Effects of the in ovo injection of vitamin D_3 and 25-hydroxyvitamin D_3 in Ross 708 broilers subsequently fed commercial or calcium and phosphorus-restricted diets. II. Immunity and small intestine morphology[1,2,3]

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ABSTRACT Effects of the in ovo injection of vitamin D_3 (**D_3**) and 25-hydroxyvitamin D_3 (**$25OHD_3$**) on the immunity and small intestine morphology of broilers fed calcium and phosphorus-restricted diets were investigated. At 18 d of incubation (**doi**), live embryonated Ross 708 broiler hatching eggs were in ovo-injected with a 50 μL solution of one of the following treatments using an Inovoject multiegg injector: 1) diluent (control); diluent containing either 2) 2.4 μg D_3; 3) 2.4 μg $25OHD_3$; or 4) 2.4 μg D_3 + 2.4 μg $25OHD_3$. At hatch, 18 randomly selected male broilers belonging to one of the 4 in ovo injection treatments were placed in each of 12 floor pens and were fed either a commercial diet or a diet restricted by 20% in calcium and available phosphorus (**ReCaP**) content for the starter, grower and finisher dietary phases. Concentrations of plasma IgG and IgM at 14 d of age (**doa**) and α-1-acid glycoprotein at 40 doa were determined. Bursa, liver, spleen, duodenum, jejunum, and ileum weights were recorded at 7, 14, and 40 doa and small intestine histology was evaluated at 14 and 40 doa. Blood and organ samples were randomly collected from 1 bird in each of the 6 replicate pens within each of the 8 (4 in ovo x 2 dietary) treatment groups. Plasma IgG levels were higher in $25OHD_3$ than in diluent or D_3 in ovo-injected birds. At 14 doa, a higher jejunal villus length (**VL**) to crypt depth (**CD**) ratio (**RVC**) was observed in birds that were in ovo-injected with $25OHD_3$ alone as compared to all other in ovo injection treatments. At 40 doa, ileal VL increased and jejunal CD decreased in commercial diet-fed birds compared to ReCaP diet-fed birds. In conclusion, the in ovo injection of $25OHD_3$ alone increased the immune response and improved the small intestine morphology and subsequent nutrient uptake of Ross 708 broilers. However, a ReCaP diet was observed to be detrimental to their small intestine morphology.

Key words: 25-hydroxyvitamin D_3, broiler, immunity, in ovo injection, small intestine morphology

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INTRODUCTION

The in ovo injection of broiler hatching eggs is widely used in the poultry industry. In comparison to the traditional method of broiler vaccination, in ovo injection has been shown to be less stressful, and is faster and provides uniform delivery of vaccines or nutrients to broiler embryos (Williams, 2007). "The poultry industry commercially uses in ovo vaccination against various diseases including Marek`s disease (Williams, 2007), while the in ovo injection of nutrients is not yet applied commercially. Rapid development of the small intestine takes place at 15 d of incubation (**doi**) and includes changes in relative intestinal weight and villi morphology. Furthermore, at that time, the expression and activity of brush-border enzymes and transporters prepare the embryo for exogenous feed ingestion

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(Uni et al., 2003). It is well documented that an earlier improvement in small intestine development has a positive long-term effect on broiler performance (Uni et al., 1999). The in ovo injection of mannan oligosaccharides has been shown to increase villus length (**VL**) and villus area (Cheled-Shoval et al., 2011). Improvements in small intestine morphology are associated with improvements in growth performance characteristics such as BW and feed conversion ratio (**FCR)** under commercial conditions (Yang et al., 2007a,b), as well as during a coccidiosis challenge (Dalloul et al., 2005). In addition, in ovo injection of different nutrients such as CpG oligodeoxynucleotides, vitamins C and E, and plant extracts have the potential to enhance immunity (Gore and Qureshi, 1997; Dalloul et al., 2005; Saki and Salary, 2015; El-Senousey et al. 2018).

Vitamin D_3 (**D_3**) is a fat soluble vitamin and its absorption in the upper region of the small intestine is facilitated by bile salts. After intestinal absorption, D_3 is transported to the liver where it is converted by 25-hydroxylase to the second metabolite of D_3, 25-hydroxyvitamin D_3 (**$25OHD_3$**). Subsequently, in renal cells, $25OHD_3$ is converted by 1α-hydroxylase to the most active form of D_3, 1,25-dihydroxyvitamin D_3 (**$1,25\text{-}(OH)_2\text{-}D_3$**; Henry, 1980). Vitamin D is crucial for proper embryo development (Narbaitz, 1987), and is involved in calcium (**Ca**) and phosphorous absorption (Bar et al, 1980), immunity (Adams and Hewison, 2008), and intestinal development (Chou et al., 2009) in chickens. When included at the same dietary level, $25OHD_3$ is approximately twice as active as D_3 in promoting Ca absorption (Myrtle and Norman, 1971), and has a higher retention in chicks compared to D_3 (93% and 80% respectively; Bar et al., 1980). Inclusion of $25OHD_3$ in broiler breeder diets has been shown to increase VL in the duodenum of broiler embryos at 19 doi and during the first 2 d of posthatch age (**doa) (**Ding et al, 2011). Additionally, supplemental dietary $25OHD_3$ at 2,760 IU/ Kg has been shown to increase VL and decrease crypt depth (**CD**) in broiler chickens (Chou et al., 2009). An increase in VL is linked to increased nutrient absorption (Onderci et al., 2006) and decreased CD is associated with less frequent epithelial cell turnover, leading to a lower energy requirement in the gut (Yang et al., 2008). Maternal $25OHD_3$ has also been shown to increase the innate immunity of chicks relative to D_3 (Saunders-Blades and Korver, 2015). Additionally, dietary supplementation of $25OHD_3$ has been shown to increase humoral immunity in early posthatch broiler chickens (Chou et al., 2009). Rodriguez-Lecompte et al. (2016) reported that the immunomodulatory function of vitamin D is associated with the levels of Ca and phosphorous in the diet.

Effects of the in ovo injection of D_3 and $25OHD_3$ on the small intestine morphology and immunity of broilers have not been previously investigated. It was hypothesized that the vitamin D_3 sources utilized in this study would positively influence the immune competency and small intestine morphology of the broilers, especially in those fed Ca and available phosphorous (**aP**)-restricted diets. Therefore, the objectives of this study were to determine the effects of the in ovo injection of D_3 and $25OHD_3$ on small intestine morphology, humoral immunity, and the inflammatory response of broilers fed either commercial diets that were and were not restricted in Ca and aP content by 20%.

MATERIALS AND METHODS

Experimental Design and Treatments

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Mississippi State University (Protocol #IACUC-17-406). Fertile broiler hatching eggs laid by 35 wk-old Ross 708 breeder hens were obtained from a commercial source and stored for 24 h. Prior to set, eggs were selected as described by Sokale et al. (2017) and a total of 2,880 eggs (average weight = 59.8 ± 0.81) were subsequently set and incubated in a Jamesway model PS 500 setter unit (Jamesway Incubator Company Inc., Cambridge, Ontario, Canada) under standard conditions (37.5° C and 29.4° C dry and wet bulb temperatures, respectively) as is described by Fatemi et al (2021b). Thirty eggs was assigned to each of 4 preassigned treatment groups on each of 24 incubator tray levels (blocks). Treatment groups on each tray were randomly placed to avoid positional effects, and monitoring of incubational conditions were as described by Fatemi et al (2020a, b). In ovo injection treatments were prepared according to the procedures of Fatemi et al. (2020a, b), and injections were administrated at 18 doi using a Zoetis Inovoject m in ovo injection machine (Zoetis Animal Health, Research Triangle Park, NC). At injection day, 8 eggs on each of the 12 tray levels were injected with Coomassie Brilliant Blue G-250 (colloidal) dye for determination of site of injection as described by Sokale et al. (2017). The sites of injections in this study were confirmed to be 97.92 and 2.7% in the amnion, and embryo body respectively. Williams (2007, 2011) has reported that amniotic and body proper injections are efficacious. In ovo injection treatments were: 1) **diluent** (control; 50 µL of commercial diluent [commercial Marek's Disease vaccine diluent; Merial Co., Duluth, GA]); 2) **D_3** (50 µL of commercial diluent containing 2.4 µg D_3), 3) **$25OHD_3$** (50 µL of commercial diluent containing 2.4 µg $25OHD_3$), and **D_3 + $25OHD_3$** (50 µL of commercial diluent containing 2.4 µg D_3 and 2.4 µg $25OHD_3$). For the posthatch period, 18 randomly selected male chicks belonging to 1 of the 4 in ovo injection treatments were placed in each of 12 floor pens and were subjected to 1 of 2 dietary treatments. Therefore, 8 treatment groups (4 in ovo injection x 2 dietary treatments) were randomly represented in each of 6 replicate blocks of pens (48 total pens) in the grow-out facility. Bird housing and husbandry during the posthatch period were as described by Fatemi et al. (2021a). Commercial diets were formulated according to Ross 708 guidelines (Aviagen, 2015). The 2 dietary treatments were: 1)

Table 1. Feed composition of the experimental diets from 0 to 41 d of age (doa).

Starter (0-14 doa)		
Item	Commercial diet	Calcium and available phosphorus restricted (ReCap) diet
Ingredient	(%)	
Yellow corn	53.23	53.23
Soybean meal	38.23	38.23
Animal fat	2.60	2.60
Dicalcium phosphate	2.23	1.71
Limestone	1.27	1.01
Salt	0.34	0.34
Choline chloride 60%	1.00	1.00
Lysine	0.28	0.28
DL-Methionine	0.37	0.37
L-threonine	0.15	0.15
Premix[1]	0.25	0.25
Coccidiostat[2]	0.05	0.05
Cellulose	0	0.78
Total	100	100
Calculated nutrients		
Crude protein	23	23
Calcium	0.96	0.768
Available phosphorus	0.48	0.384
Apparent metabolizable energy (AME; Kcal/kg)	3,000	3,000
Digestible Methionine	0.51	0.51
Digestible Lysine	1.28	1.28
Digestible Threonine	0.86	0.86
Digestible total sulfur amino acids (TSAA)	0.95	0.95
Sodium	0.16	0.16
Choline	0.16	0.16
Grower (15−28 doa)		
Item	Commercial diet	Calcium and available phosphorus restricted (ReCap) diet
Ingredient	(%)	
Yellow corn	57.13	57.13
Soybean meal	34.80	34.80
Animal fat	3.50	3.50
Dicalcium phosphate	2.00	1.52
Limestone	1.17	0.94
Salt	0.34	0.34
Choline chloride 60%	0.10	0.10
Lysine	0.21	0.21
DL-Methionine	0.32	0.32
L-threonine	0.16	0.16
Premix	0.25	0.25
Coccidiostat	0.05	0.05
Cellulose	0	0.71
Total	100	100
Calculated nutrients		
Crude protein	21.5	21.5
Calcium	0.87	0.696
Available phosphorus	0.435	0.348
AME (Kcal/kg)	3,100	3,100
Digestible Methionine	0.47	0.47
Digestible Lysine	1.15	1.15
Digestible Threonine	0.77	0.77
Digestible TSAA	0.87	0.87
Sodium	0.16	0.16
Choline	0.16	0.16
Finisher (29−45 doa)		
Item	Commercial diet	Calcium and available phosphorus restricted (ReCap) diet
Ingredient	(%)	
Yellow corn	54.23	54.23
Soybean meal	38.23	38.23
Animal fat	2.50	2.50
Dicalcium phosphate	2.23	1.71
Limestone	1.27	1.01
Salt	0.34	0.34
Choline chloride 60%	0.10	0.10
Lysine	0.28	0.28
DL-Methionine	0.37	0.37

(continued)

Table 1 (*Continued*)

Item	Commercial diet	Calcium and available phosphorus restricted (ReCap) diet
Starter (0-14 doa)		
L-threonine	0.15	0.15
Premix	0.25	0.25
Coccidiostat	0.05	0.05
Cellulose	0	0.78
Total	100	100
Calculated nutrients		
Crude protein	19.5	19.5
Calcium	0.78	0.624
Available phosphorus	0.39	0.312
AME (Kcal/kg)	3,200	3,200
Digestible methionine	0.43	0.43
Digestible lysine	1.02	1.02
Digestible threonine	0.68	0.68
Digestible TSAA	0.8	0.8
Sodium	0.16	0.16
Choline	0.16	0.16

[1]The broiler premix provided per kilogram of diet: vitamin A (retinyl acetate), 10,000 IU; cholecalciferol, 4,000 IU; vitamin E (DL-α-tocopheryl acetate), 50 IU; vitamin K, 4.0 mg; thiamine mononitrate (B_1), 4.0 mg; riboflavin (B_2), 10 mg; pyridoxine HCL (B_6), 5.0 mg; vitamin B_{12} (cobalamin), 0.02 mg; D-pantothenic acid, 15 mg; folic acid, 0.2 mg; niacin, 65 mg;biotin, 1.65 mg; iodine (ethylene diamine dihydroiodide), 1.65 mg; Mn ($MnSO_4H_2O$), 120 mg;Cu, 20 mg; Zn, 100 mg, Se, 0.3 mg; Fe ($FeSO_4.7H_2O$), 800 mg.

[2]Decocx (Zoetis, Parsippany, NJ).

commercial diet; 2) the commercial diet restricted in Ca and aP content by 20% (**ReCaP diet**) for the starter (Ca = 0.768%, aP = 0.384%), grower (Ca = 0.696%, aP = 0.348%) and finisher (Ca = 0.624%, aP = 0.312%) dietary phases (Table 1).

Blood Sampling and Immunological Assessment

One bird from each of the 48 floor pens was randomly selected, individually weighed, and euthanized for organ sampling at 0, 7, 14, and 40 doa. At 0 doa, weights of the liver, spleen, bursa and small intestine (**SI**, between terminus of the gizzard and origin of the cecal ducts) were obtained. At 7, 14, and 40 doa, individual weights of the liver, spleen, bursa, duodenum, jejunum, and ileum were obtained. All weights were subsequently expressed as percentages of BW. From the same birds that were selected for organs sampling at the aforementioned time periods, blood samples were also collected from the chorioallantoic vasculature. Plasma was subsequently extracted for immunological assay. Plasma IgG concentration was determined for samples collected at 7 and 14 doa according to the manufacturer's protocol (MyBioSource, San Diego, CA). Duplicate 100 μL volumes of standard and experimental samples were loaded into plate wells precoated with capture antibody, and were then incubated at 37°C for 90 min. Plates were washed 2 times with wash solution (50 mM Tris-buffered saline, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) and 100 μL of biotinylated chicken IgG antibody was added to each well, and plates were incubated at 37°C for 60 min. Plates were washed 3 times with wash solution and 100 μL of enzyme-conjugate liquid was added to each well in order to initiate a TMB substrate color reaction. Reactant was thoroughly washed out by PBS or TBS. Plates were incubated at 37°C for 30 min and were washed 5 times with wash solution. A 100 μL volume of color reagent was added to each well in order to produce a blue color in each sample. Samples were then incubated in a dark incubator at 37°C for 30 min. Finally, the color of each sample was changed from blue to yellow under the action of a 100 μL volume of stop solution. Plasma IgM concentration was determined at 14 doa according to the procedure of Perez-Carbajal et al. (2010) and plasma α-1-acid glycoprotien (**AGP**) was determined at 40 doa according to the procedure of Kaab et al. (2018). Optical densities (**OD**) at 450 nm (OD450) for IgG, IgM and AGP were measured with a SpectraMax M5 Microplate Reader (Molecular Devices, San Jose, CA).

Small Intestine Morphology

From each intestine sample collected from the 1 bird in each of the 48 floor pens at 14 and 40 doa, a 2 cm sample from the middle region of the duodenum, jejunum, and ileum was excised and fixed in 10% formalin. Intestinal samples were then gradually dehydrated and embedded in paraffin. The slides used for histomorphological analysis were prepared according to the procedure of Wang et al. (2015). Villus and crypt measurements from 3 different and randomly selected locations within each of the 3 intestinal regions for a particular bird were included on each slide. Slides were examined using a light microscope at 40X magnification (Micromaster, Fisher Scientific, Pittsburgh, PA) according to the method described by Fasina et al. (2010). Microscopic images were measured using ImageJ software (Wayne Rasband, NIMH, Bethesda, MD) to measure VL, villus width (**VW**) and CD. Villus length was considered as the distance from the tip to the base of the lamina propria and VW was measured between the top third and bottom third of the villus. Crypt depth was the length from the base of the villus to the mucosa layer. The ratio of VL to CD (**RVC**) was calculated by dividing VL by CD. Villus surface area was calculated for all sections using the following formula presented below by Nain et al. (2012). Villus surface area (VSA) = $2\pi \times$ (average VW/2) $\times$ VL; where average VW is the average of 3 measurements per bird (2 VW measurements from the 3 sample sections examined on each slide). Mean values of the observation variables within each of the 3 intestinal regions for each bird were subsequently subjected to statistical analysis.

Statistical Analysis

The experimental unit was floor pen, and the experimental design was a randomized complete block. A group of pens was the blocking factor with all 8 treatment combinations randomly represented in each of 6 blocks. All data were analyzed at each time period separately using 2-way analysis of variance in a 2 dietary treatment x 4 in ovo injection treatment factorial design. The general linear mixed models procedure (PROC GLIMMIX) of SAS 9.4 (SAS Institute, 2013) was employed. Differences were considered significant at $P \leq 0.05$. The following model was used for analysis of the data:

$$Y_{ijk} = \mu + B_i + D_j + I_k + (DI)_{jk} + E_{ijk},$$

Where μ was the population mean; B_i was the block factor (i = 1 to 2); D_i was the effect of each dietary treatment (j = 1 to 2); Ik was the effect of in ovo injection treatment (k = 4); (DI)ij was the interaction of each dietary treatment with in ovo injection treatment; and E_{ij} was the residual error.

RESULTS

Body and Organ Weights

No significant effects due to in ovo injection treatment were observed for BW and all relative organ data at 0 doa (Table 2), and no significant interactions were observed between diet and in ovo injection treatment for BW and relative organ weights at 7, 14, and 40 doa. Furthermore, at 7 and 14 doa, there were no significant main effects due to in ovo or dietary treatment for any of the variables examined. There were also no significant main effects due to in ovo treatment for any of the variables examined at 40 doa. However, BW was lower and relative duodenum weight was greater in birds fed ReCaP diets in comparison to those fed commercial diets (Table 2).

Immunity

No significant interactions were observed between diet and in ovo treatment for any of the immunological measurements determined at their specified time periods (Table 3). There were also no significant main effects due to diet for plasma IgG concentrations at 7 and 14 doa, IgM at 14 doa, and AGP at 14 doa, or due to in ovo injection treatment for plasma IgG concentrations at 7 doa, and AGP at 40 doa. However, at 14 doa, birds that received $25OHD_3$ alone had higher plasma IgG concentrations compared to those that were injected with diluent or D_3 alone (Table 3). An injection of $D_3 + 25OHD_3$ also resulted in plasma IgG concentrations that were higher than those in birds injected with D_3 alone. Furthermore, at 14 doa, birds that received the combination of $25OHD_3$ and D_3 had higher plasma IgM levels in comparison to those that received diluent or D_3 alone, and those that received $25OHD_3$ alone had higher IgM concentrations than those that were in ovo-injected with only diluent (Table 3).

Small Intestine Morphology

No significant interactions were observed between diet and in ovo injection treatments at any time period for any of the histomorphological measurements (Tables 4 and 5). Due to the large number of variables examined, only those variables exhibiting significant main effects due to in ovo injection and dietary treatment are discussed. At 14 doa, VL was higher in the ileum in birds that were injected with $25OHD_3$ alone compared to those that received only diluent, and duodenal CD was shallower in birds injected with $25OHD_3$ alone in comparison to all other treatments. Additionally, a lower jejunal CD was observed in birds that received $25OHD_3$ alone in comparison to those that received diluent or D_3 alone, and was lower in the $D_3 + 25OHD_3$ group in comparison to the D_3 alone group (Table 4). Greater duodenal, jejunal, and ileal RVC were observed in birds that received $25OHD_3$ alone in comparison to all other treatments. In comparison to the diluent-injected group, ileal VSA was decreased in response to the in ovo injection of $25OHD_3$ alone, and VW in the jejunum was lower in birds that received D_3 and $25OHD_3$ together than if they were received separately. At 14 doa, VL in the duodenum was decreased in birds fed ReCaP diets in comparison to those fed commercial diets. Conversely, jejunal CD was higher in birds fed ReCaP diets in comparison to those fed commercial diets (Table 4).

At 40 doa, VL in the duodenum was greater in birds injected with $25OHD_3$ alone in comparison to those that were injected with diluent or D_3 alone. However, ileal VL increased in response to the injection of $25OHD_3$ alone when compared to the D_3 alone or $25OHD_3 + D_3$ treatments. Furthermore, VL in the D_3 treatment was lower than that in the diluent control group. Crypt depth in the duodenum was shallower in birds that received $25OHD_3$ alone in comparison to those in the D_3 or diluent alone treatment groups, but ileum CD in birds

Table 2. Broiler mean BW, relative weight of the whole small intestine (**SI**), liver, spleen, bursa, duodenum, jejunum, and ileum at 0, 7, 14, and 40 d of age (**doa**) in diluent in ovo-injected (50 μL) control eggs, and eggs in ovo-injected with diluent containing 2.4 μg of vitamin D_3 (**D_3**) or 25-hydroxycholecalciferol (**$25OHD_3$**) alone, or a combination of 2.4 μg of D_3 and 2.4 μg of $25OHD_3$ (**D_3 + $25OHD_3$**), and fed a commercial diet or a diet in which calcium and available phosphorus were restricted by 20%.

Treatment	BW (g)	SI[1] (%)	Liver (%)	Spleen (%)	Bursa (%)
			0 doa		
In ovo injection					
Diluent[2]	41.3	2.88	1.02	0.017	0.087
D_3[3]	41.0	3.38	0.99	0.015	0.143
$25OHD_3$[4]	41.8	2.82	0.95	0.023	0.041
D_3+$25OHD_3$[5]	40.6	2.99	0.98	0.016	0.093
Pooled SEM	0.78	0.191	0.036	0.0029	0.0528
P value	0.759	0.177	0.588	0.158	0.599

Treatment	BW (g)	Duodenum (%)	Jejunum (%)	Ileum (%)	Liver (%)	Spleen (%)	Bursa (%)
			7 doa				
In ovo injection							
Diluent	158.8	1.63	2.83	2.46	3.12	0.076	0.36
D_3	156.8	1.77	2.54	2.62	3.18	0.077	0.17
$25OHD_3$	147.8	2.16	2.81	2.62	3.15	0.084	0.26
D_3+$25OHD_3$	157.4	1.52	2.75	2.29	3.08	0.087	0.17
Diet							
Commercial	153.5	1.88	2.74	2.62	3.08	0.077	0.16
ReCaP[6]	156.9	1.67	2.73	2.45	3.18	0.085	0.32
Pooled SEM	4.55	0.123	0.300	0.193	0.155	0.007	0.112
	P value						
In ovo	0.413	0.072	0.845	0.539	0.952	0.634	0.566
Diet	0.506	0.230	0.966	0.510	0.437	0.226	0.142
In ovo x Diet	0.177	0.132	0.742	0.881	0.451	0.887	0.530

Treatment	BW (g)	Duodenum (%)	Jejunum (%)	Ileum (%)	Liver (%)	Spleen (%)	Bursa (%)
			14 doa				
In ovo injection							
Diluent	452	1.85	1.87	1.58	2.68	0.106	0.493
D_3	449	1.15	1.76	1.53	2.93	0.169	0.178
$25OHD_3$	465	1.10	1.62	1.42	2.53	0.139	0.210
D_3+$25OHD_3$	445	1.27	1.61	1.63	2.75	0.069	0.153
Diet							
Commercial	454	1.46	1.63	1.53	2.73	0.149	0.188
ReCaP	450	1.23	1.80	1.55	2.72	0.092	0.161
Pooled SEM	9.8	0.255	0.084	0.110	0.070	0.0477	0.021
	P value						
In ovo	0.563	0.439	0.241	0.514	0.055	0.634	0.493
Diet	0.731	0.524	0.087	0.891	0.875	0.318	0.370
In ovo x Diet	0.285	0.280	0.399	0.463	0.061	0.327	0.481

Treatment	BW (g)	Duodenum (%)	Jejunum (%)	Ileum (%)	Liver (%)	Spleen (%)	Bursa (%)
			40 doa				
In ovo injection							
Diluent	2,408	0.497	1.025	0.738	2.26	0.100	0.118
D_3	2,261	0.564	1.038	0.818	2.14	0.091	0.145
$25OHD_3$	2,402	0.532	0.963	0.755	2.14	0.090	0.138
D_3+$25OHD_3$	2,254	0.510	1.021	0.657	2.50	0.112	0.119
Diet							
Commercial	2,573[a]	0.476[b]	0.969	0.718	2.30	0.096	0.125
ReCaP	2,089[b]	0.575[a]	1.055	0.766	2.22	0.101	0.136
Pooled SEM	69.3	0.0250	0.044	0.0549	0.088	0.0055	0.0120
	P value						
In ovo	0.228	0.575	0.838	0.350	0.260	0.290	0.342
Diet	0.001	0.013	0.204	0.415	0.566	0.537	0.422
In ovo x Diet	0.114	0.819	0.635	0.389	0.518	0.475	0.379

Treatment means within the same variable column within type of treatment with no common superscript differ significantly ($P < 0.05$).
[1]The weight of and gastrointestinal tract from the bottom of proventriculus to the end of the ceca.
[2]Eggs injected with 50 μL commercial diluent at d 18 of incubation.
[3]Eggs injected with 50 μL commercial diluent containing vitamin D_3 at 2.4 μg at d 18 of incubation.
[4]Eggs injected with 50 μL commercial diluent containing $25OHD_3$ at 2.4 μg at d 18 of incubation.
[5]Eggs injected with 50 μL commercial diluent containing D_3 at 2.4 and $25OHD_3$ at 2.4 μg at d 18 of incubation.
[6]A diet restricted Ca and available P by 20% throughout the rearing period.

Table 3. Broiler mean plasma IgG at 7 and 14 d of age (doa), IgM at 14 doa, and alpha-1-acid glycoprotein (AGP) at 40 doa in diluent in ovo-injected (50 μL) control eggs, and eggs in ovo-injected with diluent containing 2.4 μg of vitamin D_3 (D_3) or 25-hydroxycholecalciferol (25OHD$_3$) alone, or a combination of 2.4 μg of D_3 and 2.4 μg of 25OHD$_3$ (D_3 + 25OHD$_3$), and fed a commercial diet or a diet in which calcium and available phosphorus were restricted by 20%.

Treatment	IgG-7doa[1]	IgG-14doa[2]	IgM-14doa[3]	AGP-40doa[4]
	μM			
In ovo injection				
Diluent[5]	2.39	6.52^{bc}	2.37^{c}	2.92
D_3[6]	2.26	6.43^{c}	2.42^{bc}	2.93
25OHD$_3$[7]	2.34	6.63^{a}	2.48^{ab}	2.72
D_3+25OHD$_3$[8]	2.34	6.58^{ab}	2.57^{a}	2.86
Diet[9]				
Commercial	2.32	6.58	2.42	2.86
ReCaP	2.35	6.50	2.50	2.82
Pooled SEM	0.049	0.053	0.045	0.009
	P value			
In ovo	0.302	0.013	0.005	0.079
Diet	0.570	0.090	0.080	0.580
In ovo x Diet	0.302	0.984	0.974	0.129

[a,b]Treatment means within the same variable column within type of treatment with no common superscript differ significantly ($P < 0.05$).
[1]Plasma IgG concentrations at 7 doa.
[2]Plasma IgG concentrations at 14 doa.
[3]Plasma IgM concentrations at 14 doa.
[4]Plasma AGP concentrations at 40 doa.
[5]Eggs injected with 50 μL commercial diluent at d 18 of incubation.
[6]Eggs injected with 50 μL commercial diluent containing vitamin D_3 at 2.4 μg at d 18 of incubation.
[7]Eggs injected with 50 μL commercial diluent containing 25OHD$_3$ at 2.4 μg/ at d 18 of incubation.
[8]Eggs injected with 50 μL commercial diluent containing D_3 at 2.4 and 25OHD$_3$ at 2.4 μg at d 18 of incubation.
[9]A diet restricted Ca and available P by 20% through the rearing period.

having been in ovo- injected with 25OHD$_3$ alone or in combination with D_3 was shallower in comparison to those in the D_3 or diluent alone treatments (Table 5). In the duodenum and jejunum, CD was only lower in the 25OHD$_3$ treatment in comparison to the D_3 alone and diluent treatment groups, and in the jejunum, a higher RVC was observed in birds injected with 25OHD$_3$ alone in comparison to those injected with diluent or D_3 alone. Also, 25OHD$_3$ alone resulted in a higher RVC in the duodenum in comparison to all other treatments. In the ileum, a higher RVC was observed in the 25OHD$_3$ alone treatment in comparison to all other treatments, and the combination of 25OHD$_3$ and D_3 resulted in a higher RVC compared to D_3 alone. In the ileum, VSA was higher in the D_3 alone treatment in comparison to the 25OHD$_3$ or diluent alone treatments. At 40 doa, birds fed ReCaP diets had a lower duodenal RVC, and lower ileal VL and VW, but conversely had a deeper jejunal CD in comparison to those fed commercial diets (Table 5).

DISCUSSION

Development of the small intestine in response to vitamin D_3 occurs mainly through changes in its morphology. Inclusion of 25OHD$_3$ in breeder diets has resulted in an increased VL in broiler embryos at 19 doi as well as in broilers at 2 doa (Ding et al., 2011). In addition to its early posthatch effects, dietary supplementation of 25OHD$_3$ at 2,670 IU/kg has been shown to increase duodenal and jejunal VL and decrease jejunal CD in broilers at 28 and 35 doa (Chou et al., 2009). Various effects of the in ovo injection of vitamin D_3 sources including D_3, 25OHD$_3$, 1- α hydroxyvitamin D_3, and 1α,25-(OH)$_2$-D_3 have been reported (Bello et al., 2014; Abbasi et al., 2017; Mansour et al., 2017). However, their effects on the histomorphology of the small intestine of the embryo or posthatch broiler have not been previously reported. Nevertheless, findings in the current study reveal that the in ovo injection of 2.4 μg of 25OHD$_3$ improved the small intestine morphology of broilers in comparison to diluent or D_3 alone. In mammals, 1-α hydroxylase, which converts 25OHD$_3$ to 1α,25-(OH)$_2$-D_3, has been identified in dendritic cells, macrophages (Overbergh et al., 2000; Veldman et al., 2000), B-cells (Chen et al., 2007), T-cells (Veldman et al., 2000), and duodenal sections of the small intestine (Gawlik et al., 2015). Additionally, in contrast to D_3, 25OHD$_3$ has been shown to increase the activity of 1-α hydroxylase in the chicken (Morris et al., 2015). This suggests that cells of the immune system and those in the intestine may display a greater response to this secondary metabolite of vitamin D_3. In comparison to D_3, the half-life of 25OHD$_3$ is longer (Smith and Goodman, 1971; Hollis and Wagner, 2013) and it is absorbed at a higher rate (Bar et al., 1980). The improvement in small intestine morphology observed in response to the in ovo injection of 25OHD$_3$ alone, may be due to the longer half-life and higher rate of absorption of 25OHD$_3$. Previous findings in our laboratory revealed that breast meat yield increased (Fatemi et al., 2021 a,b) and FCR decreased (Fatemi et al., 2020a) in response to the in ovo injection of 2.4 μg of 25OHD$_3$. An improvement in small intestine morphology and humoral immunity is associated with increased breast meat yield and an improvement in the growth performance of broilers (Chou et al., 2009; Wang et al., 2019). Therefore, an enhancement of breast meat yield and growth performance observed in response to the in ovo injection of 25OHD$_3$ could be due to an enhancement of small intestine morphology and immunity in birds.

It is well documented that at least 2 wk are required for lymphocytes to be fully developed in posthatch chickens (Bar-Shira and Friedman, 2006). Additionally, various vitamin D_3 sources affect humoral immunity. In the current study, it was shown that in comparison to the injection of diluent or D_3 alone, the in ovo injection of 25OHD$_3$ resulted in increased IgG and IgM levels at 14 doa when compared to that those injected with diluent or D_3 alone. Abbasi et al. (2017) also showed that an increase in antibody titers against Newcastle Disease was observed in hatchlings in response to the in ovo injection of a 0.5 ml of solution containing 0.4 μg of 25OHD$_3$ and 6 μg of vitamin K. Thus, increased Ig levels of early-hatch broilers within the first 2 wk of life can be beneficial for the enhancement of their immunity. These results indicate that the in ovo administration of 2.4 μg of 25OHD$_3$ might be a suitable candidate for boosting the humoral immunity of early posthatch broilers. In addition to humoral immunity, the

Table 4. Broiler mean small intestine morphology measurements at 14 d of age (doa) in diluent in ovo-injected (50 μL) control eggs, and eggs in ovo-injected with diluent containing 2.4 μg of vitamin D_3 (D_3) or 25-hydroxycholecalciferol ($25OHD_3$) alone, or a combination of 2.4 μg of D_3 and 2.4 μg of $25OHD_3$ ($D_3 + 25OHD_3$), and fed a commercial diet or a diet in which calcium and available phosphorus were restricted by 20%.

Treatment	Duodenum villus length	Villus width	Crypt depth	RVC[1] length/depth	VSA[2]	Jejunum villus length	Villus width	Crypt depth	RVC length/depth	VSA	Ileum villus length	Villus width	Crypt depth	RVC length/depth	VSA
	μm				mm^2	μm				mm^2	μm				mm^2
In ovo injection															
Diluent[3]	994	89.4	136^{a}	7.7^{b}	0.28	848	89.3^{ab}	116^{b}	7.4^{b}	0.34	336^{b}	83.1	90	4.0^{b}	0.40^{a}
D_3[4]	999	93.8	137^{a}	7.4^{b}	0.29	922	98.9^{a}	137^{a}	7.0^{b}	0.34	391^{ab}	87.1	107	3.8^{b}	0.35^{ab}
$25OHD_3$[5]	1088	82.8	89^{b}	12.2^{a}	0.24	902	93.0^{a}	88^{c}	10.0^{a}	0.33	480^{a}	81.2	77	6.4^{a}	0.27^{b}
D_3+$25OHD_3$[6]	983	83.2	122^{a}	8.2^{b}	0.27	780	73.3^{b}	107^{cb}	7.5^{b}	0.30	428^{ab}	75.4	90	4.8^{b}	0.30^{ab}
Diet[7]															
Commercial	1065^{a}	93.3	124	9.5	0.28	849	85.7	104^{b}	8.6	0.32	415	85.8	88	5.0	0.34
ReCaP	967^{b}	81.4	114	9.0	0.27	877	91.5	120^{a}	7.7	0.33	403	77.5	94	4.6	0.31
Pooled SEM	50.4	9.62	10.0	0.69	0.028	45.8	6.92	6.6	0.58	0.025	39.4	10.35	8.9	0.52	0.039
				P value											
In ovo	0.092	0.549	0.001	0.001	0.195	0.168	0.050	0.001	0.001	0.543	0.020	0.864	0.067	0.001	0.039
Diet	0.005	0.062	0.129	0.311	0.644	0.555	0.380	0.021	0.150	0.778	0.746	0.362	0.455	0.376	0.409
In ovo x Diet	0.606	0.192	0.598	0.942	0.190	0.176	0.351	0.068	0.845	0.595	0.390	0.326	0.570	0.178	0.531

[a,b]Treatment means within the same variable column within type of treatment with no common superscript differ significantly ($P < 0.05$).
[1]Ratio of villus length to crypt depth.
[2]Villus surface area (VSA) calculated with average villus length and width = $2\pi \times (\text{width}/2) \times \text{length}$.
[3]Eggs injected with 50 μL commercial diluent at d 18 of incubation.
[4]Eggs injected with 50 μL commercial diluent containing vitamin D_3 at 2.4 μg at d 18 of incubation.
[5]Eggs injected with 50 μL commercial diluent containing $25OHD_3$ at 2.4 μg at d 18 of incubation.
[1]Eggs injected with 50 μL commercial diluent containing D_3 at 2.4 and $25OHD_3$ at 2.4 μg at d 18 of incubation.
[7]A diet restricted Ca and available P by 20% throughout the rearing period.

Table 5. Broiler mean small intestine morphology measurements at 40 d of age (doa) in diluent in ovo-injected (50 µL) control eggs, and eggs in ovo-injected with diluent containing 2.4 µg of vitamin D_3 (D_3) or 25-hydroxycholecalciferol (25OHD_3) alone, or with a combination of 2.4 µg of D_3 and 2.4 µg of 25OHD_3 (D_3 + 25OHD_3), and fed a commercial diet or a diet in which calcium and available phosphorus were restricted by 20%.

Treatment	Duodenum Villus length (µm)	Villus width (µm)	Crypt depth (µm)	RVC[1] Length/ Depth	VSA[2] mm²	Jejunum Villus length (µm)	Villus width (µm)	Crypt depth (µm)	RVC Length/ Depth	VSA mm²	Ileum Villus length (µm)	Villus width (µm)	Crypt depth (µm)	RVC Length/ Depth	VSA mm²
In ovo injection															
Diluent[3]	1182^{b}	101	124^{a}	9.9^{b}	0.27	975	111	123^{a}	8.2^{b}	0.36	565^{ab}	80.9	97^{a}	5.9^{bc}	0.43^{b}
D_3[4]	1200^{b}	113	126^{a}	9.7^{b}	0.30	903	107	119^{a}	7.7^{b}	0.38	458^{c}	86.1	95^{a}	4.9^{c}	0.57^{a}
25OHD_3[5]	1388^{a}	106	96^{b}	15.1^{a}	0.24	1005	110	91^{b}	11.3^{a}	0.35	658^{a}	88.2	72^{b}	9.6^{a}	0.43^{b}
D_3+25OHD_3[6]	1253^{ab}	98	112^{ab}	11.5^{b}	0.25	918	106	104^{ab}	9.5^{ab}	0.37	538^{bc}	80.1	76^{b}	7.5^{b}	0.47^{ab}
Diet[7]															
Commercial	1290	106	109	12.4^{a}	0.26	957	111	100^{b}	10.0	0.37	599^{a}	91.4^{a}	89	7.1	0.47
ReCaP	1222	103	120	10.7^{b}	0.27	944	106	118^{a}	8.4	0.36	501^{b}	76.3^{b}	80	6.9	0.48
Pooled SEM	50.4	45.9	7.7	5.8	0.72	0.025	71.4	10.37	8.5	0.98	0.031	22.9	7.61	4.1	0.60
				P value											
In ovo	0.023	0.474	0.006	0.001	0.276	0.590	0.983	0.011	0.020	0.833	0.003	0.834	0.007	0.001	0.031
Diet	0.166	0.681	0.063	0.032	0.734	0.834	0.560	0.019	0.074	0.490	0.017	0.049	0.115	0.827	0.701
In ovo x Diet	0.606	0.773	0.608	0.672	0.361	0.939	0.541	0.983	0.103	0.117	0.698	0.949	0.820	0.585	0.830

[a,b]Treatment means within the same variable column within type of treatment with no common superscript differ significantly ($P < 0.05$).
[1]Ratio of villus length to crypt depth.
[2]Villus surface area (VSA) calculated with average villus length and width = $2\pi \times (\text{width}/2) \times \text{length}$.
[3]Eggs injected with 50 µL commercial diluent at d 18 of incubation.
[4]Eggs injected with 50 µL commercial diluent containing vitamin D_3 at 2.4 µg at d 18 of incubation.
[5]Eggs injected with 50 µL commercial diluent containing 25OHD_3 at 2.4 µg at d 18 of incubation.
[6]Eggs injected with 50 µL commercial diluent containing D_3 at 2.4 and 25OHD_3 at 2.4 µg at d 18 of incubation.
[7]A diet restricted Ca and available P by 20% throughout the rearing period.

inflammatory response of the broilers was monitored by measuring AGP at 40 doa. It has been proposed in several reports that AGP levels in chickens can be used to monitor an increase in inflammation (Lee et al., 2010; Asasi et al., 2013; Fatemi, 2016). Furthermore, the AGP concentrations of modern broiler lines have been observed to be higher than those of broiler lines used in the 1990's (O'Reilly et al., 2018). However, the AGP concentrations of the birds in the current study were not significantly affected by in ovo injection or dietary treatment, indicating that a general systemic inflammatory response in the birds was not significantly affected by either treatment.

In this study, a 20% reduction in dietary Ca and aP resulted in a decline in duodenal VL at 14 doa, and other negative effects included a lower duodenal RVC and a higher jejunal CD at 40 doa. A reduction in dietary aP has been shown to exert a greater effect on broiler gut morphology than a reduction in dietary Ca. A diet containing 0.6 % of Ca and 0.3 % of aP has been shown to have no negative effects on VCR in comparison to recommended levels of Ca and aP (0.90 % of Ca and 0.45 % of aP) in broilers. However, a 30% reduction in aP in combination with a 0.90% level of Ca resulted in a lower VCR in the duodenum and ileum (Paiva et al., 2014). The inclusion of supplemental dietary phytase at 1,000 FTU/kg was sufficient to overcome the negative effects of a reduction in aP on small intestine morphology and broiler performance. In addition to this, an increase in dietary Ca levels from 0.90 to 1.05% has been shown to have no effect on small intestine morphology in broilers at different ages (Xing et al., 2020). An increase in intestine weight relative to BW is associated with a decline in small intestine morphology (Chou et al., 2009; Wu et al., 2013, 2016), and performance (Wu et al., 2016). In the current study, relative duodenal and jejunal weights increased at 41 doa, and relative jejunal weight tended to increase at 41 doa, in birds fed ReCaP rather than commercial diets. Therefore, the decline in small intestine morphology in response to a 20% reduction in Ca and aP may have been partially linked to an increase in intestine weight.

In conclusion, the influence of the in ovo injection of various vitamin D_3 sources on small intestine morphology and immunity of Ross 708 broilers fed commercial and ReCaP diets were investigated. These results showed that the ReCaP diet adversely affected broiler intestinal morphology and growth, but that it did not influence effects of the injection of these vitamin D_3 sources on their immunity and small intestine morphology. Regardless of dietary treatment, the small intestine morphology and humoral immunity of the birds were improved in response to the in ovo injection of $25OHD_3$ alone, but these effects were not observed when D_3 was injected alone. This improvement in response to the in ovo injection of 2.4 μg of $25OHD_3$ could be due to its longer half-life or greater rate of absorption. Further research is required to determine the effects of the in ovo injection of the vitamin D_3 sources on the molecular mechanisms of intestinal development and immunity in broilers.

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DISCLOSURES

There is no conflict of interest

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