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Effect of *in ovo* Injection of Calcium Carbonate Nanoparticles on Bone Post Hatched Characteristics and Broiler Chicken Performance

Research Article

J. Salary[1], H.R. Hemati Matin[2], K. Ghafari[3] and H. Hajati[4*]

[1] Department of Animal Science, Faculty of Agriculture, Bu Ali Sina University, Hamedan, Iran
[2] Department of Animal Science, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran
[3] Department of Animal Veterinary, Ferdowsi University of Mashhad, Mashhad, Iran
[4] Department of Animal Science, Faculty of Agriculture and Natural Resources, University of Tehran, Karaj, Iran

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*Correspondence E-mail: h.hajati@ut.ac.ir

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ABSTRACT

This experiment was conducted to evaluate the effect of *in ovo* injection (IOI) of calcium carbonate nanoparticles (CCN) on post-hatch bone and blood parameters and performance of broiler chickens. Fertile eggs (n=400) were distributed into 4 groups of 100 eggs. On the first day of incubation, 2 groups were injected with 0.5 mL of either 100 or 200 ppm CCN/egg dissolved in normal saline and two groups were used as sham control (injected with 0.5 mL normal saline) or un-injected control. The hatched chicks from each group were randomly assigned to 4 replications of 15 chickens and reared under standard condition. The results demonstrated that body weight, spleen weight, and bone Ca concentration of hatched chickens was higher by IOI of 200 ppm/egg CCN ($P<0.05$). The highest bursa weight was obtained by IOI of 200 ppm/egg CCN ($P<0.05$). The IOI of CCN at levels of 100 or 200 ppm/egg was significantly increased plasma alkaline phosphatase activity and bone Cu concentration of hatched chickens ($P<0.05$). Moreover, IOI had no significant effects on performance at 1 to 21 days of age ($P<0.05$). These preliminary results suggest that growth and development of bone may accelerate through IOI of CCN at levels of 100 or 200 ppm/egg on 1-d of incubation.

KEY WORDS broiler performance, calcium carbonate nanoparticles, *in ovo*.

INTRODUCTION

Modern broiler lines are intensively selected for a higher growth rate and increased size of muscles (Petracci and Cavani, 2012) which a good bone structure is need. This leads to an enhanced requirement of chicken embryos for various nutrients, and consequently the imbalance between requirement and reserves of nutrients stored within eggs may limit maximal growth and development of chicken embryos. Growth and development of bone are mainly programmed during embryogenesis. Calcium (Ca) is a critical mineral which its vital roles (e.g. bone formation, blood coagulation, and nervous system) in the body are obvious (Nordin *et al*. 1997). The major portion of Ca (almost 99%) is located in the bones (Pearson and Dutson, 1992; Bourrin *et al*. 2002). The mineralization of bone is a complicated process which needs certain Ca ions in body fluid. Triggering of bone mineralization needs adequate Ca to formation of the first structure of bone crystals (Dziedzic-Gocławska, 1995). On the other hand, Ca deficiency in poultry leads to leg broken in growth period and thereby reductions in consumed meat quality occurred (Blake and Fogelman, 2002). Moreover, leg broken leads to decreased feed intake (FI) and leads to considerable reductions in final body weight (Orban *et al*. 1999). It has been demonstrated that one possibility to supply embryos with extra nutrients could be *in*

ovo injection (IOI) of nutrients (Uni *et al*. 2005; Salary *et al*. 2014). Nutrient supplementation by IOI is more efficient when a compound is attached to nanoparticles, which deliver it inside the body tissues and cells (Zielinska *et al*. 2011; Zielinska *et al*. 2012; Sawosz *et al*. 2012). It is possible that IOI calcium carbonate nanoparticles (CCN) can affect availability and absorption of Ca in embryo and help to bone formation pre and post-embryonic life of birds. Therefore, the present study was conducted to test this hypothesis in broiler chickens.

MATERIALS AND METHODS

The Animal Ethics Committee of the Agricultural Research Center of Qom-Iran has approved the experiment.

Eggs incubation and injection

Fertile eggs (n=400) form the broiler breeders flock (35 weeks old Ross 308, average egg weight, 57.7 g, and production percentage, 83%) were allocated to 4 groups of 100 eggs. On the first day of incubation, 4 groups were injected with 100 or 200 ppm/egg CCN (Daru Pakhsh Company**,** Iran), sham control (IOI of 0.5 mL normal saline serum/egg) or un-injected control using 25 mm needle (Bhanja *et al*. 2004). Sterile normal saline were used as sham control. The injections were carried out under laminar flow system, where temperature of the chamber was maintained at 37 ˚C for avoiding any temperature stress for chicken embryo. Prior to IOI, the site of injection was disinfected with 70% ethanol and the solutions were warmed to 30 ˚C. The injected eggs were returned to the incubator after injection. Within 20 min, IOI of each treatment was completed. Immediately after the injection, the pinhole site was sealed with sterile paraffin wax and eggs were returned to the incubator. On the 19th day of incubation, the eggs were shifted to the hatcher and kept in the respective pedigree hatching boxes. On the day of hatch, chickens were weighted and hatching percentage was recorded.

Bird management and feeding

The 1-d-old chickens were evenly distributed into the same treatment groups with 4 replicates of 15 chickens per replicate. All chickens were reared under similar managerial and hygienic conditions for three weeks. The chickens were raised in clean, well-ventilated, previously disinfected room. The lighting schedule was 23 h light/1 h darkness and 32 ˚C on the first day. This was subsequently reduced 3˚C each week until the end of the third week and then maintained at 23 ˚C. The chickens were fed a basal diet containing 23.00 g CP/kg and 12800 kj metabolizable energy/kg to meet the nutrients requirements (NRC, 1994). Mash diets and fresh water offered *ad libitum*. Weight gain (WG) and FI were measured weekly and cumulatively and feed conversion ratio (FCR) was calculated accordingly.

Parameter measurements

On the day of hatch, two birds were randomly selected, weighed, slaughtered from each replicate and bursa, spleen, yolk sac and right tibia were removed. The weight of bursa, spleen, and yolk sac were recorded. Moreover, the blood of selected birds was taken. After overnight clotting of blood samples at 4 ˚C, the samples were centrifuged (1000×g for 20 min). The separated serum was transferred to a laboratory and serum alkaline phosphatase (ALP) activity was measured using commercial diagnostic kits (Biosystem-EN ISO 13485, Spain). The collected right tibias were boiled for 2 min, the surrounding meat and cartilaginous caps were removed. The bones were dried in a forced-air oven for 24 h at 105 ˚C and weighed. All tibias were ether extracted for 12 h extraction before ashing in a muffle furnace at 480 ˚C for 16 h. The mineral contents (Ca, P, and Cu) of the tibia bone samples were determined by ICP (Integra XL GBC, USA).

Statistical analysis

All data were analyzed for normal distribution using the normal option of the univariate procedure of GLM procedure of SAS software (SAS, 2008). Pen was used as the experimental unit and data were analyzed as a completely randomized design by the GLM procedure of SAS software (SAS, 2008). Statistical differences were evaluated by using a Duncan's multiple range test at the level of $P < 0.05$.

RESULTS AND DISCUSSION

Table 1 and 2 show that body weight and spleen weight of hatched chickens was higher in IOI of 200 ppm/egg CCN ($P<0.05$). Moreover, the highest bursa weight was obtained by IOI of 200 ppm/egg CCN ($P<0.05$). Table 3 show that IOI of CCN at levels of 100 or 200 ppm/egg increased plasma alkaline phosphatase (ALP) activity and bone Cu concentration of hatched chickens rather both controls ($P<0.05$), whereas the higher bone Ca concentration of hatched chickens was related to IOI of 200 ppm/egg CCN ($P<0.05$). Broiler chicken performance is present at Table 4. The results indicated that treatments had no significant effects on broiler chicken performance during 1 to 21 days of age ($P<0.05$).

The hatchability, the weight of fertile eggs, and body weight of hatched chickens are influenced by the type of injected substance and site of injection of nutrients into the eggs (Salary *et al*. 2014). The results indicated that IOI of CCN did not point to any negative effects on the hatchability percentage.

Table 1 Effect of *in ovo* injection of calcium carbonate nano-particles (CCN) on post-hatch measured parameters

Parameters	Un-injected control	Sham control	CCN, 100 ppm/egg	CCN, 200 ppm/egg	SEM	P-value
Body weight (g)	38.00^{bc}	35.70^{c}	45.17^{ab}	49.20^{a}	1.33	0.02
Hatchability (%)	74.79	67.45	72.11	66.73	4.59	0.79
Egg weight (g)	62.05	60.90	65.30	64.37	2.26	0.76
Body weight/egg weight	71.91	72.43	79.07	75.85	3.26	0.78

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.

Table 2 Effect of *in ovo* injection of calcium carbonate nano-particles (CCN) on weight (g) of immunological organs of broiler chickens

Parameters	Un-injected control	Sham control	CCN, 100 ppm/egg	CCN, 200 ppm/egg	SEM	P-value
Bursa of fabricius	0.12^{b}	0.11^{b}	0.14^{b}	0.19^{a}	0.01	0.01
Spleen	0.04^{bc}	0.03^{c}	0.06^{ab}	0.07^{a}	0.01	0.02
Yolk sac	3.34	3.64	3.63	4.11	0.27	0.61

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.

Table 3 Effect of *in ovo* injection of calcium carbonate nano-particles (CCN) on serum alkaline phosphatase (mg/dL) and bone mineralization (mg/100 g bone) of broiler chickens

Parameters	Un-injected control	Sham control	CCN, 100 ppm/egg	CCN, 200 ppm/egg	SEM	P-value
Alkaline phosphatase (ALP)	581.11^{b}	539.09^{c}	667.12^{a}	690.08^{a}	4.67	< 0.0001
Ca	9.14b^{c}	8.39^{c}	13.15^{ab}	15.65^{a}	0.82	0.031
P	3.75	3.60	5.14	5.60	0.33	0.086
Cu	4.85^{b}	4.72^{b}	6.78^{a}	7.72^{a}	0.28	0.014

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.

Table 4 Effect of *in ovo* injection of calcium carbonate nano-particles (CCN) on broiler chicken performance at 1 to 21 days of age

Parameters	Un-injected control	Sham control	CCN, 100 ppm/egg	CCN, 200 ppm/egg	SEM	P-value
Feed intake (FI) (g/d per bird)	61.20	61.42	66.49	66.13	1.22	0.25
Weight gain (WG) (g/d per bird)	38.94	39.60	42.20	45.59	1.17	0.17
Feed conversion ratio (FCR)	1.57	1.56	1.58	1.46	0.02	0.17

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.

Therefore, it can deduce that IOI of CCN is safe and had no adverse effects on hatchability percentage in broiler. However, exogenous CCN injection on the first day of incubation increased body weight of hatched chickens. It assumed that nanoparticles could cross the inner membrane and pass into the developing embryos. This assumption was based on previous investigations showing that different nanoparticles, when injected at the beginning of incubation, are affecting molecular responses and muscle development measured at the end of embryogenesis (Zielinska *et al*. 2011; Zielinska *et al*. 2012; Grodzik *et al*. 2013). Thus, the obtained higher body weight of hatched chickens in the current study may be due to the effect of tested nanoparticles on availability of nutrients and muscle development as well as its antimicrobial properties. It is confirmed by the other researchers (Sawosz *et al*. 2012; Grodzik *et al*. 2013).

Alkaline phosphatase plays important role in ossifying and calcification (Kim *et al*. 2008). In addition, Cu is essential mineral to construct of collagen (Libby and Aikawa, 2002) and to improve reactionary of bone (Gralak *et al*. 2004).

The increase in serum ALP activity is associated with accumulation of right tibia Ca and Cu contents in the present study. It is reported that layer diaphysis of bone developed in the first days of incubation (Hamburger and Hamilton, 1951), thus IOI of CCN at the first day of incubation is exactly the fast formation time of bone. On the other hand, nanotechnology improves the rate of drug absorption (Douroumis and Fahr, 2006; Chen *et al*. 2008; Merisko-Liversidge and Liversidge, 2008). Hereof, nanonized pearl powder showed higher availability in adults (Chen *et al*. 2008) and nano-calcium-enriched milk increased excretion of Ca in rats (Park *et al*. 2008).

Therefore, it is possible that CCN facilitate mineral uptake thereby raise enzyme activity and help bone forming of chicken. The use of CCN as IOI evoked an increase in bursa and spleen weights. The explanation of founded observations are difficult because of no similar study are exist in this regards.

No significant differences were found in FI, WG, and FCR by treatments which are in agreement with the previous study with IOI of vitamin E (Salary *et al*. 2014).

They declared that the degree of response to IOI depends on genetics, breeder hen age, egg size, and incubation conditions. The development of the neonatal birds is dependent on residual nutrients found in the yolk sac that have been depleted during the hatching process (Uni and Ferket, 2004). It is thought that the residual yolk is sufficient to maintain the bird until feed is offered. However, the initiation of growth may be more dependent on post-hatch feed consumption than the nutrients found in the yolk post-hatch (Nir and Levanon, 1993). Therefore, although IOI of CCN increased hatchability and bone accumulation of minerals, but offering of similar diets for all treatments lead to similar performance.

CONCLUSION

It is demonstrated that IOI of CCN at concentration of either 100 or 200 ppm/egg on 1-d of incubation had no negative effects on hatchability and embryo development. Furthermore, the main pillars (ALP activity and the concentration of Ca and Cu) of bone development enhanced by infusion of Ca source as nanoparticles in neonatal.

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