Effects of Dietary Vitamin D₃ Supplementation on AvBD-1 and chCATH-1 Genes Expression in Chicken

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In addition to the important role in maintaining calcium homeostasis, vitamin D₃ (VD₃) has also been suggested to participate in the regulation of innate and adaptive immune responses in human by enhancing antimicrobial peptides expression. In this study, we investigated the effect of supplementation of 0, 800, 1,600, 3,200 and 6,400 IU/kg of VD₃ in the basal diet on the mRNA expression of antimicrobial peptides (AvBD-1 and chCATH-1) in chicken by using quantitative real-time RT-PCR. The supplementation of VD₃ in the basal diet could significantly promote the mRNA expression of both AvBD-1 and chCATH-1 genes (P<0.01), which showed dose-dependent response from 800 to 3,200 IU/kg supplementation dose. Compared with control group (0IU/kg), 11-fold to 39-fold and 4.7-fold to 25-fold of mRNA expression difference in treatment groups were detected for AvBD-1 and chCATH-1 genes in bursa of Fabricius, respectively. In addition, VD₃ also could significantly enhance the AvBD-1 and chCATH-1 mRNA expression in thymus (P<0.05). Our results indicated that the VD₃ supplementation of 3,200 IU/kg dose in basal diet could significantly promote the AvBD-1 and chCATH-1 mRNA expression in bursa of Fabricius, which is potential to improve the innate ability to resist diseases in chicken.

Key words: chCATH-1, chicken, defensin, mRNA expression, vitamin D₃


Introduction

The sustainable development of modern poultry industry has been largely hindered by the high prevalence and high incidence of viral and bacterial diseases especially in developing countries. Compared with the intensive antibiotic treatment, it is urgent to develop one safer tactics to effectively improve the innate ability to resist diseases in farm animals. The antimicrobial peptides (AMPs) are an evolutionarily conserved component of the innate immune response and are found among all classes of life (Ganz, 2003; Bulet et al., 2004). Both exogenous and endogenous AMPs are well known as front-line participates in microbial defense (Wang et al., 2006). Hence, we could promote the disease resistance ability via enhancing the endogenous AMPs expression in domesticated chicken, which would significantly reduce the abuse of antibiotics (Yeaman and Yount, 2003).

There are two main families of AMPs, defensin and cathelicidin. To date more than 14 members (AvBD-1 to -14) have been identified in β-defensin, the only subfamily of defensin in chicken (Lynn et al., 2007). As well, there are at least four members in chicken cathelicidin, named chCATH-1 (fowlicidin-1), chCATH-2 (fowlicidin-2), chCATH-3 (fowlicidin-3) and chCATH-B1 (Xiao et al., 2006; Goitsuka et al., 2007). Among them, the expression of AvBD-1 and chCATH-1 in bursa of Fabricius and thymus have been profoundly proposed to display a wide range of microbicidal or microbistatic activities against Gram-negative and Gram-positive bacteria in chicken (Lynn et al., 2004; Xiao et al., 2006; van Dijk et al., 2008).

Vitamin D₃ (VD₃) is well known for the physiological role in regulating calcium homeostasis (Norman, 1987). However, VD₃ has also been proposed to significantly participate in the regulation of innate and adaptive immune responses in human (Adams and Hewison, 2008). The hormonal form of VD₃, 1,25-dihydroxyvitamin D₃ (1,25 (OH)₂D₃), could induce the cathelicidin antimicrobial peptide (CAMP) mRNA expression in human keratinocytes (Wang et al., 2004), myeloid cell line (Gombart et al., 2005), keratinocytes (Weber et al., 2005), and bronchial epithelial cells (Yim et al., 2007). VD₃ is commonly acknowledged as a basic nutritional component in chicken diet and would play important role in immune response (Aslam et al., 1998). However, the potential molecular mechanism of VD₃ to regulate the innate and adaptive immune response has remained unknown in chicken. In
this study, we investigated the effect of long-time supplementation of VD₃ in chicken basal diet on AvBD-1 and chCATH-1 mRNA expression, which would be significant to deduce its biological roles and the optimal supplementation dose.

**Materials and Methods**

**Animals**

Two hundred one-day-old and healthy Sichuan Mountainous Silkie were purchased from commercial company. We determined the sex by using anal swelling method for each bird and kept the male: female = 1:1 in this sample set. Feed and spring water was available *ad libitum* during the experiment. The basal diet did not contain antibiotics (Table 1). Normal hygienic measures were conducted to avoid pathogens contamination. In briefly, commercial vaccination for Marek’s disease was given at one-day-old via the intranasal routes. The chicken were vaccinated with Newcastle disease vaccine via the intracoecal routes at 7-day-old and twenty-day-old, respectively.

**Experimental Design**

After a 7-day preliminary experiment, in order to keep all the tested individuals with similar body weight, 50 birds were excluded because of the significant lower or higher body weight. Total 150 Sichuan Mountainous Silkie were finally included in the feeding experiments and randomly allocated to five treatments with obvious difference of sex ratio. The five feeding regimens were: basal diet without supplement of VD₃ (Sigma, USA) designed for control group, and basal diet added with 800 IU/kg, 1600 IU/kg, 3200 IU/kg and 6400 IU/kg VD₃ were defined as treatment groups, respectively. Body weight (BW) was measured individually at 8-day-old and 35-day-old, and feed consumption per group was measured weekly throughout the experiment. The average daily gain (ADG) and average daily feed intake (ADFI) were calculated.

At the end of the experiment (35-day-old), three male and three female individuals randomly selected from each treatment groups were euthanized. All chicken’s bursa of Fabricius and thymus gland were weighed, and calculated the bursa of Fabricius index and thymus index. The thymus gland and bursa of Fabricius were aseptically sampled and frozen immediately in liquid nitrogen. All the samples were taken within 20 min for RNA isolation, and stored at −80°C.

**Total RNA Extraction**

Total RNA was prepared from the frozen tissue samples using RNAiso Pure RNA Isolation Kit (TaKaRa, Dalian, China) according to the manufacturer’s protocol for all samples. The purified RNA was examined by 1% agarose gel electrophoresis and quantified using Agilent 2100 bioanalyzer (Agilent, USA).

**QRT-PCR**

Relative quantification analysis based on real-time RT-PCR (QRT-PCR) method must be normalized by including the reference gene to eliminate unspecific variation, which is always caused by sample preparation. Housekeeping genes, such as β-actin and GAPDH, are widely employed as endogenous references. However, Selvey and colleagues (2001) also reported that the expression level of β-actin gene considerably varied among different tissues and was unsuitable to be singly used as reference gene. In this study, we analyzed the expression distribution of β-actin gene among tissues and among VD₃ treatment groups in advance and found that there was no significant difference (data not shown), which suggested that it was acceptable to employ β-actin as reference gene.

The primers were designed according to the chicken β-actin, AvBD-1 and chCATH-1 genes reference sequences (Table 2). The specific primer sequences were tested by performing a BLAST search against the genomic NCBI database. A two-step reverse transcription PCR method was employed to generate cDNA using SYBR PrimeScript RT-PCR kit (TaKaRa, Dalian, China) according to the manufacturer’s protocol. Real-time fluorescent mensuration was conducted on the qQ5 real-time PCR detection system (Bio-Rad, USA). A 10-fold dilution series of cDNA were included in each run to determine PCR efficiency by constructing a relative standard curve. PCR efficiencies were consistently > 90% and were used to convert the cycle threshold (Ct) values into raw data. All experiments contained a negative control and the samples were analyzed in three independent runs.

### Table 1. Composition of the basal diet

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Composition (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>651.2</td>
</tr>
<tr>
<td>Soybean meal (44% CP)</td>
<td>190.0</td>
</tr>
<tr>
<td>Rapeseed meal</td>
<td>55.0</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>35.0</td>
</tr>
<tr>
<td>Fish meal</td>
<td>45.0</td>
</tr>
<tr>
<td>Salt</td>
<td>3.5</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>13.5</td>
</tr>
<tr>
<td>Limestone</td>
<td>1.6</td>
</tr>
<tr>
<td>L-Lysine · HCl</td>
<td>0.7</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.2</td>
</tr>
<tr>
<td>Choline-Cl</td>
<td>1.3</td>
</tr>
<tr>
<td>Mineral vitamin Premix¹</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Calculated composition

ME², MJ/kg

Crude Protein: 19.9

Calcium: 8.0

Available phosphorus: 5.2

Lysine: 9.8

Methionine: 3.5

¹Supplied per kilogram of diet: Fe (FeSO₄ · 7H₂O), 80 mg; Cu (CuSO₄ · 5H₂O), 8 mg; Mn (MnSO₄ · H₂O), 60 mg; Zn (ZnSO₄ · 7H₂O), 60 mg; I (KI), 0.35 mg; and Se (Na₂SeO₃), 0.15 mg; vitamin A, 4,000 IU; vitamin E, 10IU; vitamin K₃, 0.75 mg; thiamine, 2.0 mg; riboflavin, 5.0 mg; pyridoxine, 3.75 mg; cobalamin, 0.01 mg; niacin, 30 mg; D-pantothenic acid, 10 mg; biotin, 0.15 mg; and folic acid, 0.55 mg.

²ME was the calculated values.
Table 2. Information on the primers used for real-time RT-PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession No.</th>
<th>Primer sequence (5’-3’)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>L08165</td>
<td>F: 5’-GCTCCGCTCGTTGTGAC-3’</td>
<td>258</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5’-GTAAGATGCTGTGAGCATG-3’</td>
<td></td>
</tr>
<tr>
<td>AvBD-1</td>
<td>AF033335</td>
<td>F: 5’-GCGGATCCTGACCTGGCAGG-3’</td>
<td>227</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5’-GGTAGTGTGCTTGCCAGG-3’</td>
<td></td>
</tr>
<tr>
<td>chCATH-1</td>
<td>NM_001001605</td>
<td>F: 5’-CGAGTTCAAGGAGCAG-3’</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5’-GCAGGTGACATCGAGC-3’</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. The BW, ADG, ADFI, bursa of Fabricius index and thymus index of each VD₃ treatment groups in Sichuan Mountainous Silkie

<table>
<thead>
<tr>
<th>Level of VD₃ (IU/kg)</th>
<th>8-day-old BW (g)</th>
<th>35-day-old BW (g)</th>
<th>8–35d ADG (g)</th>
<th>8–35d ADFI (g)</th>
<th>Bursa index</th>
<th>Thymus index</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>57.6±0.33</td>
<td>419.0±24.29</td>
<td>12.9±0.87</td>
<td>26.2±0.71</td>
<td>1.79±0.20</td>
<td>4.80±0.68</td>
</tr>
<tr>
<td>800</td>
<td>57.6±0.38</td>
<td>503.3±26.82</td>
<td>15.9±0.94</td>
<td>27.8±2.30</td>
<td>2.84±0.07</td>
<td>5.03±0.60</td>
</tr>
<tr>
<td>1,600</td>
<td>58.1±0.93</td>
<td>478.9±25.07</td>
<td>15.0±0.86</td>
<td>28.1±1.23</td>
<td>1.78±0.38</td>
<td>5.31±0.80</td>
</tr>
<tr>
<td>3,200</td>
<td>57.7±0.05</td>
<td>412.4±34.31</td>
<td>12.7±1.23</td>
<td>26.8±1.72</td>
<td>1.65±0.11</td>
<td>4.90±0.34</td>
</tr>
<tr>
<td>6,400</td>
<td>57.6±0.43</td>
<td>402.5±25.71</td>
<td>12.3±1.57</td>
<td>26.3±2.07</td>
<td>1.58±0.21</td>
<td>4.73±0.41</td>
</tr>
</tbody>
</table>

* * *

Statistical Analysis

Relative expression was calculated as a ratio between mRNA expression of target genes (the AvBD-1 and chCATH-1) and mRNA expression of the β-actin in the same cDNA sample. The following equation was used (Pfafl, 2001):

\[
\text{ratio} = \frac{(E_{\text{target}}) \Delta C_{\text{target}}}{(E_{\text{ref}}) \Delta C_{\text{ref}}}
\]

where \( E_{\text{target}} \) and \( E_{\text{ref}} \) represent the efficiencies of real-time PCR from the target and reference genes, respectively; \( \Delta C_{\text{target}} \) is the Ct deviation of control-sample of the target gene transcript; \( \Delta C_{\text{ref}} \) means Ct deviation of control-sample of reference gene transcript.

The significance of differences in the mRNA expression of AvBD-1 and chCATH-1 after VD₃ treatment was examined by one-way ANOVA, followed by Duncan’s multiple range tests. Differences with \( P \) value of < 0.05 were considered statistically significant.

Results

The effects of dietary VD₃ supplementation on BW, ADG, ADFI, bursa of Fabricius index and thymus index of Sichuan Mountainous Silkie were listed in Table 3. This experiment showed that there was no significant difference between treatment groups in ADFI (\( P > 0.05 \)). The supplementation of 800 IU/kg to 1,600 IU/kg VD₃ in diet improved the 35-day-old BW and ADG compared with control group (\( P < 0.01 \)), while there were no significant difference between control group and other groups (\( P > 0.05 \)). When compared with control group, the supplementation of 800 IU/kg VD₃ in diet improved the bursa of Fabricius index (\( P < 0.01 \)) but not for thymus index.

Compared with control group, the supplementation of VD₃ in basal diet significantly elevated AvBD-1 mRNA expression in bursa of Fabricius with 11.5-fold (800 IU/kg), 24.1-fold (1,600 IU/kg), 39.1-fold (3,200 IU/kg) and 9.9-fold (6,400 IU/kg), respectively (\( P < 0.01 \)). In contrast, the AvBD-1 mRNA expression in thymus significantly elevated only when supplemented 3,200 and 6,400 IU/kg VD₃ in basal diet with 5.8-fold (\( P < 0.05 \)) and 21.2-fold (\( P < 0.01 \)) compared with control group (Fig. 1a). For the chCATH-1, the mRNA expression in bursa of Fabricius increased with 4.6, 15.5, 25.1 and 5.1-fold in the four treatment groups, respectively. While the chCATH-1 mRNA expression in thymus was significantly elevated when supplemented 1,600 to 6,400 IU/kg VD₃ in basal diet (\( P < 0.05 \)), with highest value occurred in 1,600 IU/kg VD₃ group (Fig. 1b).

Discussion

After the discovery of the vitamin D receptor (VDR) and key vitamin D (VD) metabolizing enzymes expressed by immune cells, the significant role of VD on the immune system regulation has been widely acknowledged, which was recently reviewed in detail (Kamen and Tangpricha, 2010). In the two major physiologically relevant forms of VD (VD₂ and VD₃), VD₃ has been proposed to induce the expression of AMPs in human immune cells with high disease resistance ability (Adams and Hewison, 2008).
farm animals, VD has long been considered as a basic nutritional component in diet with pleiotropic biological functions. The VD$_3$ deficiency in diet could constrain the thymus development and the macrophage function in chicken (Aslam et al., 1998). However, the potential molecular mechanism of VD$_3$ to regulate the innate and adaptive immune response in chicken has remained largely unknown.

In this study, we investigated the effect of long-time supplementation of VD$_3$ in chicken basal diet on AvBD-1 and chCATH-1 mRNA expression, which encode two important components of AMPs. Our results showed that the AvBD-1 and chCATH-1 mRNA expression were significantly enhanced in bursa of Fabricius when the supplementation dose ranged from 800 IU/kg to 6,400 IU/kg of VD$_3$ in basal diet and showed dose-response relationship between 800 IU/kg and 3,200 IU/kg. This observation was consistent with previous reports that VD$_3$ can significantly induce CAMP and β-defensins-2 mRNA expression in human, which are another two components of AMPs (Wang et al., 2004; Liu et al., 2007). The enhanced mRNA expressions of AvBD-1 and chCATH-1 were also observed in another important immune organ of thymus with different dose-response relationship compared with that in bursa of Fabricius. The AvBD-1 and chCATH-1 mRNA expression in bursa of Fabricius were higher than in thymus when subjected to treatments with the same supplementation dose of VD$_3$. However, the excessive supplementation of VD$_3$ above 3,200 IU/kg in diet would show the declined effect on the AvBD-1 and chCATH-1 mRNA expression, although which was also higher than control group. In fact, Yang and colleagues (1993) also reported that the excessive VD$_3$ in diet could constrain immunoglobulin production and thymic lymphocyte proliferation. The results suggested that the supplementation of VD$_3$ in diet could significantly induce the expression of AMPs, which has the potential to improve the innate ability to resist diseases in chicken.

In a large number of 1,25-(OH)$_2$-D$_3$-dependent target genes, distinct vitamin D response elements (VDREs) have been suggested to be essential in response to VD$_3$. In this process, the 1,25-(OH)$_2$-D$_3$ enters the target cell from
the circulation and binds to the vitamin D receptor (VDR) in the cytoplasm, which subsequently enters the nucleus and heterodimerizes with the retinoid X receptor (RXR); the 1,25-(OH)₂-D₃-RXR-VDR complex finally regulates the expression of target genes by binding to VDREs (Wang et al., 2004). In contrast to this classic pathway, some genes with absence of VDREs, such as neutrophil gelatinase-associated lipocalin (NGAL), could also positively respond to stimulation of 1,25-(OH)₂-D₃ (Wang et al., 2004). In this study, we tentatively analyzed the regulation elements in promoter region of AvBD-I and chCATH-I genes based on to the reference sequences deposited in GenBank (data not shown). However, we failed to detect the VDREs in the two genes, which would suggest the regulation of AvBD-I and chCATH-I mRNA expression under VD₃ treatment was also independent of VDREs.

The level of VD₃ in the diet can influence BW and feed conversion in growing broilers, and approximately 1,000 IU/kg was needed to maximize BW of birds fed with VD₃ (Fritts and Waldroup, 2003). In this study, we found that diet devoid of VD₃ affected the BW, ADG and ADFI. The result was consistent with previous studies found that VD-deficient chicks had lower growth rate and bone ash (Aslam et al., 1998).

In conclusion, our results indicated that the level of VD₃ in the diet can influence BW and feed conversion in growing broilers, and approximately 1,000 IU/kg was needed to maximize BW of birds fed with VD₃. Further, we found that diet devoid of VD₃ affected the BW, ADG and ADFI. The result was consistent with previous studies found that VD-deficient chicks had lower growth rate and bone ash (Aslam et al., 1998).

Acknowledgments

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