40[a]	51.39±0.40[a]	49.86±0.30[b]	50.12±0.40[ab]
Egg shape index	0.73±0.02	0.72±0.03	0.73±0.04	0.73±0.02	0.73±0.04
Shell quality (weight, thickness and cracked %)					
shell weight	6.71±0.22	6.84±0.24	7.23±0.25	7.08±0.24	6.94±0.23
shell thickness	156.71±4.40	155.59±5.46	155.48±4.41	157.8±4.39	159.97±5.47
Cracked shell %	1.92±0.27[a]	1.15±0.21[b]	1.44±0.23[ab]	1.52±0.25[ab]	1.55±0.24[ab]
Internal Egg quality					
Yolk weight (g)	16.29±0.43[c]	17.22±0.49[b]	17.39±0.83[b]	18.26±0.63[ab]	20.36±1.07[a]

Yolk wt. %	27.78±1.29[ab]	25.75±0.89[bc]	26.77±2.07[bc]	28.59±1.76[a]	26.71±1.89[bc]
Yolk Index	0.45±0.01	0.46±0.01	0.44±0.02	0.46±0.01	0.45±0.01
Albumen wt. (g)	40.45±1.26[a]	40.21±0.94[a]	40.93±1.74[a]	40.70±2.02[a]	37.53±0.96[b]
Albumen wt.%	61.24±2.22[ab]	63.56±3.08[a]	62.80±2.45[a]	59.75±1.99[b]	62.44±1.49[a]
Albumin Index	8.16±0.33[a]	8.04±0.36[ab]	8.48±0.39[a]	7.45±0.42[b]	7.71±0.32[b]

Values are means ± standard error. Means within the same row of different letters are significantly different at (P<0.05).

Regarding cracked shell %, higher supplementation level of organic zinc (60mg/kg diet) significantly (P<0.05) reduced it throughout the whole experimental periods from 1.92% (for laying hens received 60mg/kg diet inorganic zinc) to 1.15%. while, the lower level of organic zinc (30mg/kg diet) and nano zinc at 60 or 30mg/kg diet non-significantly reduced average cracked shell% compared with those supplemented with inorganic zinc oxide. Organic zinc supplementation (60 or 30mg/kg diet) reduced average cracked shell % compared with laying hens supplemented with the same zinc level from nano zinc source.

Replacement of inorganic zinc by organic or nano zinc (60 or 30mg/kg diet) significantly (P<0.05) increased average yolk weight (table 7) throughout the whole experimental period. On the other hand, nano zinc (60 or 30mg/kg diet) supplementation increased average yolk weight (g/yolk) compared with hens supplemented with the same level of zinc from organic source. Moreover, replacement of inorganic zinc by lower levels of organic or nano zinc had no significant effect on yolk index values.

Table 8. Effect of dietary replacement of inorganic zinc by lower levels of organic or nano zinc on antibody titer production of laying hens

Periods /week	Zinc source and supplementation levels (mg/Kg diet) Inorganic	Organic		Nano	
	60	**60**	**30**	**60**	**30**
Antibody titer (log) against avian influenza disease vaccine					
55	7.8±0.5	7.4±0.5	7.8±0.6	7.4±0.24	7.8±0.2
59	8.2±0.49[a]	8.2±0.2	8.0±0.3	8.6±0.24	7.8±0.2
63	8.2±0.58	8.8±0.5	8.2±0.4	8.8±0.8	8.6±0.24
65	8.4±0.4	8.8±0.5	8.4±0.2	8.8±0.73	8.8±0.37
Antibody titer (log) against New castle disease vaccine					
55	8.4±0.5	7.8±0.9	8.6±0.2	8.0±0.3	8.2±0.2
59	9.0±0.6	9.0±0.0	9.2±0.2	9.2±0.4	9.6±0.5
63	8.8±0.2[b]	9.0±0.0[b]	9.2±0.2[b]	9.8±0.2[a]	9.4±0.2[ab]
65	8.8±0.2[c]	9.6±0.2[ab]	9.2±0.2[bc]	10.0±0.1[a]	9.6±0.2[ab]

Values are means ± standard error. Means within the same row of different letters are significantly different at (P<0.05).

Table 9. Effect of dietary replacement of inorganic zinc by lower levels of organic or nano zinc on phagocytosis and differential leukocyte of laying hens

Items	Zinc source and supplementation levels (mg/Kg diet) Inorganic	Organic		Nano	
	60	**60**	**30**	**60**	**30**
Phagocytosis					
Phagocytic activity %	36.44±3.66[b]	43.90±0.66[a]	44.75±1.25[a]	42.89±0.92[a]	45.09±0.75[a]
Phagocytic index	1.88 ±0.34[b]	2.09±0.12[ab]	1.99±0.24[ab]	2.03±0.36[b]	2.48±0.13[a]
Differential Leukocyte					
Heterophil%	45.55±1.67	46.56±1.22	45.65±1.56	45.98±1.31	46.44±1.71
Basophil%	5.78±0.23	6.12±0.34	5.87±0.22	6.09±0.11	5.23±0.46
Eosinophil%	2.22±0.11	2.11±0.09	1.98±0.08	2.02±0.12	2.11±0.07
Lymphocyte%	38.78±1.22	37.43±1.07	38.24±0.88	38.64±0.59	37.52±1.11
Monocyte%	7.67±0.34	7.78±0.37	8.26±0.21	7.27±0.37	8.7±0.56

Values are means ± standard error. Means within the same row of different letters are significantly different at (P<0.05).

	Zinc source and supplementation levels (mg/Kg diet) Inorganic	Organic		Nano	
	60	**60**	**30**	**60**	**30**
Total protein (g/dl)	6.05±0.02	6.15±0.08	6.15±0.05	6.17±0.45	6.39±0.26
Albumin (g/dl)	3.95±0.05[b]	3.99±0.10[b]	4.16±0.04[ab]	4.40±0.15[a]	4.15±0.07[ab]

Globulin (g/dl)	2.10±0.03	2.16±0.17	1.99±0.08	1.77±0.46	2.24±0.18
Glucose (mg/dl)	199.37±1.21	198.00±3.93	200.17±2.35	199.07±3.11	196.80±2.50
Calcium (mg/dl)	19.96±0.09	18.66±3.38	19.94±0.08	19.93±0.04	19.91±0.06
P (mg/dl)	5.97±0.18	5.80±0.26	6.30±0.32	6.10±0.12	6.13±0.23
Zinc (µg/dl)	128.33±3.48	132.33±2.60	129.00±0.58	133.00±2.31	130.67±1.4

Table 10. Effect of dietary replacement of inorganic zinc by lower levels of organic or nano zinc on some blood serum units and some minerals of laying hens.

Values are means ± standard error. Means within the same row of different letters are significantly different at ($P<0.05$).

Table11. Effect of dietary replacement of inorganic zinc by lower levels of organic or nano zinc on kidney and liver function related parameters and some antioxidant enzyme activity of laying hens.

	Zinc source and supplementation levels (mg/Kg diet)				
	Inorganic	Organic		Nano	
	60	60	30	60	30
Kidney function related parameters					
Uric acid (mg/dl)	6.01±0.07	5.87±0.19	5.70±0.25	5.79±0.33	5.98±0.07
Urea (mg/dl)	49.19±4.54	55.67±2.10	51.46±8.18	54.89±0.64	52.70±2.88
Creatinine (mg/dl)	1.50±0.26	1.27±0.18	1.33±0.34	1.37±0.30	1.40±0.15
Liver function related parameters					
GOT (µ/L)	15.33±3.84	14.67±2.85	10.67±0.88	17.60±5.03	14.00±3.51
GPT (µ/L)	$52.33±3.38^{a}$	$39.00±2.31^{b}$	$45.67±2.03^{ab}$	$55.33±5.24^{a}$	$43.33±2.40^{ab}$
Some antioxidant enzyme activity					
SOD (U/dl)	$340.00±22.37^{b}$	$403.00±6.56^{a}$	$378.00±15.72^{ab}$	$413.33±3.84^{a}$	$395.67±3.93^{a}$
MDA (nmol/ml)	9.70±0.32	9.93±0.20	10.17±0.55	9.67±0.47	9.87±0.18
GPx (µ/dl)	313.67±4.18	314.67±3.18	314.67±3.53	317.67±3.53	313.00±3.79

Values are means ± standard error. Means within the same row of different letters are significantly different at ($P<0.05$).

Table 12. Effect of dietary replacement of inorganic zinc by lower levels of organic or nano zinc on blood lipids profile of laying hens.

Items	Zinc source and supplementation levels (mg/Kg diet)				
	Inorganic	Organic		Nano	
	60	60	30	60	30
Triglycerides (mg/dl)	204.55± 1.83	202.88± 0.87	201.77± 3.79	204.15±2.46	197.69±10.12
Total cholesterol (mg/dl)	190.43± 2.99	196.20± 4.75	193.20± 7.94	196.17±4.24	190.03±6.70
HDL (mg/dl)	45.43±1.45	49.03±2.55	49.77±3.25	49.60±1.59	49.40±4.28
LDL (mg/dl)	106.09±4.13	106.59±2.84	102.08±11.49	105.74±3.07	101.10±0.98
VLDL (mg/dl)	42.91±0.37	40.58±0.17	41.35±0.76	40.83±0.49	39.54±2.02

Values are means ± standard error. Means within the same row of different letters are significantly different at ($P<0.05$).

Supplementation of organic zinc at 60 or 30mg/kg diet and nano zinc at higher level (60mg/kg diet) had no significant effect on average albumen weight throughout the whole experimental period, while lower level of nano zinc (30mg/kg diet) significantly ($P<0.05$) reduced average albumen weight compared with those supplemented with inorganic zinc. Additionally, organic zinc supplementation had no significant effect on average albumen index, while nano zinc supplementation significantly reduced average albumin index compared with hen's groups fed on inorganic zinc. The best albumen index value was obtained in laying hens received 30mg organic zinc/kg diet (8.48), while the lowest values in those supplemented with 60mg nano zinc/kg diet (7.45).These obtained results are in agreement with (Abd El-Hack *et al.*, 2017) who observed that the highest values of Haugh unit score and yolk to albumin ratio were given by the control group which fed diet free of ZnO or Zn-Met. Also, our findings are in partial agreement with (Bahakaim *et al.*, 2014) who stated that Haugh unit was significantly($P<0.05$) affected by dietary levels of zinc. All quality traits of eggs were not affected by zinc addition vs values of Haugh unit score when birds fed diets enriched with ZnO (140 mg/kg diet) (Idowu *et al.*, 2011). This study obviously indicates the beneficial effects of using Zn in layers, since Zn plays a key role in formation of eggshell in the magnum and uterus during the deposition of albumen and isthmus in which membranes of eggshell are produced.

3.2. Immune Response

3.2.1. Antibody titer against New castle disease and avian influenza vaccine

Replacement of inorganic zinc by either polysaccharide zinc or zinc oxide nano-particles had no significant effect on antibody production against avian influenza disease during different periods (table 8). Adding organic or nano zinc had no clear effect on antibody production against New castle disease at 55th and 59th weeks of laying hens age, while improved it at 63rd and 65th weeks of layer age. Moreover, supplementation of Zn-NPs increased antibody production at 63rd and 65th weeks of laying hens age compared with laying hens fed the same level from polysaccharide zinc complex. These data are supported by (El-Katcha *et al.*, 2017) who reported that dietary replacement of inorganic zinc oxide with lower levels of organic zinc or zinc nano particles improved antibody titer against New castle disease vaccine at 21th, 28th, 35th and 42th day of broiler age

3.2.2. Phagocytosis and Differential leukocytic count percentage

As shown in table 9, Supplementation of 60 or 30mg of organic or nano zinc/kg diet significantly ($P<0.05$) increased phagocytic activity and improved phagocytic index compared with laying hens supplemented with 60mg of inorganic Zn. Furthermore, Zn-NPs fed groups recorded the best values of phagocytosis. These data are in contrast with (El-Katcha *et al.*, 2017) who concluded that broiler fed on different levels of organic or nano zinc non-significantly ($P\geq0.05$) improved phagocytic activity and index compared with chicks group fed on inorganic zinc supplement diet.

On the other hand, differential leukocytic counts of laying hens were non-significantly affected in replacement groups (table 8). This finding agreed with (Dönmez *et al.*, 2002) who reported that Zn supplementation did not affect peripheral blood leukocyte counts. Probably Zn supplemented at 30 ppm from organic or nano source was adequate to support optimum development of lymphocytes, which alleviated stress, as observed from the present study.

Higher immune response of broiler chicken fed on the basal diet with zinc-polysaccharide complex or zinc nanoparticles instead of inorganic source was observed through improvement of cellular and humoral immun. Broiler chicken received diets supplemented with organic or nano zinc might have increased thymulin activity, therefore, enhancing immune response through increased maturation of T-lymphocyte and activation of B lymphocytes by T-helper cells (Hudson *et al.*, 2004) . Also, the immune system is dependent on the functions of cellular metabolism. Zinc is ubiquitous in cellular metabolism and functions both structurally and catalytically in metalloenzymes (O'Dell, 1992). These data are in harmony with those obtained by (Soni *et al.*, 2013) who concluded that cellular immunity and antibody production significantly improved with organic zinc supplementation of broiler breeder.

3.3. Blood Biochemical Constituents

3.3.1 Blood serum units and some blood serum minerals

Supplemental zinc forms (organic or nano) at 60 or 30 ppm non-significantly increased ($P\geq0.05$) serum total protein concentration compared with hens supplemented with inorganic zinc (table 10). Moreover, replacement of inorganic zinc by lower levels of organic or nano zinc numerically increased blood serum albumin concentration, while decreased serum globulin concentration with 30 or 60mg of organic and nano zinc /kg diet respectively and other supplementation levels increased serum globulin concentration. The obtained data indicated that nano zinc at low level consider as immune stimulant higher than other levels and sources of zinc supplementation in laying hens diet.

Blood serum globulin level improvement in accordance with (Fawzy *et al.*, 2016) who observed that protein profile in broiler chicks supplemented with selenium and zinc showed an elevation in total proteins, albumin, and globulin levels with a decline in albumin/globulin ratio. Similarly, plasma total protein was increased with Zn supplementation in broiler diet (Feng *et al.*, 2010). These data are in contrast with (Hassan *et al.*, 2003) who indicated that feeding zinc methionine to Mandarah laying hen resulted in a significant increase in serum total protein, albumin and globulin compared to the control. This difference may be related to higher zinc supplementation than in our trial.

On the other hand, no significant effect was observed in blood serum glucose and calcium concentrations in the polysaccharide zinc or Zn-NPs fed groups while phosphorus and zinc concentrations were non-significantly ($P\geq0.05$) increased compared with laying hens received inorganic form. These data are in agreement with (Abd El-Hack *et al.*, 2017) who indicated that supplementation of ZnO or Zn-Met increased the serum content of zinc with no differences among supplemental zinc doses. Also, Güçlü and İşcan (2004) reported that organic Manganese and Zn supplementation of laying hen diets did not affect serum Ca, P, and Mg concentrations. Bahakaim *et al.* (2014) concluded that adding zinc as organic source significantly increased plasma zinc, it is may be due to higher availability of zinc in case of zinc methionine compared to zinc sulfate.

3.3.2 Blood serum parameters related to kidney and liver function & some antioxidant enzyme activity

Supplementation of organic or nano zinc had no significant effect on serum uric acid, urea and creatinine concentrations compared with laying hens supplemented with inorganic zinc (table 11). Serum GOT and GPT activities were reduced in laying hens supplemented with organic zinc and 30mg of nano zinc/kg diet, while supplementation of 60mg from nano zinc oxide particles/kg diet increased them compared with inorganic zinc supplemented group.

The present findings indicated that higher supplementation level of nano zinc may had adverse effect on hepatic cells function. These data are supported by (Fathi, 2016) who reported that nano zinc increases serum creatinine kinase activity which reflect on higher creatinine concentration. Also, El-Katcha *et al.* (2017) indicated that broiler chicks fed on different levels of organic or nano zinc had no significant ($P \geq 0.05$) effect on serum uric acid and GOT concentrations and non-significantly ($P \geq 0.05$) increased serum GPT and ALP activities when compared with broiler chicks with inorganic zinc supplementation.

Furthermore, inclusion of organic or nano zinc at 60 or 30mg/kg diet significantly ($P<0.05$) increased serum superoxide dismutase (SOD) activity while, numerically improved serum MDA and GPx activities compared with inorganic zinc fed groups. Zinc as essential trace element is important for the structure and function of Cu /Zn-SOD, which comprise more than 90% of SOD and protects the body from oxidative stress and free radicals (Noor *et al.*, 2002)**.** Using Zn in laying hen diets eliminates the free radicals by increasing the Cu /Zn-SOD content which prevents the auto oxidation of glutathione (GSH). The antioxidant function improvement in this study can be explained by removing radical O− via increment of Cu/Zn-SOD, therefore, Zn protects GSH in the blood and organs (Reid and Tervit, 1999).

Increased content of MDA is an essential index for lipid peroxidation and oxidative damage caused by reactive oxygen species (ROS) in cell (Nielsen *et al.*, 1997), where MDA is a product of degraded lipids and used to investigate the lipid peroxidation (Raharjo and Sofos, 1993). From our results, we could state that the use of organic or nano zinc oxide particles might strengthen the oxidative defenses and decrease the concentration of MDA in the blood, which would contribute to improve the productive traits and public health. Zinc is a part of more than 300 enzyme systems that play key roles in oxidative systems and protect cells from deleterious radicals like O_2^- and H_2O_2. In general, the anti-oxidation function of zinc is in improving the birds' sensitivity to some oxidative stresses (Zhao *et al.*, 2014)

3.3.3 Blood serum lipid profile

As documented in table 12, supplementation of organic or nano zinc at 60 or 30mg/kg diet non-significantly reduced serum triglycerides while, non-significantly increased serum total cholesterol and HDL concentrations except lower level of nano zinc reduced serum cholesterol concentration. Additionally, had no effect on serum LDL and VLDL concentrations compared with inorganic zinc supplemented group. The present data supported by (El-Katcha *et al.*, 2017) who found that replacement of inorganic zinc with different levels of polysaccharide zinc complex or with zinc nano particles non-significantly ($P \geq 0.05$) reduced blood serum triglycerides while increased total cholesterol and HDL and had no significant effect on blood serum LDL and VLDL concentrations of broiler chicks.

4. CONCLUSION

According to the results of the present study**,** it could be concluded that organic or zinc nanoparticles at the same level or lower (60 or 30 mg/kg diet) could be used as safe alternative sources of inorganic zinc in laying hens diet without any detrimental effect on their productive performance (through improving egg production % and reducing the cracked shell %), kidney or liver functions as well as enhancing their immune response (through improvement of oxidative defenses and phagocytic activity).

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Conflict of interest

The authors declare that they have no conflicts of interest.

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