Optimal Dietary Levels of 1α-Hydroxycholecalciferol in Broiler Chickens from 1 to 42 Days of Age

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1α-Hydroxycholecalciferol (1α-OH-D3) is an active vitamin D derivative. In this study, three experiments were conducted to evaluate the optimal dietary levels of 1α-OH-D3 in broiler chickens from 1 to 42 days of age. 1α-OH-D3 levels used were 0, 1.25, 2.5, 5, and 10 μg/kg in experiment 1, 0.625, 1.25, 2.5, 5, 7.5, and 10 μg/kg in experiment 2, and 2, 2.5, 3, 3.5, 4, 4.5, and 5 μg/kg in experiment 3. In experiment 1, the addition of 0 to 10 μg/kg of 1α-OH-D3 quadratically improved growth performance, tibia development, and mRNA expression levels of nuclear vitamin D receptor (nVDR), membrane vitamin D receptor (mVDR), and type IIb sodium-phosphate cotransporter (NaPi-IIb) in the duodenum of broiler chickens from 1 to 12 days of age. Body weight gain (BWG), the weight and ash weight of the tibia, and mRNA expression levels of mVDR and NaPi-IIb of broilers fed with 0 and 10 μg/kg of 1α-OH-D3 were lower than those of birds fed with 2.5 μg/kg of 1α-OH-D3. In experiment 2, 1α-OH-D3 levels were quadratically related to BWG and to weight and ash weight of the femur and the tibia of broiler chickens at 42 days of age. The highest values of growth performance and bone mineralization were recorded in broilers fed with 2.5 to 5 μg/kg of 1α-OH-D3. In experiment 3, there was no difference observed in BWG and the weight and ash weight of the femur and the tibia of the 42-day-old broilers fed with 2 to 5 μg/kg of 1α-OH-D3. These data suggest that the optimal dietary levels of 1α-OH-D3 were 2 to 5 μg/kg for broiler chickens from 1 to 42 days of age.

Key words: 1α-hydroxycholecalciferol, broiler chicken, growth, bone, vitamin D receptor, type IIb sodium-phosphate cotransporter

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Introduction

Vitamin D is an essential nutrient that regulates calcium (Ca) and phosphorus (P) absorption and retention in poultry diets. The vitamin D products widely used in feeds in China include cholecalciferol (vitamin D3) and 25-hydroxycholecalciferol (25-OH-D3). 1α-Hydroxycholecalciferol (1α-OH-D3) is a new vitamin D. Its relative biological value was higher than that of vitamin D3 and 25-OH-D3 (Edwards et al., 2002; Han et al., 2017). The addition of 1α-OH-D3 in feeds improved growth performance, bone mineralization, and P utilization in 1- to 21-day-old broiler chickens fed with P-deficient diets (Biehland Baker, 1997; Snow et al., 2004). Broiler chickens fed Ca- and P-inadequate diet with 5 μg/kg of 1α-OH-D3 and adequate vitamin D3 showed insufficient for bone formation (Landy and Toghyani, 2018; Ghasemi et al., 2019). Another research has shown that supplementation with 15 to 25 μg/kg of 1α-OH-D3 inhibited body weight (BW) and feed intake (FI) and resulted in kidney mineralization in 1- to 42-day-old broiler chickens fed with Ca- and P-inadequate diets (Pesti and Shivaprasad, 2010). These data suggest that the responses of broilers to 1α-OH-D3 are affected by dietary P levels. However, the optimal 1α-OH-D3 levels in P-adequate diets of broiler chickens have not been examined.

The promotion of P absorption in the small intestine by dietary 1α-OH-D3 contributes to the improvement of growth performance and bone mineralization of broiler chickens. When 1α-OH-D3 is transformed into 1,25-dihydroxycholecalciferol [1,25-(OH)2-D3], the latter binds to vitamin D
receptor (VDR) to regulate P absorption in the small intestine. Two types of VDRs are found in poultry, which are nuclear vitamin D receptor (nVDR) and membrane vitamin D receptor (mVDR). nVDR is located in the nucleus of the epithelial cells of the small intestine. mVDR, which is also called the membrane-associated rapid response steroid binding (MARRS) receptor or ERp57/GRp58, is located in the basal lateral membrane of the small intestinal cells (Khanal and Nemere, 2007; Nemere et al., 2012). Type IIb sodium-phosphate cotransporter (NaPi-IIb) is a P transporter protein in the apical membranes of the epithelial cells in the small intestine of broiler chickens (Yan et al., 2007; Forster et al., 2013). 1,25-(OH)2-D3 regulates NaPi-IIb mRNA expression levels in the small intestine of rats (Xu et al., 2002).

However, the relationships between VDR and NaPi-IIb mRNA expressions in the small intestine and 1α-OH-D3 levels in broiler diets with adequate P have not been examined.

Therefore, the objective of this study was to evaluate the effects of 1α-OH-D3 levels on growth performance, bone mineralization, and mRNA expression levels of VDR and NaPi-IIb in broiler diets with adequate P have not been examined.

Materials and Methods

**Birds, Diets and Management**

All animal care procedures adopted in this study were approved by Henan Agricultural University and Shangqiu Normal University.

The Ca and non-phytate phosphorus (NPP) levels in the basal diets were respectively 1.00% and 0.45% for broilers of 1 to 21 days old and 0.90% and 0.35% in birds of 22 to 42 days old in experiments 1, 2, and 3 (Table 1) (NRC, 1994). The basal diets did not contain supplemental vitamin D3 in the three experiments.

In experiment 1, 150 males of 1-day-old Ross 308 broilers were weighed and randomly allotted to five treatment groups with three replicate cages containing 10 birds each. Five levels of 1α-OH-D3 (0, 1.25, 2.5, 5 and 10 μg/kg) were added to the basal diet. At 12 days old, three chickens per replicate cage (9 birds per treatment) were randomly selected and euthanized by cervical dislocation to collect the tibia and the mucosa samples from the duodenum. The tibia was excised and then frozen at −20°C. The duodenal mucosa was scraped off 3 cm at half of the individual duodenal segments using a glass microscope slide on ice. This was immediately frozen in liquid nitrogen, and then stored at −80°C.

### Table 1. Ingredients and nutrient composition of the experimental diets (as-fed basis)

| Item                        | Exp. 1  | Exp. 2  | Exp. 3  |
|-----------------------------|---------|---------|---------|
|                             | 1 to 12 days | 1 to 21 days | 22 to 42 days | 1 to 21 days | 22 to 42 days |
| Ingredient (%)              |         |         |         |         |         |
| Corn                        | 58.11   | 58.12   | 63.28   | 58.10   | 63.27   |
| Soybean meal (45% CP)       | 32.07   | 32.07   | 27.52   | 32.07   | 27.52   |
| Soybean oil                 | 2.22    | 2.20    | 3.00    | 2.22    | 3.00    |
| Soy protein powder (65% CP) | 3.50    | 3.50    | 2.74    | 3.50    | 2.74    |
| Limestone                   | 1.36    | 1.36    | 1.45    | 1.36    | 1.47    |
| Dicalcium phosphate         | 1.94    | 1.94    | 1.36    | 1.94    | 1.35    |
| L-Lysine-HCl (98%)          | 0.14    | 0.14    | 0.14    | 0.14    | 0.14    |
| DL-Methionine (98%)         | 0.14    | 0.14    | 0.08    | 0.14    | 0.08    |
| Trace mineral premix¹       | 0.01    | 0.01    | 0.01    | 0.01    | 0.01    |
| Vitamin premix²             | 0.01    | 0.02    | 0.02    | 0.01    | 0.02    |
| Choline chloride (50%)      | 0.20    | 0.20    | 0.10    | 0.20    | 0.10    |
| Sodium chloride             | 0.30    | 0.30    | 0.30    | 0.30    | 0.30    |
| Nutrient composition (%)    |         |         |         |         |         |
| Metabolizable energy (kcal/kg) | 2951  | 2950    | 3054    | 2951    | 3053    |
| Crude protein               | 21.07   | 21.07   | 19.08   | 21.07   | 19.08   |
| Calcium (Ca)                | 1.00    | 1.00    | 0.90    | 1.00    | 0.90    |
| Analyzed Ca                 | 1.01    | 0.96    | 0.92    | 1.03    | 1.00    |
| Total phosphorus (tP)       | 0.69    | 0.69    | 0.58    | 0.69    | 0.57    |
| Analyzed tP                 | 0.68    | 0.69    | 0.58    | 0.70    | 0.61    |
| Non-phytate phosphorus (NPP)| 0.45    | 0.45    | 0.35    | 0.45    | 0.35    |
| Lysine                      | 1.10    | 1.10    | 0.99    | 1.10    | 0.99    |
| Methionine                  | 0.50    | 0.50    | 0.41    | 0.50    | 0.41    |

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; 0.15 mg selenium.

² The vitamin premix (without vitamin D3) 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 B12; 10.0 mg pantothenic acid; 0.55 mg folic acid; 0.18 mg biotin.
In experiment 2, 300 males of 1-day-old Ross 308 broilers were weighed and randomly allotted to six treatment groups with five replicate cages containing 10 birds each. Six different amounts of 1α-OH-D₃ (0.625, 1.25, 2.5, 5, 7.5, and 10 μg/kg) were added to the basal diet. At 42 days old, three chickens per replicate cage (15 birds per treatment) were weighed and randomly selected and euthanized by cervical dislocation for collection of the femur and the tibia. The leg bones were excised and then frozen at −20°C.

In experiment 3, 350 males of 1-day-old Ross 308 broilers were weighed and randomly allotted to seven treatment groups with five replicate cages of 10 birds each. Seven levels of 1α-OH-D₃ (2, 2.5, 3, 3.5, 4, 4.5, and 5 μg/kg) were added to the basal diet. At 42 days old, three chickens per replicate cage (15 birds per treatment) were randomly selected and euthanized. Femur and tibia samples were collected and frozen at −20°C.

The broilers were reared in stainless steel cages (190 cm × 50 cm × 35 cm). The birds were provided ad libitum access to mash feed and water during the 42 days of the experiment and were exposed to 20h of light from incandescent bulbs and 4h of darkness. The room temperature was controlled at 33°C from 0 to 3 days, 30°C from 4 to 7 days, 27°C from 8 to 21 days, and 24°C from 22 to 42 days.

1α-OH-D₃ was supplied by Taizhou Healtech Chemical Co., Ltd. (Taizhou, China). The concentration of 1α-OH-D₃ (0.625, 1.25, 2.5, 5, 7.5, and 10 μg/kg) was determined as 9.25 μg/mL. An appropriate quantity of 1α-OH-D₃ solution was pipetted and added to the diets.

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

| Gene       | Accession | Orientation | Primer sequence (5’–3’ ) | Size (bp) |
|------------|-----------|-------------|--------------------------|-----------|
| nVDR       | AF011356.1| Forward     | AAGTCATCGACACCTCTCCTG    | 173       |
| mVDR       | NM_204110.3| Forward     | GCAAAGACATCGTGAGGAGT     | 136       |
| NaPi-IIb   | NM_204474.1| Reverse     | CTACGTCGGCAACGGATTA      | 164       |
| GAPDH      | NM_204305.1| Reverse     | GAAATCATTCACACGGCCA      | 133       |

**Sample Collection and Analysis**

All broilers were weighed at 12 days (experiment 1) and 42 days of age (experiments 2 and 3). The FI, body weight gain (BWG), and feed conversion ratio (FCR) of the broilers were calculated. All the birds that died spontaneously during the experiment were weighed, and the weight was used to correct the FI.

The mineralization of the femur and tibia was analyzed as described by Hall et al. (2003). The bones were dried at 105°C for 24 h, and then weighed. The bone ash weight was determined by ashing the bone in a muffle furnace at 600°C for 48 h. The Ca and total phosphorus (tP) contents in the diets and the bones were determined as described by Han et al. (2017).

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

The total RNA was isolated from the mucosa of the duodenum of the chickens using TRizol reagent (Tiangen Biotech Co. Ltd., Beijing, China) in accordance with the manufacturer’s instructions. RNA concentration was determined spectrophotometrically. The OD260/280 values ranged from 1.8 to 2.0 to assure the purity of the total RNA. All samples were stored at −80°C.

Reverse transcription was performed with 1 μg of the total RNA using primers reverse transcription reagent kit (Takara Biotechnology Co. Ltd., Dalian, China) in accordance with manufacturer’s instructions. The primers of nuclear vitamin D receptor (nVDR), membrane vitamin D receptor (mVDR), type IIb sodium-phosphate cotransporter (NaPi-IIb) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China, Table 2).

Quantitative real-time PCR was performed using SYBR Premix PCR Kit (Takara Biotechnology Co. Ltd., Dalian, China) on a Thermo Scientific PikoReal Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The reactions were conducted in a 10 μL reaction system containing 5 μL of SYBR Green Premix I PCR mix (Tli RNaseH Plus) (2X), 0.4 μL of forward primer (10 μM), 0.4 μL of reverse primer (10 μM), 1.0 μL of cDNA, and 3.2 μL of RNase-free water. The program was set at 95°C for 60 s, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. Each gene was amplified in triplicates. The standard curve was determined using pooled samples. The gene expression levels relative to the endogenous control of GAPDH for each sample were calculated using \(2^{-ΔΔCt}\) method (Livak and Schmittgen, 2001).

**Statistical Analysis**

Replicate means are the experimental units in statistical analysis. The data were analyzed using the general linear model (GLM) procedure of the SAS software (SAS Institute,
2002). Polynomial comparisons were performed to determine the linear and quadratic effects of 1α-OH-D$_3$ levels on the growth performance, bone mineralization, and mRNA expression levels of nVDR, mVDR, and NaPi-Ⅱb.

Results

Experiment 1

The addition of 0 to 10 $\mu$g/kg of 1α-OH-D$_3$ quadratically improved the FI, BWG, and FCR of broiler chickens from 1 to 12 days of age (Table 3). Compared to the basal diet, the addition of 1.25 $\mu$g/kg of 1α-OH-D$_3$ increased the BWG. No significant differences in BWG were observed among broilers fed with 1.25, 2.5 and 5 $\mu$g/kg of 1α-OH-D$_3$. The BWG of broilers fed with 10 $\mu$g/kg of 1α-OH-D$_3$ was lower than that of birds fed with 1.25 and 2.5 $\mu$g/kg of 1α-OH-D$_3$.

Dietary 1α-OH-D$_3$ quadratically affected tibia mineralization of broiler chickens at 12 days of age (Table 3). Increasing 1α-OH-D$_3$ levels from 0 to 1.25 $\mu$g/kg enhanced the weight, length, ash weight, and P percentage of the tibia. No significant differences in tibia quality were found in birds fed with 1.25, 2.5, and 5 $\mu$g/kg of 1α-OH-D$_3$. In contrast, the weight and ash weight of the tibia of broilers fed with 10 $\mu$g/kg of 1α-OH-D$_3$ were lower than those of birds fed with 1.25, 2.5, and 5 $\mu$g/kg of 1α-OH-D$_3$.

Dietary 1α-OH-D$_3$ quadratically affected the mRNA expression levels of nVDR, mVDR, and NaPi-Ⅱb in the duodenum of broiler chickens at 12 days old (Table 3). Compared to the basal diet, the addition of 1.25 $\mu$g/kg of 1α-OH-D$_3$ enhanced the mRNA expression levels of the three genes. No significant differences in the mRNA expression levels of the three genes observed in birds fed with 1.25 and 2.5 $\mu$g/kg of 1α-OH-D$_3$. Increasing 1α-OH-D$_3$ levels from 2.5 to 10 $\mu$g/kg decreased the mRNA expression levels. The mRNA expression levels of nVDR and NaPi-Ⅱb in broilers fed with 10 $\mu$g/kg of 1α-OH-D$_3$ were lower than those of birds fed with 1.25, 2.5, and 5 $\mu$g/kg of 1α-OH-D$_3$.

Dietary 1α-OH-D$_3$ levels increased from 0 to 10 $\mu$g/kg of 1α-OH-D$_3$. The lowest FI and BWG were observed in birds fed with 10 $\mu$g/kg of 1α-OH-D$_3$. The lowest NaPi-Ⅱb mRNA expression level was detected in birds fed with 10 $\mu$g/kg of 1α-OH-D$_3$.

Supplementation with 0.625 to 10 $\mu$g/kg of 1α-OH-D$_3$ quadratically improved FI and BWG but linearly increased the mortality of broiler chickens from 1 to 42 days of age (Table 4). Growth performance of the chickens was enhanced as dietary 1α-OH-D$_3$ levels increased from 0.625 to 2.5 $\mu$g/kg. The broilers showed the highest FI and BWG when they were fed with 2.5 and 5 $\mu$g/kg of 1α-OH-D$_3$. However, the performance of the birds declined when dietary 1α-OH-D$_3$ levels increased from 5 to 10 $\mu$g/kg. The FI and BWG of broilers fed with 7.5 and 10 $\mu$g/kg of 1α-OH-D$_3$ were lower than those of birds fed with 2.5 and 5 $\mu$g/kg of 1α-OH-D$_3$. The lowest FI and BWG were observed in broilers fed with 0.625 and 10 $\mu$g/kg of 1α-OH-D$_3$. The highest mortality was recorded in birds fed with 7.5 and 10 $\mu$g/kg of 1α-OH-D$_3$.

Dietary levels of 0.625 to 10 $\mu$g/kg 1α-OH-D$_3$ were quadratically related to the weight, length, and ash weight of the femur and the tibia of the broiler chickens at 42 days old (Table 4). The lowest bone weight and ash weight was observed in broilers fed with 0.625 $\mu$g/kg of 1α-OH-D$_3$. The bone quality improved when the 1α-OH-D$_3$ levels increased from 0.625 to 2.5 $\mu$g/kg. The highest bone mineralization values were recorded in birds fed with 2.5 to 5 $\mu$g/kg of 1α-OH-D$_3$. However, the leg bone quality declined when dietary 1α-OH-D$_3$ levels increased from 5 to 10 $\mu$g/kg. The bone weight and ash weight of broilers fed with 10 $\mu$g/kg of 1α-OH-D$_3$ were lower than those of birds fed with 2.5 and 5 $\mu$g/kg of 1α-OH-D$_3$.

Table 3. Effects of dietary levels of 1α-OH-D$_3$ on growth performance, tibia mineralization, and mRNA expression levels of nVDR, mVDR, and NaPi-Ⅱb in the small intestine of broiler chickens from 1 to 12 days of age (experiment 1)

| Item               | 0        | 1.25     | 2.5      | 5        | 10       |SEM| ANOVA Linear Quadratic P-value |
|--------------------|----------|----------|----------|----------|----------|---|-------------------|
| Growth performance |          |          |          |          |          |   |                   |
| BWG (g/bird)       | 206b     | 282a     | 274a     | 249b     | 208b     | 9 | <0.001 0.376 <0.001 |
| FI (g/bird)        | 294      | 358      | 346      | 328      | 296      | 10| 0.104 0.677 0.015  |
| FCR                | 1.43     | 1.27     | 1.26     | 1.33     | 1.43     | 0.03| 0.243 0.761 0.034  |
| Tibia mineralization |          |          |          |          |          |   |                   |
| Weight (g)         | 0.44c    | 0.76a    | 0.73a    | 0.67a    | 0.56b    | 0.03| <0.001 0.050 <0.001 |
| Length (cm)        | 4.26c    | 4.83a    | 4.78b    | 4.52abc  | 4.49bc   | 0.06| 0.001 0.482 <0.001  |
| Ash (g)            | 0.15c    | 0.37a    | 0.36a    | 0.33a    | 0.26b    | 0.02| <0.001 0.001 <0.001  |
| P (%)              | 6.66c    | 8.81a    | 8.67b    | 8.56ab   | 8.01b    | 0.22| <0.001 <0.001 <0.001  |
| mRNA expression levels |          |          |          |          |          |   |                   |
| nVDR               | 1.00b    | 2.40a    | 2.00a    | 1.98a    | 1.05b    | 0.15| <0.001 0.306 <0.001  |
| mVDR               | 1.00b    | 2.33a    | 2.36a    | 1.53b    | 1.29b    | 0.15| <0.001 0.542 <0.001  |
| NaPi-Ⅱb            | 1.00c    | 2.63a    | 2.50a    | 1.83b    | 0.18d    | 0.25| <0.001 <0.001 <0.001  |

1 Values are means of 3 replicates of 10 chickens per replicate ($n=3$).
2 Values are means of 3 replicates of 3 chickens per replicate ($n=3$).
Experiment 3

Dietary levels of 2 to 5 μg/kg 1α-OH-D3 did not affect the FI, BWG, FCR, or mortality of broiler chickens from 1 to 42 days of age (Table 5). No relationship was observed between 1α-OH-D3 levels and the growth of broilers. Similarly, dietary levels of 2 to 5 μg/kg 1α-OH-D3 did not influence the weight or ash weight of the femur and the tibia of the broiler chickens at 42 days old (Table 5). In contrast, dietary 1α-OH-D3 quadratically influenced P percentages in the femur and the tibia. The lowest values of P percentages were observed in birds fed with 2 and 5 μg/kg of 1α-OH-D3.

Discussion

Growth Performance and Bone Mineralization

1α-OH-D3 was hydroxylated by 25-hydroxylase to 1,25(OH)2-D3 in the liver of chickens. 1,25(OH)2-D3 is the final active form of 1α-OH-D3. The bioactivity of 1α-OH-D3 is similar to that of 1,25(OH)2-D3 in broiler chicken diets (Edwards, 2002). Compared to basal diet without vitamin D, the addition of 2 μg/kg of 1,25(OH)2-D3 improved the BW

Table 4. Effects of dietary levels of 1α-OH-D3 on growth performance and bone mineralization of broiler chickens from 1 to 42 days of age (experiment 2)

| Item | 1α-OH-D3 (μg/kg) | SEM | ANOVA | Linear | Quadratic |
|------|------------------|-----|-------|--------|----------|
| BWG (g/bird) | 0.625 | 1.25 | 2.5 | 5 | 7.5 | 10 | 90 | <0.001 | 0.159 | <0.001 |
| FI (g/bird) | 2574 | 3934 | 4523 | 4494 | 3657 | 2665 | 150 | <0.001 | 0.641 | <0.001 |
| FCR | 2.53 | 2.05 | 1.97 | 2.07 | 1.96 | 2.16 | 0.04 | <0.001 | <0.001 | <0.001 |
| Mortality (%) | 2 | 2 | 4 | 14 | 16 | 2 | 0.054 | 0.004 | 0.194 |

Femur mineralization at 42 days of age

| Item | 1α-OH-D3 (μg/kg) | SEM | ANOVA | Linear | Quadratic |
|------|------------------|-----|-------|--------|----------|
| Weight (g) | 2.34 | 3.21 | 4.33 | 4.41 | 3.85 | 2.53 | 0.17 | <0.001 | 0.119 | <0.001 |
| Length (cm) | 5.43 | 6.63 | 6.93 | 6.81 | 6.62 | 5.92 | 0.11 | <0.001 | 0.032 | <0.001 |
| Ash (g) | 0.80 | 1.28 | 1.75 | 1.85 | 1.58 | 0.99 | 0.08 | <0.001 | 0.015 | <0.001 |
| P (%) | 5.44 | 6.72 | 7.19 | 6.86 | 6.33 | 6.98 | 0.19 | 0.070 | 0.085 | 0.069 |

Tibia mineralization at 42 days of age

| Item | 1α-OH-D3 (μg/kg) | SEM | ANOVA | Linear | Quadratic |
|------|------------------|-----|-------|--------|----------|
| Weight (g) | 2.63 | 3.89 | 5.60 | 5.87 | 5.13 | 3.77 | 0.23 | <0.001 | <0.001 | <0.001 |
| Length (cm) | 7.24 | 8.84 | 9.47 | 9.30 | 9.09 | 8.21 | 0.16 | <0.001 | 0.001 | <0.001 |
| Ash (g) | 1.13 | 1.69 | 2.48 | 2.67 | 2.28 | 1.67 | 0.11 | <0.001 | <0.001 | <0.001 |
| P (%) | 7.45 | 7.59 | 7.85 | 7.92 | 7.84 | 7.10 | 0.10 | 0.154 | 0.627 | 0.013 |

Table 5. Effects of dietary levels of 1α-OH-D3 on growth performance and bone mineralization of broiler chickens from 1 to 42 days of age (experiment 3)

| Item | 1α-OH-D3 (μg/kg) | SEM | ANOVA | Linear | Quadratic |
|------|------------------|-----|-------|--------|----------|
| BWG (g/bird) | 2 2.5 3 3.5 4 4.5 5 | 21 | 0.208 | 0.094 | 0.567 |
| FI (g/bird) | 4654 | 4592 | 4337 | 4409 | 4293 | 4525 | 4458 | 57 | 0.448 | 0.113 | 0.154 |
| FCR | 1.97 | 1.90 | 1.88 | 1.86 | 1.93 | 1.88 | 1.92 | 0.02 | 0.935 | 0.705 | 0.355 |
| Mortality (%) | 2 | 2 | 4 | 2 | 4 | 0 | 1 | 0.820 | 0.852 | 0.355 |

Femur mineralization at 42 days of age

| Item | 1α-OH-D3 (μg/kg) | SEM | ANOVA | Linear | Quadratic |
|------|------------------|-----|-------|--------|----------|
| Weight (g) | 4.58 | 4.76 | 4.20 | 4.35 | 4.61 | 4.57 | 4.36 | 0.08 | 0.512 | 0.570 | 0.649 |
| Length (cm) | 6.91 | 6.84 | 6.43 | 6.72 | 6.48 | 6.62 | 6.69 | 0.05 | 0.039 | 0.073 | 0.023 |
| Ash (g) | 2.06 | 2.12 | 1.98 | 2.16 | 2.10 | 2.09 | 2.03 | 0.04 | 0.640 | 0.978 | 0.427 |
| P (%) | 7.65 | 8.00 | 8.56 | 7.80 | 8.23 | 7.95 | 7.70 | 0.08 | 0.025 | 0.778 | 0.010 |

Tibia mineralization at 42 days of age

| Item | 1α-OH-D3 (μg/kg) | SEM | ANOVA | Linear | Quadratic |
|------|------------------|-----|-------|--------|----------|
| Weight (g) | 6.02 | 6.13 | 5.76 | 5.95 | 5.96 | 6.36 | 5.83 | 0.11 | 0.878 | 0.966 | 0.917 |
| Length (cm) | 9.33 | 9.34 | 8.89 | 9.13 | 8.80 | 8.93 | 9.17 | 0.06 | 0.089 | 0.088 | 0.042 |
| Ash (g) | 2.81 | 2.73 | 2.83 | 2.69 | 2.82 | 3.07 | 2.71 | 0.06 | 0.721 | 0.685 | 0.950 |
| P (%) | 7.47 | 7.97 | 8.56 | 7.91 | 8.66 | 8.42 | 7.35 | 0.11 | <0.001 | 0.578 | <0.001 |

1 Values are means for 5 replicate cages of 10 chickens per cage (n=5).

2 Values are means for 5 replicate cages of 3 chickens per cage (n=5).
and tibia ash percentage of 16-day-old broilers (Aburto et al., 1998). Similar results were obtained in the present study. The BWG and tibia ash weight of 1- to 12-day-old broilers was enhanced when dietary 1α-OH-D₃ levels increased from 0 to 2.5 μg/kg (experiment 1). The BWG, FI, and the weight and ash weight of the femur and the tibia of the 42-day-old broilers fed with 0.625 and 1.25 μg/kg of 1α-OH-D₃ were lower than those of the birds fed with 2.5 μg/kg of 1α-OH-D₃ (experiment 2). These data suggest that 1.25 μg/kg of 1α-OH-D₃ failed to satisfy the required level for growth performance and bone mineralization of the broilers.

The BW of 16-day-old broilers fed with 16 μg/kg of 1,25-(OH)₂-D₃ was lower than that of birds fed with 2 to 4 μg/kg of 1,25-(OH)₂-D₃ (Aburto et al., 1998). Moreover, compared to the control diet with adequate vitamin D, the addition of 10 and 15 μg/kg of 1,25-(OH)₂-D₃ decreased the BW in 1- to 21-day-old chickens (Rennie et al., 1993; Mitchell et al., 1997). Similar results were observed in 1α-OH-D₃ studies. High 1α-OH-D₃ levels (25 μg/kg) resulted in a severe kidney mineralization and growth inhibition in broiler chickens (Pesti and Shivaprasad, 2010). In the present study, the BWG and FI of the 42-day-old broilers fed with 7.5 and 10 μg/kg of 1α-OH-D₃ were lower than those of birds fed with 2.5 and 5 μg/kg of 1α-OH-D₃ (experiment 2). The highest mortality and the poor leg bone mineralization were recorded for the 42-day-old birds fed with 10 μg/kg of 1α-OH-D₃ (experiment 2). These data suggest that 7.5 and 10 μg/kg of 1α-OH-D₃ exceeded the optimal amount for growth and bone quality of broiler chickens from 1 to 42 days of age.

Research has shown that the addition of 5 μg/kg of 1,25-(OH)₂-D₃ increased the tibia ash percentage of 21-day-old broilers (Elliot et al., 1995; Mitchell et al., 1997; Han et al., 2009). In contrast, the addition of 5 μg/kg of 1,25-(OH)₂-D₃ (Elliot et al., 1995) or 1α-OH-D₃ (Pesti and Shivaprasad, 2010) did not affect the growth of broilers. These data suggest that 5 μg/kg of 1α-OH-D₃ or 1,25-(OH)₂-D₃ may be the maximum required amount for chickens. Aburto et al. (1998) did not find significant differences in growth between birds fed with 2 and 4 μg/kg of 1,25-(OH)₂-D₃. In the present study, the highest growth performance and bone mineralization values were detected in the 42-day-old chickens fed with 2.5 to 5 μg/kg of 1α-OH-D₃ (experiment 2). No difference was observed in the growth and leg bone quality of the 42-day-old birds fed with 2 to 5 μg/kg of 1α-OH-D₃, (experiment 3). Thus, the optimal 1α-OH-D₃ level for growth performance and bone mineralization in chickens should range from 2 to 5 μg/kg.

**mRNA Expression Levels of nVDR, mVDR, and NaPi-IIb**

The improvement of growth performance and bone development by 1α-OH-D₃ is related to P absorption and the P transporter gene expressions. 25-OH-D₃ is a derivative of vitamin D₃. Research has shown that the required 25-OH-D₃ amount is 12.5 μg/kg for broiler chickens from 1 to 42 days of age (Chen et al., 2017). Compared to the basal diet without vitamin D₃, the addition of 12.5 μg/kg of 25-OH-D₃ increased the mRNA expression levels of nVDR, mVDR, and NaPi-IIb in the duodenum of broilers (Han et al., 2018).

In the present study, the mRNA expression levels of nVDR, mVDR, and NaPi-IIb in the duodenum were quadratically affected by 1α-OH-D₃ levels. The addition of 1.25 and 2.5 μg/kg of 1α-OH-D₃ enhanced the mRNA expression levels compared to the basal diet. Increasing the 1α-OH-D₃ levels from 2.5 to 10 μg/kg decreased the mRNA expression levels of nVDR, mVDR, and NaPi-IIb. These data suggest that the addition of 1.25 and 2.5 μg/kg of 1α-OH-D₃ promoted the mRNA expression levels of nVDR, mVDR, and NaPi-IIb in the duodenum.

**Optimal 1α-OH-D₃ Levels**

Vitamin D₃ and 25-OH-D₃ are used to regulate Ca and P metabolism in poultry. The recommended levels of vitamin D₃ in China are 25 and 19 μg/kg for broilers from 1 to 21 days and 22 to 42 days of age, respectively. The estimated 25-OH-D₃ levels are from 10 to 20 μg/kg in 1- to 21-day-old broiler chickens (Goodgame et al., 2011; Chen et al., 2017). The presence of 1α-OH-D₃ in broiler chicken diets is more active than vitamin D₃ and 25-OH-D₃ (Edwards et al., 2002; Han et al., 2017). Thus, the optimal dietary 1α-OH-D₃ level (2–5 μg/kg) is lower than those of the two vitamin D.

In conclusion, the 1α-OH-D₃ levels were quadratically related to growth, leg bone mineralization, and the mRNA expression levels of nVDR, mVDR, and NaPi-IIb in the small intestine of broilers. The highest values of BWG, bone weight, and mRNA expression levels of the three genes were detected in chickens fed with 2.5 to 5 μg/kg of 1α-OH-D₃. No significant difference was observed in growth or bone quality of the 42-day-old birds fed with 2 to 5 μg/kg of 1α-OH-D₃. These data indicated that 2 to 5 μg/kg of 1α-OH-D₃ was sufficient for growth performance and bone mineralization of broiler chickens from 1 to 42 days of age.

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**Conflicts of Interest**

The authors declare no conflict of interest.

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