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The Effect of *in ovo* Supplementation of Nano Zinc Oxide Particles on Hatchability and Post-Hatch Immune System of Broiler Chicken

Research Article

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ABSTRACT

The aim of this study was to evaluate the effects of *in ovo* injection of zinc oxide nanoparticles on the hatchability, production performance and immune responses of broilers. A total of 192 fertile eggs obtained from a Ross 308 broiler breeder flock were used. The eggs were divided into 4 experimental groups including a positive control group with normal saline injection, and injection of 50, 75 and 100 ppm nano-ZnO on the first day of incubation period in the air cell and were placed in the incubator. The hatched chicks were allocated to a completely randomized design with 4 treatments and 4 replicates for a 42-day experiment. *In ovo* injection of nano-ZnO increased hatchability of eggs compared to the control group ($P<0.05$). The daily feed intake during the starter phase significantly increased in the birds in 50 and 75 ppm nano-ZnO groups ($P<0.05$). At the grower phase in rearing period the growth rate and feed intake of all the nano-ZnO injected groups were higher than the control group ($P<0.05$). There was no significant difference in the feed conversion ratio between different treatment groups ($P>0.05$). The results of antibody titer against the influenza virus at 10 days of age did not differ between the experimental treatments ($P>0.05$). The heterophil, lymphocytes, monocytes and hematocrit number in the blood of broiler chickens at 10 days of age did not show any significant difference ($P>0.05$). The results of the present study suggest that *in ovo* injection of nano ZnO particles had positive effects on early embryo mortality rate, total white blood cells, however the performance traits of the hatched chicks were not significantly affected through *in ovo* injection of nano ZnO particles.

KEY WORDS antibody titer, broiler, hatchability, immune system, *in ovo* injection, nanoparticles of zinc, performance.

INTRODUCTION

In ovo injection of nanoparticles, could be a new tool of nano-feeding, providing embryos with a supplementary amount of nutrients. *In ovo* injection method designed by Uni and Ferket (2004), safely transfer external nutrients into fertile eggs. At the final steps of embryogenesis, nutrients transferred into the amniotic fluid are then ingested, and absorbed by the developing embryo (Uni *et al*. 2005). The increased nutrient requirement of the fast-growing embryo, particularly at the late embryogenesis, could make *in ovo* method beneficial to birds. *In ovo* injection of nutrients could improve the performance of chicks through enhancing digestive capacity, decreasing post-hatch mortality, improved immune system characteristics and improvement in skeletal and muscle development (Ferket, 2011). There are limited mineral reserves in yolk and this may leave the embryo with insufficient mineral supply at the end of the incubation period (Yair and Uni, 2011) and cause metabolic disorders in fast-growing embryos of broiler chickens (Angel, 2007). Zinc has a well-known role in the development of the immune system of the chicks (Kidd, 2003) and

dietary zinc supplementation improve the antibody synthesis (Cardoso *et al*. 2007) and performance of non-specific immunity system for instance neutrophils and natural killer cells (Shankar and Prasad, 1998) in broilers. By the 10th day of chicken embryonic development, the first signs of the immune system are occurred. On days 11 of incubation, T cells have appeared and B cell differentiation is carried out after the 15th day of incubation. By the 18th day of incubation, the chicken embryo is capable of show innate and adaptive responses to pathogens (Davison, 2003; Ribatti, 2010). The nanotechnology significantly increases the surface to volume ratio, physical activity and chemical stability in nanoparticles which leads to obvious changes in the physical and chemical characteristics (Joshua *et al.* 2016). The nanoparticles can penetrate into the cell membranes and tissues, improve nutrient supply across tissues, and keep nutrients, safe against damages before reaching the target cells (Joshua *et al.* 2016). This study was designed to examine the effect of *in ovo* injection of nano-forms of zinc oxide on the hatchability and post-hatch immune responses in broiler chicken.

MATERIALS AND METHODS

In this research, ZnO nanoparticles were synthesized by sol-gel method as described by Mahdavi and Ashrafi Talash (2017). Briefly, zinc oxide sol was produced by adding 0.15 molar zinc acetate into methanol. Then, the homogeneous solution the mixture was stirred for 90 minutes by a magnetic stirrer. Diaphanous sol changed to white color by adding of 1.5 molar sodium hydroxide and pH of solution was tuned to 10. The milky white solution was stirred again for 60 minutes. In the next step the sol was centrifuged for 20 minutes at 10000 rpm. A two-step washing with a 60-40 mixture of ethanol and water was used to remove whole organic materials from the surface. The residue was dried and calcined for 2 h at 60 ˚C and 200 ˚C, respectively, and a white color powder was obtained (Mahdavi and Ashrafi Talash, 2017).

First, a pre-test carried out to find the effect of the dosage of nano-ZnO *in ovo* injection on the egg's hatchability. In most of the previous reports, *in ovo* injection has been done on the 18th day of the incubation period, when eggs are moved from setter to hatcher machine, however, because the development of the immune system in chicken embryo begins before this time and on the other hand the mineral compounds are stable substances, in this experiment it was decided to inject the eggs on the first day of the incubation.

The results showed that the dosages more than 100 ppm can adversely affect the hatchability of eggs and there was no difference in the hatchability of eggs injected with normal saline and the non-injected eggs.

In the main study, the effect *in ovo* injection of nano zinc oxide at three graded levels, 50, 75 and 100 ppm in Ross 308 broiler breeder (46 week age, 3240-3365 g live weight range) fertile eggs (average weight of 62.9 g) were tested. The room thempretaure for *in ovo* injections was 18 to 20 ˚C with 75 to 80% humidity. Because of the compareable hatchability observed for normal saline injected eggs and the non injected eggs in the pre-test, only a control group with *in ovo* injection of normal saline as the positive control group was included in the main experiment. The weight of each egg was recorded and eggs randomly allocated into experimental groups, to have comparable average weights for all the experimental groups. A total of 192 fertile eggs from Ross 308 broiler breeder flock were disinfected using ethyl alcohol (70% purity). In each experimental group, 48 eggs were set in the incubator in three replicates (16 eggs each).

The eggs were incubated at 37.7 ˚C and relative humidity of 70-75% from day 0 to 18 and for day 19 to 21, the incubator re-adjusted on 37.2 ˚C and 75-82% relative humidity and the eggs were set in a horizontal position. On the first day of incubation, a small hole was made in eggshell on the broad end of the egg to remove the eggshell by using a sharp dental instrument as egg driller and *in ovo* injection was done in the air cell using a 22G hypodermic needle (20 mm long) and melted paraffin was used to block the pore.

The unhatched eggs were broken to determine the embryo mortality during the incubation period and the step of embryo mortality was determined based on fetal growth according to the guidelines of Aviagen (2014). The hatchability (survival rate) of experimental groups, post-hatch performance, and immune responses were recorded. Because of the different hatchability rate of experiment, the post-hatch section of the experiment was done using unequal chick number for each experimental group and the survived chickens of each treatment were divided into three replicates.

The ethical committee of the University of Mohaghegh Ardabili approved the procedure of the experiment. The chicks were reared for 6 weeks. The diets were similar for all treatments. The diets were formulated using the WUFFDA software for starter (1 to 10 days), grower (11 to 24 days) and finisher (25 to 42 days) phases of rearing period, based on the nutrient requirements of Ross 308 broilers. The trace mineral premix used in the experimental diet formulation was contained the normal Zn dosage because inducing a Zn deficiency was not in the aims of the present study. The components and chemical analysis of the diets are presented in Table 1.

At 12, 24 and 42 d of age, body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) of chickens from each pen were calculated for feeding period.

Table 1 The ingredients and the chemical composition of the basal experimental diet

Ingredient (g/kg)	Starter (0-10 days)	Grower (11-24 days)	Finisher (24-42 days)
Corn	483.3	508.6	541.1
Soybean meal	425.2	393.4	353.1
Soybean oil	46.7	57.1	66.4
Dicalcium phosphate	18.7	17.2	16.5
Limestone	11.0	10.0	9.3
Vitamin and mineral premix[1]	5	5	5
Methionine	3.7	3.1	3.0
Lysine	2.3	1.5	1.5
Salt	3.6	3.6	3.6
Salinomycin 12%	0.5	0.5	0.5
Calculated analysis			
Metabolizable energy (kcal/kg)	3000	3100	3200
Crude protein	230	215	200
Calcium	9.8	9.0	8.5
Available phosphorus	4.9	4.5	4.3
Na	1.6	1.6	1.6
Lysine	14.4	13.0	12.0
Methionine	7.1	6.0	1.6
Methionine + cystine	10.8	9.9	9.4

[1] Supplied per kilogram of diet: vitamin A: 900 IU; vitamin D_3: 2000 IU; vitamin E: 18 IU; vitamin K_3: 2 mg; Thiamine: 1.8 mg; Riboflavin: 6.6 mg; Pyridoxine: 3 mg; Cobalamin: 15 µg; Niacin: 30 mg; Pantothenic acid: 10 mg; Biotin: 0.1 mg; Folic acid: 1.25 mg; Choline chloride: 200 mg; Fe: 50 mg; Cu: 10 mg; Mn: 100 mg; Zn: 85 mg; I: 0.8: mg and Se: 0.2 mg.

FI and FCR were all corrected for mortality rate. At 35 d of the experiment, two series blood samples were taken from the wing vein of two birds per replicate, following approximately eight hours fasting.

The first samples were collected into vials containing ethylenediaminetetraacetic acid (EDTA) and centrifuged for 10 min at 3000 × g, then plasma was collected and stored at −20 ˚C until analysis. The serum samples were used to measure the serum biochemical parameters and antibody titer against the Newcastle disease virus (NDV) and avian influenza virus (AIV) by hemagglutination inhibition test according to the International Epizootic Office (OIE) recommendation. The blood samples with EDTA anticoagulant were used to determine the number of white blood cells (WBC). One hundred white blood cells were counted to record the differential WBC, and to determine the H/L ratio.

The concentrations of triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL) and low-density lipoprotein cholesterol (LDL) in serum samples were analyzed with an automatic biochemical analyzer (HITACHI912. Japan), following the instructions of the corresponding reagent kit (Audit Diagnostics Co. Ireland). Statistical analysis was carried out using the procedure general linear models (GLM), SAS 9.1 statistical software (SAS, 2003) and the means were compared using Duncan's multiple range test at the 5% level.

RESULTS AND DISCUSSION

Figure 1 demonstrates the X-ray diffraction pattern of synthesized ZnO nanoparticles. The XRD results showed a good harmony with Hexagonal structure base on.The effect of *in ovo* injection of zinc oxide nanoparticles on embryo mortality and hatchability of incubating eggs is shown in Table 2. The mortality rates in the initial days (0-11 days) in all three nano ZnO injected groups were significantly lower than the control group ($P<0.05$), but there was no significant differences in days 11-18 and 19-21 of the incubation period. There was no significant difference in hatchability of eggs injected with different levels of zinc oxide nanoparticles. Joshua *et al* (2016) have reported that *in ovo* injection of nano form of zinc oxide at 20, 40, 60 and 80 µg/egg, were not influenced the hatchability. As a matter of fact the hatchability rates obtained in present research were more than 20% less than that previous report by Bakyaraj *et al*. (2012) and Joshua *et al*. (2016). The effect of *in ovo* feeding may vary depending on the strain, breeder hen age, egg size, and incubation conditions (Uni and Ferket, 2004). Salmanzadeh (2012) found a lower hatchability on *in ovo* injection of glucose and attributed it 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.

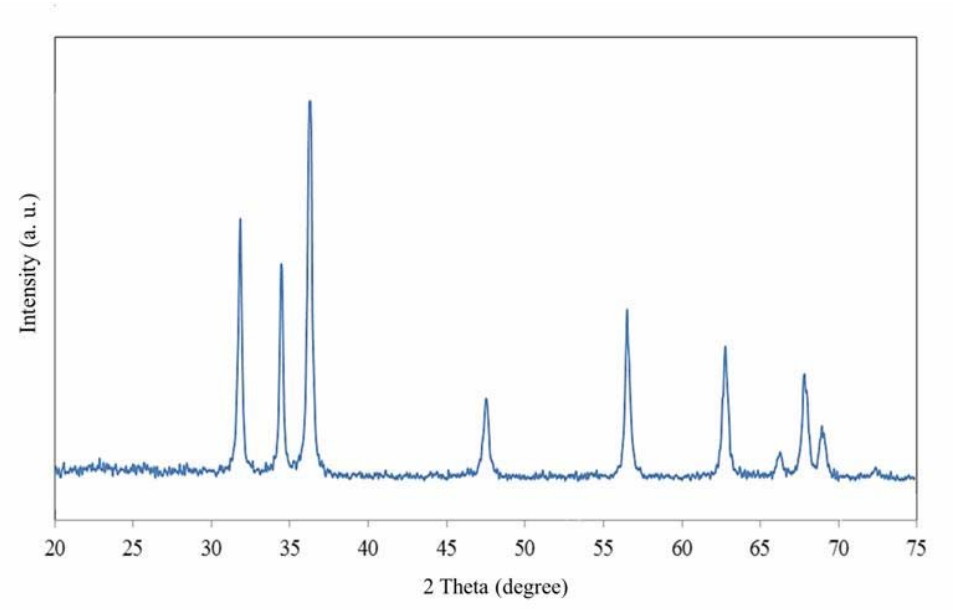

Figure 1 X-ray diffraction pattern of the ZnO nanoparticles

Table 2 Effect of different levels of *in ovo* injection of zinc oxide nanoparticles on the embryo mortality and hatchability of broiler breeder eggs

The level of *in ovo* nano-ZnO injection	Embryo mortality at different periods of incubation %			Hatchability %
	0-11 d	11-18 d	18-21 d	
Control	21.26[a]	11.02	8.91	58.81
50 ppm	16.87[b]	13.65	7.89	61.59
75 ppm	15.91[b]	15.05	8.13	62.91
100 ppm	16.57[b]	16.62	6.49	60.32
SEM	1.65	4.35	3.76	2.54
P-value	0.01	0.72	0.32	0.82

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.

The effect of *in ovo* injection of zinc oxide nanoparticles on the performance of broilers is presented in Table 3. The daily feed intake in the starter phase for birds from 50 and 75 ppm nano zinc oxide groups were higher than the control group.

No significant variation (P>0.05) observed in the daily weight gain and feed conversion ratio of the control chicks and the chicks received graded levels of nano form of zinc oxide. In the grower phase, higher daily feed intake was observed in the nano zinc oxide treated groups than the control group (P<0.05). The nano ZnO inclusion also resulted in the best daily weight gain in the grower phase (P<0.05), however, no significant variation (P>0.05) existed in the feed conversion ratio. No significant variation existed in daily feed intake, daily weight gain and feed conversion ratio between control and graded levels of nano form of zinc oxide during the finisher phase and whole experimental period (P>0.05). There were no significant difference in mortality rate of chicks hatched from the *in ovo* injected eggs.

Joshua *et al*. (2016) found a better body weight gain and feed efficiency in broiler chickens hatched from *in ovo* injected of nano ZnO particles. Bakyaraj et al. (2012) also found no difference in the feed conversion ratio of *in ovo* Zn injected chicks. Reports of dietary supplementation of zinc on the performance of broiler chickens confirmed its beneficial effects. Huang *et al*. (2007) used zero, 20, 40, 60, 80, 100, 120 and 140 mg zinc per kg of broiler chicken feed; while the base diet contains 28 mg of zinc. They found that dietary zinc supplementation improved daily gain and daily feed intake, but did not affect the feed conversion ratio. Hegazy and Adachi (2000) and Huang *et al.* (2007) also reported positive effects of dietary zinc supplementation on performance of broiler chickens, however, in the studies of Pimental *et al.* (1991) and Sunder *et al.* (2008) no positive effect was found.

Zn deficiency reduces animal appetite and growth rate (Ensminger *et al.* 1990). Studies have shown that zinc-deficient embryos have abnormal body skeletons (Van Campen and Scaife, 1967; Selling *et al.* 1975).

Table 3 Effect of *in ovo* feeding of broiler eggs with nano form of zinc oxide at graded levels on feed intake, weight gain and feed conversion ratio of broilers (0-6 weeks)

Item	Control	50 ppm nano-ZnO	75 ppm nano-ZnO	100 ppm nano-ZnO	SEM	P-value
Feed intake (g/bird/d)						
Starter	17.55[b]	21.46[a]	21.52[a]	17.80[b]	0.67	0.01
Grower	60.63[b]	89.40[a]	87.54[a]	87.91[a]	2.12	0.0001
Finisher	165.82	143.84	141.89	146.50	7.10	0.36
Total	95.45	96.55	95.11	96.32	3.15	0.76
Weight gain (g/bird/d)						
Starter	13.95	15.56	15.26	14.40	0.91	0.58
Grower	36.20[b]	53.91[a]	52.91[a]	56.75[a]	2.62	0.0005
Finisher	87.27	75.79	82.63	81.53	4.82	0.57
Total	52.78	54.15	56.68	57.28	2.23	0.19
Feed conversion						
Starter	1.25	1.39	1.42	1.25	0.07	0.26
Grower	1.67	166	165	1.57	0.06	0.58
Finisher	1.90	1.90	1.72	1.79	0.08	0.37
Total	1.80	1.79	1.68	1.68	0.058	0.18
Mortality rate % (1-42 d)						
Total	7.08	6.75	5.31	6.90	0.07	0.33

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Emmert and Baker (1995) stated that the onset of symptoms of zinc deficiency (Zn) is mainly affected by previous Zn nutrition or zinc (Zn) reserves of the bird body. In birds previously fed with marginal levels of zinc (Zn), deficiency symptoms occur after 5 days, while in birds that have received more zinc supplement, the symptoms occur at least after 8 days.

The effect of *in ovo* feeding of broiler eggs with a nano form of zinc oxide at graded levels of Immune system responses of broilers is presented in Table 4. The lowest antibody titer against NDV was observed in the control group compared to nano zinc oxide treated groups (P<0.05). The 50 ppm nano zinc oxide inclusion level also resulted in the higher NDV titer than the 75 and 100 nano zinc oxide groups (P<0.05). No significant variation existed in the serum antibody level against influenza virus between the control and graded levels of nano form of zinc oxide studied (P>0.05). The highest total white blood cells were observed in nano zinc oxide treated birds than the control group (P<0.05), however, the heterophil, lymphocyte, monocyte count and hematocrit percentage were comparable between experimental groups.

NRC (1994) has recommended 40 Zn ppm in broiler chicken's diet, which seems just considered the growth performance (Burrell *et al.* 2004). However, higher levels of dietary zinc (60 to 180 ppm) have resulted in better immunity to broiler chicks (Bartlett and Smith, 2003). Zinc affects T lymphocytes and is involved in non-covalent reactions of cytoplasmic components with tyrosine kinase, an essential protein in the early stages of lymphocyte activity (Walsh *et al.* 1994).

This effect is due to improving the production and function of lymphocytes, thereby increasing the titer and improving the immune response. When the bird survives after Newcastle disease, the antibody against the virus can be measured in serum after 6 to 10 days and its amount depends on the virus strain.

Zinc deficiency has been shown to increase susceptibility to infectious diseases, which indicates the importance of zinc in the immune system. It has been shown that a zinc deficiency can lead to a defect at various levels of the host defense from the first defensive barrier of the body, the skin to the humoral and cellular immunity (Walsh *et al.* 1994).

Sunder *et al.* (2008) reported that cellular and hemorrhagic immunity in broiler chickens that received 80 mg/kg zinc or more was significantly higher than in chickens fed less than 80 mg/kg of zinc supplement. Zinc is required for metabolic function and the activity of three hundred enzymes in the body (Prasad and Kucuk, 2002) and its deficiency in the diet increases the oxidation damage of the cell membrane due to the free radicals (Prasad and Kucuk, 2002; Oteiza *et al.* 1996).

Table 5 shows the effect of *in ovo* feeding of broiler eggs with a nano form of zinc oxide at graded levels on blood lipid fractions. Among the various graded levels of inclusion of a nano form of zinc oxide, 75 ppm/egg inclusion reduced the serum triglyceride (TG) level and the 100 ppm/egg increased the serum cholesterol concentration (P<0.05).

The serum LDL and HDL concentrations were higher in 75 and 100 ppm/egg inclusion level of nano form of zinc oxide (P<0.05).

Table 4 Effect of *in ovo* feeding of broiler eggs with nano form of zinc oxide at graded levels on antibody titers against Newcastle and influenza viruses and total ($\times 10^3/mm^3$) and differential (%) leukocyte count in 35-d-old chicks

Item	Antibody titer against (log_2)		Total white blood cells	Monocytes	Heterophyles	Lymphocytes	Hematocrit (%)
	NDV	AIV					
Control	6.75[a]	3.25	23.33[b]	1.66	43.00	55.33	36.16
50 ppm	6.00[b]	2.75	32.35[a]	1.75	42.75	55.50	30.87
75 ppm	5.75[bc]	3.50	31.07[a]	2.00	39.50	57.50	37.55
100 ppm	5.25[c]	3.00	33.90[a]	2.25	38.75	59.00	34.40
SEM	0.21	0.42	2.33	0.43	4.49	4.59	2.60
P-value	0.003	0.63	0.06	0.79	0.88	0.93	0.35

NDV: Newcastle disease virus and AIV: Influenza disease virus.
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 Effect of *in ovo* feeding of broiler eggs with nano form of zinc oxide at graded levels of blood lipoproteins in broilers (10 d of age, mg/dL)

Item	TG	Cholesterol	LDL	HDL
Control	125.0[a]	191.7[c]	19.6[b]	166.3[b]
50 ppm	94.3[bc]	150.5[d]	13.3[c]	138.0[c]
75 ppm	85.1[c]	212.3[b]	28.2[a]	190.5[a]
100 ppm	98.6[b]	231.0[a]	30.8[a]	206.4[a]
SEM	3.40	5.3	1.6	7.4
P-value	0.04	0.04	0.04	0.02

TG: triglyceride; LDL: low density lipoprotein and HDL: high density lipoprotein.
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.

Zinc supplementation reduces blood glucose and cholesterol concentrations in broiler chicks under thermal stress conditions (Kucuk *et al.* 2003). Onderci *et al.* (2003) reported that 30 mg/kg of zinc sulfate supplement reduces serum malondialdehyde (MDA) concentrations. Zinc also produces metallothionein, which is an effective agent to control hydroxyl radicals (Oteiza *et al.* 1996).

CONCLUSION

In ovo feeding of nanoforms of zinc oxide does not harm the developing embryo and does not affect hatchability. The results suggest that *in ovo* injection of nano ZnO particles had positive effects on early embryo mortality rate, total white blood cells, however the performance traits of the hatched chicks were not significantly affected through *in ovo* injection of nano ZnO particles. However, further many advanced researchers are required to explore further beneficial effects and safety of nanoforms of minerals.

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