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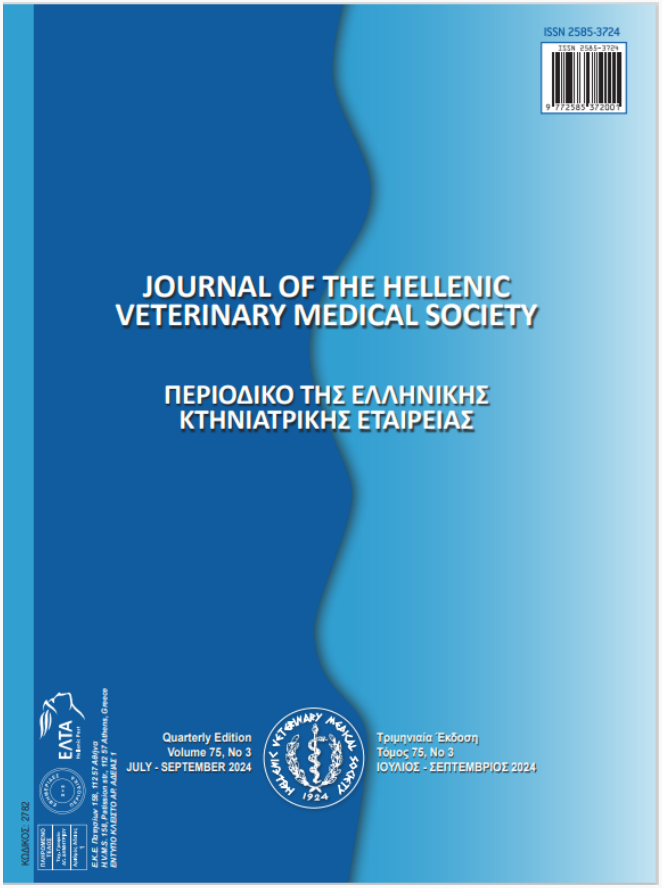

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Research article
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In ovo injection of organic zinc effects on hatching rate of broiler breeder hen eggs and productivity of broilers

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ABSTRACT: The aim of this study was to investigate the effect of *in ovo* injection of organic zinc on the characteristics of broiler chickens and the performance of broiler chickens A total of 320 eggs from Ross 308 strain with an average weight of 65 grams were randomly divided into four groups, negative control (no injection, T1), positive control (*in ovo* injection of 0.272 ml of normal saline solution, T2) and two experimental groups: eggs that received *in ovo* injection of 0.272 mg of organic zinc (T3); and eggs that were injected with 0.544 mg of the same organic zinc (T4). Injections were done in the amniotic sac on the 10th day of incubation. The effect of injection on hatching traits and then on functional traits, blood parameters, immune response, carcass characteristics and cecal microbial population of resulting broilers were measured. The results showed that the percentage of hatched broiler chickens in the zinc-treated groups was significantly lower compared to the two control groups ($P<0.05$). However, the intraocular injection of zinc caused a significant increase in the final weight gain of the birds and their feed intake. Blood parameters were also affected in experimental treatments and blood glucose increased and blood triglyceride, cholesterol and lipoproteins decreased compared to negative and positive control ($P<0.05$). No statistically significant difference was observed in the immune response, microbial population and carcass characteristics among the experimental groups ($P>0.05$). In conclusion, the intra egg injection of organic zinc can show favorable results in the performance indicators of broiler chickens, although it had no apparent effect on the immune response and microbial population as tested.

Keywords: *in ovo* injection; zinc; broiler; hatching; humoral immunity; breast weight

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INTRODUCTION

The consumption of poultry products in the world is increasing and the broiler industry needs healthy birds with fast growth and good muscles to meet the needs of consumers (Alvar *et al*, 2017). A modern and integrated poultry industry can provide a useful source of protein for the growing world population. Improving breeding and management of short-term broiler chickens are among the advances of this industry, which can also benefit from reducing feed costs. There are novel reports on positive effects of feed additives in human (Liu *et al*., 2023; Chuai *et al*., 2023; Zhen *et al*., 2024; Liang *et al*., 2024) and animals (Poorghasemi *et al*., 2018; Azizi *et al*., 2018; Sigolo *et al*., 2019).

Zinc is a mineral found in all cells and is involved in a wide range of metabolic and biochemical processes. This element is considered essential in the nutrition of humans and animals, as it is necessary for the stabilization and functioning of many metalloenzymes involved in the metabolic processes of the body. However, the amount of this element in the basic components of food rations does not meet the needs of the animals' body, because most of the food ingredients that make up the ration are deficient in this element. In addition, the zinc element in plant sources is largely provided as the phytate complex. Therefore, it is necessary to add zinc supplements in the poultry diet to prevent the negative consequences of its deficiency (Huang *et al*., 2019). Zinc deficiency in breeder laying hen diet's leads to reduced hatching, abnormal embryo growth and poor growth performance of offspring, while zinc supplements in breeder's diet can eliminate these negative effects. (Yu *et al*., 2017). Zinc plays an important role in improving the proper functioning of the immune system through increasing the number of T lymphocyte cells, neutrophils, macrophages, antibody and interferon production, as well as reducing cell permeability against viruses and increasing the size of lymphatic organs. Zinc is also required for the function and proliferation of lymphocytes and the production of metalloenzymes such as DNA and RNA polymerases. Dietary zinc deficiency protects the integrity of lymphoid organs and the function of T cells. The lack of zinc in the diet of mother hens increases the antibody titer against sheep red blood cell antigen in the next generation (Akhavan-Salamat and Ghasemi, 2019). By increasing the amount of zinc, the activity of enzymes involved in lipid metabolism increases and their concentration in the blood decreases. Oocytes may absorb more lipids from the blood to compensate for the reduction of lipids and cause a decrease in their plasma concentration (Zhao *et al*., 2016).

Traditionally, inorganic minerals such as zinc oxide and zinc sulfate are used to achieve maximum performance levels in the supplementation of broiler chickens (Abd El-Haliem *et al*., 2020). As a result of the low pH of the upper environment of the digestive tract, when inorganic components of minerals are used, they tend to separate, thus can communicate with other minerals and dietary components in the digestive tract, which absorb and disrupts them throughout the small intestine (Yan and Waldroup, 2006). As a result, these unabsorbed minerals are excreted with the feces of the bird, which may cause environmental concerns, especially when poultry manure is used to fertilize agricultural land (Kibet *et al*., 2013). One of the nutritional strategies to reduce the wastage of mineral supplements in the diet is to use organic sources of minerals instead of inorganic sources. The results of various studies show that the use of organic components of minerals in the diet of broiler chickens can increase the consumption and absorption of minerals and improve the body weight gain index and reduce the excretion of minerals (Nollet *et al*., 2007). The high levels of bioavailability of the organic mineral fraction can be a good explanation for their stability in the upper parts of the digestive tract, leading to the small intestine, where they are absorbed. However, not all organic minerals are stable at low pH, which may affect their bioavailability (Vieira *et al*., 2020).

One of the significant developments in the poultry industry is the emergence and implementation of *in ovo* injection technology. The substances that are injected into the egg to increase production performance and immunity, must be safe for the embryo and do not interfere with maternal antibodies and cause immunity in the flock with a single injection during hatching (Neves *et al*., 2017). During the final stages of incubation, high energy levels are required for proper development of the embryo. In addition, the lack of access to food after hatching, which often occurs with a delay of 24-48 hours in broilers, shows the necessity of storing energy and nutrients before hatching to maintain body metabolism and regulate body temperature (Nouri *et al*., 2018).

During the incubation period, embryonic cells, tissues and organs are differentiated and begin to develop, so the incubation period is a vital part and *in ovo* nutrition can play an important role in the life of broil-

er chickens. During the 21 days of embryo growth and development, the chick embryo takes advantage of essential nutrients inside the egg chamber for tissue growth, and its energy needs (Subramaniyan *et al*., 2019).

Alternatively *in ovo* injection of some nutrients such as minerals and vitamins may lead to reducing the negative effects of stress, along with creating positive effects in the post-hatching period and functional characteristics of broiler chickens. (Hajati *et al*., 2014). Zinc effectively affects feed intake by interfering with the mechanism of proteins, lipids and carbohydrates (Kim and Kang, 2022).

Recent studies on the use of organic minerals have shown that their intake in the diet in the early stages of rearing can improve the amount of restriction of intake at the end of the rearing period (Mwangi *et al*., 2017). When the mineral injection is without a negative effect on hatching rate then increasing the availability of organic matter through *in ovo* injection may subsequently lead to an increase in overall body growth, (Peebles *et al*., 2021).

Joshua *et al*. (2016) reported that the *in ovo* injection of zinc, copper and selenium nanoparticles was not harmful for the growth and development of the embryo and did not have a negative effect on the hatching chick. (Joshua *et al*., 2016). Biria *et al*. (2020) did not observe a significant difference in weight gain and conversion factor by *in ovo* injection of different concentrations of zinc (50, 75 and 100 ppm) and only in the rearing phase did the amount of feed consumed in the injected treatments increase significantly. It was found and in the finisher phase, this distance disappeared (Biria *et al*, 2020).

.MATERIALS AND METHODS

This experiment was carried out in the hatchery and broiler farm of Navid Morgh Guilan Company (Rasht, Iran) in 2022, and involved 320 fertilized eggs from Ross 308 strain with an average weight of 65±1 grams. The eggs were assigned at random to 4 experimental groups each consisting of 8 replications with 10 eggs in each replication. (Table 1). The experiment covered a 21-day incubation followed by 42-day rearing periods.

The eggs were incubated for 18 days, then transferred to a hatcher for 3 days (Jamesway multistage, model PT-100, Canada) The temperature and humidity in the setter and hatcher were 37.6°C and 56%, of and 37°C and 58.5%, respectively.

Injection into the eggs

On the 10th day of incubation, selenium was injected into the amniotic sac. First, the injection site was disinfected with betadine, and then a round pin was used to make a hole in the cork so that the tip of the needle protruded from the other side of the cork (1 mm) and the hole was in the desired location. is created Injection was done using HELMA insulin syringes (Syringe, China).

The fertilized eggs were taken under a strong light and the needle was inserted in the middle of the chicken's back, where there were the least blood vessels, to the right and parallel to the shell.

Experimental Treatments

Table 1 presents details of the four experimental groups. In summary:

Treatment 1: negative control without any *in ovo* injection

Treatment 2: positive control injection of 0.272 ml of normal saline solution into each egg

Treatment 3: injection of 0.272 ml of a solution whose in which the concentration of organic zinc element is was 100 micrograms per ml. In this way, 0272/ for so each egg received 0.0272 mg of organic zinc. was injected

Treatment 4: injection of 0.272 ml of a solution whose in which the organic zinc concentration is was 200 µg/ml. In this way, each egg was injected with so

Table 1. Summary of the treatments employed to examine the effect of in ovo administration of zinc

Treatment	Injection	Volume injected (ml)	Zinc content (mg)	Number of replicates (eggs/replicate)
T1(control 1)	NO INJECTION	0	0	8 (10)
T2(control 2)	Saline	0.272	0	8 (10)
T3	zinc	0. 272	0.0272	8 (10)
T4	zinc	0. 272	0.0544	8 (10)

Table 2. Diets of experimental groups

Diet components (g/kg)	Days of age					
	1-4	5-11	12-19	20-29	30-38	39-42
Ingredients, (%)						
Corn	505	517.5	538	551	565	571
Wheat	20	25	30	35	35	37
Corn gluten feed	29	29	34	45	45	54
Iranian soybean meal (5.44%)	328	327	313	300	287	274
Corn gluten (64%)	35	25	17.5	0	0	0
Fish powder	20	10	0	0	0	0
Calcium carbonate	9	8.5	6.6	6.4	5.9	5.4
D-calcium phosphate	13	12.2	11.1	9.3	7.7	6.4
Bentonite	4.6	5.1	5.85	5.9	6.2	6.5
Soy oil	16	21	25	29.5	31	31
Vitamin supplement[1]	1.25	1.1	1	1	1	1
Mineral supplement[1]	3	2.5	2.5	2.5	2.5	2.5
Salt	1.4	1.5	1.8	1.9	2	2.1
Bicarbonate	2.2	2.2	2.2	2.2	2.2	2.2
DL-methionine	3	2.8	2.7	2.5	2.4	2.2
L-lysine chloride	3.4	3	2.8	2.3	1.9	1.7
L-threonine	1.1	0.9	0.9	0.7	0.5	0.3
L-arginine	0.6	0.3	0.2	0	0	0
Choline chloride 60%	0.85	0.8	0.75	0.7	0.6	0.6
Toxinbinder	2	2	2	2	2	2
Multienzyme (containing phytase)	0.1	1	0.1	0.1	0.1	0.1
Monensin sodium 10%	0	1	1	1	1	0
Acidfire	1.5	1.5	1	1	1	0
Total	1000	1000	1000	1000	1000	1000
Energy Kcal/kg	3025	3060	3100	3125	3150	3175
Protein (%)	23.9	22.6	21.2	20.2	19.3	18.8
Calcium (%)	1.01	0.93	0.79	0.7	0.68	0.63
Available phosphorous(%)	0.5	0.47	0.43	0.39	0.38	0.35
Sodium (%)	0.16	0.15	0.15	0.15	0.155	0.16
Lysine (%)	1.43	1.34	1.25	1.17	1.11	1.06
Lysine (digestible ileum) (%)	1.29	1.22	1.14	1.06	1.01	0.97

[1]The amount of vitamins and minerals per kg of the final diet: Vitamin A, 9000 IU; vitamin D3, 3000 IU; vitamin E, 18 IU; vitamin K3, 3 mg; vitamin B1 (Thiamine), 1.8 mg; vitamin B2 (Riboflavin), 6 mg; vitamin B6 (Pyridoxine), 3 mg; vitamin B12 (Cobalamin), 0.012 mg; vitamin B3 (Niacin), 30 mg; vitamin B9 (Folic acid), 1 mg; vitamin H3 or B7 (Biotin), 0.24 mg; vitamin B5 (Pantothenic acid), 10 mg; 500 mg; Choline,100 mg; Mn, 100 mg; Zinc, 80 mg; Iron, 10 mg; Cu, 1 mg; I,0.2 mg.

each egg received 0.0544 mg (54.4 micrograms) of organic zinc.

The organic zinc used in this research was prepared with the brand name zinc-Glycinate (BASF Germany) and with a purity of 26% in powder form, which was combined with amino acid glycine in the form of chelate.

Measurement of traits at hatching

On day 21, the number and weight of live broiler chickens collected from each experimental group was recorded and survival rate was calculated. The broiler chickens were later transferred to rearing facilities. Broiler chickens that did not hatch were also examined and classified based on the cause and time of embryonic death.

Rearing conditions

The surviving birds were distributed at random among the treatments as shown in Table 1. In each of the 16 pens broiler chickens had easy access to feeding and drinking nipples.

The vaccination program of the chick started on the first day, double Newcastle-influenza plus Bron-

chitis clone 4.91 was given by injection and in drinking water, on the eighth day, the clone vaccine was sprayed, on the fourteenth and twentieth days, Gamboro vaccine, and on the sixteenth and twenty fourth, the Lasota vaccine was administered to the birds in the water.

During the growing period, pens were separated by a fence with dimensions of 1×1 m. Exposure conditions were carried out according to the recommended program of Ross 308 strain. Environmental parameters such as temperature, humidity, light, ventilation, etc., were considered the same according to the proposed program of Ross 308 strain and the amount of feed consumed was assumed to be *ad libitum.* The food ration and chemical composition used is presented in Table 2. Lighting schedule used was according to that proposed for Ross 308 (Aviagen, 2021).

Performance

The feed for each experimental unit was placed in special bags on which the pen number and type of treatment were recorded. The consumed feed was measured periodically and if there were losses feed and/or chick during the period, the corrected feed was calculated. At the end of each period, the broiler chickens of each experimental unit were weighed as a group and their number was recorded. The weight gain of each chick was calculated in grams in each period. The feed conversion ratio was calculated according to the weight gain and feed intake of each experimental pen in each period.

Carcass characteristics

Two chickens at the end of the experiment period (42 days old) were selected from each experimental unit in such a way that their weight was close to the average. Then the chickens were slaughtered after 4 hours of starvation and the carcass yield was determined. Carcass characteristics and weight of internal organs of recorded. The measured traits were; weights of live body, full carcass, carcass with full stomach, carcass with empty stomach, breast, thigh, wing, abdominal fat, pancreas, heart, gizzard, duodenum, jejunum, ileum, and cecum. Weights were measured using a digital scale with an accuracy of 0.001 g (Kern-PFB, Germany).

Blood parameters and liver enzymes

On day 42, two broiler chickens from each replicate were picked at random, and blood from the wing vein was withdrawn. The two blood samples were mixed together and was immediately sent to the laboratory. The parameters determined were the levels of glucose, total cholesterol, and triglyceride, very low density lipoproteins, high density lipoproteins, low density lipoproteins, ratio of total cholesterol to high density lipoproteins, ratio of low density lipoproteins to high density lipoproteins and liver enzymes using diagnostic kits of Pars Azmoun company (Iran). Liver enzymes aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase were also measured with diagnostic kits of Pars Azmoun company and photometrically with a spectrophotometer (223337-S2100 UV-UNICO, USA). Serum cholesterol was measured using the kit of Man company (Iran) and by enzymatic-colorimetric method.

Immune system measurement

The method of Lerner *et al*. (1971) was applied to determine humoral immunity. Broiler chickens were immunized against sheep red blood cells (SRBC). On days 28 and 36, 0.2 cc of SRBC was injected into the wing vein of two birds in each repetition. On days 35 and 42, blood was collected from the injected broiler chickens, and the number of antibodies against SRBC was measured by the hemagglutination method (Seidavi *et al*., 2014). To measure the antibody titer, V micro hemagglutination pellets (MAXWELL-NINGBO FUCHUN CO LTD, China) were used.

In order to check the titer of Newcastle (NDV) and influenza (AIV) on days 35 and 42 of each treatment, two chickens were selected and blood was taken from their wing veins. Hemagglutination inhibition (HI) test was used based on OIE standard (Maxwell-Ningbo Fuchun Co LTD) to check Newcastle and influenza serum titers. For this purpose, first 25 microliters of PBS were poured into all the wells, then 25 microliters of bird serum were added to the first well and its dilution was done until the last well. It was placed on a mechanical shaker for 1 minute and then kept at 25°C for 30 minutes. In the next step, 25 microliters of 1% red blood cells were added to all the wells and the microplate was again placed on a mechanical shaker for 15 seconds. After this stage, the microplates were kept at 25 degrees for 30 minutes and then the results were recorded.

To measure the response of the cellular immune system of 4 chickens from each repetition, subcutaneous injections of 0.1 ml of PBS and phytohemagglutinin were performed in the left and right wings, respectively, on the 40th day of the experiment. The thickness of the injection site of the wing before and

18 hours after injection was measured with a caliper, and the difference in thickness after injection of the left wing (saline) and thickness after injection of the right wing (PHA) was considered as a measurement criterion with a caliper (Guanglu, China). Also, the weight of spleen, thymus and bursa of Fabricius was measured for two samples of each repetition with a digital scale with an accuracy of 0.001 grams (Kern-PFB, Germany).

Selenium concentration in tibia bone

To obtain ash samples, bones were dried at 105°C for 24 hours. Then the samples were ground and placed in a porcelain crucible in a furnace at 600 ºC. To determine bone mineral concentration, one bone ash sample was selected from each treatment. Using the methods specified by US-EPA (1986), the samples were dissolved and digested, and the manganese concentration in each ash sample was analyzed using a spectrophotometer method (223337-S2100 UV, unico, USA).

Fatty acid profile of breast muscle

Carcass fatty acid profile was determined by extracting 10 grams of breast fat of two birds of each treatment. At first, the fat samples were well mixed with 100 ml of methanol: chloroform solution (2:1) for about 3-4 hours. The samples were then filtered and mixed with 25 ml saturated sodium chloride solution in the decanter funnel. In the next step, the chloroform phase containing fat was filtered by a filter paper soaked in anhydrous potassium sulfate. The smoothed samples were dried under vacuum by rotating operator to leave only fat. After this step, 10 mg of extracted fat was mixed well with 2 ml of potassium hydroxide, 2 ml of normal methanol and 7 ml of n-hexane, then the resulting samples were centrifuged for 10 minutes. In the next step, the samples remained stationary for 5 minutes to separate its upper phase. Then, about one microliter of the supernatant phase was injected to evaluate the profile of fatty acids inside the gas chromatography machine (hp6890-US00000397-Agilent-hp-USA) and the number of fatty acids was expressed as a percentage.

Cecal microbial population

To determine the microbial population of cecum of chickens, on day 42, two birds of each treatment whose weight was close to the average of the group were selected, weighed and slaughtered. After opening the abdominal cavity, the cecum was separated with sterile scissors and its contents. From the last two centimeters of it, it was drained into sterile microtubes and the contents of two birds of each repetition were merged together and then to check the microbial population of dominant bacteria including lactobacillus, *Escherichia coli*, coliform, *Bifidobacterium* sp. and coliform population. Anaerobic bacteria were stored at -70°C until microbial culture. To count *Lactobacillus sp*. bacteria from MRS-agar (Man rogosasharpe agar) culture medium, to count *Escherichia coli* bacteria from EMB-agar (Eosine methylene blue agar) culture medium, to count coliform bacteria from Mac culture medium Mac Conkey agar, and to count *Bifidobacterium sp*. and the total population of anaerobic bacteria, the corresponding specific culture medium was used. Finally, the colonies related to each culture medium were counted as colony forming units (CFU Colony Forming Unit) in one gram of sample and the CFU data were reported in Log10 format to be used for data analysis.

Statistical analysis

The data were analyzed as a completely random design with treatment as the main factor using SPSS (2008) software, and the mean comparisons of the treatments were evaluated using Duncan's multiple range test.

RESULTS

In ovo injection of organic zinc caused a significant decrease in chick hatching index of the zinc treated and positive control groups compared to the negative control group (Table 3). However, it did not have a significant effect on the weight of day-old broiler chickens. The late infection related to the injected treatments was significantly higher than un-injected treatment (Table 3).

The effect of organic zinc injection on final weight, feed intake and conversion factor are reported in Table 4. The results showed that the final weight of birds in zinc-treated groups was significantly higher than the control groups (negative and positive), but not significant. The treatment effect on conversion coefficient was non-significant ($P>0.05$). Zinc treatment significantly improved production index as shown in Table 4.

The effect of *in ovo* injection of organic zinc on carcass characteristics and the relative weight of body organs are shown in Tables 5 and 6. Differences among groups were mainly non-significant (Tables 5 and 6).

In ovo injection of organic zinc improved some of the blood parameters of the experimental treatments.

As shown in Table 7, the glucose levels increased in the zinc-treated groups, while cholesterol metabolites, triglycerides, VLDL, LDL and HDL lipoproteins significantly decreased (P<0.05).

The results on the function of liver enzymes are shown in table 7. In general, no significant differences were observed, although the treatment with 0.0544 mg zinc significantly improved the function of alanine transaminase enzyme compared to the control group (P<0.05). Also, in the treatment of 0.0272 mg zinc, the level of alkaline phosphate decreased significantly compared to the positive control group (P<0.05).

The degree of swelling resulting of injecting phytohemagglutinin in the right and left leg of broiler chickens are given in Table 8 and showed that the differences were non-significant (P>0.05). There was no statistically significant difference in the immune response index and intestinal microbial population between the experimental treatments and the negative and positive control groups (P>0.05) (Tables 8 and 9).

The results of the fatty acid profile of the chest muscle are shown in Table 10. The results showed that there was no significant relationship between the injected selenium and the fatty acid profile of the breast muscle. The variation in the fatty acids profiles between groups was minor and not significant (Table 10).

Table 11 reports the concentration of zinc in the tibia of the left leg. No significant effect of *in ovo* injection on selenium concentration was found.

Table 3. Effect of in ovo injection of different levels of zinc on the mean of some hatching traits

Items	T1	T2	T3	T4	SEM	*P-value*
Hatchability (%)	95.0^{a}	55.0^{b}	61.250 ^{b}	46.250 ^{b}	6.838	0.000
Chick weight (g)	46.485 ^{a}	44.493 ^{a}	44.996 ^{b}	47.994 ^{a}	0.661	0.004
Chicken for sale	9.125 ^{a}	5.000^{b}	5.625 ^{b}	4.500 ^{b}	0.643	<0.0001
Cull chick	0.375 ^{b}	0.500 ^{b}	0.500 ^{a}	0.125 ^{a}	0.239	0.643
Unhatched eggs	0.375 ^{b}	4.500 ^{a}	3.750 ^{a}	5.375 ^{a}	0.679	<0.0001
Early contamination	0.000 ^{a}	0.000 ^{a}	0.000 ^{a}	0.000 ^{a}	0.000	1.00
Late contamination	0.000^{b}	1.000 ^{a}	0.000 ^{b}	0.625 ^{a}	0.162	0.000
Broken egg	0.000 ^{a}	0.000 ^{a}	0.000 ^{a}	0.000 ^{a}	0.000	1.00
Inconclusive eggs	0.000 ^{a}	0.500 ^{a}	0.125 ^{a}	0.125 ^{a}	0.160	0.165

T1: Negative control (without injection), T2: Positive control (injection of 0.272 ml of normal saline solution), T3: Injection of 0.0272 mg zinc, T4: Injection of 0.0544 mg zinc.
^{a,b} Means within each row of treatments with no common different superscript differ significantly at P<0.05. (SEM, standard error of means)

Table 4. Effect of *in ovo* injection of different levels of zinc on growth, feed intake and feed conversion ratio and economical indices

Items	T1	T2	T3	T4	SEM	***P-value***
Feed Intake (g)						
1-21d	1259.025^{a}	1212.775 ^{a}	1142.575 ^{a}	1189.150 ^{a}	26.552	0.056
22-42d	2670.100^{b}	2911.900 ^{b}	3424.550 ^{a}	3673.525 ^{a}	132.193	0.001
1-42d	3929.125^{b}	4124.500 ^{b}	4567.300 ^{a}	4862.675 ^{a}	127.891	0.001
Weight Gain (g)						
1-21d	935.743 ^{a}	942.515 ^{a}	892.790 ^{a}	934.517 ^{a}	23.197	0.445
22-42d	1257.043^{b}	1428.200 ^{b}	1772.450 ^{a}	1846.400 ^{a}	67.289	0.000
1-42d	2192.770 ^{c}	2370.715 ^{b}	2665.215 ^{a}	2780.792 ^{a}	62.126	<0.0001
Feed Conversion Ratio (g/g)						
1-21d	1.280 ^{a}	1.280 ^{a}	1.218 ^{b}	1.212 ^{b}	0.010	0.002
22-42d	2.128 ^{a}	2.038 ^{ab}	1.933 ^{c}	1.990 ^{bc}	0.029	0.004
1-42d	1.753 ^{a}	1.708 ^{a}	1.690 ^{a}	1.717 ^{a}	0.015	0.075
Production index	304.300 ^{c}	328.540 ^{b}	379.975 ^{a}	371.300 ^{a}	7.669	<0.0001
Feed price per kg of live weight (Rials/kg)	290395.940^{a}	284171.425^{ab}	268017.565^{c}	277269.745^{b}	2599.929	0.000

T1: Negative control (without injection), T2: Positive control (injection of 0.272 ml of normal saline solution), T3: Injection of 0.0272 mg zinc, T4: Injection of 0.0544 mg zinc.
^{a,b} Means within each row of treatments with no common different superscript differ significantly at P<0.05. (SEM, standard error of means)

Table 5. Effect of *in ovo* injection of different levels of zinc on carcass characteristics of broiler chickens at market weight

Items	T1	T2	T3	T4	SEM	*P-value*
Live body weight (g)	2238.125^{b}	2400.625^{b}	2778.750^{ab}	2890.000^{a}	73.235	0.000
Defeather body weight (g)	2093.875^{b}	2313.750^{b}	2673.750 ^{a}	2795.625^{a}	73.388	<0.0001
Full abdomen carcass weight (g)	1793.125^{b}	1993.750^{b}	2323.125 ^{a}	2367.500^{a}	64.306	<0.0001
Empty abdomen carcass weight (g)	1491.875^{a}	1640.625^{a}	1925.625 ^{a}	1786.250^{a}	135.418	0.183
Breast weight (g)	558.650 ^{b}	589.155 ^{b}	817.775^{a}	741.775^{ab}	58.932	0.026
Relative weight of breast (%)	31.043 ^{a}	25.740 ^{a}	34.838 ^{a}	30.463 ^{a}	2.775	0.199
Drumsticks (thighs) weight (g)	429.907 ^{b}	484.582 ^{b}	546.790^{a}	581.610 ^{a}	15.253	<0.0001
Relative weight of drumsticks (thighs) (%)	24.000 ^{a}	25.565 ^{a}	23.517 ^{a}	23.995 ^{a}	0.627	0.164
Wings weight (g)	120.300 ^{b}	131.610 ^{b}	150.103^{a}	155.173 ^{a}	3.869	0.000
Relative weight of wings (%)	6.710 ^{a}	6.595 ^{a}	6.455 ^{a}	6.405 ^{a}	0.087	0.104
Abdominal fat weight (g)	36.390 ^{b}	35.378 ^{b}	53.850 ^{a}	49.885 ^{a}	6.186	0.129
Relative weight of abdominal fat (%)	2.025 ^{a}	1.765 ^{a}	2.300 ^{a}	2.057 ^{a}	0.250	0.535
Pancreas weight (g)	4.763 ^{a}	5.435 ^{a}	5.445 ^{a}	5.138 ^{a}	0.433	0.657
Relative weight of pancreas (%)	0.263 ^{a}	0.270 ^{a}	0.232 ^{a}	0.215 ^{a}	0.022	0.316
Gizzard (ventriculus) weight (g)	22.390 ^{a}	23.243 ^{a}	23.273 ^{a}	22.735 ^{a}	0.877	0.870
Relative weight of gizzard (ventriculus) (%)	1.255 ^{a}	1.180 ^{ab}	1.003 ^{b}	0.937 ^{b}	0.047	0.002
Heart weight (g)	10.765 ^{a}	10.300 ^{a}	11.048 ^{a}	11.065 ^{a}	0.694	0.780
Relative weight of heart (%)	0.600 ^{a}	0.520 ^{a}	0.505 ^{a}	0.455 ^{a}	0.037	0.100
Proventriculus weight (g)	8.330 ^{a}	9.385 ^{a}	11.683 ^{a}	10.288 ^{a}	0.940	0.131
Relative weight of proventriculus (%)	0.460 ^{a}	0.465 ^{a}	0.495 ^{a}	0.423 ^{a}	0.039	0.645
Crop weight (g)	8.082 ^{a}	8.650 ^{a}	9.910 ^{a}	9.208 ^{a}	0.978	0.605
Relative weight of crop (%)	0.445 ^{a}	0.430 ^{a}	0.430 ^{a}	0.380 ^{a}	0.044	0.747

T1: Negative control (without injection), T2: Positive control (injection of 0.272 ml of normal saline solution), T3: Injection of 0.0272 mg zinc, T4: Injection of 0.0544 mg zinc.
^{a,b} Means within each row of treatments with no common different superscript differ significantly at P<0.05. (SEM, standard error of means)

Table 6. Effect of *in ovo* injection of different levels of zinc on gastro-intestinal characteristics at market weight

Items	T1	T2	T3	T4	SEM	*P-value*
Duodenum weight (g)	15.550^{a}	14.980^{a}	15.942 ^{a}	16.690 ^{a}	1.263	0.809
Relative weight of duodenum (%)	0.875 ^{a}	0.750 ^{ab}	0.670 ^{a}	0.690 ^{a}	0.066	0.177
Jejunum weight (g)	34.063^{a}	41.800^{a}	39.248 ^{a}	39.870 ^{a}	2.700	0.265
Relative weight of jejunum (%)	1.903 ^{ab}	2.073 ^{a}	1.690 ^{a}	1.648 ^{a}	0.118	0.087
Ileum weight (g)	26.770^{a}	30.705^{a}	33.278 ^{a}	34.895 ^{a}	2.627	0.197
Relative weight of ileum (%)	1.483 ^{a}	1.533 ^{a}	1.430 ^{a}	1.435 ^{a}	0.122	0.926
cecum weight (g)	13.588^{a}	10.045^{a}	11.868 ^{a}	12.758 ^{a}	1.017	0.138
Relative weight of cecum (%)	0.768 ^{a}	0.505 ^{b}	0.455 ^{b}	0.528 ^{b}	0.067	0.027

T1: Negative control (without injection), T2: Positive control (injection of 0.272 ml of normal saline solution), T3: Injection of 0.0272 mg zinc, T4: Injection of 0.0544 mg zinc.
^{a,b} Means within each row of treatments with no common different superscript differ significantly at P<0.05. (SEM, standard error of means)

Table 7. Effect of *in ovo* injection of different levels of zinc on blood parameters at market weight

Items	T1	T2	T3	T4	SEM	*P-value*
Glucose (mg/dl)	54.025 ^{b}	62.575 ^{b}	77.225 ^{a}	84.925 ^{a}	3.518	0.000
Total cholesterol (mg/dl)	152.225 ^{a}	139.975 ^{b}	132.100 ^{b}	120.525 ^{c}	2.618	<0.0001
Triglycerides (mg/dl)	140.900 ^{a}	134.075 ^{a}	90.200 ^{b}	86.775 ^{b}	5.415	<0.0001
VLDL (mg/dl)	28.325 ^{a}	27.025 ^{a}	18.075 ^{b}	17.600 ^{b}	1.119	<0.0001
HDL (mg/dl)	42.400 ^{a}	40.200 ^{ab}	39.650 ^{b}	36.900 ^{c}	0.830	0.005
LDL (mg/dl)	72.125 ^{a}	64.825 ^{b}	65.825 ^{b}	57.400 ^{c}	2.018	0.002
LDL/HDL	1.698 ^{a}	1.610 ^{a}	1.663 ^{a}	1.552 ^{a}	0.046	0.184
SGOT (AST) (U/L)	237.250 ^{b}	227.500 ^{b}	232.750 ^{a}	230.000 ^{a}	4.426	0.474
SGPT (ALT) (U/L)	41.950 ^{a}	35.425 ^{b}	37.700 ^{b}	45.200 ^{a}	2.158	0.033
Cholesterol/HDL	3.585 ^{a}	3.483 ^{a}	3.338 ^{bc}	3.265 ^{c}	0.057	0.008
Alkaline phosphate (U/L)	7180.000^{a}	8025.000^{a}	5937.50 ^{b}	6907.50 ^{ab}	402.902	0.024

T1: Negative control (without injection), T2: Positive control (injection of 0.272 ml of normal saline solution), T3: Injection of 0.0272 mg zinc, T4: Injection of 0.0544 mg zinc.
^{a,b} Means within each row of treatments with no common different superscript differ significantly at P<0.05. (SEM, standard error of means)
VLDL: very-low-density lipoprotein, HDL: high-density lipoprotein, LDL: low-density lipoprotein, SGOT: serum glutamic-oxaloacetic transaminase, AST: aspartate amino transferase, SGPT: serum glutamic pyruvic transaminase, ALT: alanine amino transferase.

Table 8. Effect of *in ovo* injection of different levels of zinc on immune parameters at market weight

Items	T1	T2	T3	T4	SEM	*P-value*
Antiseratiter against influenza at 21 days (lg2)	4.500 ^{a}	4.250 ^{a}	3.750 ^{a}	3.500 ^{a}	0.395	0.310
Antisera titer against influenza at 28 days (lg2)	3.000 ^{a}	3.000 ^{a}	3.000 ^{a}	3.250 ^{a}	0.375	0.952
Antisera titer against first injection of Newcastle after7 days (lg2)	4.500 ^{a}	4.500 ^{a}	4.500 ^{a}	4.750 ^{a}	0.402	0.960
Antisera titer against second injection of Newcastle after7 days (lg2)	3.750 ^{a}	3.750 ^{a}	4.000 ^{a}	4.000 ^{a}	0.270	0.835
The number of antibodies produced against sheep red blood cells (TSRBS)	4.50^{ab}	3.500 ^{b}	4.000 ^{a}	4.250 ^{a}	0.375	0.321
Inflation index of the skin of the foot membrane						
Right foot before phytohemagglutinin injection (ml)	18.18^{a}	17.515 ^{a}	4.500 ^{a}	4.750 ^{a}	0.402	0.960
Right foot after phytohemagglutinin injection (ml)	19.79^{a}	19.723 ^{a}	4.000 ^{a}	4.000 ^{a}	0.270	0.835
left foot before phytohemagglutinin injection (ml)	18.25^{a}	17.110 ^{a}	3.750 ^{a}	3.500 ^{a}	0.395	0.310
left foot after phytohemagglutinin injection (ml)	20.72^{a}	20.878 ^{a}	3.000 ^{a}	3.250 ^{a}	0.375	0.952
Thymus weight (g)	3.510 ^{a}	4.573 ^{a}	5.063 ^{a}	5.270 ^{a}	0.775	0.415
Relative weight of thymus (%)	0.195 ^{a}	0.230 ^{a}	0.218 ^{a}	0.220 ^{a}	0.037	0.922
Liver weight (g)	54.26^{a}	53.062 ^{a}	63.585 ^{ab}	70.257 ^{a}	3.960	0.030
Relative weight of liver (%)	2.570 ^{a}	2.355 ^{a}	2.735 ^{a}	2.895 ^{a}	0.264	0.534
Spleen weight (g)	3.000 ^{a}	3.008 ^{a}	3.050 ^{a}	3.370 ^{a}	0.306	0.801
Relative weight of spleen (%)	0.170 ^{a}	0.150 ^{a}	0.133 ^{a}	0.140 ^{a}	0.016	0.406
Bursa of Fabricius weight (g)	1.375 ^{a}	2.460 ^{a}	1.880 ^{a}	2.140 ^{a}	0.352	0.220
Relative weight of bursa of Fabricius (%)	0.075 ^{a}	0.118 ^{a}	0.085 ^{a}	0.083 ^{a}	0.016	0.297

T1: Negative control (without injection), T2: Positive control (injection of 0.272 ml of normal saline solution), T3: Injection of 0.0272 mg zinc, T4: Injection of 0.0544 mg zinc.
^{a,b} Means within each row of treatments with no common different superscript differ significantly at P<0.05. (SEM, standard error of means)

Table 9. Effect of *in ovo* injection of different levels of zinc on caecal microflora (CFU/g) at market weight

Items	T1	T2	T3	T4	SEM	*P-value*
Coliform	9.970[a]	9.670[a]	9.583[a]	9.548[a]	0.199	0.455
Escherichia coli	9.715[a]	9.830[a]	9.705[a]	9.942[a]	0.224	0.860
Lactobacillus sp.	9.583[a]	9.593[a]	9.033[a]	8.640[a]	0.265	0.070

T1: Negative control (without injection), T2: Positive control (injection of 0.272 ml of normal saline solution), T3: Injection of 0.0272 mg zinc, T4: Injection of 0.0544 mg zinc.
[a,b] Means within each row of treatments with no common different superscript differ significantly at P<0.05. (SEM, standard error of means)

Table 10. Effect of *in ovo* injection of different levels of zinc on the mean of meat fatty acids profile of broiler chickens (%)

Items	T1	T2	T3	T4
Myristic acid (C14:0)	0.385	0.45	0.48	0.4
Pentadecanoic acid (C15:0)	0.055	0.085	0.08	0.1
Palmitic acid (C16:0)	24.71	24.44	23.71	23.54
Heptadecanoic acid (C17:0)	0.055	0.065	0.07	0.03
Stearic acid (C18:0)	6.75	6.70	6.54	6.37
Arachidic acid (C20:0)	0.18	0.19	0.18	0.15
Heneicosanoic acid (C21:0)	0.135	0.22	0.2	0.21
Behenic acid or Docosanoic acid (C22:0)	0.185	0.235	0.25	0.16
Myristoleic acid (C14:1)	0.055	0.12	0.1	0.11
Palmitoleic acid (C16:1)	3.88	3.80	3.61	3.83
Heptadecenoic acid (C17:1)	0	0	0.08	0.07
Oleic acid (C18:1, n-9)	38.04	36.43	37.46	36.72
Elaidic acid (C18:1t)	0	0.07	0.06	0.05
Gondoic acid or Eicosenoic acids (C20:1)	0.095	0.175	0.1	0.06
Linoleic acid (C18:2, n-6)	23.04	24.04	24.01	25.18
Linolenic acid (C18:3)	1.59	1.87	1.99	2.05
Dihomo-gamma linoleic acid (C20:3)	0.615	0.915	0.91	0.91

T1: Negative control (without injection), T2: Positive control (injection of 0.272 ml of normal saline solution), T3: Injection of 0.0272 mg zinc, T4: Injection of 0.0544 mg zinc.

Table 11. Effect of *in ovo* injection of different levels of zinc on the mean of zinc consternation in tibia of broiler

Items	T1	T2	T3	T4
Concentration (mg/kg)	149.28	148.9	149.42	150.11

T1: Negative control (without injection), T2: Positive control (injection of 0.272 ml of normal saline solution), T3: Injection of 0.0272 mg zinc, T4: Injection of 0.0544 mg zinc.

DISCUSSION

The time and place of nutrient injection *in ovo* have an effect on the percentage of hatching broiler chickens and performance traits. In the present study there was a significant decrease in the chicken index and a significant increase in the late contamination index in the injected treatments (positive control and zinc). In addition, the absence of significant differences in the weight of one-day-old broiler chickens among the experimental groups indicates that there is no negative effect of the injection pos-hatching. Joshua *et al*. (2016) reported that the *in ovo* injection of zinc, copper and selenium nanoparticles is not harmful for the growth and development of the embryo and does not have a negative effect on the hatching chicken. In the study of Biria *et al* (2020), *in ovo* injection did not have a significant effect on hatching broiler chickens. The effect of in-egg feeding may vary depending on the strain and incubation conditions. The age of the chicken and the size of the egg are also influential in this case. Salmanzadeh *et al*. (2012) reported that with *in ovo* glucose injection, hatchability of broiler chickens decreased and they attributed that to a possible allergic reaction in the air sac and stated that this disorder may have prevented the embryos from breathing. The authors added that this type of allergic reaction occurs less due to the *in ovo* injection of mineral particles.

In the present study, two different levels of organic

zinc (0.0272 mg and 0.0544 mg) were injected into the amniotic sac on the 10th day of incubation, and the results were consistent with the results of other researchers regarding performance indices. Joshua *et al*. (2016) stated that the most significant effect on the feed conversion ratio was observed for nanoparticles of zinc (2.16), copper (2.46) and selenium (2.51) for concentrations of 40, 4 and 0.225 micrograms per egg, respectively. Jose *et al*. (2018) reported that *in ovo* injection of 0.25 and 0.5 mg zinc per egg (zinc sulfate, zinc methionine, and Nano zinc oxide) did not increase bird growth performance after hatching, whereas in the research of Kim *et al*. (2022), higher concentrations of intra egg zinc injection (60 mg per egg) improved bird weight gain. In addition, authors showed that intra egg injection of zinc and supplementation in the diet did not affect the conversion factor, but significantly improved body weight (Kim and Kang, 2022). In a similar study, Biria *et al*. (2020) did not observe a significant difference in weight gain and conversion rate with *in ovo* injection of different concentrations of zinc (50, 75 and 100 ppm) and only in the breeding phase; the amount of feed consumed in the injection treatments increased significantly but in the final growth phase consumption differences were not significant. The effect of injection of zinc on blood parameters results were consistent with those of the study of Biria *et al*. (2020).

Sharideh *et al*. (2016) investigated the effect of four increasing levels of zinc oxide supplementation (40, 80, 120 and 160 mg/kg in the diet) on humoral immune response in broilers and stated that the diet containing 120 mg gram/kg zinc had the best performance in terms of improving growth and feed conversion ratio. The control diet had the lowest while that containing 120 mg/kg of zinc had the highest weight.

Zhu *et al*. (2017) investigated zinc supplementation effect on indices related to hatchability, growth and development of embryos and growth performance under heat stress conditions on breeder flocks and stated that the use of surface 110 mg/kg of zinc sulfate and zinc oxide diet at 32 °C temperature improved weight gain, increased embryo viability, increased chick hatching and improved thigh meat quality in progeny (broiler chickens). Their results were consistent with the present study regarding weight gain and thigh weight.

Functional indices of growth, meat quality and the amount of minerals in the tissues in broiler chickens using three levels (zero, 60 and 120 mg/kg of diet) of zinc and three levels (zero, 150 and 300 mg/kg of kg of diet) alpha-tocopherol acetate were investigated. In general, the level of 120 mg/kg of organic zinc diet along with alpha-tocopherol acetate improved the oxidative stability of breast and thigh muscles and enriched these tissues with zinc element, but it did not have a significant effect on the feed conversion ratio and loss percentage. (Akbari Moghaddam Kakhki *et al*., 2018). In another study, the effect of replacing inorganic zinc with organic zinc on performance factors in broilers was investigated. In that experiment, 400 roosters were placed in two groups with different food rations. The first group had a corn and soybean base diet and 25 mg/kg diet as an inorganic zinc source, while the second group was fed a base diet supplemented with 40 mg/kg organic zinc source. The results showed that the organic zinc source caused more weight gain than the inorganic zinc at the age of 14 to 21 days, and the amount of zinc in the tibia was higher (Mwangi *et al*., 2017).

Changing the source of minerals used in poultry feed can be a useful strategy to improve the growth of embryos and broilers. In research with *in ovo* injection of zinc-methionine in nano and organic forms, they reported that the highest body weight was related to zinc-methionine and nano zinc-methionine treatment (one-day-old chicken weight). Also, the weight of the small intestine and liver in these two treatments was higher than the other treatments on days 1 and 7, but at the end of the sixth week, no significant difference was observed in the weight of the chickens among the treatments. Nano zinc-methionine treatment had higher alkaline phosphatase and maltase activity than other treatments on days 1, 3 and 7, and in addition, the amount of Zn-T1 gene expression was also higher in this treatment than other treatments, as well as the expression of mRNA related to metallothionein. Hepatic and intestinal responses also increased significantly in these treatments (Razani *et al*, 2017).

Peebles *et al*. (2020) studied the effect on body temperature of growing broiler chickens after that had received *in ovo* injection of organic zinc, manganese and copper. Jahanian Najafabadi *et al*., (2008) showed that supplementation with zinc (28-88 mg/kg diet) had no significant effect on body weight, feed intake and conversion rate of broiler chickens. In a similar report, supplementation with zinc-methionine (40, 80 and 120 mg/kg diet) did not have a beneficial effect on body weight in any of the initial and final periods. The possible value for this difference can be

attributed to the difference in the zinc content used and the physicochemical characteristics of the zinc source (Pimentel *et al*., 1991). Hudson *et al*. (2005) and Huang *et al*. (2007) who used 160 mg of zinc sulfate per kg of diet and 20-140 mg of heptahydrate zinc sulfate, respectively, and stated that zinc sources have an effect on the weight gain of chickens. Zakaria *et al*. (2017), reported that the functional response of broiler chickens was similar when fed 80 mg or 122mg of inorganic zinc along with zinc methionine per kilogram of diet. Saenmahayak *et al*. (2010) found that dietary supplementation of zinc, regardless of its source, in an amount of more than 40 mg/kg diet had no effect on the growth of broiler chickens. On the other hand, adding inorganic zinc in the amount of 0-1500 ppm or adding its organic form in the amount of 0-60 ppm or adding a combination of both forms in the diet. Broiler did not affect conversion rate (Kim and Patterson, 2004, Rossi *et al*., 2007).

Zinc is an important factor in the synthesis, secretion and storage of insulin, which can subsequently affect the blood glucose content (Søndergaard *et al*., 2006). It was found that zinc supplementation decreased serum glucose levels (Uyanik *et al*., 2001, Kim and Kang, 2022), which is not consistent with the results of this study and may be due to the form of zinc supplementation (intravenous injection) egg in broilers.

The observed differences in the indicators investigated of eggs of mother hens in this research with those findings of previous studies may be attributed to genetics, age of the mother hens, egg size and/or incubation conditions. The growth and development of newly hatched birds depends on the amount of nutrients remaining in the yolk sac. However, initiation of growth may depend more on post-hatch nutrition than on yolk nutrients.

CONCLUSION

In ovo injection of organic zinc can show favorable results in performance indicators of broiler chickens including feed intake, weight gain and final weight, although it has no effect on immune response and microbial population. On the other hand, the causes of the negative effect on hatching rate should be investigated further to ensure an acceptable economic benefit to the producer.

CONFLICT OF INTEREST

None declared

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