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Effects of *in ovo* Injection of Nano Zinc Oxide on the Hatchability, Immunity and Antioxidant Responses, and Relative Gene Expressions of Interleukin 2 and 12 in Broiler Chickens

Research Article

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ABSTRACT

This experiment was conducted to examine the effects of *in ovo* injection of nano zinc oxide (Nano-ZnO) on hatchability, immunity responses, and performance in broiler chickens. A total of 432 fertilized eggs (Ross 308) were assigned to a completely randomized design with four treatments by four replications and 27 eggs in each replicate. Treatments included: 1) negative control (no injection), 2) positive control- no treatments with injection of solutions (20 µL saline), 3) 20 µL of Nano-ZnO (0.5 mg of Nano-ZnO in 50 mL saline), and 4) 20 µL of Nano-ZnO (0.6 mg of Nano-ZnO in 50 mL saline). Results showed that hatchability (P=0.002) and weight of hatched chicks (P=0.0001) decreased in treatments two and three compared to negative control. However, embryonic mortality were increased in early (≤7 d) and late (17-21 d) stages in comparison with the negative control (P=0.008). Fourteen days after vaccination, antibody titers of avian Influenza (P=0.025) and Newcastle (P=0.002) disease were increased in chickens that received Nano-ZnO compared to control groups. Relative gene expression levels of interleukin-2 (IL-2) (P=0.002) and 12 (P<0.05) were greater in treatment 3 compared to other treatments. Although *in ovo* injection of Nano-ZnO reduced hatchability at first glance, this was the result of injection, but it did not reduce the hatchability and chickens' weight compared to positive control. *In ovo* injection of Nano-ZnO increased embryonic mortality, and improved humoral and cellular immune responses and weight of the spleen, which indicates the positive effects of injections and confirms the hypothesis of the study.

KEY WORDS broiler chick, immune responses, *in ovo* injection, nano zinc oxide, performance.

INTRODUCTION

Zinc plays an important role in many vital functions of the body (Suttle, 2010). Zinc requirements in poultry influenced with increasing in growth and production or presence of limiting factors in the diet in zinc stores in the body, therefore, zinc storage in body can easily be reduced. Its deficiency in breeders reduces hatch rates, increases mortality, and decreases chicken growth (Rossi *et al*. 2007). Addition of zinc to the diet of breeders increases fertility and egg production, and decreases mortality in various embryonic stages (Favero *et al*. 2013). Nutrition plays a key role during embryonic period through affecting the development and survival of birds; hence, lack of attention to it causes delays and decreases growth leading to economic loss. Suitable nutrition is crucial for breeders to ensure adequate nutrient transfer to eggs and nutrient supply for natural embryonic growth. In adequate or excessive nutrients in eggs cause embryonic death. Therefore, properly implemented chick's nutrition programs can improve production and

reproductive performance. The content of minerals in the yolk significantly was reported to reduce during the incubation of broiler embryos resulting in complications of nutritional deficiencies (Yair and Uni, 2011).

Nano particles have higher surface area to volume ratio than larger particles, thus, rendering a higher absorption capacity. These materials are rapidly distributed to most organs and tissues after injection into the body, with a very high cellular absorption (Salata, 2004). Nanoparticles can bypass conventional physiological ways of nutrient distribution and transport across tissue and cell membranes, as well as protect compounds against destruction prior to reaching their targets. *In ovo* administration of nanoparticles, may be seen as a new method of nano-nutrition, providing embryos with an additional quantity of nutrients.

Based on the research hypothesis, an experiment was conducted to evaluate the effects of *in ovo* enjection of Nano-ZnO on the hatchability, immune responses, oxidative enzymes, and relative genes expression levels of interleukin 2 and 12 in broiler chickens.

MATERIALS AND METHODS

Experimental design and diets

The present study was approved by the Animal Care Committee of the Sari Agricultural Sciences and Natural Resources University (SANRU), northern Iran. A total of 432 fertile eggs (Ross 308) was assigned to a completely randomized design with four treatments and four replications with 27 eggs in each replicate. Treatments included: 1) negative control (no injection), 2) positive control (20 µL saline), 3) 20 µL Nano-ZnO (0.5 mg of Nano-ZnO in 50 mL saline), and 4) 20 µL Nano-ZnO (0.6 mg of Nano-ZnO in 50 mL saline). In order to investigate the effects of different Nano-ZnO concentrations on the immunity and performance of broilers, nine male chicks from each replicate were randomly selected and assigned to four experimental groups with four replicates for 42 days. All birds were housed in pens and received feed and water *ad libitum*. The diet was formulated according to Ross 308 broiler nutrition specification handbook (Hosseini *et al*. 2016) (Table 1).

In ovo injection

On the 10th day of the experiment, *in ovo* injection was performed into the air sac. To this end, all the injection areas were disinfected with 70% ethanol. Then a hole was then punched using a 21-gauge needle and then 20 µL of (*in ovo* injection) solution was injected into the air sac using a 22-gauge needle. After injection, the injection area was sealed with paraffin tape and the eggs transferred to hatching baskets.

Hatch characteristics and embryonic mortality

On hatching days, the number of chicks was counted in each replicate to calculate the hatchability percentage. Then, the embryonic mortality, and fertile and infertile eggs were examined by breaking the unhatched eggs. All infertile eggs were opened and examined macroscopically for evidence of embryonic mortality. All unhatched eggs were analyzed for developmental stage of dead embryos. The time of embryonic death was assigned to one of four categories: early dead (≤7 d), mid-dead (8-16 d), late dead (17-21 d), and pips (Cobb, 2001). The percentage of embryonic mortality and hatching percentage were expressed based on the eggs placed into the incubator. The percentage of fertile eggs and efficiency of converting eggs to chick were presented as described by (Joshua *et al*. 2016).

Breeding and feeding

On hatching days, nine chicks were randomly selected from each replicate and transferred to the experimental pens for rearing. A control corn-soybean meal based diet formulated according to Ross308 broiler nutrition specification to meet all nutrient requirements (Table 1).

General immunity

In order to investigate the immune response, vaccination against Newcastle disease and influenza were carried out in 28 d of the experiment. On days 35 and 42 after vaccination, two chickens from each treatment were randomly selected and blood samples (3 mL) were collected from the left brachial vein. The presence of Newcastle and influenza antibodies in sera samples was assessed by hem agglutination inhibition (HI) method (Poorghasemi *et al*. 2015).

Cell-mediated immunity

An inter-digital skin test was made to evaluate the cutaneous basophil hypersensitivity (CBH) at 28-d of age as described by (Corrier and Deloach, 1990).

RNA isolation and cDNA synthesis

On the first day of breeding, two chicks were selected from each replicate and the spleen samples were collected, washed in sterile phosphate buffer saline, and immediately snap frozen in liquid nitrogen. Samples were stored at -80 ˚C until RNA extraction. Total RNA was isolated from spleen samples using the RNXTM-Plus solution (CinnaGen, Tehran, Iran) according to the manufacturer's instructions, and stored at -80 ˚C pending cDNA synthesis. For each spleen sample, cDNA synthesis was carried out using the Omni script reverse transcriptase kit (Qiagen Inc., Tehran, Iran). The cDNA synthesis reaction conditions were 42 ˚C for 30 min and 95 ˚C for 3 min.

Table 1 Chemical compositions and nutrient contents of the experimental diet

Ingredients (%)	Starter	Grower	Finisher
	0-10 d	11-24 d	25-42 d
Corn grain	47.12	50.62	55.87
Soybean meal	43.78	40.25	34.66
Soybean oil	4.37	5.25	5.82
DCP	1.74	1.50	1.40
Limestone	1.10	1.03	0.95
Mineral premix[1]	0.25	0.25	0.25
Vitamin premix[2]	0.25	0.25	0.25
Salt	0.23	0.24	0.24
$NaHCO_3$	0.15	0.15	0.15
DL-Met	0.33	0.27	0.24
L-Lys	0.21	0.12	0.14
L-Theronine	0.07	0.04	0.03
Calculate feed analysis			
Metabolizable energy (kcal)*	2950	3050	3150
Crude protein (%)	22.62	21.15	19.20
Ca (%)	0.94	0.85	0.78
Av. P (%)	0.47	0.42	0.39
Lys (%)	1.42	1.26	1.14
Met + Cys (%)	1.06	0.97	0.89
Theronine (%)	0.95	0.86	0.77

[1] Composition (per kg): Mn: 75000 mg; Fe: 50000 mg; Cu: 8,000 mg; I: 750 mg and Se: 60000 µg.
[2] Composition (per kg): vitamin A: 8000 IU; vitamin D_3: 2000 IU; vitamin K_3: 1800 mg; vitamin B_1: 1800 mg; vitamin B_2: 6000 mg; vitamin B_6: 2800 mg; vitamin B_{12}: 12000 µg; Pantothenic acid: 10000 mg; Niacin: 40000 mg; Folic acid: 1000 mg and Biotin: 0.3 mg/kg.
* Metabolisable energy (ME) was calculated according to Bourdillon *et al*. (1990).

Quantitative real-time PCR (qPCR)

Quantitative real-time polymerase chain reaction (qPCR) was implemented to determine the relative transcripts of interleukin 2 (IL2) and interleukin 12 (IL12) in the spleen tissue. Details of primer sequences are provided in Table 2. Expression of beta-actin transcript was used as an internal housekeeping gene. All transcripts were quantified using Quanti Fast SYBR Green PCR Kit (Cat. No. 204052; Qiagen, GmbH, Germany) in a 20 µL reaction volume containing 1 µL of single-strand cDNA, 10 µL of master mix, 0.5 µL of each forward and reverse primers, and 8 µL of distilled H_2O in 20 µL by Rotor-Gene 6000 real-time PCR software (Corbett Research, Sydney, Australia). The program used for the amplification of genes consisted of a denaturing cycle of 5 minutes at 95 ˚C, followed by 40 cycles (95 ˚C for 15 seconds, annealing, and extension at 60 ˚C for 40 seconds). At the end of each PCR, a melting curve analysis was performed at arate of 0.1˚C/s for all genes to check the specificity of the products. Standard curves of each primer pair efficiency were determined with five series of 10-fold dilution of positive control cDNA as a template.

The efficiency of the assays (E) was ≥ 95%, and standard curve R^2 was ≥ 0.999. The relative levels of mRNA were analyzed by the $2^{-\Delta\Delta C}{}_t$ method (Livak, 2001).

Gene expressions of interleukin 2 and 12

On the first day of breeding, two chicks were selected from each replicate and the spleen samples were collected, washed in sterile phosphate buffer saline, and immediately snap frozen in liquid nitrogen. Samples were stored at -80 ˚C until RNA extraction. Total RNA was extracted from spleen samples using the RNXTM-Plus solution (Cinna Gen, Tehran, Iran) according to the manufacturer's instruction sand was then reverse transcribed in the presence of 1 µM oligo-primer and 4 U Omni script RTase (Qiagen Inc., Tehran, Iran). Real-time PCR reactions were carried out in a total volume of 20 ml with 1 ml of complementary DNA (50 ng/mL), 10 mL of SYBR green master mix (Quanti-Nova SYBR Green PCR Kit; Qiagen Inc), 0.5 mL of forward and 0.5 mL reverse primers (20 ng of each), and 8 Ml of nuclease-free H_2O. Samples were run in duplicate and expressed relative to β-actin as housekeeping gene, which was stable under the culture conditions used.

Table 2 Primer sequences used for real-time quantitative PCR[1]

Gene[2]	Primer sequence (5′-3′)3	Annealing temperature (˚C)	Fragment size (bp)	GenBank accession number
IL-2	F:CCCGTGGCTAACTAATCTGCTG	66.1	287	HE608819
	R:TGAGACACCAGTGGGAAACAGT	60.2		
IL-12	F:GCCGACTGAGATGTTCCTGG	61.4	227	JN942590
	R:CCTTGCTTTTGTATTTCTTTGTGC	57.7		
β -actin	F:AGCCAACAGAGAGAAGATGACAC	60.0	134	L08165.1
	R:CATCACCAGAGTCCATCACAATA			

[1] The listed oligonucleotides were used to analyze gene expression via real-time quantitative PCR.
[2] IL-2: interleukin 2 and IL-12: Interleukin 12.

Data were normalized to a calibrator sample using the ΔCt method with correction for amplification efficiency (Livak, 2001).

Carcass quality

Chickens were slaughtered at 42 days of age and the weight of individual carcass components was evaluated and calculated as the percentage of each carcass component.

Antioxidant enzyme activities

In order to evaluate the antioxidant enzyme activity, two samples from each replicate were randomly selected on hatching days and blood samples were taken from their hearts and collected in plastic tubes containing ethylenediaminetetraacetic acid (EDTA). Samples were maintained on ice until plasma was separated by centrifugation (1500×g for 20 minutes) within 1h of collection. Plasma was harvested and stored at -20 ˚C until further analysis. Concentrations of glutathione peroxidase (GPX) and superoxide dismutase (SOD) were determined using commercial RANSEL and RANSOD kits (RANDOX Laboratories Ltd., London, UK) according to the manufacturer's instructions.

Statistical analysis

This research was conducted in a completely randomized design with four treatments, four replicates, and 27 observations per replication using the Generalized Linear model (GLM) procedure of SAS (2003) software. Differences among treatment means were measured by Duncan's multiple range test and considered significant at $P < 0.05$. Gene expression fold changes, standard error, and statistical significance were calculated by the software based on the formula developed by (Pfaffl and Hageleit, 2001). Data were normalized to a calibrator sample using the ΔC t method with correction for amplification efficiency (Livak, 2001). A P-value of less than 0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

Data of hatchability, embryonic mortality, and relative chick weights are presented in Table 3. The highest andlowest values for hatchability and fertile hatchability were obtained in T1 and T4, respectively. *In ovo* injection of 20 µg of Nano-ZnO (T4) resulted in increased embryonic mortality in an early stage. The results showed that *in ovo* injection reduced hatchability and increased early and late embryonic mortality compared to the negative control ($P<0.05$), but this difference was not significant compared to the positive control (T2).

Titers against influenza and Newcastle disease viruses measured at 35 and 42-d after vaccination are presented in Table 4. There were no significant differences in influenza and Newcastle antibody titers between treatments ($P<0.05$) seven days after vaccination. However, both antibody titers increased significantly 14 days after vaccination, indicating that Nano-ZnO increased blood immunity. The data showed that cutaneous basophil hypersensitivity was not affected by the treatments (Table 4).

The relative gene expression levels of interleukin 2 and 12 are presented (Table 5) in T3 were significantly higher than those of other treatments ($P<0.05$). Also, increasing doses of injection T4 reduced IL-2 and 12 gene expressions compared to T3, indicating that T3 has the most appropriate concentration of Nano-ZnO to increase the expression of IL 2 and 12. The concentrations of antioxidant enzymes (SOD and GPX) and the percentages of organs that involved in immunity response (spleen, bursa, and liver) are presented in Table 6. Table 7 shows that SOD and GPX activities were not influenced by the treatments. Mean concentrations of GPX and SOD in plasma was not influenced by the treatments, but the spleen relative weight increased in T4 ($P=0.023$).

The percentages of carcass components are shown (Table 7) did not differ between treatments ($P>0.05$). Adding Nano-ZnO to the diet did not affect ($P>0.05$) body weight and feed conversion ratio, but feed intake decreased in all treatments compared to T1 (Table 8) ($P=0.030$).

In the current study, *in ovo* injection of Nano-ZnO reduced percentage of hatchability, but it is not only reason for the increased embryo mortality, because *in ovo* injection of saline also increased embryo mortality in this stage.

Table 3 Effects of *in ovo* injection of zinc oxidnano particles on hatchability, embryonic mortality, and body weight on the 1thd and relative body weight of broiler chickens

Treatments	Hatchability (%)	Fertile hatchability (%)	Embryonic mortality (%)			BW (g)	Relative weight (%)*
			Early	Mid	Late		
T1	90.7[a]	98.0[a]	1.04[b]	0.000	0.93[c]	41.2[a]	63.5[a]
T2	58.3[b]	66.3[b]	16.9[a]	1.04	15.7[ab]	38.7[c]	60.2[b]
T3	60.1[b]	66.6[b]	9.55[ab]	1.96	21.8[a]	39.8[b]	61.5[ab]
T4	63.8[b]	69.4[b]	19.7[a]	2.97	7.79[c]	39.8[b]	61.8[ab]
P-value	0.002	0.003	0.008	0.18	0.008	0.0001	0.026
SEM	1.95	2.04	1.67	0.45	1.83	0.005	0.324

T1: no injection; T2: injection of 20 µL of saline; T3: injection of 20 µL of Nano-ZnO (0.5 g/mL of Nano-ZnO in 50 mL of saline) and T4: injection of 20 µL of Nano-ZnO (0.6 g/mL of Nano-ZnO in 50 mL of saline).
BW: body weight.
* (chicken weight / egg weight) × 100.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 4 Effects of *in ovo* injection of zinc oxidnano particles on humoral [anti-log_2] and cellular immunity responses

Treatments	Antibody titers[1]					
	AI35 (log_2)[*]	AI42 (log_2)[†]	ND35 (log_2)[#]	ND42 (log_2)[§]	CBH (mm)12[‡]	CBH (mm)24[¥]
T1	1.75	2.00[b]	1.75	2.75[b]	0.09	0.08
T2	1.75	2.25[b]	2.00	2.50[b]	0.28	0.05
T3	1.50	2.50[b]	1.75	4.75[a]	0.13	0.09
T4	1.75	3.75[a]	2.00	3.50[b]	0.17	0.11
P-value	0.87	0.025	0.83	0.002	0.26	0.55
SEM	0.13	0.18	0.13	0.16	0.033	0.015

[1] Antibody titers against avian influenza [log_2] after 7 days (*), and 14 days (†) of vaccination. Antibody titers against Newcastle disease [log_2] after 7 days (#) and 14 days (§) of vaccination. Cutaneous basophil hypersensitivity after12 h (‡) and 24 h (¥). Hypersensitivity test after 24 hours.
T1: no injection; T2: injection of 20 µL of saline; T3: injection of 20 µL of Nano-ZnO (0.5 g/mL of Nano-ZnO in 50 mL of saline) and T4: injection of 20 µL of Nano-ZnO (0.6 g/mL of Nano-ZnO in 50 mL of saline).
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 5 Effects of *in ovo* injection of zinc oxidenanoparticles on relative gene expressions of interleukin-2 and 12

Treatments	IL-2	IL-12
T1	1.03[b]	1.05[b]
T2	0.17[b]	0.08[b]
T3	4.62[a]	2.45[a]
T4	1.27[b]	1.49[a]
P-value	0.002	0.001
SEM	0.31	0.079

T1: no injection; T2: injection of 20 µL of saline; T3: injection of 20 µL of Nano-ZnO (0.5 g/mL of Nano-ZnO in 50 mL of saline) and T4: injection of 20 µL of Nano-ZnO (0.6 g/mL of Nano-ZnO in 50 mL of saline).
IL-2: interleukin 2 and IL-12: Interleukin 12.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 6 Effects of *in ovo* injection of zinc oxidenanoparticles on concentrations of antioxidant enzymes and relative immunity organs of broiler chickens

Treatments	Antioxidant enzymes		Spleen (%)	Bursa (%)	Liver (%)
	SOD (u/mL)	GPX (u/grH)			
T1	151	50.2	0.09[b]	0.19	0.02
T2	142	45.9	0.08[b]	0.18	1.93
T3	150	54.9	0.09[b]	0.22	1.59
T4	147	42.3	0.13[a]	0.22	2.05
P-value	0.24	0.64	0.02	0.39	0.10
SEM	1.60	0.63	0.005	0.009	0.066

T1: no injection; T2: injection of 20 µL of saline; T3: injection of 20 µL of Nano-ZnO (0.5 g/mL of Nano-ZnO in 50 mL of saline) and T4: injection of 20 µL of Nano-ZnO (0.6 g/mL of Nano-ZnO in 50 mL of saline).
SOD: super oxide dismutase and GPX: glutathion peroxidase.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 7 Effect of *in ovo* injection of zinc oxidenanoparticles on relative weight of carcass components

Treatments	Heart (%)	Abdominal fat (%)	Gizzard (%)	Gallbladder (%)	Thigh (%)	Breast (%)
T1	0.39	0.07	1.48[a]	0.10	19.9	24.7
T2	0.43	1.53	1.12[b]	0.09	19.7	26.6
T3	0.41	1.57	1.15[b]	0.09	18.7	27.9
T4	0.51	1.19	1.22[b]	0.11	19.4	28.5
P-value	0.086	0.28	0.061	0.66	0.59	0.11
SEM	0.015	0.10	0.045	0.006	0.32	0.54

T1: no injection; T2: injection of 20 µL of saline; T3: injection of 20 µL of Nano-ZnO (0.5 g/mL of Nano-ZnO in 50 mL of saline) and T4: injection of 20 µL of Nano-ZnO (0.6 g/mL of Nano-ZnO in 50 mL of saline).
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 8 Effect of *in ovo* injection of zinc oxidenanoparticles on chickens' performance at 42 days of age

Treatments	Body weight (g)	Feed intake (g)	Feed conversion ratio
T1	1687	2963[a]	1.75
T2	1677	2865[b]	1.71
T3	1652	2889[b]	1.75
T4	1693	2877[b]	1.70
P-value	0.78	0.030	0.56
SEM	15.2	10.8	0.017

T1: no injection; T2: injection of 20 µL of saline; T3: injection of 20 µL of Nano-ZnO (0.5 g/mL of Nano-ZnO in 50 mL of saline) and T4: injection of 20 µL of Nano-ZnO (0.6 g/mL of Nano-ZnO in 50 mL of saline).
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Hence many of reasons related to drop in hatchability such as type of injection; location and time of injection is well visible. But hatchability was not significantly different in the treatments administrated with Nano-ZnO. El-Rayes *et al*. (2019) has been reported that, *in ovo* zinc injection at a proper level has no adverse effect on developing chicken embryo or hatchability. Meanwhile, high levels of zinc showed a reduction in the hatchability and this may be attributed to the imbalance of amnion minerals content that interfered with embryogenesis during the late incubation or the toxicity of zinc nano-form due to its high availability (Jose *et al*. 2018).

The amount of Nano-ZnO and injection site might be the factors that increased mortality. Injection of nutrients into the air sac reduces hatchability and leads to the sensitivity of the chorioallantois membrane, interruption of respiration, and embryonic death (Ohta *et al*. 1999). Salmanzadeh *et al*. (2012) was conducted an experiment with glucose injection, the results of this experiment showed that hatchability had been decreased. They assumed that a lower hatchability of *in ovo* injection of glucose attributed to a possible allergic reaction in air sac that prevents the respiration of the embryo, however, it must be confirmed that such type of allergic reaction occurs with regard to *in ovo* injection of nanoparticles of trace minerals.

Biria *et al*. (2020) indicated that *in ovo* injection of nano ZnO particles had positive effects on early embryo mortality rate in broiler chicken.

Optimal consumption of Zn is very important in the development and maintenance of the immune system and its related cells. Sunder *et al*. (2008) reported that cellular and hum oral immune responses in broilers supplemented with 80 mg/kg of Zn were significantly higher than those received lower levels of Zn. By activating the thymus, Nano-ZnO causes maturation of T lymphocytes and increases the functioning of B lymphocyte leading to secretion of macrophages. Interleukin 2 is a type of cytokine signaling molecule in the immune system that stimulates the growth and differentiation of T lymphocytes and plays a vital role in the functions of B lymphocyte, natural killer cells (NK cells), macrophages, and oligodendrocytes (Kidd, 2003). Interleukin 12 is one of the most important cytokines that improves cellular immunity to respond to viral infections (Takei *et al*. 2008). Immunologists' believe that interleukin 12 is a key point to create a balance between Thelper-1 and Thelper-2 (Romani, 2008). The toxicity of nano-selenium is 7 times lower than that of inorganic selenium and 3 times lower than that of organic selenium (Peng *et al*. 2007). Trace minerals are important nutritional components for imparting immunity and *in ovo* enrichment can be a way for improving the immune system of the birds (Hassan, 2018). El-Bahr *et al*. (2020) reported that inclusion of ZnO-NPs, particularly 60 mg/kg, in the diet of Japanese quails to improve antioxidant and immune status.

In the current study, the relative expression levels of IL-2 and IL-12 genes in T3 were significantly higher than other treatments. Obminska-Mrukowicz and Szczypka, (2005) reported that the use of sodium di-ethyldithiocarbamate with zinc sulfate in human increased interleukin 2, and subsequently promoted bodily immunity, which is the same as

the current research. Goel *et al.* (2012) reported that injection of Zn (0.5 mg) into eggs caused a significant increase in interleukin 2 and 12 concentrations. Prasad *et al.* (1988) reported that secretion of interleukin-12 was affected by various concentrations of Zn. Similarly, increasing doses of injection in T4 reduced IL-2 and 12 gene expressions compared to T3 in here, suggesting that T3 has the most appropriate concentration of Nano-ZnO to increase the expressions of ILs 2 and 12. Goel *et al.* (2012) stated that *in ovo* injection of Nano-ZnO could increase cellular immunity and attributed this to the role of zinc in the proliferation and differentiation of lymphocytes and increased expression of IL-2 gene. A bird fed with zinc-rich diet was reported to have more active macrophages than a bird fed with Zn-poor diet (Kidd, 2003). As zinc plays an important role in the structure of transcriptional enzymes (DNA polymerase and RNA polymerase), it probably increases gene expression of interleukin 2 receptors as a result of increased secretion of interleukin 2. Jose *et al.* (2018) reported that Nano Zn-administered group showed a non-significant down regulation of *MUC2* gene.

Fathi *et al.* (2016) reported that SOD activity in the serum increased in broiler chickens received 20 mg/kg of Nano-ZnO. Zinc is a cofactor and involved in more than 240 enzymes and can influence oxidative processes. Zinc is also necessary for the structure and function of Cu-Zn-SOD, which contains 90 percent of the total SOD and protects tissues from the oxidative lesion (Noor *et al.* 2002). In agreement with our findings, Liu *et al.* (2015) reported that dietary supplemental Zn increased Cu-Zn-SOD activities in the breast and thigh muscles of broilers. These different results might be due to the different ways of Zn injection and Zn-adequate eggs used in the present study. Another insignificant reason that can be that the enzyme concentration was evaluated on the hatch day, which might have been better to be evaluated at the middle or end of the experiment as the relative weights of the spleen and the liver were affected by Nano Zn oxide injection at these two time points.

Shokraneh *et al.* (2020) reported that injection of Nano-Se and Nano-ZnO increased antioxidant activity and reduced oxidative stress in broiler hatchlings. It has been shown that Zn sources can substantially enhance antioxidant capacity and resistance against oxidative stress in developing embryos and hatchlings (Zhang *et al.* 2018). Zhu *et al.* (2017) also indicated that maternal dietary zinc could protect chick embryos against maternal heat stress by elevating antioxidant capacity. Oxidative stress induced upregulation of SOD-1 gene expression, which is usually followed by upregulation of the expression of CAT and GPX genes (Ahmad *et al.* 2012).

The significant upregulation of antioxidant enzymes because of ZnO-NP supplementation has been confirmed in broilers (Ahmad *et al.* 2012). Nanoparticles have been reported to be more efficient for animals than larger particles at low doses due to the easy absorption and biological availability as well as appear to interact better with other materials due to the significance of the active surface (El-Basuini *et al.* 2017).

Liu *et al.* (2011) examined the effect of different zinc supplementation levels on carcass characteristics and meat quality in broiler chickens and reported that various levels of zinc increased breast weight. Sahin *et al.* (2005) stated that dietary supplementation with zinc sulphate could increase carcass weight in Japanese quail. MyoD expression may increase in the breast tissue as in the embryonic tissue and possibly, zinc has a direct effect on the expression of this gene. Liu *et al.* (2011) stated that addition of zinc in the diet reduced breast and leg fats of broiler chickens, which is not in agreement with the results of this research. In the present study adding Nano-ZnO to the diet did not affect ($P>0.05$) body weight and feed convert ratio (FCR), which may be related to the amount or the form of Nano-ZnO injection, which is consistent with previous studies (Sunder *et al.* 2008). Zinc deficiency in poultry causes weight loss, therefore, dietary supplementation with zinc often causes a positive response in the performance of broiler chicks (Blamberg *et al.* 1960). Łukasiewicz *et al.* (2020) reported an absence of effects on dressing percentage and the carcass content of breast muscle and giblets at 42 days. Several reports show enhanced growth performance (Fathi *et al.* 2016; Sahoo *et al.* 2016; Hassan, 2018), but others show no effects of Zn nanoparticles on BW (Asheer, 2017; Bami *et al.* 2018). After absorption in the intestine, nanoparticles can enter the bloodstream and be stored in various organs and be distributed independently of the blood circulation (Anjum *et al.* 2016). Badawi *et al.* (2017) also indicated that the chickens supplemented with NanoZnO had a higher body gain and better feed conversion rate (FCR) than birds without Zn supplement.

CONCLUSION

As a conclusion, our findings showed that although *in ovo* injection of Nano-ZnO reduced hatchability at first glance, this was the result of injection effect, because it did not reduce the hatchability and chickens' weight compared to positive control. *In ovo* injection of 20 µl of Nano-ZnO increased embryo mortality, and improved humoral and cellular immune responses and weight of the spleen, which indicates the positive effects of injections and confirms the hypothesis of the study.

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