Non-ruminants

Effect of different vitamin D_3 metabolites on intestinal calcium homeostasis-related gene expression in broiler chickens

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ABSTRACT - The purpose of this study was to investigate the effects of vitamin D_3 metabolites 1α-hydroxycholecalciferol $(1α(OH)D_3)$, 25-hydroxycholecalciferol $(25(OH)_2D_3)$, and 1,25-dihydroxycholecalciferol $(1,25(OH)_2D_3)$ on growth performance, bone quality, and intestinal calcium homeostasis-related gene expression in broiler chickens. One-day-old broilers were fed a basal diet and basal diet containing different vitamin D_3 metabolites. The body weight, feed intake, and feed conversion ratio in control and experimental broilers were measured to assess the growth performance, mineral levels, and bone breaking strength. The duodenum was used to assess calcium homeostasis-related gene expressions by quantitative reverse transcription-PCR. No statistically significant difference was found in growth performance, mineral deposition, or bone breaking strength in broiler chickens after three weeks feeding with vitamin D_3. However, supplementation of vitamin D_3 metabolites tended to improve feed conversion rate, bone mineral deposition, and breaking strength in broiler chickens. The results demonstrated that vitamin D_3 metabolites significantly upregulated calcium homeostasis-related genes, including calbindin, β-glucuronidase, TRPV6, and Na/Pi IIb cotransporter, mRNA levels after 12 h of feeding. The vitamin D_3 metabolite $1,25(OH)_2D_3$ was the most effective at regulating calcium homeostasis-associated gene expression after 6 h of feeding. Dietary vitamin D3 metabolites may alleviate the development of TD in broiler chickens and these effects probably occur through regulation of intestinal calcium homeostasis-related gene expression.

Key Words: gene, 1α-hydroxycholecalciferol, 25-hydroxycholecalciferol, 1,25-dihydroxycholecalciferol

Introduction

Tibial dyschondroplasia (TD) is an extremely common skeletal abnormality associated with rapid growth rate in broiler chickens (Leach and Lilburn, 1992). It leads to enormous economic losses worldwide and to animal welfare problems (Pines et al., 2005). Tibial dyschondroplasia is characterized by the formation of a lesion composed of non-vascularized, non-mineralized cartilage that extends from the epiphyseal growth plate into the metaphysic (Leach and Nesheim, 1965). It has been reported that TD is influenced by several factors, including genetics and nutrition (Waldenstedt, 2006). Nutrition plays a major role in the development and maintenance of bone structure, such as calcium, phosphorus, and vitamin D (Fleming, 2008).

Vitamin D_3 (cholecalciferol) has been widely used as a feed additive to improve calcium and phosphorus metabolism and bone development in poultry (Baker et al., 1998). It has also been demonstrated that supplementation of vitamin D metabolites could efficiently prevent TD in broiler chickens (Edwards, 1990; Roberson and Edwards, 1996; Whitehead et al., 2004).

Conversion of vitamin D_3 to 25-hydroxycholecalciferol $(25(OH)D_3)$ is catalyzed by 25-hydroxylase in livers. $25(OH)D_3$ is further converted to 1,25-dihydroxycholecalciferol $(1,25(OH)_2D_3)$ by 1α-hydroxylase in kidney. $1,25(OH)_2D_3$ is the biologically active form of vitamin D *in vivo* (Henry et al., 1979; Henry et al., 1985). It has been shown that $25(OH)D_3$ significantly improved body weight and feed efficiency of broiler chickens compared with those of the birds fed vitamin D_3 (Yarger et al., 1995; Fritts and Waldroup, 2003; Koreleski and Swiatkiewicz, 2005). Recently, we also demonstrated that broilers fed 1α-hydroxycholecalciferol $(1α(OH)D_3)$ had higher body weight gain, feed intake, and tibia breaking strength compared with birds fed $25(OH)D_3$ at 42 days of age (Han et al., 2016). However, no significant effects were

found on body weight or feed efficiency in broiler chickens after supplementation of diet with $1,25(OH)_2D_3$ (Roberson and Edwards, 1996).

Vitamin D_3 is involved in the regulation of levels of minerals such as calcium and phosphorous. Several mechanisms have been proposed to elucidate the vitamin D_3 metabolism and mechanisms of calcium transport in the small intestine (Norman et al., 1982; Kumar, 1990). However, little is known about the transcriptional level of intestinal calcium homeostasis-related genes in response to different vitamin D_3 metabolites in broiler chickens. Therefore, the present study was conducted to investigate the effects of different vitamin D metabolites including $1\alpha(OH)D_3$, $25(OH)D_3$, and $1,25(OH)_2D_3$ on intestinal calcium homeostasis-related gene expression in broiler chickens.

Material and Methods

All experiments were performed in accordance with approved guidelines. The animal protocol was approved by local Institutional Animal Care and Use Committee (IACUC case No. 104-14). The concentration of vitamin D metabolites described earlier (Yarger et al., 1995; Roberson and Edwards, 1996; Chou et al., 2009; Han et al., 2009) was used in the present study. For examination of growth performance, mineral levels, and breaking strength, forty-eight one-day-old broilers (Avian) were randomly allocated to four different treatment groups (n = 12 per group) that were fed: basal diet (control), basal diet plus 5 µg/kg of $1\alpha(OH)D_3$, basal diet plus 69 µg/kg of $25(OH)D_3$, and basal diet plus 5 µg/kg of $1,25(OH)_2D_3$. The basal diets were formulated based on National Research Council recommendations (NRC, 1994) (Table 1). Water and feed was provided *ad libitum* over the entire experimental period. Total individual body weight, feed intake, and feed conversion ratio were recorded every week. At the end of the experiment, broilers were sacrificed by cervical dislocation. The blood was collected and centrifuged to harvest serum for biochemistry. The left tibia was removed for determination of mineral deposition and breaking strength measurement. For examination of calcium homeostasis-related gene expression, sixteen one-day-old broilers (Avian) were randomly allocated to four different treatment groups (n = 4 per group): basal diet (control), basal diet plus 5 µg/kg of $1\alpha(OH)D_3$, basal diet plus 69 µg/kg of $25(OH)D_3$, and basal diet plus 5 µg/kg of $1,25(OH)_2D_3$. After 0, 6, and 12 h of feeding, the duodenum was excised for RNA extraction.

Tissue RNA was extracted from the duodenum of broilers using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and resuspended in diethyl pyrocarbonate-treated water. Reverse transcription was performed with a Transcriptor Reverse Transcriptase kit (Roche Applied Science, Indianapolis, IN, USA). Quantitative reverse transcriptase-polymerase chain reaction (PCR) for each gene (Table 2) was performed using Miniopticon Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Boston, MA, USA). Polymerase chain reaction was performed by 40 cycles at 95 °C for 30 s, 58-60 °C for 60 s, and 72 °C for 30s. Beta-actin mRNA was determined as the internal control gene. The mRNA expression of each gene was normalized to its β-actin mRNA expression in the same sample. Threshold cycle (Ct) values were obtained and relative gene expression was calculated using the formula: $(1/2)$Ct target genes-Ct β-actin.

The left tibias were collected, cleaned of adhering tissue, weighed, dried to constant weight at 105 °C, defatted, and then ashed in a muffle furnace at 600 °C. Ash was dissolved in concentrated HCl for mineral determination. Calcium and phosphorus content was measured by atomic absorption spectrophotometry (AOAC, 1995a,b). Tibia breaking strength (breaking force divided by bone weight) was measured using TA.XT plus Texture Analyser (Mason Technology, Dublin, Ireland) with the use of a probe (HDP/3PB). Tibia was placed at the central position with 10 cm clearance, allowing the comparison of breaking strength values.

Table 1 - Nutrient composition of basal diet

Ingredient (g/kg)	
Corn	608.7
Soybean meal (43% CP)	320.0
Soybean oil	15.0
Soy protein isolate	34.1
Dicalcium phophate	13.1
L-lysine HCl	1.4
DL-methionine	1.4
Trace mineral premix[1]	1.0
Vitamin premix[2]	0.3
Choline chloride (50%)	2.0
Sodium chloride	3.0
Nutrient composition	
Metabolizable energy (kcal/kg)	2972
Analyzed crude protein (g/kg)	205.8
Analyzed calcium (g/kg)	3.8
Analyzed total phosphorous (g/kg)	5.4
Non-phytate phosphorous (g/kg)	3.5
Lysine (g/kg)	11.0
Methionine (g/kg)	5.0

CP - crude protein.
[1] The trace mineral premix provided the following (per kg of diet): iron, 100 mg; zinc, 100 mg; copper, 8 mg; manganese, 120 mg; iodine, 0.7 mg; selenium, 0.3 mg.
[2] The vitamin premix provided the following (per kg of diet): vitamin A, 8,000 IU; vitamin E, 20 IU; menadione, 0.5 mg; thiamine, 2.0 mg; riboflavin, 8.0 mg; niacin, 35 mg; pyridoxine, 3.5 mg; vitamin B12, 0.01 mg; pantothenic acid, 10.0 mg; folic acid, 0.55 mg; biotin, 0.18 mg.

Table 2 - Primer sequences for quantitative reverse transcription-PCR

Gene	Primer sequence (5′to3′)	Product size
Calbindin	Forward: GGTCTTGGCATGTTATGGA Reverse: CGAACAAGCAGGTGAGAA	136 bp
β-glucuronidase	Forward: ACCTGTGGTGGCCTTATCTC Reverse: GTTGACCCCGTGGAAGTAGA	191 bp
TRPV6	Forward: CACAAGATGATGCGATACACAGAA Reverse: GCCTCGTGACAGTGATGGA	135 bp
Na/Pi llb	Forward: TGTCACAATTCCACCTTCAGAGA Reverse:AAGTATGAGACCGATGGCAAGAT	166 bp
24-hydroxylase	Forward: AACATTTCACGCAATCCACA Reverse: AATGGCACAGATGGTGTCAA	158 bp
VDR	Forward: CGTGAGAAGCAAATTCAGCA Reverse: GAGGTCCAGGTTGGAAAACA	157 bp
β-actin	Forward: CCACCGCAAATGCTTCTAAAC Reverse: GAAGGTGTCAGCAGTCTT	175 bp

PCR - polymerase chain reaction; VDR - vitamin D receptor.

Data were subjected to one-way ANOVA using the General Linear Model procedure of SAS statistical package (Statistical Analysis System, version 9.2) for completely randomized designs. Duncan's new multiple range test was used to evaluate differences between means (SAS Institute, 2008). P-values ≤ 0.05 were considered statistically significant.

Results

To examine the effect of vitamin D$_3$ metabolites on growth performance and bone quality, broilers were fed different vitamin D$_3$ metabolites (1α(OH)D$_3$, 25(OH)D$_3$, and 1,25(OH)$_2$D$_3$) for three weeks. However, there were no statistically confirmed differences between the growth performance and vitamin D$_3$ metabolite supplementation during the entire feeding period (Table 3). Although the changes in tibia calcium and phosphorus content were not statistically significant, a trend of increased tibia calcium and phosphorus content was observed with the supplementation of vitamin D$_3$ metabolites (Table 4). Dietary supplementation of vitamin D$_3$ metabolites had no significant effect on serum calcium and serum phosphorus content (Table 4). Although they did not reach statistical significance, vitamin D$_3$ metabolites caused a similar trend in increasing bone breaking strength in broiler chickens after feeding for three weeks (Table 4). These results indicate that supplementation of vitamin D$_3$ metabolites did not significantly improve growth performance and bone quality of broiler chickens.

To examine the effect of different vitamin D$_3$ metabolites on calcium homeostasis-related gene expression in the duodenum, broiler chickens were fed 5 μg/kg of 1α(OH)D$_3$, 69 μg/kg of 25(OH)D$_3$, and 5 μg/kg of 1,25(OH)$_2$D$_3$ for 0, 6, and 12 h. The level of calbindin mRNA was rapidly increased at 6 h after feeding broiler chickens additional amounts of vitamin D$_3$ metabolites (Figure 1A). Among vitamin D$_3$ metabolites, 1,25(OH)$_2$D$_3$ appeared to be more efficient than 1α(OH)D$_3$ and 25(OH)D$_3$ at regulating calbindin mRNA expression at 6 and 12 h (Figure 1A). The level of β-glucuronidase mRNA was induced at 6 h after feeding 1,25(OH)$_2$D$_3$ compared with other vitamin D$_3$ metabolites (Figure 1B). After 12 h of feeding, 1α(OH)D$_3$ and 25(OH)D$_3$ also promoted β-glucuronidase mRNA expression and 1,25(OH)$_2$D$_3$ increased the β-glucuronidase mRNA even further (Figure 1B). Similarly, 1,25(OH)$_2$D$_3$ rapidly induced the level of transient receptor potential cation channel, subfamily V, member 6 (TRPV6) mRNA at 6 h (Figure 1C). After 12 h of feeding, all vitamin D$_3$ metabolites exhibited increased TRPV6 mRNA expression (Figure 1C). The level of type IIb sodium-dependent phosphate (Na/Pi IIb) cotransporter mRNA was rapidly increased at 6 and 12 h after feeding broiler chickens additional amounts of vitamin D$_3$ metabolites (Figure 2A). The level of 24-hydroxylase mRNA was reduced at 12 h after feeding 25(OH)D$_3$ compared with the control diet (Figure 2B). However, none of the vitamin D$_3$ metabolites changed the vitamin D receptor (VDR) mRNA levels in the duodenum of broilers (Figure 2C). Taken together, these results indicate that vitamin D$_3$ metabolites were able to rapidly modulate calcium homeostasis-related gene expression in the duodenum and 1,25(OH)$_2$D$_3$ was the most effective vitamin D$_3$ metabolite in regulating calcium homeostasis-associated genes.
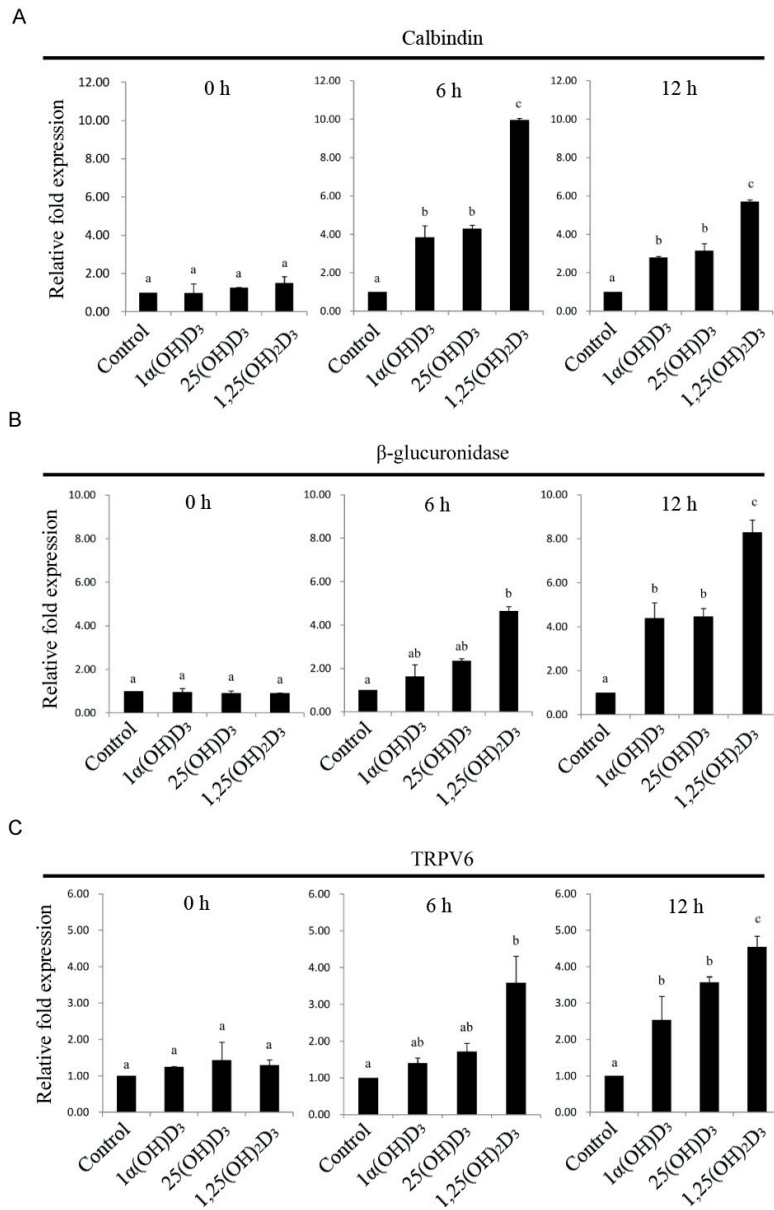
A

B

C

1α(OH)D$_3$ - 1α-hydroxycholecalciferol; 25(OH)D$_3$ - 25-hydroxycholecalciferol; 1,25(OH)$_2$D$_3$ - 1,25-dihydroxycholecalciferol; TRPV6 - transient receptor potential cation channel, subfamily V, member 6.
Values were expressed as mean ± standard deviation (n = 4).
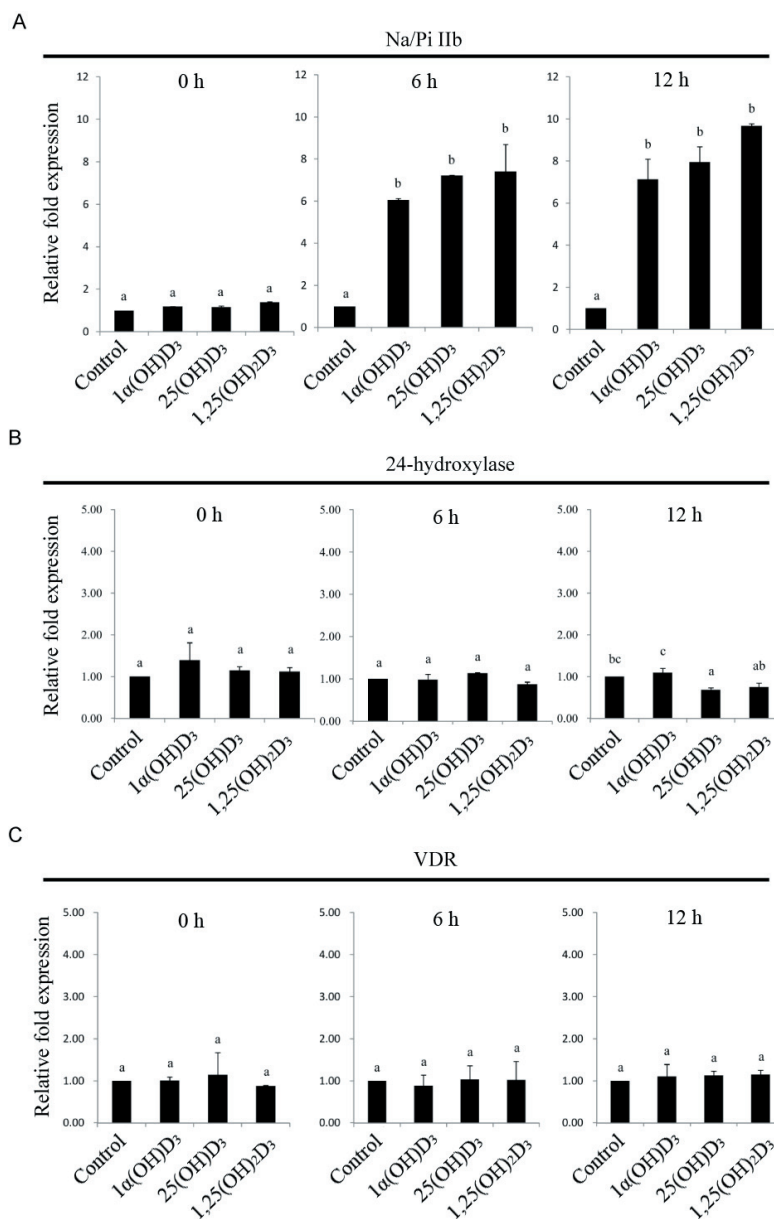a-c - Means followed by different letters are significantly different (P<0.05).

Figure 1 - Effects of different vitamin D$_3$ metabolites on duodenal (A) calbindin, (B) β-glucuronidase, and (C) TRPV6 gene expression in broilers.

Table 3 - Effect of different vitamin D$_3$ metabolites on growth performance of 21-day-old broilers

	Control (n = 12)		1α(OH)D$_3$ (n = 12)		25(OH)D$_3$ (n = 12)		1,25(OH)$_2$D$_3$ (n = 12)		P-value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Initial body weight (g)	43	0.3	43	0.3	43	0.4	43	0.2	NS
Final body weight (g)	750	270	800	80	730	5	780	19	NS
0-21days									
ADG (g/day)	35	2	38	1	34	0.4	37	2	NS
ADFI (g/day)	45	1	46	0.08	43	1	44	1	NS
FCR (gain/feed)	0.787	0.035	0.825	0.012	0.797	0.006	0.835	0.016	NS

1α(OH)D$_3$ - 1α-hydroxycholecalciferol; 25(OH)D$_3$ - 25-hydroxycholecalciferol; 1,25(OH)$_2$D$_3$ - 1,25-dihydroxycholecalciferol; ADG - average daily gain; ADFI - average daily feed intake; FCR - feed conversion ratio; SD - standard deviation; NS - no significant difference (P>0.05).

1α(OH)D$_3$ - 1α-hydroxycholecalciferol; 25(OH)D$_3$ - 25-hydroxycholecalciferol; 1,25(OH)$_2$D$_3$ - 1,25-dihydroxycholecalciferol; Na/Pi IIb - type IIb sodium-dependent phosphate; VDR - vitamin D receptor.
Values were expressed as mean ± standard deviation (n = 4).
a-c - Means followed by different letters are significantly different (P<0.05).

Figure 2 - Effects of different vitamin D$_3$ metabolites on duodenal (A) Na/Pi IIb, (B) 24-hydroxylase, and (C) VDR gene expression in broilers.

Table 4 - Effect of different vitamin D$_3$ metabolites on tibia, serum calcium, and phosphate levels and breaking strength in broilers

	Control (n = 12)		1α(OH)D$_3$ (n = 12)		25(OH)D$_3$ (n = 12)		1,25(OH)$_2$D$_3$ (n = 12)		P-value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Calcium of bone (%)	13.00	2.36	14.24	1.89	14.62	2.71	13.37	0.83	NS
Phosphate of bone (%)	6.06	1.17	6.99	0.73	7.04	1.42	6.32	0.37	NS
Serum calcium (mg/dL)	8.77	0.17	9.60	0.83	9.13	0.24	8.58	0.40	NS
Serum phosphate (mg/dL)	8.58	1.07	8.67	0.26	7.60	0.08	8.67	0.19	NS
Breaking strength (N)	187.43	18.31	197.67	20.51	208.66	19.92	193.48	8.65	NS

1α(OH)D$_3$ - 1α-hydroxycholecalciferol; 25(OH)D$_3$ - 25-hydroxycholecalciferol; 1,25(OH)$_2$D$_3$ - 1,25-dihydroxycholecalciferol; SD - standard deviation; NS - no significant difference (P>0.05).

Discussion

In this study, we demonstrated that vitamin D_3 metabolites could rapidly regulate calcium homeostasis-related gene expression in the duodenum from 6-12 h after feeding, although the growth performance and bone quality of broiler chickens at 21 days of age were not significantly changed. Among these vitamin D_3 metabolites, $1,25(OH)_2D_3$ was the most effective in modulating calcium homeostasis-associated gene expression.

It has been reported that feeding broilers 69 μg/kg of $25(OH)D_3$ for three weeks did not affect body weight gain, feed conversion ratio, or feed intake (Chou et al., 2009). There were no significant effects on body weight or feed efficiency at 21 days of age in broiler chickens after supplementation of the diet with $1,25(OH)_2D_3$ (Roberson and Edwards, 1996). Similarly, our results also confirm the previous reports that no significant effects were observed on growth performance of broiler chickens at 21 days of age after supplementation of the diet with $1α(OH)D_3$, $25(OH)D_3$, or $1,25(OH)_2D_3$. Furthermore, $1α(OH)D_3$ did not alter bone breaking strength, tibia calcium, and phosphorus content in broiler chickens at 21 days of age (Han et al., 2009). Consistently, we also found that vitamin D_3 metabolites did not improve bone breaking strength and bone mineral deposition after feeding for three weeks. In contrast, broilers fed 6 μg/kg of $1,25(OH)_2D_3$ for three weeks exhibited significantly increased bone ash content (Roberson and Edwards, 1996). The contradiction may be due to the differences between composition of basal diet and chicken strains. Together, these findings demonstrate that vitamin D_3 metabolites do not have a significant effect on growth performance, but mineral deposition and bone breaking strength in broiler chickens may vary after feeding for three weeks.

Calbindin is a vitamin D-induced calcium-binding protein that plays a key role in intestinal intracellular calcium transport. The expression of calbindin in the intestine and kidney has been shown to be regulated by $1,25(OH)_2D_3$ in rats and chickens (Brehier and Thomasset, 1990; Hall and Norman, 1990; Ferrari et al., 1992). Supplementation of $1,25(OH)_2D_3$ could induce calbindin mRNA expression in the duodenum of 1α-hydroxylase knockout mice (Hoenderop et al., 2004). Here, we further demonstrated that vitamin D_3 metabolites are able to induce intestinal calbindin mRNA expression *in vivo*. Particularly, the expression of calbindin peaked at 6 h and declined afterwards, 12 h after $1,25(OH)_2D_3$ treatment. The intestinal epithelial Ca^{2+} channel, TRPV6, is primarily activated by β-glucuronidase. It has been found that β-glucuronidase secretion from

isolated intestinal epithelial cells was remarkably increased by supplementation of $1,25(OH)_2D_3$ (Khanal et al., 2008). The intestinal TRPV6 gene expression was significantly increased after incubation for 6 h with $25(OH)D_3$ or $1,25(OH)_2D_3$ (Balesaria et al., 2009). Supplementation of $1,25(OH)_2D_3$ significantly upregulated the TRPV6 mRNA expression in the duodenum of 1α-hydroxylase knockout mice (Hoenderop et al., 2004). Our findings further suggested that vitamin D_3 metabolites, including $1,25(OH)_2D_3$, transcriptionally regulate β-glucuronidase and TRPV6 mRNA expression *in vivo*. The NaPi-IIb cotransporter is primarily expressed in the brush-border membranes of the small intestinal epithelium, where it is considered the major sodium-Pi cotransporter (Hilfiker et al., 1998). Dietary supplementation of $1,25(OH)_2D_3$ significantly regulates the expression of NaPi-IIb cotransporter in the intestine of rats (Katai et al., 1999). In broilers, $1α(OH)D_3$ also has a similar effect on regulation of intestinal NaPi-IIb cotransporter gene expression (Han et al., 2009). Here, we also demonstrated that vitamin D_3 metabolites could rapidly induce the levels of NaPi-IIb cotransporter mRNA in the intestine of broiler chickens. Several genes associated with calcium homeostasis have been shown to be regulated by VDR, such as calbindin and TRPV6 (Bolt et al., 2005; Meyer et al., 2006). Since dietary $1,25(OH)_2D_3$ could be absorbed and bound to intestinal VDR and then regulate its downstream gene transcriptionally, we speculate that intestinal gene expression may be directly regulated by VDR in response to vitamin D_3 metabolites.

The 24-hydroxylase belongs to cytochrome P450-containing enzyme (CYP24) that is important in the regulation of vitamin D metabolism (Minghetti and Norman, 1988). The expression of 24-hydroxylase in the kidney has been demonstrated to be regulated by $1,25(OH)_2D_3$ and to initiate the inactivation of $1,25(OH)_2D_3$ (Armbrecht et al., 1997). Interestingly, we found that intestinal 24-hydroxylase mRNA expression was reduced by dietary supplementation of $25(OH)D_3$ in broiler chickens. However, $1α(OH)D_3$ and $1,25(OH)_2D_3$ did not cause a significant effect on intestinal 24-hydroxylase mRNA expression. The metabolite $1,25(OH)_2D_3$ plays a central role in regulating mineral metabolism through direct interaction with intracellular VDR in intestinal and kidney epithelial cells (Haussler et al., 1998). It has been reported that vitamin D analogs auto-regulate the expression of the VDR gene expression in cultured kidney cells (Costa et al., 1985; Santiso-Mere et al., 1993). In contrast, intestinal VDR gene expression was not affected by $25(OH)D_3$ or $1,25(OH)_2D_3$ in human cells (Balesaria et al., 2009). Similarly, we also found that vitamin D_3 metabolites did not have an effect on the

regulation of VDR gene expression in broiler chickens. How vitamin D$_3$ metabolites differently regulate intestinal calcium homeostasis-associated gene expression in broiler chickens remains to be investigated in the further studies.

Conclusions

Calbindin, β-glucuronidase, TRPV6, and NaPi-IIb cotransporter gene expression is rapidly responsive to dietary vitamin D$_3$ metabolites in the duodenum of broilers. Among vitamin D metabolites, 1,25(OH)$_2$D$_3$ is the most effective at regulating calcium homeostasis-associated genes in broilers.

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