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≪Research Note≫

Effects of In Ovo Vitamin D_3 Injection on Subsequent Growth of Broilers

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This study was conducted to investigate the influence of *in ovo* vitamin D_3 (Vit D_3) administration on growth of broiler chickens when Vit D_3 was dissolved in soybean oil. Sixty Ross broiler eggs were incubated at 37.8℃ and ＞60% relative humidity. Distilled water, soybean oil, or Vit D_3 (60 IU / 0.5 mL) dissolved in soybean oil, was administered *in ovo* on Day 18 of incubation. Seven days after hatching, chicks were sexed, and 12 birds (six female and six male) close to the average body weight (BW) of each treatment were selected and their BW continuously recorded until 28 days of age, then sacrificed. Liver and pectoral muscle were collected to determine the mRNA expression of IGF-1 and IGF-1 receptor, and the length of tibia was measured. There were no significant differences in BW, liver weight, or pectoral muscle weight between the groups. However, an interaction was observed between treatments and sexes in the tibia length. In comparison among only males, tibia length in the Vit D_3 with oil group was longer than that of the control, but not different from that of the oil group. The same tendency was observed in the hepatic IGF-1 mRNA expression in chicks of either sex, with this effect only being observed after the treatments and not in the control. On the other hand, there was an interaction between treatments and sexes in the mRNA expression of IGF-1 receptor, which was highest in the Vit D_3 with oil group in females, but not in males. These results indicated that the *in ovo* administration of Vit D_3 affected IGF-1 receptor mRNA expression without growth.

Key words: broiler embryo, IGF-1, IGF-1 receptor, vitamin D_3

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Introduction

Vitamin D_3 (Vit D_3) is known to be involved in the promotion of calcium absorption in the small intestine (Bronner, 2003), maintenance of calcium homeostasis (DeLuca, 2004; Fleet, 2017; Soares, 1984), the proliferation of osteoblasts in bone metabolism (Bronner and Stein, 1995; Nordin, 2010), and affects bone growth and formation (Biely J and March BE, 1967; Saunders-Blades and Korver, 2014). In addition, it has been suggested that Vit D_3 is multifunctional in relation to immunity, metabolism, proliferation, differentiation, and apoptosis in various cell types, though little is known about its involvement in regulating tissue growth.

Nutritional supplementation with Vit D_3 is becoming popular in animal husbandry, using 25-hydroxycholecalciferol (25 (OH) D_3), a metabolite of Vit D_3, as its efficacy and cost are better than those of Vit D_3. When *in ovo* 25 (OH) D_3 was dissolved in vaccine diluent buffer and injected into broiler eggs, hatchability was improved, but no adverse effects on growth were seen (Bello *et al*., 2014). However, the 25 (OH) D_3 was dissolved in ethanol and vaccination buffer, while Vit D_3 is usually an oil soluble vitamin. In addition, 25 (OH) D_3 is an intermediate hormone form of Vit D_3 family. Thus, the conclusions of Bello *et al*. (2014) might be affected by injection of a solvent-dissolved form of Vit D_3.

In addition, dietary administration of 25 (OH) D_3 improved the development of satellite cell activity, and growth of skeletal muscles (Hutton *et al*., 2014). However, the effects of Vit D_3 over 25 (OH) D_3 were not evaluated in embryonic nutrition.

Thus, this study was conducted to evaluate growth-promoting effects of *in ovo* administration of Vit D_3, using commercially available soybean oil as a solvent.

Materials and Methods

Animals

One hundred fertilized eggs of Ross broiler breeder were used. All eggs were obtained from the same breeder flock and laid within a 24-h period. Eggs were incubated at 37.8℃ and over 60% relative humidity (RH).

Sixty eggs were selected by candling on Day 17 of incubation, and eggs were divided into 3 groups with 20 eggs each. Egg shells were drilled at the large end and were administered *in ovo* with distilled water (control), 0.5 mL soybean oil (Oil group), or 60 IU (same as the egg contents) /0.5 mL Vit D_3 dissolved in soybean oil (Vit D_3 with oil group) on Day 18 of incubation. At hatching, chicks were sorted by

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Table 1. **Sequence of Real-time PCR primers**

Gene		Primer
IGF-1	Forward (5′-3′)	TGCTCCAATAAAGCCACCTAAATC
	Reverse (5′-3′)	TTCTGTTTCCTGTGTTCCCTCTAC
IGF-1 receptor	Forward (5′-3′)	TGATCTGGCTGCGAGAAACT
	Reverse (5′-3′)	CAGACGTCGGAGTGTGTTGT
GAPDH	Forward (5′-3′)	GCCGTCCTCTCTGGCAAA
	Reverse (5′-3′)	TGTAAACCATGTAGTTCAGATCGATGA

Table 2. **Effects of composition of *in ovo* administration of control, soybean oil, and vitamin D_3 at Day 18 of incubation, and sex differences in body weights of broiler breeder chicks at 0, 3, 7, 14, 21, and 28 days of age**

Treatment	Sex	Initial egg weights	Body weights					
			Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
		(g)						
Control	Female	60.4	45.6	68.2	144.1	427.4	770.0	1095.7
	Male	58.8	45.3	68.0	145.6	433.4	778.0	1164.0
Soybean oil	Female	59.0	45.1	67.5	142.0	420.4	761.7	1096.7
	Male	58.9	46.6	68.8	145.1	444.0	806.7	1286.7
Vit D_3 + oil	Female	58.9	43.9	70.3	154.1	441.8	783.3	1178.3
	Male	59.6	45.9	66.5	143.1	435.0	778.3	1206.7
	Pooled SE	1.1	0.9	2.8	5.3	16.5	35.4	44.7

Values are means for 6 birds.

sex and body weight (BW) was measured. Twelve chicks (6 female and 6 male) were selected for near-average BW. These chicks were then housed in the same floor pen, and were fed a commercial starter diet (ME 3,100 kcal /kg, CP 21%) for 4 weeks. BW was recorded every week.

At 28 days of age, chicks were sacrificed by cervical dislocation, and livers and pectoral muscles were collected to determine the mRNA expression levels of IGF-1 and IGF-1 receptor. In addition, the tibia lengths were measured.

Total RNA Isolation

Total RNA of the liver and pectoral muscle (about 50−100 mg) was extracted using 1 mL of Trizol™ reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's procedure. After incubation of the homogenized samples with 200 μL of chloroform on ice for 10 min, the samples were centrifuged at 15,000 rpm for 30 min at 4℃. The supernatant of each sample was then transferred to new tube and mixed with 500 μL of isopropyl alcohol. After incubation for 5 min on ice, samples were centrifuged at 4℃ at 15,000 rpm for 10 min. The supernatant was removed, and the RNA pellet was washed once with 80% ethanol. The pellet was air dried and dissolved in 20 μL of diethyl pyrocarbonate (DEPC)-treated water. The RNA quantity was determined by spectrophotometry at 260 nm. Samples were stored at −80℃ until use.

Reverse Transcription (RT) and Real-time PCR Analysis

Reverse transcription (RT) and real-time PCR was carried out by the method described in Furuta *et al*. (2009). The primer pairs of IGF-1, IGF-I receptor, and GAPDH were reported for real time PCR (Table 1; Yun *et al*., 2005; Furuta *et al*., 2009).

Statistics

The results obtained were analyzed by two-way ANOVA, by considering Vit D_3 treatments and sexes as the main effects, using the General Linear Models procedure of SAS® software (SAS Institute, 2001). When differences among means were significant, means were compared using Tukey's multiple range test with statistical significance considered at $P<0.05$.

Results and Discussion

Although there was a significant difference only in age, data for BW of each sex and each age are shown in Table 2. In this study, the average egg weights were equal in all three groups. Hatchability was 75% in the control group and 95% in both the Oil and Vit D_3 groups.

The difference in the tibia length between Vit D_3 with oil and control groups might be due to the combination of Vit D_3 and soybean oil, as there was no clear influence of Vit D_3 alone on tibia length (Table 3).

There was no significant difference in BW, liver weight, or pectoral muscle weight. However, the interaction was observed between treatments and sexes in the tibia length ($P<0.05$). In comparison among only males, tibia length in the Vit D_3 with oil group was longer than that of the control, but not significantly different from that of the oil group.

The same tendency was observed in the hepatic IGF-1 mRNA expression of chicks in both sexes (Table 4), with this

effect only being observed after the treatments and not in the control ($P<0.05$).

On the other hand, there was an interaction between treatments and sex in IGF-1 receptor mRNA expression level ($P<0.05$). The Vit D_3 with oil group showed the highest expression among all three treatments in females (Table 5; $P<0.05$), but not in males ($P>0.05$).

Bello *et al*. (2014) reported that 25 (OH) D_3 dissolved in vaccine diluent buffer, did not negatively affect growth performance after hatching, while the hatching rate in the 25 (OH) D_3-treated group was higher than in the control group, suggesting the possibility of improving hatching rates by administration of 25 (OH) D_3. In addition, Hutton *et al*. (2014) showed that dietary supplementation of 25 (OH) D_3 improved pectoral muscle development in broiler chicks.

There was no significant difference in body weight, liver weight, and pectoral muscle weight in the present study, though interactions were observed between treatments and sexes in tibia length. The tibia length of males in the Vit D3 with oil group was significantly longer than that of males in the control group ($P<0.05$). The effect of sexes wwas not observed in the results of hepatic IGF-1 mRNA expression, but differed significantly among treatments ($P<0.05$). IGF-1 mRNA expression was significantly higher in the Vit D_3 with oil group than in the control group ($P<0.05$). These changes in IGF-1 mRNA expression were therefore similar to the changes observed in tibia length in male chicks.

The mRNA expression levels of IGF-1 receptor in the shallow pectoral muscle, which is an indicator of growth, increased only in females, while in the liver, the expression levels of IGF-1 mRNA increased in the area of Vit D_3. Although multiple functions of Vit D_3 are reported, the detailed mechanisms involved in this phenomenon remain unknown. In humans, estrogen, a female hormone has been reported to be involved in bone metabolism (Cauley, 2015). From this, it is considered that sex steroid hormones may be responsible for sexual dimorphism in chickens.

The above phenomenon was not consistent with previous results (Bello *et al*., 2014). The vitamin D receptor is thought to be involved in all vitamin D functions. This receptor is regulated by 1,25-dihydroxy-vitamin D_3 and 25 (OH) D_3 (Darwish and DeLuca, 1993), though some of the regulatory mechanisms which govern this are unclear. These results indicate that Vit D_3 affects embryonic and subsequent growth, with this function probably being related to the form of Vit

Table 3. **Effects of composition of *in ovo* administration of control, soybean oil, and vitamin D_3 at Day 18 of incubation, and sex differences in shank length in broilers at 28 days of age**

		Tibia length
		(mm)
Control	Female	108.4±1.4[ab]
	Male	102.2±3.7[b]
Soybean oil	Female	105.3±3.6[ab]
	Male	106.5±1.8[ab]
Vit D_3 + oil	Female	101.2±4.3[b]
	Male	108.7±2.6[a]
P value	Treatments	NS
	Sex	NS
	Interaction	0.05

Values are means±SE for 6 birds.
[a, b] Means in the same column with no common superscript differ significantly ($P<0.05$).

Table 4. **Effects of composition of *in ovo* administration of control, soybean oil, and vitamin D_3 at Day 18 of incubation on weights, and insulin-like growth factor 1 (IGF-1) mRNA expression of livers in broilers at 28 days of age**

Treatment		Sex	Liver	
			Weights	IGF-1 mRNA expression
			(g)	(/GAPDH)
Control		Female[1]	25.5±1.7	1.6±0.2
		Male[1]	29.2±1.8	2.1±0.3
Soybean oil		Female[1]	26.6±1.7	2.1±0.2
		Male[1]	28.9±1.3	2.5±0.3
Vit D_3 + oil		Female[1]	26.3±2.1	2.1±0.2
		Male[1]	28.1±1.5	2.8±0.5
Control[2]			27.3±1.3	1.8±0.1[b]
Soybean oil[2]			27.5±1.2	2.3±0.2[ab]
Vit D_3 + oil[2]			27.5±1.1	2.4±0.3[a]
P value	Treatments		NS	0.05
	Sex		NS	NS
	Interaction		NS	NS

[1] Values are means±SE for 6 birds.
[2] Values are means±SE for 12 birds.
[a, b] Means in the same column with no common superscript differ significantly ($P<0.05$).

Table 5. **Effects of composition of i*n ovo* administration of control, soybean oil, and vitamin D_3 at Day 18 of incubation, and sex difference on weights and insulin-like growth factor 1 receptor (IGF-1R) mRNA expression of pectoral muscle in broilers at 28 days of age**

Treatment	Sex	Pectoral muscle	
		Weights	IGF-1R mRNA expression
		(g)	(/GAPDH)
Control	Female	79.8±1.8	0.91±0.09[b]
	Male	84.8±5.0	0.92±0.10[b]
Soybean oil	Female	76.6±10.0	1.16±0.08[b]
	Male	92.8±3.8	1.20±0.15[ab]
Oil + Vit D	Female	82.3±3.2	1.61±0.13[a]
	Male	81.1±2.4	1.22±0.09[b]
P value	Treatments	NS	NS
	Sex	NS	NS
	Interaction	NS	0.05

Values are means±SE for 6 birds.
[a, b] Means in the same column with no common superscript differ significantly ($P<0.05$).

D_3, or interactions between Vit D_3, 25 (OH) D_3, and 1,25-dihydroxy-vitamin D_3.

References

Bello A, Zhai, W., Gerard PD, Peebles ED. Effects of the commercial in ovo injection of 25-hydroxycholecalciferol on broiler posthatch performance and carcass characteristics. Poultry Science, 93: 155−162. 2014.

Biely J and March BE. Calcium and vitamin D in broiler rations. Poultry Science, 46: 223−232. 1967.

Bronner F. Mechanisms and functional aspects of intestinal calcium absorption. Journal of Experimental Zoology, 300: 47−52. 2003.

Bronner F and Stein WD. Calcium homeostasis – an old problem revisited. Journal of Nutrition, 125: 1987S−1995S. 1995.

Cauley JA. Estrogen and bone health in men and women. Steroids, 99: 11−15. 2015.

Darwish H and DeLuca HF. Vitamin D-regulated gene expression. critical reviews in Eukaryotic Gene Expression, 3: 89−116. 1993.

DeLuca HF. Overview of general physiologic features and functions of vitamin D. The American Journal of Clinical Nutrition, 80: 1689S−1696S. 2004

Fleet JC. The role of vitamin D in the endocrinology controlling calcium homeostasis. Molecular and Cellular Endocrinology, 453: 36−45. 2017.

Furuta H, Yoshimi Y, and Ohta Y. Expression of mRNA for 3HADH in manipulated embryos to produce germline chimeric chickens. Cytotechnology, 60: 165−168. 2009.

Hutton KC, Vaughn MA, Litta G, Turner BJ and Starkey JD. Effect of vitamin D status improvement with 25-hydroxycholecalciferol on skeletal muscle growth characteristics and satellite cell activity in broiler chickens. Journal of Animal Science, 92: 3291−3299. 2014.

Nordin BE. Evolution of the calcium paradigm: the relation between vitamin D, serum calcium and calcium absorption. Nutrients, 2: 997−1004. 2010.

Saunders-Blades JL and Korver DR. The effect f maternal vitamin D source on broiler hatching egg quality, hatchability, and progeny bone mineral density and performance. The Journal of Applied Poultry Research, 23: 773−783. 2014.

Soares JH Jr. Calcium metabolism and its control – a review. Poultry Science, 63: 2075−2083. 1984

Yun JS, Seo DS, Kim WK, Ko Y. Expression and relationship of the Insulin-like growth factor system with posthatch growth in the Korean native ogol chicken. Poultry Science, 84: 83−90. 2005.