Journal of Animal and Veterinary Advances 12 (3): 316-322, 2013
ISSN: 1680-5593
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Effects of Dietary Vitamin D_3 on the Apoptosis, Morphological Structure and Antibacterial Peptide *Hepcidin* Gene Expression in the Spleen of *Monopterus albus*

De-Bing Li, Shan-Shan Shao, Gong-Wei Zhang, Ding-Gang Zhou and Xue-Wei Li
College of Animal Science and Technology,
Sichuan Agricultural University, 625014 Ya'an, China

Abstract: To investigate the effects of dietary Vitamin D_3 (VD_3) on the apoptosis, morphological structure and antibacterial peptide *hepcidin* gene expression in the spleen of *Monopterus albus* (*M. albus*), so as to provide theoretical basis for investigating the immunoregulatory functions and mechanisms of VD_3. A total of 540 healthy *M. albus* (weigh, 21.7±2.1 g) were randomly assigned into six groups (3 replicates per group, 30 *M. albus* per replicate) and fed with dietary VD_3 at various concentrations (0 (control), 250, 500, 1000, 2000 and 4000 IU kg^{-1}). The experiment lasted for 60 days. After feeding experiment, the spleen was collected from 6 *M. albus* selected randomly from each group for the detection of apoptosis using FCM and antibacterial peptide *hepcidin* gene using fluorescent quantitative RT-PCR, besides morphological structure was observed using histological method. The Proliferation Index (PI) was significantly increased ($p<0.05$), the apoptotic rate was significantly reduced ($p<0.05$), *hepcidin* gene expression was significantly up-regulated ($p<0.05$), the percentage of white pulp was increased and more splenic corpuscles were clearly visible in the 1000 IU kg^{-1} group compared to the control group. There were no significant differences in *hepcidin* gene expression in the 4000 IU kg^{-1} group and the number of splenic corpuscle was reduced, gradually degenerating and disappearing. The results demonstrate that appropriate dose dietary provision of VD_3 significantly reduced the cell apoptosis rate in the spleen of *M. albus* whereas promoted the growth of peripheral lymphoid organ and significantly increased *hepcidin* gene expression, enhancing immunity. However, when the dietary provision of VD_3 was excessive or deficient, the apoptosis was increased, cell proliferation was blocked, growth of spleen was inhibited, *hepcidin* gene expression was down-regulated and immune function was impaired.

Key words: *Monopterus albus*, vitamin D_3, apoptosis, *hepcidin* gene, morphological structure

INTRODUCTION

Spleen is primary peripheral immune organ in fish, where immune cells differentiate, maturate, settle, proliferate and where the immune response is elicited. Vitamin D_3 is one of the vitamins actively studied currently. Besides its classical regulation of calcium and phosphorus, VD_3 also participates in immune regulation, regulating proliferation and differentiation, improving glucose metabolism, etc. Especially the effect of VD_3 on immune regulation has become a hot research area of immune nutrition, yet the related regulation mechanism is not well known (Li *et al*., 2001; Chen, 2011). Apoptosis is an active process of programmed cell death regulated by gene which plays a crucial role in the normal development and homeostasis of multi-cellular organisms and resisting various interferences external. Studies have confirmed that VDRs are distributed in most immune cells including monocytes and activated lymphocytes which indicates that these cells are also the targets of VD_3 It is known that hundreds of genes regulating cell cycle, proliferation, differentiation and apoptosis directly or indirectly have Vitamin D Responsive Element (VDRE) indicating that VD_3 can also have an important effect on proliferation, differentiation and apoptosis (Nemere and Farach-Carson, 1998; Samuel and Sitrin, 2008).

Fish is vertebrate that has both specific and non-specific immunity. Though, specific immunity of fish is underdeveloped compared to that of mammals, non-specific immunity plays an important role. As a typical molecule of nonspecific immunity in fish, *hepcidin* gene plays an important role in maintaining normal immune function of fish (He *et al*., 2011). Present studies show that *hepcidin* gene expresses stably in the spleen of fish including *Monopterus albus*, largemouth bass and Red Seabream (Chen *et al*., 2005; Bai *et al*., 2008; Li *et al*., 2009).

Corresponding Author: Ding-Gang Zhou, College of Animal Science and Technology, Sichuan Agricultural University, 625014 Ya'an, China

In the animal husbandry, researches about VD lay particular stress on the effect of that on calcium-phosphorus metabolism and growth performance of animals while few researches about VD related to the regulation of immune functions (Li *et al*., 2001) and the effect of VD on immune functions of *M. albus* has not been reported. The study took *Monopterus albus* as research object to investigate the effects of dietary Vitamin D_3 (VD_3) on the apoptosis, morphological structure and antibacterial peptide *hepcidin* gene expression in the spleen of *Monopterus albus*, so as to provide theoretical basis for investigating the immunoregulatory functions and mechanisms of VD_3.

MATERIALS AND METHODS

Experimental diets: The basal diet, prepared according to the feed formula reported by Cheng *et al*. (2009), contained fishmeal and soybean meal as protein sources, fish oil as a fat source and inorganic salts and vitamin (excluding VD_3) as supplements. The composition and nutrition levels of basal diet are shown in Table 1. The six experimental diets were then prepared by the addition of VD_3 (Bayer Animal Health Co., Ltd. Sichuan, China) to the basal diet at various concentrations (0 (control), 250, 500, 1000, 2000 and 4000 IU kg^{-1}). All ingredients were sieved (80-mesh), mixed well, extruded into 2 mm long feed, dried naturally and preserved for future use.

Experimental animals and feeding management: A total of 540 wild and healthy *M. albus* (weigh, 21.7±2.1g) were obtained from Caoba town, Yucheng District, Ya'an, Sichuan. *M. albus* were randomly assigned to the six experimental groups designated (0 (control), 250, 500, 1000, 2000 and 4000 IU kg^{-1}) group with three replicates per group and 30 *M. albus* per replicate. Aquarium (100×60×60 cm) and aerated tab water (height of the water surface was 15-20 cm^2) were used to still-culture *M. albus* in the Fish Physiology Laboratory of Sichuan Agriculture University for 15 days. *M. albus* experiments began after a period of adjustment to the aquarium environment and the experimental diet. The water conditions were as follows: temperature, 20±3°C; pH, 6.8-7.5, dissolved oxygen concentration, >5 mg L^{-1}. *M. albus* were fed with an amount equivalent to 2% of the body weight at 18:00 each day and the feeding amount was adjusted according to their intake conditions to ensure food were consumed within 30 min. Aquarium water (approximately 2/3 of the total volume) was changed at 09:00 each day to allow the removal of contaminants. The experiment lasted for 60 days.

Table 1: Composition and nutrient levels of basal diets (air dry basis) (%)

Items	Contents
Ingredients	
Corn	5.27
α-starch	15.50
Wheat middlings	4.00
Soybean meal	26.00
Fish meal	46.00
Fish oil	2.00
$Ca(H_2PO_4)_2$	0.20
Vitamin premix[1]	0.03
Choline	0.50
Mineral premix[2]	0.50
Total	100.00
Nutrient levels[3]	
CP	42.00
Ca	2.00
P	1.53
Lys	3.07
Met + Cys	1.42

[1]Contained the following per kilogram of the diet; VA: 6000 IU; VE: 50 mg; VK: 5 mg; VB_1: 15 mg; VB_2: 15 mg; VB_3: 25 mg; VB_5: 30 mg; VB_6: 10 mg; VB_7: 0.2 mg; VB_{11}: 3 mg; VB_{12}: 0.03 mg; [2]Contained the following per kilogram of the diet: $Fe(FeSO_4 \cdot 7H_2O)$, 150 mg; $Cu(CuSO_4 \cdot 5H_2O)$, 30 mg; $Mn(MnSO_4 \cdot H_2O)$, 120 mg; $Zn(ZnSO_4 \cdot 7H_2O)$, 150 mg; $Se(NaSeO_3)$, 0.15 mg; I(KI), 0.35 mg. [3]Nutrient levels were calculated value

Sample collection and preparation: After 12 h feeding at 60 days of the experiment, six *M. albus* were randomly selected from each group for tissue (spleen) collection. Tissues were snap-frozen in liquid nitrogen and preserved at -80°C for future use. About 2 g spleen tissue was ground for apoptosis detection according to the method reported by Chen *et al*. (2009). Besides, one or two pieces of spleen, head kidney and hindgut tissues were collected, fixed by 10% neutral formalin, embedded in paraffin, sectioned, stained conventionally with HE and mounted with neutral gum then histologic and morphological structure was observed using light microscope.

Antibacterial peptide *hepcidin* gene expression detection using RT-PCR: According to the *M. albus hepcidin* gene sequence (GenBank: No. GU997139), the primer was designed for *hepcidin* gene as: 5'-CTCCGCCA TTCCATTCAA-3' and 5'-GCTGTAACGCTTCTGTCT-3'. The *HPRT* (GenBank: NO. DQ218476) expression was analyzed as an internal control for reaction efficiency, the primers were 5'-ACTCAAGTGGCGACAATC-3' and 5'-TGGCTCTATCTAAGACAATCAAT-3'. Primers were synthesized using Shanghai Invitrogen Biological Engineering Co., Ltd.

Total RNA were isolated using TRIzol® reagent. Extracted total RNA were detected using the nucleic acid analyzer NanoVue (GE, USA) and the OD260/OD280 value was between 1.82-1.89. The 28S and 18S mRNA showed clear and bright strips with clean edges after 1% agarose gel electrophoresis and the brightness of 28S band approximately twice that of the 18S band which met the

requirement of RT-PCR. The RNA concentration of each sample was then adjusted to approximately 480 ng μL^{-1} and cDNA was synthesized using PrimeScript RT reagent kit according to the instructions (TaKaRa, Dalian, China).

Real-time PCR amplification was performed using the CFX96 PCR System (Bio-Rad, USA) with the synthesized cDNA as templates. The reaction system comprised: 10 μL SYBR Premix Ex Taq II (2×) (TaKaRa, Dalian, China), 0.8 μL upstream and downstream primers (10 μM), 1.6 μL cDNA template and 6.8 μL ddH_2O. The reaction conditions were: pre-denaturation at 95°C for 3 min, denaturation at 95°C for 10 sec, annealing at 57°C for 30 sec, 40 cycles. Dissociation curve temperatures were set between 65-95°C heating rate was set at 0.5°C sec^{-1} and the plate was read once per second. Each sample contained two replicates and ddH_2O was used as a blank control.

Purified and sequenced PCR product was serialy diluted (10 fold) 1:10^5 to construct the standard curve. Gene amplification efficiency was set above 95% (amplification efficiency: HPRT, 97.1%; Hepcidin, 99.8%). The HPRT was set as internal reference and samples were quantified using the Ct value according to the liner calculation formula obtained from the standard curve.

Apoptosis detection of spleen using FCM: About 1 mL cell homogenate prepared was added to propidium iodide dye liquor (5 μL mL^{-1} of propidium iodide, 0.5% Tritonx-100, 0.5 mg of RNAase), stained at normal temperature for about 20 min then centrifuged and washed with PBS one time re-suspended with 1 mL PBS, the cells were detected using FACSCalibur flow cytometry (BD Pharmingen, USA). Then, the results were printed after the analysis with the software modifit and the cell Proliferation Index (PI) was calculated using the following equation (Chen *et al*., 2009):

$$PI\ (\%) = \frac{S+(G_2+M)}{G_0/G_1+S+(G_2+M)} \times 100$$

Where:

S = Cells in synthesis phase of DNA
G_2+M = Cells in mitotic phase
G_0/G_1 = Cells in stationary phase

About 100 μL cell homogenate of spleen in pipe of flow cytometry was mixed with 5 μL annexins V-FITC and 5 μL propidium iodide, standing for 15 min at room temperature away from light then mixed with 400 mL PBS, detected using FACSCalibur FCM and the Apoptotic Index (AI) was obtained after the analysis with CellQuest Software. In the scatter-plot of FCM, the left lower quadrant showed live cells. The right upper quadrant showed unliving cells, namely necrotic cells and non-viable apoptotic cells. The right lower quadrant showed viable apoptotic cells. The left upper quadrant showed mechanical damaged cells:

$$AI\ (\%) = \frac{\text{Cell number in right upper quadrant} + \text{Cell number in right lower quadrant}}{\text{Cell number in the four quadrants}} \times 100$$

Toxicity tests with live bacteria: After formal experiments, 20 *M. albus* selected randomly from each group were injected with *Aeromonas hydrophila*. As standard pathogen, *Aeromonas hydrophila* (*A. hydrophila*, AS1.927) was purchased from Beijing Institute of Microbiology of Chinese Academy of Sciences. Cultured with ordinary broth, *Aeromonas Hydrophila* with stroke-physiological saline solution was prepared into bacterium fluid with the half lethal concentration of 3.18×10^{10} CFU mL^{-1} according to the method reported by He (2010). About 0.3 mL bacterium fluid prepared was injected into the abdominal cavity of each fish. At 6 h after the toxicity test began, clinic symptoms of *M. albus* begun to be observed and the deaths and mortality of *M. albus* were recorded at 1-4 days after the toxicity test. Immune protection rate was calculated according to the equation (Bai *et al*., 2011) as follows:

$$\text{Immune protection rate}\ (\%) = \left(\frac{\text{1-Mortality of test group}}{\text{Mortality of control}}\right) \times 100$$

Statistical analysis: Gene expression data are presented as mean±SD. SPSS 17.0 Software was used for one-way analysis of variance. Significance was defined at $p<0.05$ and extreme significance was defined at $p<0.01$.

RESULTS AND DISCUSSION

Effects of dietary vitamin D_3 on *hepcidin* gene expression in spleen of *M. albus*: The *hepcidin* gene expression level in the spleen of the control group was set as the basal control for all experimental groups and the effects of dietary vitamin D_3 on *hepcidin* gene expression of *M. ablus* were investigated. Hepcidin expression levels in 500, 1000 and 2000 IU kg^{-1} groups were differentially elevated by VD_3 compared to control ($p<0.05$) and that especially in the 500 IU kg^{-1} group (1.81 fold vs. control) and 1000 IU kg^{-1} group (1.94 fold vs. control) were significantly increased (Table 2). There were non-significant differences in the 250 and 4000 IU kg^{-1}

Table 2: Effects of dietary VD_3 on hepcidin gene expression in the spleen of *Monopterus albus* (n = 6)

Added VD_3 levels (IU kg^{-1})					
0	250	500	1000	2000	4000
1.00±0.31^{c}	0.92±0.25^{c}	1.81±0.11^{a}	1.94±0.17^{a}	1.23±0.19^{b}	0.96±0.22^{c}

Different superscript lowercase letters indicate extremely significant differences ($p<0.05$) between data within the same row

Table 3: Effects of dietary VD_3 on the proliferation and apoptosis in the spleen of *Monopterus albus* (n = 6)

	Vitamin D_3 supplemental level/(IU/kg)					
Items	0	250	500	1000	2000	4000
Cell percentage in G_0/G_1 period	89.25±2.71^{a}	87.86±2.02^{a}	86.25±3.21^{c}	85.59±4.38^{c}	86.12±1.41^{c}	87.55±2.41^{ab}
Cell percentage in S period	5.45±0.21^{d}	6.03±0.27^{c}	7.28±0.19^{a}	7.41±0.13^{a}	7.01±0.24^{ab}	6.54±0.12^{c}
Cell percentage in G_2+M period	4.81±0.22^{d}	6.12±0.25^{c}	6.46±0.14^{b}	7.02±0.32^{a}	6.88±0.15^{a}	5.92±0.36^{b}
PI (%)	10.74±0.52^{b}	9.42±0.28^{c}	10.86±0.33^{ab}	11.28±0.25^{a}	10.21±0.32^{b}	9.31±0.17^{c}
AI (%)	9.71±0.42^{a}	7.76±0.32^{b}	6.37±0.13^{cd}	5.27±0.18^{e}	5.59±0.38^{d}	7.84±0.32^{b}

Different superscript lowercase letters indicate significant differences ($p<0.05$) between data within the same column different superscript uppercase letters indicate significant differences ($p<0.05$) between data within the same row

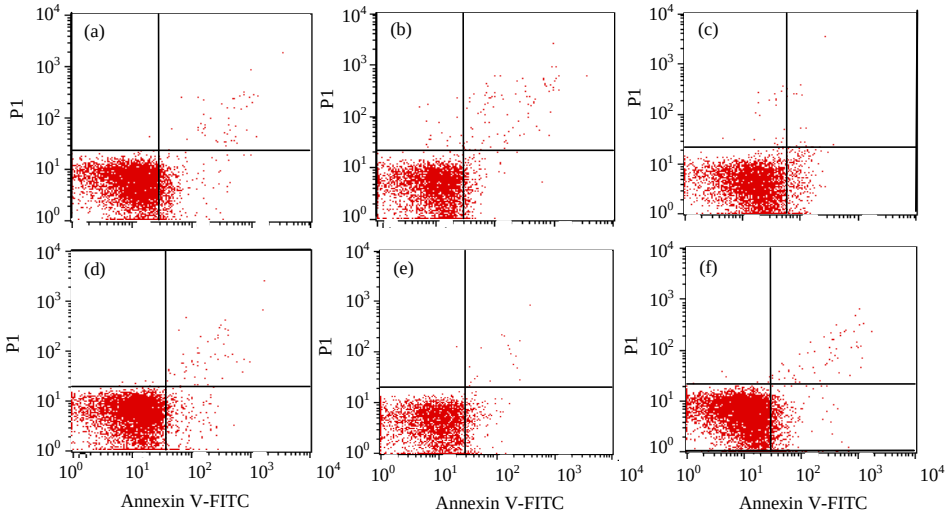

Fig. 1: Scattergram of cell apoptosis in the spleen; compared to the control group, the number of apoptotic cells in the 1000 IU kg^{-1} group was obviously reduced; a) 0 IU kg^{-1} group; b) 250 IU kg^{-1} group; c) 500 IU kg^{-1} group; d) 1000 IU kg^{-1} group; e) 2000 IU kg^{-1} group; f) 4000 IU kg^{-1} group

groups compared to the control ($p>0.05$). On the whole, *hepcidin* gene expression levels were first decreased and then were decreased with the increase of dietary VD_3 levels.

Effects of dietary vitamin D_3 on the cell proliferation and apoptosis in spleen of *M. albus*: The percentage of cells in stationary phase was first decreased and then increased while that in mitosis and proliferative stage was first increased and then decreased with the increase of dietary VD_3 levels (Table 3). PI in the 1000 IU kg^{-1} group was significantly increased ($p<0.05$) while that in the 4000 IU kg^{-1} group was significantly decreased compared to the control ($p<0.05$). AI was first decreased and then increased with the increase of dietary VD_3 levels (Fig. 1). AI in the groups with the supplementation of VD_3 were all significantly lower than that in the control group ($p<0.05$) with the lowest AI in the 1000 IU kg^{-1} group, significantly lower than the other groups ($p<0.05$).

Effects of dietary vitamin D_3 on morphological structure in the spleen of *Monopterus albus*: Normal spleen consists of capsula and splenic pulp. Capsula consists of flattened epithelial tissue and thin layer of connective tissue. Splenic pulp consists of red pulp and white pulp and the percentage of the former is higher than that of the latter. Red pulp consists of splenic cord and splenic sinus. Splenic cord is reticular tissue scaffold matching each other where there are lymphocytes, plasma cells, other types of blood cells and red cells besides. Splenic sinus is lacuna saving blood. White pulp whose shape is irregularly like the structure of lymphoid nodule is formed with the accumulation of very dense lymphocytes. Actually white pulp consists of lymphocytes around artery.

According to the results of *hepcidin* gene expression and the apoptosis in the spleen, the morphological structures of spleen in the 250, 1000 and 4000 IU kg^{-1} groups were observed. In the 250 IU kg^{-1} group the number of splenic corpuscle was few or the volume visible became decreased and lymphocytes were sparse, leading to the red-stained field in vision (Fig. 2a). In the 1000 IU kg^{-1} group most splenic corpuscles were clearly visible, the proportion of white pulp (lymphoid tissue) and red pulp was about 1:1 and different amounts of macrophages were visible where brownish yellow particle sediment was massive. In the 4000 IU kg^{-1} group, the number of splenic corpuscle gradually degenerating and disappearing was decreased and lymphoid tissue with sparse and disordered permutation (Fig. 2c) was loose.

Effects of dietary vitamin D_3 on the mortality and immune protection rate of *M. albus* after the toxicity tests with live bacteria: At 1 day after *Aeromonas hydrophila* was injected into the abdominal cavity of *M. albus*, death broke out in the control group and the 4000 IU kg^{-1} group with seven *M. albus* dead both in the two groups (Table 4). At 4 day, the mortality in the control group was up to 65% and that in the 4 000 IU kg^{-1} group was up to 60%. The mortality was lower and immune protection rate was induced in the other groups with the supplementation of VD_3 compared to the control group especially the effect in the 500 and 1000 IU kg^{-1} groups where the immune protection rates both were up to 62%.

With the discovery of VDR in premyelocyte, researches about proliferation and differentiation have being conducted continuously. The effects of 1, 25 $(OH)_2D_3$ with its analogues on the immunological reaction of normal mice were observed by Yang *et al.* (1993), further more it was found that whether VD_3 was deficient or excessive, the proliferation of thymic lymphocytes was inhibited, the number of thymic lymphocytes was reduced and DNFB stimulation index was decreased, suppressing T cell immune regulation. Results of most researches done through the model where cancer cells were cultured *in vitro* show that active vitamins *in vivo* play a role in inhibiting the proliferation inducing differentiation and apoptosis. Accordingly investigation of active VD_3 has been used to the cancer treatment with some achievements (Deeb *et al.*, 2007; Grant *et al.*, 2007; Perez-Lopez *et al.*, 2012). The study by Cao *et al.* (2007) confirmed that dietary vitamin D_3 significantly increased the number of T cell subset and white blood cells, enhancing immune function of dairy cow in perinatal period besides. It was reported by Zhang and Wang (2010) that indexes of thymus and spleen in broilers were

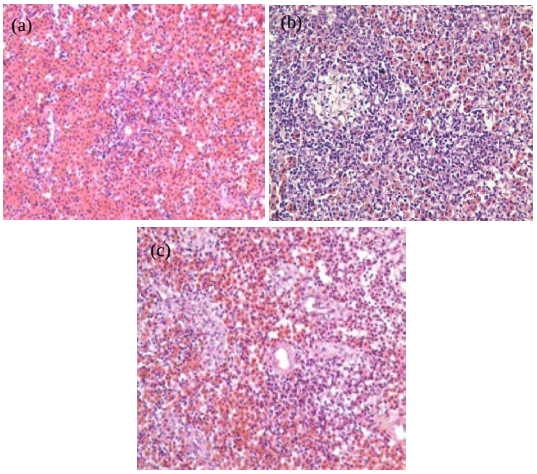

Fig. 2: Effects of dietary VD_3 on the morphologic structure in the spleen of *Monopterus albus*; a) 250 IU kg^{-1} group white pulp reduced and lymphocytes arranged loose. HE x200; b) 1000 IU kg^{-1} group splenic corpuscle could be clearly seen. "a" indicated splenic corpuscle, "b" indicated red pulp. HE x200; c) 4000 IU kg^{-1} group splenic corpuscle dissolved and disappeared. HE x200

Table 4: Effects of dietary VD_3 on the disease resistance of *Monopterus albus* (n = 6)

		Added VD_3 levels (IU kg^{-1})					
Items	Days	0	250	500	1000	2000	4000
Mortality after toxicity test	1	7	5	2	1	4	7
	2	5	3	2	3	3	3
	3	0	2	1	1	1	2
	4	1	1	0	0	0	0
Total mortality	-	13	11	5	5	8	12
Total death rate (%)	-	65	55	25	25	40	60
Immune protection rate (%)	-	-	15	62	62	38	8

increased with the increase of dietary VD_3 level. The study conducted using FCM is the first to reveal that dietary vitamin D_3 levels had significant effect on proliferation and apoptosis in the spleen of *M. albus*. It is embodied in that appropriate VD_3 level (1000 IU kg^{-1}) promoted cell proliferation and reduced cell apoptosis while deficient or excessive VD_3 level inhibited proliferation and increased apoptotic rate.

Though spleen of bony fish has no obvious red pulp and white pulp, it has both hematopoiesis and immune function. White pulp consisting of dense T cells or B cells scatters in spleen parenchyma or distributes itself along central artery structure of splenic corpuscle like that of lymphoid nodule was made of b lymphocyte mostly and macrophage besides (Xie and Yuan, 2010). The results of the study suggest that the number of splenic

corpuscle visible was very small when VD_3 was deficient (250 IU kg^{-1}). In the groups with average VD_3 level (1000 IU kg^{-1}) percentage of white pulp was increased and more splenic corpuscles were clearly visible. In the group with high VD_3 level the number of splenic corpuscle was decreased, degenerating and disappearing. It can be seen that dietary vitamin D_3 levels have a significant effect on the histologic structure of spleen and the main peripheral immune organ of fish which is consistent with the result of apoptosis.

Studies by Zhang *et al.* (2011), Li *et al.* (2009) and Lei (2009) suggested that appropriate VD_3 level could induce the expression of antimicrobial peptide, β-defensin and cathelicidin-1 with improving immune functions and growth performance in chicken. The results of the study suggest that when dietary VD_3 was 500 and 1000IU kg^{-1}, *hepcidin* gene expression was significantly increased, reconfirming the feasibility of enhancing resistance of *M. albus* to pathogenic microorganism with dietary vitamin D_3.

Clinical medical cases of human show that infants with rickets are easily infected in respiratory system indicating that immunity of those patients is lowered Besides many references of epidemiology and clinical data show that when the VD_3 content decreases, humans are easily infected with various chronic and acute infectious diseases while with the supplementation of VD_3 the incidence of those diseases was significantly reduced (Chen, 2011). The above references indicate that the VD_3 level *in vivo* is closely related to the immune function. The study of toxicity test directly showed that appropriate VD_3 increased the resistance to diseases.

According, to the comprehensive analysis of various indexes, it can be showed that with excessive or deficient VD_3 cell proliferation was inhibited, cell apoptosis rate was increased, the number of splenic corpuscle and white cells in spleen of *M. albus* was decreased and antibacterial peptide *hepcidin* gene expression was decreased which may lead to the reduction of immune function.

Thus, VD_3 could regulate immune function by affecting cell proliferation, apoptosis and then by regulating the growth of immune organ and immune related gene.

CONCLUSION

Under the condition of this experiment, dietary VD_3 (1000 IU kg^{-1}) significantly promoted cell proliferation and growth of spleen, reduced apoptosis, up-regulated antibacterial peptide *hepcidin* gene expression and increased immunity. When VD_3 was deficient or excessive (4000 IU kg^{-1}), immune function was reduced.

ACKNOWLEDGEMENTS

This project was sponsored by the Natural Science Fund of Education Department, Sichuan Province (0914ZA146).

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