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In ovo feeding with 25-hydroxycholecalciferol influences bone mineral density of chicks

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ABSTRACT - The purpose of this study was to determine if *in ovo* feeding with 25(OH)D3 could influence the incubation parameters of bone mineral composition, mineral density, and bone breaking strength in post-hatch broilers. Fertile eggs from Cobb® broiler breeders were either non-injected or injected with 0, 1.2, 2.4, or 3.6 µg of 25(OH)D3/100 µL olive oil at eight days of incubation. The data were subjected to variance analysis using the General Linear Model procedure (GLM) of SAS®. There was a contrast-1 effect (non-injected eggs vs. injected eggs) for tibial bone mineral density and egg mass loss, with higher bone mineral density in non-injected eggs (0.836 mmAl) compared with injected eggs (0.790 mmAl) and lower egg mass loss in non-injected eggs (11.25%) compared with injected eggs (12.10%). Tibial and femur bone mineral density responded quadratically, and injections of 0.47 and 0.68 µg of 25(OH)D3 increased tibia and femur bone mineral density, respectively. Egg mass loss responded quadratically, and 25(OH)D3 injections of 2.21 µg reduced egg mass loss (11.60%). *In ovo* feeding of 25(OH)D3 can compromise bone mineral density of post-hatch chicken.

Keywords: bone strength, incubation, vitamin D3, 25(OH)D3

1. Introduction

Modern broiler chickens have been specially bred for rapid growth. However, this accelerated growth may lead to metabolic disorders resulting in high financial losses. Examples of these disorders include locomotion problems from weak legs, bone deformities, fractures and related structural lesions.

The occurrence of bone problems during broiler rearing is responsible for economic losses worldwide (Shim et al., 2012). Bone deformities and incidence of fractures pose serious problems for poultry production and causes yield decrease due to the birds' difficulties accessing water and feed.

According to Oviedo-Rondón et al. (2013), egg nutritional composition can benefit the embryonic development, so *in ovo* feeding is a strategy used to improve the development of specific embryo systems or increase nutritional reserves of chicks post-hatch (Roto et al., 2016). This strategy utilizes the injection of *in ovo* solutions, with the content varying according to purpose, but is based on the use of vitamins, minerals, amino acids, probiotics, or a combination of nutrients (Yair and Uni, 2011; Roto et al., 2016; Sgavioli et al., 2016a).

Studies have shown that *in ovo* inoculation of 1.25(OH)2D3, 25(OH)D3, and 24.25(OH)2D3 during incubation improves hatching, bone development, and yolk calcium concentration and interferes with receptor mRNA expression by IGF-1 (Yair et al., 2013; Bello et al., 2014a,b,c; Bello et al., 2015; Yair et al., 2015).

The studies conducted recommend *in ovo* feeding at the 17th or 18th day of incubation – the same time as *in ovo* vaccination. However, physiologically, bone mineralization begins on the eighth day of incubation by solubilizing the calcium present in the egg yolk (Freeman and Vince, 1974). The effects of this *in ovo* administration of 25(OH)D3 on the eighth day of incubation on post-hatch bone development have not been previously investigated.

For this study, we examined the effects of 25-hydroxycholecalciferol (25(OH)D3) *in ovo* feeding on the eighth day of incubation on bone mineral composition, mineral density, and bone breaking strength of broilers. This study is significant because it investigates the potential use of 25(OH)D3 for the prevention of broiler locomotion problems by promoting structural improvement of the locomotor system.

2. Material and Methods

This study was conducted in accordance with the ethical principles for animal experimentation, adopted by the Brazilian College of Animal Experimentation (COBEA), and the experimental procedures were approved by the local Committee for Ethical Animal Use (CEUA; case no. 021889/13), in Jaboticabal, SP, Brazil (21°14'05" South latitude, 48°17'09" West longitude, and average altitude of 615.01 m).

Six hundred (600) fertile eggs from 43-week-old Cobb® 500 broiler hens were distributed (67±2 g) into ten trays (replicates) at 12 eggs/treatment/tray (120 eggs/treatment). Eggs were incubated in incubators (CASP®, IHM Line e-V A 06, Amparo, SP, Brazil) equipped with automatic temperature, humidity, and egg-turning control. The initial and final temperatures and humidity were 37.8 and 36.9 °C and 85.7 and 84.0%, respectively, with 504 h of incubation.

Eggs were distributed into five treatments, in a completely randomized experimental design, as follows: eggs non-injected (unperforated) and eggs injected with 0, 1.2, 2.4, or 3.6 µg of 25(OH)D3/100 µL of olive oil (Aydin et al., 2001; Ahmad et al., 2018) on the eighth day of incubation. The eight day of incubation was chosen because, according to Shim et al. (2012), the broiler embryo bone system starts to develop on the fourth day of incubation.

Pure crystalline 25(OH)D3 (Hy·D; DSM Nutritional Products, Basel, Switzerland) was dissolved in olive oil. The 25(OH)D3 injected treatments corresponded to vitamin D3 activity levels of 0, 48, 96, and 144 IU, respectively (DSM, 2016).

For the injection, eggs were held horizontally and, after cleaning with 70% ethanol, the shell was perforated near the thin end (the end opposite to the air cell) with a sterile needle [Injex, 13 × 0.38 (27.5 G1/2″)], through which 100 µL aqueous additive solution were injected into the albumen, approximately 6 mm below the membrane (Sgavioli et al., 2016a). After injection, the hole was sealed with a label identifying the treatment and replicate.

Hatchability (number of hatched chicks/number of incubated fertile eggs) and embryo mortality were determined according to embryo diagnosis phases (initial: 1-7 days, intermediate: 8-14 days, and late: 15-21 days of incubation).

Egg mass loss (EML) was calculated as the difference in the egg weight before incubation and the weight at the 18th day of incubation, expressed as a percentage of initial egg weight.

Ten birds per treatment whose average body weight was close to the average weight of the experimental unit were sacrificed at one day of age, for a total of 50 animals. Birds were stunned by individual exposure to CO_2 gas for 2 min in a flow-through system and then slaughtered by cervical dislocation.

Bone mineral density (BMD; mmAl) was determined for the left femur and tibia. Muscles, cartilage, and fibula were removed prior to densitometry evaluation. The evaluation was performed with the assistance of an aluminum step wedge (6063 alloy, ABNT) with 12 steps. Steps 1 to 10 were 0.5 mm thick. Steps 11 and 12 were 0.6 and 8.0 mm thick, respectively. Each step measured 5×25 mm^2, and the scale was radiographed together with the bones. The scale was placed parallel to the bone, with the highest steps at the top of the frame. The x-ray equipment was calibrated using the technique relating kilovoltage (kV), milliamperage (mA), and milliamperage-second (mAs) with the thickness of the analyzed bone area. A standard x-ray machine (Siemens, model Tridoro 812 E) was calibrated at 30kV, 400mA, and 4mAs (Sgavioli et al., 2016b).

The right femur and tibia were used to determine bone calcium, phosphorus, and ash contents. The soft tissue was removed, and bones were boiled in deionized water for 5 min. After drying at room temperature, samples were immersed in petroleum ether for 48 h, dried in a forced-ventilation oven at 60 °C for 48 h, and then ground in a ball mill. Bone mineral content was determined using wet analyses. Ash content was determined by burning the samples at 600 °C. The methods were applied according to Silva and Queiroz (2002) and expressed as a percentage of defatted dry matter or mineral matter.

The left femur and tibia were used for the mechanical bone-strength test (three-point bending). The test was conducted using an EMIC® (DL 3000) universal testing machine. The load was applied at a rate of 5 mm/min with a force of 2000 N to determine the maximum permissible force (Fmax) of the bone. Bones were fixed on two supports (two points), with the span adjusted according to the size of the smallest bone. The force was then applied at the geometric mean point of the bone between the two supports (middle third of the bone), and the equipment recorded the results. These variables express bone strength at the middle third.

Bones were radiographed using Kodak P-MATG/RA film and 18×24, 24×30, and 30×40 cm frames. The metal frame was set with intensifying screens (Kodak, Lanex Regular). Bones were placed 2 cm distant from the borders of the frame to allow uniform penetration of the x-rays. The film was previously identified by light impression, then developed and fixed in an automatic processor (Kodak X-OMAT 200).

Densitometry was read using a digital scanner (Scanion A3). The digitalized images were analyzed using the Image-Pro Plus software program (Image-Pro Plus, Media Cybernetics, version 4.1) calibrated for BMD. Bone mineral density was calculated on the diaphysis. Values were expressed as millimeters of aluminum (mmAl).

The effects of incubation treatments (non-injected eggs; eggs injected with 0, 1.2, 2.4, or 3.6 µg of 25(OH)D3/100 µL of olive oil) on all studied parameters were analyzed statistically using the model described below:

$$Y_{ij} = \mu + T_i + e_{ij},$$

in which Y = studied parameter, µ = mean value of the parameter, T_i = treatment (eggs non-injected and eggs injected with 0, 1.2, 2.4, or 3.6 µg of 25(OH)D3/100 µL of olive oil); and e_{ij} = residual error.

Data were analyzed for the presence of outliers (Box-and-Whisker plot), normal distribution of studentized errors (Cramer-Von-Mises test), and homogeneity of variances (Brown-Forsythe).

Means were compared by 5% probability polynomial orthogonal contrasts, as follows: contrast 1 – comparison between non-injected egg treatment versus the average of treatments with 0, 1.2, 2.4, or 3.6 µg of 25(OH)D3/100 µL of olive oil; contrasts 2 and 3 – comparisons using linear and quadratic regression models (0, 1.2, 2.4, or 3.6 µg of 25(OH)D3, for checking the effects of additive application. For embryonic mortality and hatchability, data were analyzed for frequency by Fisher's exact test at the 5% probability level.

3. Results

There was a contrast-1 effect (non-injected eggs vs. injected eggs) for the tibial BMD (P<0.0001). Higher tibial BMD were observed in non-injected eggs (0.836 mmAl) when compared with injected eggs (0.790 mmAl) (Table 1). Tibial and femur BMD responded quadratically (P<0.001) (Table 1), and injections of 0.47 and 0.68 µg increased the tibial and femur BMD, respectively.

There was a contrast-1 effect (non-injected eggs vs. injected eggs) for EML (P = 0.0079). Lower EML were observed in non-injected eggs (11.25%) when compared with injected eggs (12.10%) (Table 1). Egg mass loss responded quadratically (P = 0.0110) (Table 1), and injections of 2.21 µg reduced EML (11.60%).

Femur and tibia concentration of ash, calcium, and phosphorus and bone strength did not differ (P>0.05) (Tables 2 and 3). Hatchability and embryonic mortality (Table 4) did not differ (P>0.05).

Table 1 - *In ovo* feeding with 25(OH)D3 levels on femur and tibia bone mineral density of post-hatch chicks and egg mass loss

Treatment	Femur bone mineral density (mmAl)	Tibia bone mineral density (mmAl)	Egg mass loss[2] (%)
Non-injected eggs	0.841±0.001	0.836±0.002	11.25±1.67
0 µg of 25(OH)D3	0.840±0.001	0.837±0.001	12.14±1.72
1.2 µg of 25(OH)D3	0.813±0.006	0.806±0.005	11.67±1.49
2.4 µg of 25(OH)D3	0.822±0.004	0.808±0.004	11.83±1.27
3.6 µg of 25(OH)D3	0.719±0.003	0.707±0.005	12.79±2.12
CV%	1.07	1.18	14.09
		Contrast probability	
Non-injected vs. injected eggs	<0.0001*	<0.0001*	0.008*
Linear effect[1]	<0.0001*	<0.0001*	0.09
Quadratic effect[1]	<0.0001*	<0.0001*	0.011*

±standard error; CV - coefficient of variation.
[1] Treatments used for the regression calculation: 0, 1.2, 2.4, and 3.6 µg of 25(OH)D3.
[2] Egg mass loss: difference in egg weight before incubation and at the 18th day of incubation, expressed as a percentage of initial egg weight.
* Significant at P<0.05.

Table 2 - *In ovo* feeding with 25(OH)D3 levels on femur and tibia weight, ash, calcium, and phosphorus contents of post-hatch chicks

Treatment	Weight (g)	Ash (%)	Calcium (%)	Phosphorus (%)	Weight (g)	Ash (%)	Calcium (%)	Phosphorus (%)
	Femur				Tibia			
Non-injected eggs	0.062±0.030	29.59±0.41	11.92±0.33	6.28±0.17	0.097±0.080	29.18±0.60	11.08±0.19	5.77±0.12
0 µg of 25(OH)D3	0.080±0.010	27.22±1.23	10.89±0.51	5.92±0.32	0.118±0.016	29.24±0.73	11.47±0.36	5.80±0.20
1.2 µg of 25(OH)D3	0.080±0.009	28.98±0.70	11.90±0.52	6.03±0.30	0.116±0.013	28.77±0.58	10.91±0.27	5.77±0.17
2.4 µg of 25(OH)D3	0.068±0.010	29.46±1.35	11.23±0.27	5.96±0.38	0.097±0.004	29.57±0.46	11.06±0.10	5.92±0.07
3.6 µg of 25(OH)D3	0.069±0.008	28.68±0.58	11.40±0.27	6.42±0.15	0.097±0.005	29.44±0.73	10.82±0.38	6.04±0.21
CV%	28.46	7.59	8.10	10.78	24.25	4.00	5.84	6.45
				Contrast probability				
Non-injected vs. injected eggs	0.19	0.33	0.20	0.72	0.40	0.69	0.97	0.53
Linear effect[1]	0.23	0.26	0.63	0.31	0.09	0.49	0.16	0.25
Quadratic effect[1]	0.93	0.18	0.31	0.56	0.96	0.38	0.57	0.63

±standard error; CV - coefficient of variation.
[1] Treatments used for the regression calculation: 0, 1.2, 2.4, and 3.6 µg of 25(OH)D3.

Table 3 - *In ovo* feeding with 25(OH)D3 levels on femur and tibia bone strength of post-hatch chicks

Treatment	Femur bone strength (N)	Tibia bone strength (N)
Non-injected eggs	12.92±1.40	9.42±0.86
0 µg of 25(OH)D3	13.40±1.41	9.60±0.86
1.2 µg of 25(OH)D3	14.09±1.42	10.03±0.87
2.4 µg of 25(OH)D3	12.02±1.40	11.71±0.88
3.6 µg of 25(OH)D3	13.73±1.42	11.25±0.86
CV%	26.28	20.51
	Contrast probability	
Non-injected vs. injected eggs	0.81	0.21
Linear effect[1]	0.87	0.10
Quadratic effect[1]	0.72	0.61

±standard error; CV - coefficient of variation.
[1] Treatments used for the regression calculation: 0, 1.2, 2.4, and 3.6 µg of 25(OH)D3.

Table 4 - *In ovo* feeding with 25(OH)D3 levels on hatchability and embryonic mortality frequency

Treatment	Hatchability (%)	Embryonic mortality (%)		
		Initial (1-7 days)	Intermediate (8-14 days)	Late (15-21 days)
Non-injected eggs	91.23	5.44	0.00	3.33
0 µg of 25(OH)D3	87.18	6.99	0.00	5.83
1.2 µg of 25(OH)D3	89.47	4.70	0.83	5.83
2.4 µg of 25(OH)D3	86.84	5.66	0.00	7.50
3.6 µg of 25(OH)D3	82.61	9.06	0.00	8.33
Probability[1]	0.22	0.24	0.29	0.24

[1] Significant at P<0.05 (Chi-square).

4. Discussion

In ovo feeding with 25(OH)D3 was evaluated in terms of ability to promote changes in bone development post-hatch. Despite the increase in tibia and femur BMD and a decrease on EML observed with *in ovo* feeding (25(OH)D3), non-injected eggs had higher BMD and lower EML compared with injected eggs.

Egg mass loss up at the time of transference to the hatcher is used in commercial settings to determine embryo development stage and is related with hatchling weight (Noy and Pinchasov, 1993). Excessive egg water loss (>14%) causes embryo death by dehydration (Romanoff, 1930); on the other hand, EML between 11 and 12% up to 18 days of incubation increases hatchability.

In the present study, *in ovo* feeding increased EML. One hypothesis for this result is that the opening made to perform the procedure at the eighth day of incubation may have favored EML. However, in a similar experiment, Sgavioli et al. (2015) performed *in ovo* feeding with vitamin C before the beginning of the incubation using the same techniques and there was no influence on EML. *In ovo* feeding with 2.21 µg of 25(OH)D3 reduced this effect and caused EML to be 11.60%, therefore, below 12% and close to control (11.25%). This shows that the effect of *in ovo* feeding on hatchability varies with the concentration of the solution and stage of embryonic development.

Despite the variation in EML, treatments did not influence hatchability and embryonic mortality. The lack of treatment effects on hatchability and mortality indicates that *in ovo* feeding with 25(OH)D3 at the eighth day of incubation did not result in compromised embryo development. Similar to the present study, Bello et al. (2015) found no difference in EML with *in ovo* feeding with 0.6 µg of 25(OH) D3 at 18 days of incubation. However, Sgavioli et al. (2015) reported a decrease in egg hatchability after injecting increasing levels of vitamin C before incubation.

In addition, according to Uni and Ferket (2003), high concentrations of solutions can interfere with osmotic equilibrium and affect embryo development. These authors described that the maximum osmolarity of the solution to be injected *in ovo* is 800 mOsm. The solution applied *in ovo* in the present study was 384 mOsm.

In the egg, the active form of vitamin D is supplied to the embryo by the yolk, acting on bone metabolism for skeleton formation. Thus, *in ovo* feeding in the embryonic period can positively influence bone growth in the first weeks of bird life, since, at this stage, the metabolism of bone tissue is intense, and failure to meet nutritional needs for this function will result in poor bone formation (Gonçalves et al., 2013).

However, in this study, higher BMD of tibia and femur occurred for non-injected eggs, when compared with injected eggs. Also, *in ovo* feeding with vitamin D3 resulted in quadratic effects to tibia and femur. However, when replacing the levels found as ideal (0.47 and 0. 68 µg of 25(OH)D3, respectively) in the equations, we found lower BMD values (0.824 and 0.831 mmAl, respectively) when compared with values for the non-injected eggs and eggs injected only with olive oil.

The *in ovo* feeding with olive oil caused significant decline in embryonic mortality with a simultaneous improvement in hatchability (Aydin et al., 2001). Olive oil has been shown to contain natural anti-oxidative and anti-ageing potentials (Fitó et al., 2007). However, olive oil contains oleocanthal that inhibits the cyclooxygenase activity in prostaglandin biosynthesis (Beauchamp et al., 2005). Further research is needed to access the effects of *in ovo* feeding with olive oil on bone development of broilers.

Yair et al. (2013) and Yair et al. (2015) demonstrated that changing embryonic nutrition by *in ovo* feeding with a vitamin D3 enrichment solution affects structural and mechanical properties of tibia and femur and changes the dynamics of skeletal development. However, Bello et al. (2014b) found no difference in BMD of eggs with *in ovo* feeding with 0.2, 0.6, 1.8, and 5.4 µg of 25(OH)D3 at 18 days of incubation. The authors reported that the amniotic injection of 0.2, 0.6, and 1.8 µg 25(OH)D3, when compared with diluent-injected controls, resulted in an increase in bone breaking strength of male broilers at 28 days post-hatch.

Future studies should be conducted regarding bone and cartilaginous development of broilers with *in ovo* feeding with 25(OH)D3 either at 8, 17, or 18 days of incubation, as studies on the subject are scarce, and among those performed (Yair et al., 2013; Yair et al., 2015; Bello et al., 2014a,b), the results are conflicting.

5. Conclusions

In ovo feeding of 25(OH)D3 can compromise bone mineral density of chicken post-hatch.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: T.C.O. Quadros and S. Sgavioli. Data curation: T.C.O. Quadros, S. Sgavioli, D.M.C. Castiblanco, E.T. Santos, G.M. Andrade, L.L. Borges and A.R. Almeida. Formal analysis: S. Sgavioli. Funding acquisition: S.M. Baraldi-Artoni. Investigation: T.C.O. Quadros, S. Sgavioli, D.M.C. Castiblanco, E.T. Santos, G.M. Andrade, L.L. Borges and A.R. Almeida. Methodology: T.C.O. Quadros, S. Sgavioli, D.M.C. Castiblanco, E.T. Santos, G.M. Andrade, L.L. Borges and A.R. Almeida. Project administration: S. Sgavioli. Supervision: S. Sgavioli. Writing-original draft: A.R. Almeida. Writing-review & editing: S. Sgavioli.

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