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1,25-Dihydroxycholecalciferol Improved the Growth Performance and Upregulated the Calcium Transporter Gene Expression Levels in the Small Intestine of Broiler Chickens

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1,25-Dihydroxycholecalciferol (1,25-$(OH)_2$-D_3) is the final active product of vitamin D. This study aimed to investigate the effects of 1,25-$(OH)_2$-D_3 on growth performance, bone development, and calcium (Ca) transporter gene expression levels in the small intestine of broiler chickens. On the day of hatching, 140 female Ross 308 broilers were randomly allotted into two treatments with five replicates (14 birds per replicate). Two levels of 1,25-$(OH)_2$-D_3 (0 and 1.25 μg/kg) were added to the basal diet without vitamin D. Results showed that the addition of 1.25 μg/kg 1,25-$(OH)_2$-D_3 increased the average daily feed intake and the average daily gain and decreased the feed conversion ratio and mortality in 1- to 19-day-old broiler chickens compared with the basal diet without vitamin D ($P<0.05$). 1,25-$(OH)_2$-D_3 also enhanced the length, weight, ash weight, and the percentage contents of ash, Ca, and P in the tibia and femur of broilers ($P<0.05$). The mRNA expression levels of the Ca-binding protein (CaBP-D28k) in the duodenum, jejunum, and ileum of 19-day-old broilers increased to 88.1-, 109.1-, and 2.7-fold, respectively, after adding 1,25-$(OH)_2$-D_3 ($P<0.05$). The mRNA expression levels of the plasma membrane Ca ATPase 1b (PMCAlb) in the duodenum and the sodium (Na)/Ca exchanger 1 (NCX1) in the duodenum and the jejunum were also enhanced to 1.57−2.86 times with the addition of 1,25-$(OH)_2$-D_3 ($P<0.05$). In contrast, the mRNA expression levels of PMCA1b and NCX1 in the ileum and that of vitamin D receptor (VDR) in the small intestine were not affected by 1,25-$(OH)_2$-D_3 ($P>0.05$). These data indicate that 1,25-$(OH)_2$-D_3 upregulated Ca transporter gene transcription and promoted Ca^{2+} absorption in the small intestine, especially in the proximal intestine (duodenum and jejunum), thereby improving growth performance and bone mineralization in broiler chickens.

Key words: broiler chicken, CaBP-D28k, 1,25-dihydroxycholecalciferol, NCX1, PMCAlb, VDR

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Introduction

Vitamin D_3 (VD_3) is used as an essential feed additive to regulate calcium (Ca) absorption in poultry. Vitamin D deficiency inhibits growth and decreases bone mineralization in chickens (Baker *et al*., 1998; Chen *et al*., 2017). Supplemental VD_3 has been shown to improve the average daily feed intake (ADFI) and average daily gain (ADG) in broilers (Baker *et al*., 1998). The optimal requirement of VD_3 is about 25 μg/kg feed in broilers from 1 to 21 days of age (Chinese Feeding Standard of Chicken, Ministry of Agriculture of China, 2004). VD_3 undergoes 25-hydroxylation in the liver to form 25-hydroxycholecalciferol (25-OH-D_3) and 1α-hydroxylation in the kidney to form the final product 1,25-dihydroxycholecalciferol (1,25-$(OH)_2$-D_3). The bioactivity of 1,25-$(OH)_2$-D_3 is higher than that of VD_3 and 25-OH-D_3 (Soares *et al*., 1995).

1,25-$(OH)_2$-D_3 binds to the vitamin D receptor (VDR) to regulate Ca absorption in the intestine. Ca absorption in the small intestine of animals includes transcellular and paracellular pathways. The paracellular pathway allows the direct exchange of Ca^{2+} between the intestine and blood via tight

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junctions (Hoenderop *et al*., 2005). The Ca^{2+} transcellular transport consists of three major steps: entry of Ca^{2+} through the brush border to intestinal cells, movement from the apical membrane to the basal membrane, and extrusion through the basal membrane to the blood (Bar, 2008; Fleet and Schoch, 2010). The first step proceeds down the chemical gradient of Ca^{2+}, which is facilitated by the transient receptor potential cation channels (TRPV5 and TRPV6). The second step is facilitated by the intracellular Ca-binding protein CaBP-D9k (mammals) or CaBP-D28k (poultry). The energy-dependent third step proceeds up the chemical gradient of Ca^{2+} and is facilitated by the plasma membrane Ca ATPase 1b (PMCA1b) and sodium (Na)/calcium exchanger (NCX1) (Hoenderop *et al*., 2005; Lytton, 2007). The components of all three steps of Ca^{2+} transcellular transport are dependent on vitamin D (Bar, 2008).

Studies in rodents have shown that TRPV6, CaBP-D9k, PMCA1b, and NCX1 exist in the small intestine of mammals and that the injection of 1,25-$(OH)_2$-D_3 increased the mRNA expression levels of four Ca transporter genes and promoted Ca absorption in the small intestine of mice (Okano *et al*., 2004; Benn *et al*., 2008; Khuituan *et al*., 2012; Chow *et al*., 2013; Wongdee and Charoenphandhu, 2015).

CaBP-D9k is found in the small intestine of mammals, whereas CaBP-D28k is expressed in the poultry intestine. Four Ca transporter genes (i.e., TRPV6, CaBP-D28k, PMCAlb, and NCX1) were successfully cloned in the small intestine of laying hens (Sugiyama *et al*., 2007; Yang *et al*., 2011; Li *et al*., 2018). The highest expression levels of TRPV6 mRNA were found in the duodenum of laying hens, followed by the jejunum and ileum (Yang *et al*., 2011). The protein expression levels of CaBP-D28k and PMCA1b were higher in the duodenum than in the jejunum and ileum (Sugiyama *et al*., 2007; Li *et al*., 2018). Vitamin D regulates Ca transporter gene expression levels after binding VDR. The addition or injection of 1,25-$(OH)_2$-D_3 increased the mRNA expression levels of CaBP-D28k in the small intestine of white Leghorn cockerels (Clemens *et al*., 1988; Hall and Norman, 1990) and laying hens (Bar *et al*., 1990).

However, reports on Ca absorption in broilers are lacking. The relationship between dietary 1,25-$(OH)_2$-D_3 and Ca transporter gene expression levels in broiler chickens has not been evaluated. Therefore, this study aimed to investigate the effects of dietary 1,25-$(OH)_2$-D_3 levels on growth performance, leg bone mineralization, and Ca transporter gene expression levels in the small intestine of broiler chickens.

Materials and Methods

Birds, Diets, and Management

All procedures used in this study were approved by the Animal Welfare and Ethics Committee of Henan Agricultural University and Shangqiu Normal University (Permit Number: 2019-1023).

On the day of hatching, 140 female Ross 308 broilers were randomly allotted to two treatments with five replicates (14 birds per replicate). Two levels of 1,25-$(OH)_2$-D_3 (0 and 1.25 μg/kg) were added to the basal diet without vitamin D (Table 1). The basal diet contained 1.00% Ca and 0.45% non-phytate phosphorus (NPP) in accordance with the recommendations of the NRC (1994).

Table 1. **Ingredients and nutrient composition of the basal diet (as-fed basis)**

Ingredient (%)	Basal diet
Corn	58.10
Soybean meal (44% CP)	32.07
Soybean oil	2.22
Soybean protein powder (65% CP)	3.50
Limestone	1.36
Dicalcium phosphate	1.94
L-Lysine·HCl (98%)	0.14
DL-Methionine (98%)	0.14
Trace mineral premix[1]	0.01
Vitamin premix[2]	0.02
Choline chloride (50%)	0.20
Sodium chloride	0.30
Nutrient composition (%)	
Metabolizable energy (kcal/kg)	2951
Crude protein (CP)	21.07
Calcium (Ca)	1.00
Analyzed Ca	1.05
Total phosphorus (tP)	0.69
Analyzed tP	0.69
Non-phytate phosphorus (NPP)	0.45
Lysine	1.10
Methionine	0.50

[1] The trace mineral premix provided the following (per kg of diet): 80 mg iron, 40 mg zinc, 8 mg copper, 60 mg manganese, 0.35 mg iodine, and 0.15 mg selenium.

[2] The vitamin premix (without vitamin D) provided the following (per kg of diet): 8,000 IU vitamin A, 20 IU vitamin E, 0.5 mg menadione, 2.0 mg thiamine, 8.0 mg riboflavin, 35 mg niacin, 3.5 mg pyridoxine, 0.01 mg vitamin B_{12}, 10.0 mg pantothenic acid, 0.55 mg folic acid, and 0.18 mg biotin.

Crystalline 1,25-$(OH)_2$-D_3 was supplied by Changzhou Book Chemical Co., Ltd. (Changzhou, China). The 1,25-$(OH)_2$-D_3 solution was prepared as described by Han *et al*. (2018). The crystalline 1,25-$(OH)_2$-D_3 was weighed, dissolved in ethanol, and diluted using propylene glycol (5% ethanol: 95% propylene glycol). The solution concentration was analyzed using high-performance liquid chromatography (HPLC) at the Shanghai Fuxin Analysis Technology Center (Shanghai, China). The concentration of 1,25-$(OH)_2$-D_3 solution was 10.39 μg/mL.

Broiler chickens aged 1−19 days were reared in stainless-steel cages (140 cm×70 cm×35 cm). Birds were provided ad libitum access to mash feed and water during experiments with 23 h of light and 1 h of darkness from days 1 to 3 and with 20 h of light and 4 h of darkness from days 4 to 19. Room temperature was controlled at 33℃ from days 1 to 3, 30℃ from days 4 to 7, and 27℃ from days 8 to 19.

Sample Collection and Analysis

At 19 days of age, all chickens were weighed, and the ADFI, ADG, feed conversion ratio (FCR), and mortality were determined. Two birds per replicate (10 birds per treatment)

Table 2. **Primer sequences for quantitative real-time PCR**

Gene	Accession	Orientation	Primer sequence (5′−3′)	Size (bp)
CaBP-D28k	NM-205513.1	Forward	AGATCTGGCACCACTACGAC	187
		Reverse	TGAGCAAGCTCAACGATTCCT	
PMCAlb	NM-001168002.3	Forward	AGCTCAAGATGGTGCAGCTA	165
		Reverse	AACAAACCTGCTTTGCCAATCT	
NCX1	NM-001079473.1	Forward	TCACCTTCTTCTTCTTCCCAATCT	158
		Reverse	GCAACCTTTCCGTCCATCTC	
VDR	AF011356.1	Forward	AAGTCATCGACACCCTCCTG	173
		Reverse	GCCAAAGACATCGTTGGAGT	
GAPDH	NM-204305.1	Forward	GAACATCATCCCAGCGTCCA	133
		Reverse	ACGGCAGGTCAGGTCAACAA	

CaBP-D28k, calcium-binding protein 28-kDa; PMCA1b, plasma membrane calcium ATPase 1b; NCX1, sodium/calcium exchanger 1; VDR, vitamin D receptor; and GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

were randomly selected and euthanized through cervical dislocation to collect the tibia, femur, and mucosal samples from the duodenum, jejunum, and ileum.

Randomly selected chickens were euthanized, and the whole small intestine was isolated immediately from the gastrointestinal tract and cut into three pieces: duodenum (pancreatic loop), jejunum (from the distal duodenal loop to the Meckel's diverticulum), and ileum (from Meckel's diverticulum to the ileocecal junction) (de Verdal *et al*., 2010). These segments were rinsed with 0.9% ice-cold NaCl solution. The mucosa was scraped off 3 cm at the center of individual segments (i.e., duodenum, jejunum, and ileum) using a glass microscope slide, immediately frozen in liquid nitrogen, and kept at −80℃.

Tibia and femur bones were collected and stored at −20℃. The length and leg bone weight were determined after drying for 24 h at 105℃. The ash weight was determined after ashing for 48 h at 600℃. The percentage contents of ash, Ca, and P were measured as percentages of bone weight. Ca and total P (tP) contents in the diets and bones were determined as described by Han *et al*. (2018).

Total RNA Extraction, Reverse Transcription, and Quantitative Real-time Polymerase Chain Reaction (PCR)

Total RNA was isolated from the duodenal, jejunal, and ileal mucosae of chickens using the Trizol reagent (Takara Biotechnology Co. Ltd., Dalian, China) in accordance with the manufacturer's instructions. RNA concentration was determined using a spectrophotometer. OD260/280 values ranged from 1.8 to 2.0 to assure the purity of the total RNA. All samples were stored at −80℃. Reverse transcription was performed using 1 μg total RNA with the PrimeScript Reverse Transcription Reagent Kit (Takara Biotechnology Co. Ltd., Dalian, China) in accordance with the manufacturer's instructions. The primers for CaBP-D28k, PMCAlb, NCX1, VDR, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China, Table 2). Quantitative real-time PCR was performed using the SYBR Premix PCR Kit (Takara Biotechnology Co. Ltd., Dalian, China) on a Roche Lightcycler® 480 Real-Time PCR system (Roche Diagnostics, Risch, Switzerland). Reactions were conducted in a 10 μL reaction system containing 5 μL SYBR Green Premix I PCR mix (Tli RNaseH Plus, 2×), 0.4 μL forward primer (10 μM), 0.4 μL reverse primer (10 μM), 1.0 μL cDNA, and 3.2 μL RNase-free water. The program was set at 95℃ for 60 s, followed by 40 cycles of 95℃ for 10 s, 60℃ for 30 s, and 72℃ for 30 s. Each gene was amplified in triplicate. The standard curve was determined using the pooled samples. The gene expression levels relative to the endogenous control of GAPDH for each sample were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical Analysis

Replicates served as experimental units in the statistical analysis. All data were analyzed by Student's t-test using the SAS software (SAS Institute, 2002). Statistical significance was set at $P<0.05$.

Results

Growth Performance

Vitamin D deficiency in the basal diet resulted in a lower growth rate and higher mortality in broilers from 1 to 19 days of age (Table 3). 1,25-$(OH)_2$-D_3, the final active form of vitamin D, improved the growth of broiler chickens. The addition of 1.25 μg/kg 1,25-$(OH)_2$-D_3 increased the ADFI and ADG by 16.6% and 60.9%, respectively. 1,25-$(OH)_2$-D_3 decreased the FCR and mortality of broilers compared to the basal diet without vitamin D ($P<0.05$).

Bone Mineralization

Dietary vitamin D deficiency resulted in bone deformity and lower length of the tibia and femur in 19-day-old broilers fed the basal diet without vitamin D (Tables 4 and 5). 1,25-$(OH)_2$-D_3 improved bone mineralization in broilers. The addition of 1.25 μg/kg 1,25-$(OH)_2$-D_3 enhanced the percentage contents of ash, Ca, and P in the tibia and femur of 19-day-old broilers compared with the basal diet ($P<0.05$). The length, weight, and ash weight of the leg bones were increased by 1,25-$(OH)_2$-D_3 ($P<0.05$).

Ca Transporter Gene Expression Levels

Five Ca absorption-related genes (TRPV6, CaBP-D28k, PMCAlb, NCX1, and VDR) were examined. TRPV6, an apical membrane Ca channel in intestinal cells, was not successfully cloned in this study.

Table 3. **Effects of dietary 1,25-$(OH)_2$-D_3 levels on the growth performance of broiler chickens from 1 to 19 days of age[1]**

1,25-$(OH)_2$-D_3 (μg/kg)	ADFI (g/chick)	ADG (g/chick)	FCR (g/g)	Mortality (%)
0	34.37[b]	19.10[b]	1.82[a]	10.00[a]
1.25	40.08[a]	30.74[a]	1.31[b]	2.86[b]
SEM	1.39	2.15	0.10	1.67
P value	0.029	<0.001	0.001	0.020

[a–b] Means in the same column without a common superscript differ ($P<0.05$).
[1] Values are means of five replicates of 14 chickens per replicate ($n=5$).
1,25-$(OH)_2$-D_3, 1,25-dihydroxycholecalciferol; ADFI, average daily feed intake; ADG, average daily gain; FCR, feed conversion ratio; and SEM, standard error of the mean.

Table 4. **Effects of dietary 1,25-$(OH)_2$-D_3 levels on the tibia mineralization of broiler chickens at 19 days of age[1]**

1,25-$(OH)_2$-D_3 (μg/kg)	Length (cm)	Weight (g/bone)	Ash (g/bone)	Ash (%)	Ca (%)	P (%)
0	4.90[b]	0.94[b]	0.33[b]	34.66[b]	12.35[b]	5.70[b]
1.25	5.83[a]	1.47[a]	0.75[a]	50.61[a]	18.91[a]	8.82[a]
SEM	0.18	0.10	0.08	2.70	1.12	0.53
P value	0.002	0.002	<0.001	<0.001	<0.001	<0.001

[a–b] Means in the same column without a common superscript differ ($P<0.05$).
[1] Values are means of five replicates of two chickens per replicate ($n=5$).
1,25-$(OH)_2$-D_3, 1,25-dihydroxycholecalciferol; Ca, calcium; P, phosphorus; and SEM, standard error of the mean.

Table 5. **Effects of dietary 1,25-$(OH)_2$-D_3 levels on the femur mineralization of broiler chickens at 19 days of age[1]**

1,25-$(OH)_2$-D_3 (μg/kg)	Length (cm)	Weight (g/bone)	Ash (g/bone)	Ash (%)	Ca (%)	P (%)
0	3.72[b]	0.72[b]	0.25[b]	35.60[b]	12.90[b]	5.93[b]
1.25	4.48[a]	1.15[a]	0.58[a]	50.30[a]	18.40[a]	8.64[a]
SEM	0.14	0.08	0.06	2.53	0.93	0.46
P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[a–b] Means in the same column without a common superscript differ ($P<0.05$).
[1] Values are means of five replicates of two chickens per replicate ($n=5$).
1,25-$(OH)_2$-D_3, 1,25-dihydroxycholecalciferol; Ca, calcium; P, phosphorus; and SEM, standard error of the mean.

CaBP-D28k is expressed in the cytoplasm of intestinal cells and transports Ca from the apical membrane to the basolateral membrane. The addition of 1,25-$(OH)_2$-D_3 increased the mRNA expression levels of CaBP-D28k in the small intestine of broilers at 19 days of age ($P<0.05$, Tables 6–8). The mRNA expression levels of CaBP-D28k in the duodenum, jejunum, and ileum of birds fed 1.25 μg/kg 1,25-$(OH)_2$-D_3 were enhanced to 88.1, 109.1, and 2.7 times, respectively, compared with birds fed the basal diet without vitamin D.

PMCAlb and NCX1 are located in the basolateral membrane of the intestinal cells. Ca^{2+} extrusion from enterocytes to the blood is performed by PMCA1b and NCX1. The addition of 1,25-$(OH)_2$-D_3 increased the mRNA expression levels of PMCA1b in the duodenum of 19-day-old birds by 57% compared with those in birds fed the basal diet ($P<0.05$) (Tables 6–8). In contrast, 1,25-$(OH)_2$-D_3 did not affect the mRNA expression levels of PMCA1b in the jejunum and ileum ($P>0.05$). The mRNA expression levels of NCX1 in the duodenum and jejunum of broilers fed 1.25 μg/kg 1,25-$(OH)_2$-D_3 were higher than those in birds fed the basal diet ($P<0.05$). The mRNA expression levels of NCX1 in the ileum were not influenced by 1,25-$(OH)_2$-D_3 ($P>0.05$).

1,25-$(OH)_2$-D_3 binds to the VDR to regulate Ca absorption in the small intestine. The mRNA expression levels of VDR in the small intestine were not affected by 1,25-$(OH)_2$-D_3 ($P>0.05$) (Tables 6–8).

Table 6. **Effects of dietary 1,25-$(OH)_2$-D_3 levels on Ca transporter mRNA expression levels in the duodenum of broiler chickens at 19 days of age[1]**

1,25-$(OH)_2$-D_3 (μg/kg)	CaBP-D28k	PMCA1b	NCX1	VDR
0	1.00[b]	1.00[b]	1.00[b]	1.00
1.25	88.12[a]	1.57[a]	1.75[a]	0.91
SEM	16.04	0.10	0.16	0.06
P value	<0.001	<0.001	0.014	0.246

[a–b] Means in the same column without a common superscript differ ($P<0.05$).
[1] Values are means of five replicates of two chickens per replicate ($n=5$).
1,25-$(OH)_2$-D_3, 1,25-dihydroxycholecalciferol; Ca, calcium; CaBP-D28k, calcium-binding protein 28-kDa; PMCA1b, plasma membrane calcium ATPase 1b; NCX1, sodium/calcium exchanger 1; VDR, vitamin D receptor; and SEM, standard error of the mean.

Table 7. **Effects of dietary 1,25-$(OH)_2$-D_3 levels on Ca transporter mRNA expression levels in the jejunum of broiler chickens at 19 days of age[1]**

1,25-$(OH)_2$-D_3 (μg/kg)	CaBP-D28k	PMCA1b	NCX1	VDR
0	1.00[b]	1.00	1.00[b]	1.00
1.25	109.10[a]	1.23	2.86[a]	0.86
SEM	19.87	0.06	0.36	0.05
P value	<0.001	0.095	0.002	0.110

[a–b] Means in the same column without a common superscript differ ($P<0.05$).
[1] Values are means of five replicates of two chickens per replicate ($n=5$).
1,25-$(OH)_2$-D_3, 1,25-dihydroxycholecalciferol; Ca, calcium; CaBP-D28k, calcium-binding protein 28-kDa; PMCA1b, plasma membrane calcium ATPase 1b; NCX1, sodium/calcium exchanger 1; VDR, vitamin D receptor; and SEM, standard error of the mean.

Table 8. **Effects of dietary 1,25-$(OH)_2$-D_3 levels on Ca transporter mRNA expression levels in the ileum of broiler chickens at 19 days of age[1]**

1,25-$(OH)_2$-D_3 (μg/kg)	CaBP-D28k	PMCA1b	NCX1	VDR
0	1.00[b]	1.00	1.00	1.00
1.25	2.71[a]	1.11	0.97	1.18
SEM	0.32	0.06	0.14	0.09
P value	<0.001	0.492	0.998	0.330

[a–b] Means in the same column without a common superscript differ ($P<0.05$).
[1] Values are means of five replicates of two chickens per replicate ($n=5$).
1,25-$(OH)_2$-D_3, 1,25-dihydroxycholecalciferol; Ca, calcium; CaBP-D28k, calcium-binding protein 28-kDa; PMCA1b, plasma membrane calcium ATPase 1b; NCX1, sodium/calcium exchanger 1; VDR, vitamin D receptor; and SEM, standard error of the mean.

Discussion

Growth Performance

Vitamin D is an essential nutrient in poultry diets and its derivatives include VD_3, 25-OH-D_3, and 1,25-$(OH)_2$-D_3. 1,25-$(OH)_2$-D_3 is the final product of vitamin D, and its relative bioactivity is higher than that of VD_3 and 25-OH-D_3 (Soares *et al*., 1995). Severe vitamin D deficiency results in the slow growth of broilers (Baker *et al*., 1998; Chen *et al*., 2017). The growth performance of chicken was improved after adding VD_3, 25-OH-D_3, or 1,25-$(OH)_2$-D_3 to the diets (Aburto *et al*., 1998; Baker *et al*., 1998; Fritts and Waldroup, 2003; Chen *et al*., 2017). Similar results were observed in the present study. Poor performance was observed in birds fed the basal diet without vitamin D. The addition of 1.25 μg/kg 1,25-$(OH)_2$-D_3 increased the ADFI and ADG of broilers compared with the basal diet. These data indicate that vitamin D deficiency damaged the growth performance of broilers. Growth of chickens recovered after vitamin D supplementation.

Bone Mineralization

Vitamin D stimulates Ca absorption in the small intestine and promotes Ca retention in the bones of poultry. Vitamin D deficiency causes leg bone deformation and mineralization abnormalities in broilers (Baker *et al*., 1998; Chen *et al*.,

2017). The leg bone quality improved when VD_3, 25-OH-D_3, or 1,25-$(OH)_2$-D_3 was added to the diets of broiler chickens (Aburto *et al*., 1998; Baker *et al*., 1998; Chen *et al*., 2017). Similar results were observed in the present study. Broilers fed the basal diet without vitamin D had lower bone and ash weights. The addition of 1.25 μg/kg 1,25-$(OH)_2$-D_3 increased the percentage contents of Ca and P in the tibia and femur compared with the basal diet. Accordingly, the weight and ash weight in the tibia and femur were enhanced by 1,25-$(OH)_2$-D_3. These data indicate that vitamin D deficiency inhibited leg bone development in broilers. Vitamin D supplementation improved bone growth and mineralization in chickens.

Ca Transporter Gene Expression Levels

Improvements in growth performance and bone mineralization are attributed to increased Ca absorption in the small intestine of broilers. Thus, Ca transporter gene expression levels were examined in the present study.

TRPV6 is an apical membrane Ca channel in intestinal cells and is expressed in the small intestine of mammals and poultry. The injection of 1,25-$(OH)_2$-D_3 increased the mRNA expression levels of TRPV6 in the duodenum of male mice (Benn *et al*., 2008; Khuituan *et al*., 2012). TRPV6 was detected in the small intestine of laying hens (Yang *et al*., 2011), but has not been cloned in the small intestine of broilers (Rousseau *et al*., 2016; Proszkowiec-Weglarz *et al*., 2019). The mRNA bands of TRPV6 in the small intestine of chickens were not observed in the present study. The difference in TRPV6 gene expression levels between laying hens and broiler chickens should be further clarified.

CaBP-D9k is expressed in the mammalian intestine, whereas CaBP-D28k is expressed in the poultry intestine. CaBP-D9k or CaBP-D28 moves Ca from the apical membrane to the basolateral membrane of intestinal cells. The injection of 1,25-$(OH)_2$-D_3 increased the mRNA expression levels of CaBP-D9k in the duodenum of male mice (Okano *et al*., 2004; Benn *et al*., 2008; Khuituan *et al*., 2012). Similarly, the addition or the injection of 1,25-$(OH)_2$-D_3 enhanced the mRNA expression levels of CaBP-D28k in the small intestine of cockerels (Clemens *et al*., 1988; Hall and Norman, 1990; Sechman *et al*., 1996), laying hens (Bar *et al*., 1990), and broiler chickens (Yang *et al*., 2019). The mRNA expression levels of intestinal CaBP-D28k increased to 50.0 and 433.7 times after the injection and addition of 1,25-$(OH)_2$-D_3, respectively, in vitamin D-deficient chicks (Clemens *et al*., 1988; Yang *et al*., 2019). Similar results were observed in the present study. The addition of 1.25 μg/kg 1,25-$(OH)_2$-D_3 enhanced the mRNA expression levels of CaBP-D28k in the duodenum, jejunum, and ileum to 88.1-, 109.1-, and 2.7-fold, respectively, compared to the basal diet. These data indicated that 1,25-$(OH)_2$-D_3 upregulated the mRNA expression levels of CaBP-D28k in the small intestine especially in the proximal intestine (duodenum and jejunum). Dietary Ca levels also affected CaBP-D28k gene expression (Li *et al*., 2012; Rousseau *et al*., 2016), in which low Ca levels upregulated the mRNA expression levels of CaBP-D28k in the duodenum of broilers.

PMCA1b and NCX1 are located in the basolateral membrane of the intestinal cells. Ca is extruded from the basolateral membrane into the blood via the action of PMCA1 and NCX1 (Hoenderop *et al*., 2005). Vitamin D deficiency decreased the mRNA expression levels of PMCA1b and NCX1 in the duodenum of rats (Zhu, 1995). The injection of 1,25-$(OH)_2$-D_3 increased the mRNA expression levels of PMCA1b and NCX1 in the duodenum of rats and mice by 69.5%−166.0% (Zhu, 1995; Khuituan *et al*., 2012; Wongdee and Charoenphandhu, 2015). Similar results have been observed in poultry. Vitamin D deficiency reduced, but 1,25-$(OH)_2$-D_3 repletion elevated the mRNA and protein expression levels of NCX1 in Cobb Harding chick duodenum (Centeno *et al*., 2011). The injection of 1,25-$(OH)_2$-D_3 also enhanced the mRNA expression levels of PMCA1b in the duodenum of white leghorn cockerels fed vitamin D-deficient diets (Cai *et al*., 1993). Similar results were observed in the present study. The addition of 1,25-$(OH)_2$-D_3 increased the mRNA expression levels of PMCA1b and NCX1 in the duodenum of broilers. These data indicate that vitamin D stimulates PMCA1b and NCX1 gene expression levels and promotes the extrusion of Ca from the basolateral membrane into the blood. The gene expression levels of PMCA1b and NCX1 were also affected by dietary Ca levels (Centeno *et al*., 2004; Rousseau *et al*., 2016), in which low dietary Ca levels increased the mRNA and protein expression levels of PMCA1b and NCX1 in the duodenum of chickens.

The mRNA expression levels of CaBP-D28k in the duodenum and jejunum of broilers increased to 88.1−109.1 times after adding 1,25-$(OH)_2$-D_3, whereas the mRNA expression levels of PMCAlb and NCX1 were enhanced to 1.57−2.86 times with the addition of 1,25-$(OH)_2$-D_3. Hence, the response of PMCA1b and NCX1 gene expression to vitamin D was not as sensitive as that of CaBP-D28k.

Vitamin D binds to VDR to regulate Ca absorption in the small intestine. VDR is located in the nuclei of intestinal epithelial cells. The mRNA expression levels of VDR are highest in the duodenum of broiler chickens, lower in the jejunum, and lowest in the ileum (Han *et al*., 2018). Vitamin D deficiency decreased the mRNA and protein expression levels of VDR in the duodenum of rats (Zineb *et al*., 1998) and chicks (Centeno *et al*., 2011). The optimal levels of 25-OH-D_3, 1α-hydroxycholecalciferol (1α-OH-D_3), and 1,25-$(OH)_2$-D_3 increased the mRNA expression levels of VDR in the duodenum of rats (Zineb *et al*., 1998), Cobb Harding chicks (Centeno *et al*., 2011), and broiler chickens (Han *et al*., 2018; Yang *et al*., 2020). In contrast, the addition of 1,25-$(OH)_2$-D_3 did not affect the mRNA expression levels of VDR in the small intestine in the present study. The differences between our study and previous research may be due to the dosages of 1,25-$(OH)_2$-D_3.

In conclusion, the addition of 1,25-$(OH)_2$-D_3 upregulated Ca transporter gene expression levels and stimulated Ca absorption in the small intestine, especially in the proximal intestine (duodenum and jejunum), thereby improving growth performance and bone mineralization in broiler chickens.

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Conflict of Interest

The authors declare no conflict of interest.

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