G.J.A.S.R

Global Journal of Animal Scientific Research

Journal homepage: www.gjasr.com

Print ISSN:2345-4377 *Online ISSN:2345-4385*

Original Article

Response of Broiler Chicks to *in ovo* Injection of Calcium, Phosphorus, and Vitamin D Complex (CaDPhos)

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ARTICLE INFO

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How to Cite this Article:
Ghobadi, N., and Hemati Matin, H.R (2015). Response of Broiler Chicks to *in ovo* Injection of Calcium, Phosphorus, and Vitamin D Complex (CaDPhos). *Global Journal of Animal Scientific Research*, 3(2), 544-549.

Article History:
Received: 9 May 2015
Revised: 11 May 2015
Accepted: 13 May 2015

ABSTRACT

The effect of *in ovo* injection (IOI) of calcium, phosphorus, and vitamin D complex (CaDPhos) was studied on post-hatch bone parameters and broiler chick's performance. Fertilized eggs (n=480) were distributed into 4 groups of 120 eggs. On 1st day of incubation, two groups were used as sham control (injected with 0.5 mL physiology serum) (T1) and un-injected control (T2). The other 2 groups were injected with 0.5 mL of 50 or 100% concentration CaDPhos complex/egg (T3 and T4, respectively). The hatched chicks from each group were randomly assigned to 4 replications of 20 chicks and reared similarly. The hatchability percentage and body weight of hatched chicks was greater for T4 compared with other treatments ($P<0.05$), although had no significant differences with T3. Moreover, alkaline phosphatase activity was greater for T4 chicks at d 1 and for T3 and T4 at d 42 compared to other treatments ($P<0.05$). The broiler chicks with IOI of CaDPhos complex have significantly higher bone dry matter, P, and Cu concentration at d 1, as well as bone Ca and P concentrations at d 21 rather both controls ($P<0.05$), whereas, the lowest bone dry matter was obtained for T2 ($P<0.05$). The results indicated that T3 and T4 increased feed intake of broiler chicks from d 1 to 21 ($P<0.05$). These preliminary results suggest that growth and maturation of bone cells may be accelerated through IOI of 50% or 100% concentration CaDPhos complex /egg on 1-d of incubation.

Keywords: Bone, Broiler, CaDPhos complex, *In ovo*.

INTRODUCTION

Modern broiler lines are intensively selected for higher growth rate and increased size of muscles (Petracci and Cavani, 2012), which need good formation of bone structure. 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. Several approaches toward finding remedies for bone related conditions due to the rapid growth rate of broilers have been explored (Bello *et al*., 2014b).

Calcium (Ca) is the most abundant mineral in the body of animals (McDonald *et al*., 1995; Ayasan and Okan, 1999). Well over 90% of Ca is found in the bones where it combines with phosphorus (P), the second most abundant mineral in bone, to form calcium phosphate crystals or hydroxyapatite (Scott *et al*., 1982; Bello *et al*., 2014b). They, also, have vital roles over bone formation in the body (McDonald *et al*., 1995; Nordin *et al*., 1997). Cholecalciferol (vitamin D3) is absorbed from the diet or synthesized in the skin from dehydrocholesterol (de Matos, 2008) and is required in avian species for proper bone and eggshell mineralization (Bello *et al*., 2014b). Vitamin D, Ca, and P have close interlock with each other. Vitamin D and several of its carbon-1-α-hydroxylated derivatives have been shown to improve P uptake in chickens and rats. The mechanism(s) by which are as yet unclear; however, several theories have been suggested [e.g. vitamin D stimulate Ca-dependant P pump in cells lining the intestine and vitamin D acts as a P transport hormone stimulating P transport in many tissues in the body including the intestine (Shim *et al*., 2008)]. Therefore, the mineralization of bone is complicated process which needs certain Ca and P ions as well as vitamin D in body fluid to formation of first structure of bone crystals (Dziedzic-Gocławska, 1995). On the other hand, Ca, P, or vitamin D deficiency in broilers leads to many problems in growth period and thereby reductions in consumed meat quality were occurred (Blake and Fogelman, 2002; Bennett, 2008).

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). In this regards IOI of 25-hydroxycholecalciferol improved bone development (Bello *et al*., 2014a). It is suggested that a single IOI of a nutrients at egg transfer may adequately provide all the benefits that the dietary supplementation of nutrients provides (Bello *et al*., 2014a). On the other hand, it is hypothesized that IOI of multi-nutrient supplementation could be more efficient rather individually. So, IOI of a complex of Ca, P, and vitamin D might affect availability and absorption of Ca, P along with vitamin D in embryo and help bone formation during pre and post embryonic life of birds. Therefore, the present study was conducted to test this hypothesis in broiler chicks.

MATERIALS AND METHODS

Eggs Incubation and Injection

A total of 480 fertilized eggs, form the broiler breeders flock (Ross 308; broiler breeders age, 40 weeks, first cycle of production, average egg weight, 61.5 g, and production percentage, 79%) maintained on the breeders adequate nutritional plan were collected, weighed and distributed into 4 groups of 120 eggs each. On the 1st day of incubation, two groups were used as sham control (injected with 0.5 mL physiological serum) (T1) and un-injected control (T2). The other 2 groups were injected with 0.5 mL of 50 or 100% calcium, phosphorus, and vitamin D complex (CaDPhos) (Mahdeamin Group Company**,** Iran) per egg (T3 and T4, respectively) using 25 mm needle as the standardized method (Bhanja *et al*., 2004). Sterile physiological serum was included as sham control, primarily to rule out a possible negative response caused by the stress of injection and handling. 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. Each treatment of IOI was completed within 20 min out from incubator. 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, eggs were shifted to the hatchery and kept in the respective pedigree hatching boxes. On the day of hatching, chicks were weighed and hatching percentage was recorded.

Bird Management and Feeding

The 1-d-old chicks were evenly distributed into the same treatment groups with 4 replicates of 20 chicks per replicate. All chicks were reared under similar managerial and hygienic conditions for 6 weeks. The chicks were raised in clean, well-ventilated, previously disinfected room. The lighting schedule was 23 h light / 1 h darkness at 32°C at the first day. This was subsequently reduced 3°C each week until the

end of the third week, then after was constant. The chicks were fed a basal diet to meet the nutrient requirements formulated by UFFDA software based on NRC (1994) (Table 1). Mash diets and fresh water offered *ad libitum*. Weight gain (WG) and feed intake (FI) were measured weekly and feed conversion ratio (FCR) was calculated accordingly.

Table 1: Diet composition at different periods of the experiment

Ingredient (% or as stated)	1 to 21 d	22 to 42 d
Corn grain	59.15	60.67
Soybean meal (42% CP)	34.28	34.11
Soybean oil	1.66	1.29
Limestone	1.64	1.36
Dicalcium phosphate	1.88	1.61
Premix[2]	0.50	0.50
Common salt	0.33	0.33
$_{DL}$-Met	0.29	0.12
$_{L}$-Lys	0.19	-
Thr	0.07	-
NaHCO3	0.01	0.01
Calculated		
Metabolizable energy (kcal/kg)	3,000	3,000
Crude protein	21.54	21.54
Calcium	1.00	0.85
Available phosphorus	0.47	0.42
Digestible Met	0.46	0.37
Digestible Lys	1.19	0.94

[2]Supplied the following per kilogram of diet: vitamin A (retinyl acetate), 8,000 IU; vitamin D_3 (cholecalciferol), 3,000 IU; vitamin E ($_{DL}$-alpha-tocopheryl acetate), 25 IU; menadione , 1.5 mg; vitamin B_{12} (cyanocobalamin), 0.02 mg; biotin, 0.1 mg; folacin (folic acid), 1 mg; niacin (nicotinic acid), 50 mg; pantothenic acid, 15 mg; pyridoxine (pyridoxine_HCl), 4 mg; riboflavin, 10 mg; and thiamin, 3 mg (thiamin mononitrate); 10 mg of copper ($CuSO_4$); 1.0 mg of iodine Ca (IO3) 2; 80 mg of iron ($FeSO_4\_H_2O$); 100 mg of manganese ($MnSO_4\_H_2O$); 0.15 mg of selenium ($NaSeO_3$); 80 mg of zinc ($ZnSO_4\_H_2O$); and 0.5 mg of cobalt ($CoSO_4$).

Parameter Measurements

On the day of hatch, 2 birds from each replicate were randomly selected and the blood of selected birds was taken**.** After slaughtering the right tibia was removed. After overnight clotting of blood samples at 4°C, the samples were centrifuged (1,000 × *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) (Nouri Sanami *et al*., 2014). This procedure was repeated on 21 and 42 days of age.

Statistical Analysis

All data were analyzed for normal distribution using the NORMAL option of the UNIVARIATE 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. Statistical differences were established using a Duncan's Multiple Range Test at the level of $P<0.05$.

RESULTS

Tables 2 showed that hatchability percentage and body weight of hatched chicks were greater for IOI of 100% CaDPhos complex/egg than other treatments. Moreover, ALP activity was greater for IOI of 100% CaDPhos complex/egg at d 1 and for IOI of 50% or 100% CaDPhos complex/egg at d 42 than other treatments (Table 3, $P<0.05$). The IOI of CaDPhos complex significantly increased bone dry matter, P and Cu concentration at d 1, as well as bone Ca and P concentrations at d 21 of broiler chickens rather both controls (Table 4, $P<0.05$), whereas, the lowest bone dry matter of broiler chickens was obtained by un-injected control at d 42 ($P<0.05$). The results indicated that IOI of CaDPhos complex led to increase FI of broiler chicks from d 1 to 21 (Table 5, $P<0.05$). There were no significant differences between other treatments.

Table 2: Effect of *in ovo* injection of CaDPhos complex on measured post-hatch parameters

Measurements	Sham control	Un-injected control	Injection of 50% of CaDPhos /egg	Injection of 100% of CaDPhos /egg
Hatchability (%)	67.67	79.67	84.33	88.67
Body weight (g)	44.57	45.22	46.85	50.07

CaDPhos is complex of Calcium, Phosphorus, and Vitamin D.

Table 3: Effect of *in ovo* injection of CaDPhos complex on alkaline phosphatase activity of broiler chicks

Measurement	Sham control	Un-injected control	50% of CaDPhos /egg	100% of CaDPhos /egg	SEM	P-value
d 1	628.00^{bc}	605.33^{c}	644.66^{b}	682.33^{a}	9.28	0.002
d 21	272.00	269.00	287.67	293.67	5.20	0.301
d 42	260.00^{b}	260.67^{b}	299.33^{a}	313.33^{a}	8.08	0.006

Means with common letters in the same row are not significantly different (P<0.05). SEM: Standard error of the means.

Table 4: Effect of treatments on bone mineralization (mg/100g bone) of broiler chicks at different ages

Treatments	d 1				d 21				d 42			
	Dry matter	Ca	P	Cu	Dry matter	Ca	P	Cu	Dry matter	Ca	P	Cu
Sham control	45.76^{b}	7.23	3.96^{b}	3.23^{b}	44.06	15.54^{b}	7.10^{b}	1.02	52.85^{a}	16.25	7.16	1.05
Un-injected control	44.29^{b}	7.53	3.83^{b}	3.09^{c}	43.32	14.81^{b}	7.05^{b}	0.98	44.16^{b}	15.91	7.82	1.01
50% of CaDPhos /egg	51.19^{a}	7.49	4.07^{ab}	3.47^{b}	50.76	17.75^{a}	7.98^{ab}	1.07	50.52^{a}	16.78	8.30	1.17
100% of CaDPhos /egg	53.13^{a}	7.98	4.49^{a}	4.19^{a}	52.12	17.76^{a}	8.74^{a}	1.12	51.65^{a}	17.75	8.60	1.15
SEM	1.26	0.12	0.10	0.14	1.68	0.47	0.28	0.03	1.18	0.43	0.28	0.03
P-value	0.01	0.18	0.01	0.01	0.12	0.01	0.04	0.52	0.01	0.51	0.36	0.52

Means with common letters in the same column are not significantly different (P<0.05). SEM: Standard error of the means.

Table 5: Effect of treatments on weight gain (WG, g/d per bird), feed intake (FI, g/d per bird), and feed conversion ratio (FCR) of broiler chicken from 1 to 42 days of age

Treatments	d 1 to 21			d 22 to 42			d 1 to 42		
	WG	FI	FCR	WG	FI	FCR	WG	FI	FCR
Sham control	523.06	929.10^{b}	1.76	1562.60	3176.45	2.03	2091.66	4105.57	1.96
Un-injected control	492.48	941.65^{b}	1.95	1549.27	3154.10	2.04	2041.75	4095.75	2.01
50% of CaDPhos /egg	608.41	1041.23^{ab}	1.72	1660.60	3127.90	1.89	2269.01	4169.13	1.85
100% of CaDPhos /egg	640.11	1080.03^{a}	1.72	1619.51	3169.33	1.96	2259.62	4249.37	1.88
SEM	29.71	26.48	0.06	31.92	22.53	0.04	46.04	33.09	0.03
P-value	0.28	0.04	0.48	0.64	0.91	0.49	0.19	0.37	0.25

Means with common letters in the same column are not significantly different (P<0.05). SEM: Standard error of the means.

DISCUSSION

The hatchability percentage and body weight of hatched chicks are influenced by the type of injected substance and site of injection of nutrients into the eggs (Salary *et al*., 2014), which obtained results are in agreement with other researchers (Selim *et al*., 2012). The IOI of CaDPhos complex did not point to any negative effects on the hatchability percentage. Therefore, it can deduce that IOI of CaDPhos complex is safe and had no adverse effects on hatchability percentage in broiler breeder eggs. In companion studies, a one-time IOI of a dose of 25(OH)-D3 improved the hatchability of embryonated broiler hatching eggs (Bello *et al*., 2013) without having any detrimental effects on overall growth performance (Bello *et al*., 2014a). However, exogenous CaDPhos complex injection on 1st day of incubation (time of bone formation system, Proszkowiec-Weglarz and Angel R. 2013) increased body weight of hatched chicks. It was assumed that CaDPhos complex could cross the inner membrane and pass into the developing embryos. This assumption was based on previous investigations showing that different particles, when injected at the beginning of incubation, are affecting molecular responses, bone and muscle development measured at the end of embryogenesis (Zielinska *et al*., 2011, 2012; Grodzik *et al*., 2013). Thus, the obtained greater body weight of hatched chicks in the current study may be due to effect of tested complex on availability of nutrients, bone and muscle development, the findings confirmed by other researchers (Sawosz *et al*., 2012; Grodzik *et al*., 2013).

The significant improvement in growth rate of commercial broilers reflects increases in gross meat yield, with little or no accompanying compensatory changes in their skeletal structure (Bello *et al*., 2014b). Less dense and more porous bones may subsequently lead to increases in the incidences of a wide range of bone-related conditions in the birds. Although Ca and P are

the main mineral constituents of bone, other elements including microminerals can also be found in the profile of bone minerals, which are vital in the determination of percentage residual bone ash (Kim *et al*., 2012).

Alkaline phosphatase play important role in ossify and calcification (Kim *et al*., 2008). In addition, Cu is essential mineral to construct collagen (Libby and Aikawa, 2002) and to improve reactionary of bone (Gralak *et al*., 2004). Increases in serum ALP activity is associated with accumulation of right tibia Ca and Cu contents in present study. It is reported that layer diaphysis of bone developed in the first day of incubation (Hamburger and Hamilton, 1951), thus it is possible that IOI of CaDPhos complex at the first day of incubation facilitate mineral uptake and thereby increased ALP activity and help bone formation of broiler chicks (Pratt and Kaplan, 2000). The IOI vitamin D leads to higher systemic concentrations of Ca which is associated with greater in bone Ca concentration (Bello *et al*., 2013; Bello *et al*., 2014b).

Except FI at d 1 to 21, no significant differences were found in FI, WG, and FCR by treatments, which are in agreement with a previous study (Salary *et al*., 2014). They declared that the degree of response to IOI depend 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 CaDPhos complex increased hatchability and bone accumulation of minerals, but offering similar diets for all treatments lead to similar growth performance.

CONCLUSION

It is demonstrated that IOI of CaDPhos complex at concentration of 50 or 100% /egg on 1-d of incubation increased hatchability and embryo development. In addition, the alkaline phosphatase activity, the accumulation of Ca, P, and Cu enhanced by infusion of CaDPhos complex in neonatal.

ACKNOWLEDGEMENTS

The authors are thankful to Agricultural Research Center of Qom for providing facilities and financial support of this study.

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