Effects of normal and low calcium and phosphorus levels and 25-hydroxycholecalciferol supplementation on performance, serum antioxidant status, meat quality, and bone properties of broilers

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ABSTRACT To determine the effects of normal and low dietary calcium (**Ca**) and phosphorus (**P**) levels and 25-hydroxycholecalciferol (**25-OH-D_3**) supplementation on performance, serum antioxidant status, meat quality, and bone properties of broilers, 224 1-day-old Arbor Acre male broilers were used in this study. Broilers were allotted randomly to 1 of 4 treatments in a 2 × 2 factorial arrangement that included normal or low Ca and P diet with or without 69 μg/kg 25-OH-D_3. The trial consists of a starter phase from day 1 to 21 and a grower phase from day 22 to 42. Dietary 25-OH-D_3 supplementation increased ($P < 0.05$) average daily weight gain from day 22 to 42 and decreased feed conversation ratio from day 22 to 42 and day 0 to 42. On day 21, 25-OH-D_3 increased serum concentrations of total antioxidant capacity (**T-AOC**), catalase (**CAT**), and glutathione peroxidase in broilers fed low Ca and P diet (Interaction, $P < 0.05$). 25-hydroxy cholecalciferol significantly decreased serum malondialdehyde concentration. Dietary Ca and P deficiencies significantly decreased serum Ca and P concentrations and increased serum parathyroid hormone (**PTH**) concentration, and serum Ca and 25-OH-D_3 concentrations were significantly increased by 25-OH-D_3 supplementation. On day 42, serum T-AOC and CAT concentrations were decreased by dietary Ca and P deficiencies without 25-OH-D_3 (Interaction, $P < 0.05$) and unaffected by dietary Ca and P deficiencies with 25-OH-D_3. Dietary Ca and P deficiencies significantly decreased Ca, P, and alkaline phosphatase concentrations and increased PTH concentration in serum. Dietary 25-OH-D_3 increased ($P < 0.05$) serum Ca and 25-OH-D_3 concentrations and decreased ($P < 0.05$) serum tartrate-resistant acid phosphatase concentration. The interaction between CaP level and 25-OH-D_3 was observed ($P < 0.05$) for tibial Ca content and femoral bone density. 25-hydroxycholecalciferol significantly increased tibial breaking strength. These data indicated that 25-OH-D_3 supplementation at 69 μg/kg increased growth performance in some periods, enhanced serum antioxidant capacity, and improved bone mineralization and breaking strength of broilers.

Key words: 25-hydroxycholecalciferol, performance, antioxidant status, bone properties, broiler

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INTRODUCTION

Calcium (**Ca**) and phosphorus (**P**) play important roles in bone health and skeletal muscle development (Li et al., 2012; Kiefer-Hecker et al., 2018). Although the growth rate and feed efficiency have been improved in the process of selective breeding, broiler chickens often suffer from bone abnormalities and disorders, which results in commercial economic loss (Bello et al., 2014; Abbasi et al., 2017). Bone development and mineralization are closely related to the storage of Ca and P and are directly influenced by their respective dietary levels (Regassa et al., 2015). Dietary Ca and P deficiencies can hinder bone formation and result in bone abnormalities and bloody meat during the processing of the carcass (Chen and Moran, 1995). Driver et al. (2006) suggested that carcass quality of broilers depends on the age and the dietary levels of Ca and P.

Vitamin D_3 regulates the absorption of Ca and P in the small intestine (Haussler et al., 2013; Proszkowiec-Weglarz and Angel, 2013) and maintain optimal serum Ca and P homeostasis for bone mineralization and remodeling (Dal Jang et al., 2018). In addition, vitamin D_3 has

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also been reported to reduce oxidative stress by upregulating the antioxidative defense systems including glutathione (**GSH**), glutathione peroxidase (**GSH-Px**), and superoxide dismutase (**SOD**) in rats (Sardar et al., 1995; Garcion et al., 1999). Lower bone mineral density (**BMD**), a common feature of osteoporosis, was shown to be related with higher oxidative stress index and total plasma oxidant status in osteoporotic population (Altindag et al., 2008). Therefore, vitamin D_3 could contribute to bone health and development by relieving oxidative stress status. Owing to the addition of a hydroxyl group, the use of 25-hydroxycholecalciferol (**25-OH-D_3**) rather than its precursor can circumvent the 25-hydroxylation reaction in the liver (Susanna et al., 2010). Therefore, 25-OH-D_3 has a higher biological activity compared with the regular vitamin D_3 (Bar et al., 1980) and has less adverse effects and is more stable compared with 1,25-dihydroxycholecalciferol [**1,25-$(OH)_2$-D_3**] (Soares et al., 1995). Previous studies have investigated the effects of dietary 25-OH-D_3 supplementation on bone growth and mineralization of broilers (Koreleski and Swiątkiewicz, 2005; Bello et al., 2014). 25-hydroxycholecalciferol, the major circulating form of vitamin D_3, has been shown to increase body weight gain and feed efficiency and decrease the incidence of leg disorders (Whitehead et al., 2004; Garcia et al., 2013). Dietary 25-OH-D_3 supplementation could improve breast meat production because of increased muscle protein synthesis (Vignale et al., 2015). Vitamin D_3 metabolites have been shown to improve Ca and P utilization in broiler chicken (Liem, 2009). Therefore, the effects of 25-OH-D_3 on bone health and skeletal muscle development are an interesting area of research in broilers fed with diets deficient in Ca and P. Although it is generally accepted that the biopotency of 25-OH-D_3 is higher than regular vitamin D_3, little work has been done on the effects of 25-OH-D_3 supplementation on antioxidant capacity, meat quality, and bone properties of broilers fed diets adequate or deficient in Ca and P levels.

Given this background, the objective of this study was to determine the effects of 25-OH-D_3 supplementation on performance, serum antioxidant capacity, meat quality, and bone mineralization and biomechanical parameters of broilers fed normal or low Ca and P diets.

MATERIALS AND METHODS

Experimental Birds

The experimental protocol used in this study was approved by the Institutional Animal Care and Use Committee of China Agricultural University (Beijing, China). A total of 224 1-day-old male Arbor Acre broilers (weighing 46.5 ± 0.2 g) were purchased from Arbor Acres Poultry Breeding Company (Beijing, China). All broilers were raised on wire-floored cages in an environmentally controlled room without windows and had ad libitum access to feed and water. Incandescent lighting was continuous in the room, and the light bulbs had plastic filters designed to block ultraviolet radiation under conditions of the experiment. The ambient temperature was maintained at 33°C at the beginning and decreased as the birds progressed in age to ensure a final temperature of 24°C until the end of the 42-D experiment. All broilers were inoculated with inactivated infectious bursa disease vaccine on day 14 and 21 and Newcastle disease vaccine on day 7 and 28. The trial was conducted in 2 phases, consisting of a starter phase from day 1 to 21 and a grower phase from day 22 to 42.

Experimental Design and Diets

The broilers were assigned randomly to 1 of 4 treatments in a 2 × 2 factorial arrangement that included normal or low Ca and P diet, as well as that diet supplemented without or with 69 μg 25-OH-D_3/kg feed (**NCaP**, **LCaP**, **NCaP + 25-OH-D_3** or **LCaP + 25-OH-D_3**, respectively). There were 7 replicate pens per treatment with 8 broilers per pen. All starter and grower diets were based on corn–soybean meal (Table 1). The level of supplemental vitamin D_3 in both normal and low Ca and P diets was maintained at 2,760 IU/kg of diet, which is the commercial level used in this study. The Ca-P levels in the normal diets were 1.00% Ca, 0.45% nonphytate phosphorus (**NPP**), and 0.90% Ca, 0.35% NPP for the starter and grower phases, respectively. The Ca-P levels in the inadequate diets were 0.70% Ca, 0.25% NPP, and 0.62% Ca, 0.19% NPP for the starter and grower phases, respectively. Treatment diets were supplemented with 69 μg/kg 25-OH-D_3 (Morris et al., 2014), calculated to be equal to 2,760 IU/kg provided by vitamin D_3 based on the conversion of 0.025 μg of vitamin D_3 into 1 IU according to the National Research Council (NRC, 1994). Feed-grade 25-OH-D_3 was obtained from Haineng Bioengineering Co., Ltd. (Rizhao, China). Decreases in dietary Ca and P levels were carried out by reducing dicalcium phosphate and limestone levels in the diets. Corn starch was used to balance the energy of each treatment. Other essential nutrients in diets met recommendations (NRC, 1994).

The contents of vitamin D_3 and 25-OH-D_3 in diets were determined in duplicate according to the methods described by Mattila et al. (1992) and Jakobsen et al. (2004) with slight modifications. Diets were analyzed in duplicate for Ca (method 968.08) and P (method 985.01) content according to the Association of Official Analytical Chemists (AOAC, 2006).

Sample Collection and Processing

On day 21 and 42, broilers were fasted for 12 h and then weighed to determine average daily weight gain (**ADG**), average daily feed intake, and feed conversation ratio (**FCR**). All birds that died spontaneously during the experiment were weighed, and the weight was used to correct the FCR. On day 21 and 42, one bird per pen with body weight close to the average body weight of each pen (6 birds per treatment) was selected. Blood samples (5 mL) were collected from the wing veins into a 10-mL anticoagulant-free Vacutainer tube (Greiner

Table 1. Composition and nutrient levels of the experimental diets (%, as-fed basis).

Item	Starter phase (days 1–21)				Grower phase (days 22–42)			
	NCaP[1]	LCaP[1]	NCaP + 25-OH-D_3[1]	LCaP + 25-OH-D_3[1]	NCaP	LCaP	NCaP + 25-OH-D_3	LCaP + 25-OH-D_3
Ingredient								
Corn	58.17	58.17	58.17	58.17	64.26	64.26	64.26	64.26
Soybean meal	30.44	30.44	30.44	30.44	24.05	24.05	24.05	24.05
Corn starch	-	2.28	-	2.28	-	1.99	-	1.99
Corn gluten meal	2.00	2.00	2.00	2.00	2.50	2.50	2.50	2.50
Fish meal	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Soybean oil	3.38	2.35	3.38	2.35	3.60	2.70	3.60	2.70
Dicalcium phosphate	1.50	0.45	1.50	0.45	1.04	0.20	1.04	0.20
Limestone	1.30	1.10	1.30	1.10	1.35	1.10	1.35	1.10
Salt	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
L-Lys HCl, 98%	0.01	0.01	0.01	0.01	0.08	0.08	0.08	0.08
DL-Met, 98%	0.14	0.14	0.14	0.14	0.04	0.04	0.04	0.04
L-Thr, 98%	0.01	0.01	0.01	0.01	0.03	0.03	0.03	0.03
Chromium oxide	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Premix[2]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
25-OH-D_3, μg/kg	-	-	69	69	-	-	69	69
Calculated levels								
ME, Mcal/kg	3.05	3.05	3.05	3.05	3.15	3.15	3.15	3.15
CP	21.00	21.00	21.00	21.00	19.00	19.00	19.00	19.00
Lys	1.10	1.10	1.10	1.10	1.00	1.00	1.00	1.00
Ca	1.00	0.70	1.00	0.70	0.90	0.62	0.90	0.62
Total P	0.70	0.50	0.70	0.50	0.59	0.43	0.59	0.43
NPP	0.45	0.25	0.45	0.25	0.35	0.19	0.35	0.19
Analyzed levels								
Ca	0.97	0.68	0.98	0.69	0.88	0.60	0.87	0.61
Total P	0.68	0.48	0.68	0.49	0.57	0.43	0.56	0.42
VD_3, μg/kg	71.9	72.0	71.8	71.8	71.7	71.8	71.7	71.7
25-OH-D_3, μg/kg	ND[3]	ND	70.5	70.1	ND	ND	69.8	69.9

[1]NCaP, normal calcium and phosphorus diet; LCaP, low calcium and phosphorus diet; NCaP + 25-OH-D_3, normal calcium and phosphorus diet supplemented with 69 μg/kg 25-hydroxycholecalciferol; LCaP + 25-OH-D_3, low calcium and phosphorus diet supplemented with 69 μg/kg 25-hydroxycholecalciferol.

[2]The premix provided the following per kilogram of diet: zinc, 60 mg; iron, 100 mg; manganese, 80 mg; copper, 10 mg; iodine, 0.35 mg; selenium, 0.3 mg; vitamin A, 10,000 IU; vitamin D_3, 2,760 IU; vitamin E, 30 IU; vitamin K_3, 2 mg; vitamin B_{12}, 1.2 mg; riboflavin, 6 mg; nicotinic acid, 40 mg; pantothenic acid, 12 mg; pyridoxine, 3 mg; biotin, 0.2 mg; and choline chloride, 800 mg.

[3]Not detected.

Bio-One GmbH, Kremsmunster, Austria) and then centrifuged at 3,000 × *g* for 10 min at 4°C. Serum samples were withdrawn and then stored at −80°C until further analysis.

On day 42, one bird (close to the average body weight per pen) was slaughtered by cervical dislocation. Immediately after death, the left breast muscle samples were rapidly collected into self-sealing bags with labels and frozen at 4°C for further analysis of meat color, pH values, and drip loss. Tibia and femur from the left leg of each bird were excised and freed of surrounding soft tissues. The length of each bone was recorded using a digital caliper, as described in Cromwell et al. (1972). Bones were frozen at −20°C for analysis of bone quality.

Meat Quality Measurement

At the respective time postmortem, the values of $pH_{45\ min}$ and $pH_{24\ h}$ were measured using a glass penetration pH electrode (pH-star, Matthaus, Germany) and the pH decline (%) was calculated according to the following formula: $[(pH_{45\ min} - pH_{24\ h})/pH_{45\ min}] \times 100$. Meat color was measured using a Chromameter (CR-410, Konica Minota, Tokyo, Japan) and expressed as lightness (L*), redness (a*), and yellowness (b*) values. The meat color and pH value were measured in triplicate at 3 different orientations (middle, medial, and lateral) for each sample. Drip loss was determined using the plastic bag method as described by Straadt et al. (2007). Briefly, a slice of 1-cm thickness of muscle was cut from the left breast muscle and weighed and then placed in a nitrogen filled container to avoid evaporation and oxidation. After 24 h at 4°C, the slice of meat was carefully cleaned using filter paper and weighed.

Serum Measurement

The content of total antioxidant capacity (**T-AOC**) and activities of catalase (**CAT**), GSH-Px, SOD, and malondialdehyde (**MDA**) in serum samples were determined in duplicate using a spectrophotometer methods with colorimetric kits according to manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Serum Ca, P, alkaline phosphatase (**ALP**), and tartrate-resistant acid phosphatase (**TRAP**) were analyzed in duplicate using commercial kits (Zhongsheng Beikong Bio-technology & Science Inc., Beijing, China). Serum parathyroid hormone (**PTH**) was assessed in duplicate using commercially available ELISA test kit (Sino-UK institute of Biological Technology, Beijing, China). Serum 25-OH-D_3 was determined using liquid chromatography-tandem mass spectrometry system (LC-MS/MS 6430, Agilent Technologies, Santa Clara, CA) according to the methods described by Priego Capote et al. (2007). Serum 25-OH-D_3 concentration was expressed as ng/mL in the samples.

Table 2. Effects of 25-hydroxycholecalciferol supplementation on growth performance of broilers fed normal or low calcium and phosphorus diet.[1]

	BW (g)		ADG (g/D)			ADFI (g/D)			FCR (g/g)		
Item	day 21	day 42	days 1–21	days 22–42	days 1–42	days 1–21	days 22–42	days 1–42	days 1–21	days 22–42	days 1–42
Main effect											
CaP level											
Normal	682	2,475	30.26	85.37	57.58	38.43	126.66	82.11	1.27	1.48	1.43
Low	623	2,217	27.43	75.93	51.58	35.89	128.31	81.90	1.31	1.71	1.59
25-OH-D_3											
−	653	2,287	28.86	77.82	53.21	37.62	129.26	83.22	1.31	1.68	1.58
+	652	2,405	28.83	83.48	55.95	36.71	125.71	80.79	1.27	1.51	1.44
SEM	16.22	60.58	0.77	2.73	1.43	1.69	6.09	3.31	0.05	0.07	0.05
P-value											
CaP level	0.001	<0.001	0.001	0.002	<0.001	0.146	0.789	0.952	0.478	0.003	0.002
25-OH-D_3	0.940	0.063	0.959	0.047	0.068	0.597	0.565	0.469	0.564	0.015	0.010
CaP × 25-OH-D_3	0.796	0.135	0.817	0.131	0.128	0.691	0.553	0.403	0.757	0.327	0.580

Data were the means and SEM.
[1]BW, body weight; ADFI, average daily feed intake; ADG, average daily weight gain; FCR, feed conversion ratio; 25-OH-D3, 25-hydroxycholecalciferol.

Bone Analysis

Total bone density of the tibias and femurs was calculated using the formula according to the Archimedes principle (Keenan et al., 1997): bone density = (A/A–B) × P, where A is the weight of the hydrated bone out of water, B is the weight of the hydrated bone submerged in water, A–B is the difference in weight, and P is the density of distilled water. The bone biomechanical properties were analyzed via a 3-point bending test using an MTS Material Testing Apparatus (Model 810, MTS Systems Corporation, Eden Prairie, MN). Force was applied to the midpoint of the bone, which was supported by 2 fulcrums spaced 30 mm apart and loaded at a constant speed of 10 mm/min until the bone broke. Breaking strength (N), failure deflection (mm), stiffness (N/mm), and absorbed energy (J) were measured.

To analyze Ca and P contents of bones, the tibia and femur samples were placed in a ethanol container for 2 D and then extracted with ethyl ether for 4 D. The bones were dried at 105°C for 24 h and then weighed. The ash weight was measured after the dried fat-free bones were ashed at 600°C for 16 h in a muffle furnace. Bone ash was used to analyze Ca (method 968.08) and P (method 985.01) according to the Association of Official Analytical Chemists (AOAC, 2006) procedure.

Statistical Analysis

All data were analyzed as a 2 × 2 factorial design using the General Linear Model procedure of SAS 9.4 (SAS Institute Inc., Cary, NC). The cage was the experimental unit for growth performance, and the selected bird from each cage was the experimental unit for the other parameters. The model included the effects of CaP level and 25-OH-D_3 and the interaction between CaP level and 25-OH-D_3. Least squares means were derived for all treatments and compared using the PDIFF (Adjust Tukey) and STDERR options of SAS. Values were expressed as least squares means and SEM. Significant differences were declared at $P < 0.05$.

RESULTS

Bird Performance

Bird performance is shown in Table 2. There were no significant interactions on growth performance of broilers between CaP level and 25-OH-D_3. Dietary Ca and P deficiencies decreased ($P < 0.05$) ADG from day 1 to 21, day 22 to 42, and day 0 to 42 and increased FCR from day 22 to 42 and day 0 to 42. Dietary 25-OH-D_3 supplementation increased ($P < 0.05$) ADG from day 22 to 42 and decreased FCR from day 22 to 42 and day 0 to 42.

Meat Quality

No significant interactions were detected in meat quality of broilers between CaP level and 25-OH-D_3. The main effects of CaP level and 25-OH-D_3 did not significantly influence pH values at 45 min and 24 h, pH decline, L*, a*, b*, and drop loss in breast muscle of broilers.

Serum Antioxidant Capacity

Serum antioxidant capacity was shown in Table 3. On day 21, dietary 25-OH-D_3 supplementation increased serum concentrations of T-AOC, CAT, and GSH-Px in broilers fed low Ca and P diet but had no effect in broilers fed normal Ca and P diet (CaP level × 25-OH-D_3, $P < 0.05$). In addition, dietary 25-OH-D_3 supplementation decreased ($P < 0.05$) serum MDA concentration in broilers.

On day 42, serum T-AOC and CAT concentrations were decreased by dietary Ca and P deficiencies without 25-OH-D_3 supplementation and unaffected by dietary Ca and P deficiencies with 25-OH-D_3 supplementation (CaP level × 25-OH-D_3, $P < 0.05$).

Bone Biochemical Markers

On day 21, serum Ca and P concentrations were decreased ($P < 0.05$), and serum PTH concentration was increased ($P < 0.05$) by dietary Ca and P

Table 3. Effects of 25-hydroxycholecalciferol supplementation on antioxidant enzyme levels in serum of broilers fed normal or low calcium and phosphorus diet.[1]

Item	T-AOC (U/mL)	CAT (U/mL)	GSH-Px (U/mL)	SOD (U/mL)	MDA (nmol/mL)
Day 21					
Main effect					
CaP level					
Normal	45.09	58.90	892	35.88	4.51
Low	34.14	54.24	814	33.47	4.12
25-OH-D_3					
−	35.69	54.62	768	33.68	5.17
+	43.54	58.51	937	35.67	3.47
Interaction effect					
NCaP	45.34[a]	60.39[a]	869[a]		
LCaP	26.03[b]	48.85[b]	667[b]		
NCaP + 25-OH-D_3	44.84[a]	57.40[a]	914[a]		
LCaP + 25-OH-D_3	42.23[a]	59.62[a]	961[a]		
SEM	1.23	1.88	40.60	4.18	0.37
P-value					
CaP level	<0.001	0.025	0.074	0.567	0.227
25-OH-D_3	<0.001	0.055	<0.001	0.643	<0.001
CaP × 25-OH-D_3	<0.001	0.002	0.007	0.232	0.920
Day 42					
Main effect					
CaP level					
Normal	9.20	58.44	871	29.12	5.37
Low	8.78	49.91	858	28.42	5.01
25-OH-D_3					
−	8.87	52.60	868	28.22	5.54
+	9.11	55.75	861	29.33	4.83
Interaction effect					
NCaP	9.28[a]	60.84[a]			
LCaP	8.47[b]	44.37[b]			
NCaP + 25-OH-D_3	9.13[a,b]	56.05[a,b]			
LCaP + 25-OH-D_3	9.08[a,b]	55.46[a,b]			
SEM	0.21	3.36	16.50	0.64	0.46
P-value					
CaP level	0.025	0.022	0.462	0.354	0.457
25-OH-D_3	0.195	0.363	0.658	0.153	0.149
CaP × 25-OH-D_3	0.041	0.031	0.510	0.089	0.407

[a,b]Within comparisons, means in the same column with different superscripts differ significantly ($P < 0.05$).

Data were the means and SEM, n = 6 broilers per treatment for data on interaction effect, and n = 12 broilers per treatment for data on main effect.

[1]NCaP, normal calcium and phosphorus diet; LCaP, low calcium and phosphorus diet; NCaP +25-OH-D_3, normal calcium and phosphorus diet supplemented with 69 μg/kg 25-hydroxycholecalciferol; LCaP +25-OH-D_3, low calcium and phosphorus diet supplemented with 69 μg/kg 25-hydroxycholecalciferol; T-AOC, total antioxidant capacity; CAT, catalase; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; MDA, malondialdehyde.

deficiencies. Concentrations of Ca and 25-OH-D_3 in serum of broilers were increased ($P < 0.05$) by dietary 25-OH-D_3 supplementation (Table 4).

On day 42, serum concentrations of Ca, P, and ALP were decreased ($P < 0.05$), and serum PTH concentration was increased ($P < 0.05$) by dietary Ca and P deficiencies. Dietary 25-OH-D_3 supplementation increased ($P < 0.05$) serum Ca and 25-OH-D_3 concentrations and decreased ($P < 0.05$) serum TRAP concentration in broilers.

Bone Mineralization

In tibias, bone ash and P contents were decreased ($P < 0.05$) by dietary Ca and P deficiencies. Dietary supplementation with 25-OH-D_3 improved ($P < 0.05$) bone density of broilers (Table 5). The interaction between CaP level and 25-OH-D_3 was observed ($P < 0.05$) for Ca content in tibias of broilers, in that dietary 25-OH-D_3 supplementation increased Ca content in tibias of broilers fed low Ca and P diet but had no effect in broilers fed normal Ca and P diet (CaP level × 25-OH-D_3, $P < 0.05$).

In femurs, bone Ca and P contents were decreased ($P < 0.05$) by dietary Ca and P deficiencies. Dietary supplementation with 25-OH-D_3 increased ($P < 0.05$) Ca content in broilers (Table 6). Dietary 25-OH-D_3 supplementation increased bone density in femurs of broilers subjected to low Ca and P diet (CaP level × 25-OH-D_3, $P < 0.05$).

Bone Biomechanical Properties

Tables 7 and 8 show the bone biomechanical properties through 3-point bending tests at the mid-diaphysis of tibias and femurs. There were no significant interactions between CaP level and 25-OH-D_3 on bone biomechanical properties of tibial and femurs. In tibias and femurs, breaking strength, stiffness, and absorbed energy were decreased ($P < 0.05$) by dietary Ca and P deficiencies. Dietary supplementation with 25-OH-D_3

Table 4. Effects of 25-hydroxycholecalciferol supplementation on bone biochemical markers in serum of broilers fed normal or low calcium and phosphorus diet.[1]

Item	Ca (mmol/L)	P (mmol/L)	ALP (U/L)	TRAP (U/L)	PTH (U/L)	25-OH-D_3 (ng/mL)
Day 21						
Main effect						
CaP level						
Normal	1.88	1.54	4,327	372	61.93	28.47
Low	1.48	0.97	4,314	324	67.64	28.13
25-OH-D_3						
−	1.61	1.07	3,975	335	65.54	25.11
+	1.75	1.45	4,576	361	64.03	31.49
SEM	0.09	0.15	705	32.33	2.00	1.06
P-value						
CaP level	0.016	0.001	0.914	0.150	0.010	0.750
25-OH-D_3	0.002	0.130	0.405	0.436	0.459	<0.001
CaP × 25-OH-D_3	0.062	0.327	0.910	0.795	0.488	0.134
Day 42						
Main effect						
CaP level						
Normal	1.84	1.64	4,498	283	56.11	26.86
Low	1.52	1.01	2,836	268	67.32	25.72
25-OH-D_3						
−	1.51	1.15	3,437	317	62.10	23.77
+	1.85	1.50	3,898	235	61.33	28.81
SEM	0.12	0.16	274	20.84	5.18	1.52
P-value						
CaP level	0.011	0.003	<0.001	0.477	0.041	0.466
25-OH-D_3	0.007	0.103	0.108	<0.001	0.876	0.004
CaP × 25-OH-D_3	0.195	0.274	0.429	0.652	0.907	0.799

Data were the means and SEM, n = 6 broilers per treatment for data on interaction effect, and n = 12 broilers per treatment for data on main effect.

[1]Ca, calcium; P, phosphorus; ALP, alkaline phosphatase; TRAP, tartrate-resistant acid phosphatase; PTH, parathyroid hormone; 25-OH-D_3, 25-hydroxycholecalciferol.

increased ($P < 0.05$) breaking strength in tibias of broilers.

DISCUSSION

Feeding low Ca and P diet damaged growth performance such as decreased ADG from day 1 to 21, day 22 to 42, and day 0 to 42 as well as increased FCR from day 22 to 42 and day 0 to 42, indicating that lower dietary Ca and P levels used in the current experiment had negative effects on the growth rate of broilers. Similar results have been previously reported in broilers (Boiling et al., 2000; Johnston and Southern, 2000; Watson et al., 2006). These results support the importance of adequate Ca and available P intake to achieve normal growth performance and bone mineralization in

Table 5. Effects of 25-hydroxycholecalciferol supplementation on tibia mineralization of broilers fed normal or low calcium and phosphorus diet.[1]

Item	Weight (g)	Length (cm)	Bone density (g/cm^3)	Ash (%)	Ca (%)	P (%)
Main effect						
CaP level						
Normal	4.38	10.26	1.38	36.84	17.14	8.22
Low	4.13	10.09	1.27	32.89	16.26	6.98
25-OH-D_3						
−	4.16	10.14	1.23	34.16	16.44	7.28
+	4.36	10.21	1.42	36.27	16.96	7.92
Interaction effect						
NCaP					17.27[a]	
LCaP					15.60[b]	
NCaP + 25-OH-D_3					17.00[a]	
LCaP + 25-OH-D_3					16.92[a]	
SEM	0.25	0.16	0.07	1.60	0.30	0.46
P-value						
CaP level	0.334	0.291	0.106	0.023	0.008	0.013
25-OH-D_3	0.431	0.647	0.010	0.094	0.092	0.172
CaP × 25-OH-D_3	0.384	0.150	0.103	0.848	0.014	0.242

[a,b]Within comparisons, means in the same column with different superscripts differ significantly ($P < 0.05$). Ash, Ca and P contents are calculated by dividing the ash, Ca, and P weights by fat-free dry weight of tibias.

Data were the means and SEM, n = 6 broilers per treatment for data on interaction effect, and n = 12 broilers per treatment for data on main effect.

[1]NCaP, normal calcium and phosphorus diet; LCaP, low calcium and phosphorus diet; NCaP +25-OH-D_3, normal calcium and phosphorus diet supplemented with 69 μg/kg 25-hydroxycholecalciferol; LCaP +25-OH-D_3, low calcium and phosphorus diet supplemented with 69 μg/kg 25-hydroxycholecalciferol; Ca, calcium; P, phosphorus.

Table 6. Effects of 25-hydroxycholecalciferol supplementation on femur mineralization of broilers fed normal or low calcium and phosphorus diet.[1]

Item	Weight (g)	Length (cm)	Bone density (g/cm^3)	Ash (%)	Ca (%)	P (%)
Main effect						
CaP level						
Normal	3.25	7.42	1.50	36.04	17.29	8.33
Low	2.97	7.34	1.33	32.10	15.93	6.97
25-OH-D$_3$						
−	3.14	7.38	1.33	33.37	16.12	7.30
+	3.08	7.39	1.50	34.77	17.10	7.99
Interaction effect						
NCaP			1.51[a]			
LCaP			1.15[b]			
NCaP + 25-OH-D$_3$			1.48[a]			
LCaP + 25-OH-D$_3$			1.51[a]			
SEM	0.15	0.10	0.06	1.91	0.44	0.37
P-value						
CaP level	0.083	0.448	0.007	0.053	0.006	0.002
25-OH-D$_3$	0.672	0.932	0.008	0.472	0.037	0.074
CaP × 25-OH-D$_3$	0.698	0.932	0.003	0.067	0.100	0.829

[a,b]Within comparisons, means in the same column with different superscripts differ significantly ($P < 0.05$). Ash, Ca, and P contents are calculated by dividing the ash, Ca, and P weights by fat-free dry weight of femurs.

Data were the means and SEM, n = 6 broilers per treatment for data on interaction effect, and n = 12 broilers per treatment for data on main effect.

[1]NCaP, normal calcium and phosphorus diet; LCaP, low calcium and phosphorus diet; NCaP +25-OH-D$_3$, normal calcium and phosphorus diet supplemented with 69 μg/kg 25-hydroxycholecalciferol; LCaP +25-OH-D$_3$, low calcium and phosphorus diet supplemented with 69 μg/kg 25-hydroxycholecalciferol; Ca, calcium; P, phosphorus.

growing animals. In this study, significant improvements were observed on ADG and FCR of broilers fed diets supplemented with 25-OH-D$_3$ on day 22 to 42. As reported by Vazquez et al. (2018), supplementation of 25-OH-D$_3$ at a rate of 69 μg/kg to broiler chickens significantly increased growth performance. However, Hutton et al. (2014) showed that growth performance and feed efficiency were not influenced by dietary 25-OH-D$_3$ supplementation. Although the effects of supplementation of 25-OH-D$_3$ to diets on growth performance of broilers are controversial, it is a consistent result that addition of 25-OH-D$_3$ increased serum 25-OH-D$_3$ concentration in broilers (Hutton et al., 2014; Bozkurt et al., 2017). Until recently, the cost of production of 25-OH-D$_3$ is an important factor which prevents the use of this more polar form of vitamin D$_3$ in poultry diets. Owing to technological advances, 25-OH-D$_3$ has become available to the industry at a reasonable price (Keshavarz, 2003).

Chen and Moran (1995) demonstrated that lower dietary Ca and P could result in broken bones and bloody meat during the processing of broiler production. Previous results indicated that vitamin D status could affect skeletal muscle metabolism and function, and vitamin D$_3$ metabolites have been identified to improve Ca utilization in the growing body (Girgis et al., 2013; Regassa et al., 2015). Recently, information about the mechanisms by which 25-OH-D$_3$ affects skeletal muscle hypertrophy and muscle fiber composition and size has emerged (Hutton et al., 2014; Vignale et al., 2015). These mechanisms could affect color and sensorial meat characteristics of meat. However, our results showed that the parameters of meat quality in breast muscle such as pH decline, L*, a*, b*, and drop loss values were unaffected by CaP level and 25-OH-D$_3$ and their interaction and did not provide specific evidence to support the relationship between dietary vitamin D status and meat quality.

Table 7. Effects of 25-hydroxycholecalciferol supplementation on tibia biomechanical parameters of broilers fed normal or low calcium and phosphorus diet.

Item	Breaking strength (N)	Failure deflection (mm)	Stiffness (N/mm)	Absorbed energy (J)
Main effect				
CaP level				
Normal	305	2.04	188	0.36
Low	179	2.29	108	0.25
25-OH-D$_3$				
−	215	2.16	134	0.27
+	269	2.16	162	0.34
SEM	23.76	0.15	17.69	0.04
P-value				
CaP level	<0.001	0.125	<0.001	0.017
25-OH-D$_3$	0.034	0.988	0.124	0.097
CaP × 25-OH-D$_3$	0.200	0.184	0.144	0.684

Data were the means and SEM, n = 6 broilers per treatment for data on interaction effect, and n = 12 broilers per treatment for data on main effect.

Abbreviation: 25-OH-D$_3$, 25-hydroxycholecalciferol.

Table 8. Effects of 25-hydroxycholecalciferol supplementation on femur biomechanical parameters of broilers fed normal or low calcium and phosphorus diet.

Item	Breaking strength (N)	Failure deflection (mm)	Stiffness (N/mm)	Absorbed energy (J)
Main effect				
CaP level				
Normal	267	2.47	124	0.37
Low	154	2.77	63.03	0.26
25-OH-D_3				
−	196	2.51	86.63	0.29
+	225	2.73	99.90	0.34
SEM	14.26	0.23	11.06	0.03
P-value				
CaP level	<0.001	0.218	<0.001	0.003
25-OH-D_3	0.063	0.347	0.236	0.155
CaP × 25-OH-D_3	0.127	0.545	0.163	0.314

Data were the means and SEM, n = 6 broilers per treatment for data on interaction effect, and n = 12 broilers per treatment for data on main effect.

Abbreviation: 25-OH-D_3, 25-hydroxycholecalciferol.

As a result of the nutritional and physiological properties, broilers are susceptible to lipid peroxidation, leading to the production of peroxidative metabolites. Oxidative stress is an important predisposing factor for the pathological status of many bone diseases including osteoporosis (Wauquier et al., 2009). Excessive reactive oxygen species could disturb the balance between oxidant and antioxidant systems in the body, promote lipid peroxidation, and reduce antioxidant enzyme activities, which results in osteocyte apoptosis and inhibits bone formation (Wauquier et al., 2009; Savasky et al., 2018). Normally, excessive oxidative radicals are eliminated by antioxidant systems including nonenzymatic antioxidant defensive components and a series of antioxidant enzymes. The deficiency in calcium leads to a marked decrease of antioxidant enzymes including SOD (Choi and Jung, 2017). Previous studies reported that P deficiency depressed GSH content and antioxidant enzymes activities and downregulated the mRNA levels of antioxidant enzymes in young grass carp (Chen et al., 2017). It is known that vitamin D protects cells against oxidative damages in rats (Choi and Jung, 2017), and 25-OH-D_3 has been shown as effective in improving body antioxidant status via enhancing the enzymatic antioxidant system in rats and ducks (Sardar et al., 1995; Garcion et al., 1999; Ren et al., 2018). In the present study, our findings showed that broilers fed low Ca and P diet reduced serum antioxidant capacity such as decreased concentrations of serum T-AOC, CAT, and GSH-Px. When subjected to low Ca and P diet, the broilers supplemented with 25-OH-D_3 had higher serum contents of T-AOC, CAT, and GSH-Px, which indicates preventive changes to counteract the oxidative damages induced by dietary Ca and P deficiencies. Enzymatic inactivation of reactive oxygen species is achieved by the higher activities of antioxidant enzymes mainly including SOD, CAT, and GSH-Px. Superoxide dismutase acts as the first line of defense against the adverse effects of oxygen radicals by catalyzing the dismutation of the endogenous superoxide radical to H_2O_2, which is detoxified into H_2O and O_2 by CAT and GSH-Px (Bai et al., 2018), whereas GSH-Px alleviates the oxidative damage by eliminating the excessively generated free radicals (Delles et al., 2014). The nonenzymatic antioxidant defense system is reflected by the increased level of T-AOC (Long et al., 2018). Our result suggested that 25-OH-D_3 supplementation improved body antioxidant status via activating the enzymatic and nonenzymatic antioxidant defensive systems when broilers were subjected to low Ca and P diet on day 21. Lipid peroxidation can be directly reflected by the changes in MDA concentration because MDA is the major end product of peroxidation of polyunsaturated fatty acid, which is associated with the oxidative damage (Perai et al., 2014). In our study, dietary 25-OH-D_3 supplementation prevented the increase of MDA level in serum of broilers, suggesting that lipid peroxidation could be prevented by dietary 25-OH-D_3 supplementation.

The ability of 25-OH-D_3 to alleviate bone deformation is related to its main role in maintaining Ca and P homeostasis so that these minerals can promote bone mineralization (Bozkurt et al., 2017). However, we found that dietary 25-OH-D_3 supplementation increased serum Ca concentration but did not significantly influence serum P concentrations of broilers. Although further researches should be conducted to clarify these results, there was an important point that changes of serum Ca and P levels were in concert with Ca and P contents in tibias and femurs. Among bone remodeling markers, ALP is regarded as one of bone formation markers, which reflects osteoblastic activities (Van Straalen et al., 1991), whereas TRAP is regarded as one of bone resorption markers, which reflects osteoclastic activities (Minkin, 1982). Dietary Ca and P deficiencies decreased serum ALP activity, suggesting that bone formation was suppressed when boilers were subjected to low Ca and P diet. 25-hydroxycholecalciferol supplementation decreased serum TRAP activity of boilers, which indicated that dietary supplementation of 25-OH-D_3 could improve bone development via suppressing bone resorption in this study. On day 21 and 42, our results showed that serum PTH concentration was significantly increased by dietary Ca and P deficiencies. Previous studies in rats reported that feeding a low Ca diet induced hypocalcemia and elevated serum PTH concentration (Iwamoto et al., 2004), which the changes in response to Ca deficiency are considered to be physiologically reasonable.

Accurate measurements for bone status are critical to develop nutritional strategies that can reduce structural bone loss in broilers. There are several bone parameters to evaluate bone status: bone ash, bone mineral contents, bone density, bone breaking strength, and stiffness, etc. In the present study, decreases of bone ash, Ca, and P contents and bone biomechanical properties including breaking strength, stiffness, and absorbed energy were observed in tibias and femurs of broilers fed low Ca and P diet. In addition, feeding low Ca and P diet to broilers decreased femoral bone density but had no significant influences on tibial bone density. These results indicated that lower dietary Ca and P levels used in the current research had negative effects on the bone quality of broilers, and Ca and P play important roles in bone health and development. The bone mineral density and bone breaking strength appeared to be good indicators of Ca nutrition, with breaking strength being more reliable than bone mineral density in monitoring Ca deficiency (Bai et al., 2017). In the present study, we found that dietary 25-OH-D_3 supplementation increased bone density and breaking strength in tibias and femurs, regardless of whether broilers were fed with normal or low Ca and P diets. In addition, significant interaction effect was observed for bone density in femurs of broilers, in that 25-OH-D_3 supplementation increased bone density of broilers fed with low Ca and P diet. According to our results, 25-OH-D_3 supplementation could improve bone quality such as bone density and breaking strength of broilers, which was not dependent on dietary Ca and P levels. However, this effect was more marked in low Ca and P diet for bone density. These results indicated that feeding diets supplemented with 25-OH-D_3 to broilers had a potential to improve bone fracture resistance and decrease incidence of leg disorders.

In conclusion, these data indicated that 25-OH-D_3 supplementation at 69 μg/kg increased growth performance in some periods, enhanced serum antioxidant status, and improved bone mineralization and breaking strength of broilers. Moreover, significant interaction effects between CaP level and 25-OH-D_3 were observed for specific serum antioxidants and bone properties, and effects of dietary 25-OH-D_3 supplementation were more marked in low Ca and P diet for serum T-AOC content, serum CAT activity, and bone density.

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