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Vitamin D_3 modulates yellow catfish (*Pelteobagrus fulvidraco*) immune function *in vivo* and *in vitro* and this involves the vitamin D_3/VDR-type I interferon axis

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ABSTRACT

Vitamin D_3 (VD_3) has been shown to regulate immune function in mammals. 1,25-dihydroxyvitamin ($1,25(OH)_2D_3$) is the active form of vitamin D_3, which is also known as calcitriol. The current study investigated the immunomodulatory effects of $1,25(OH)_2D_3$ on the innate immune response of yellow catfish (*Pelteobagrus fulvidraco*) after *in vivo* and *in vitro* immune challenge. The *in vivo* results showed that increasing dietary vitamin D_3 decreased mortality, enhanced the immune protective rate, and increased serum lysozyme, catalase and SOD activities in yellow catfish infected with *Edwardsiella Ictaluri* ($p < 0.05$). The *in vitro* results showed that $1,25(OH)_2D_3$ (0, 1, 10, 100, 200 pM) dose-dependently attenuated the rate of apoptosis and production or reactive oxygen species and increased the phagocytic activity of head kidney macrophages stimulated with 10 mg/L lipopolysaccharide (LPS) and 100 mg/L of Poly(I:C) ($p < 0.05$). Real-time quantitative PCR results showed that increasing dietary vitamin D_3 content *in vivo* and increasing the level of $1,25(OH)_2D_3$ *in vitro* partially regulated the expression of VD_3/VDR-type I interferon axis genes (*vdr, irf-3, ifn-a, jak1, stat1, ifi56* and *ifp35*) after immune challenge. These results indicated that vitamin D_3 content helped yellow catfish to resist oxidative stress and inflammation caused by immune challenge, and immunomodulation involved the VD_3/VDR-type I interferon action axis.

1. Introduction

Inactive vitamin D is widely found in animals and plants and requires conversion to 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) in animals to be bioactive. Fish cannot synthesize vitamin D and rely entirely on diet to meet their needs (Dusso et al., 2005). Vitamin D_3 (VD_3) in fish can be hydroxylated by the liver and kidney to form $1,25(OH)_2D_3$ (Lock et al., 2010), which is also known as calcitriol. $1,25(OH)_2D_3$ binds to the intracellular vitamin D receptor (nVDR) to exert its biological effects and the activated nVDR heterodimerizes with retinoid X receptor (RXR), which then collectively bind to nVDR-RXR response elements within the target gene promoter to affect target gene expression (Haussler et al., 2013; Lock et al., 2010). The mammalian VDR is present in immune cells such as macrophages (Calton et al., 2015; Harvey et al., 2013), and all immune cells can respond to signaling via the VDR (Han et al., 2016). Vitamin D_3 has been found to regulate the innate and adaptive immune system of mammals (Hewison, 2012; Aranow, 2011). However, there are few reports about vitamin D_3 regulating the fish immune system.

In recent years, studies have found that $1,25(OH)_2D_3$ regulates type I interferon (IFN) production in humans. $1,25(OH)_2D_3$ can increase levels of IFN-β that may inhibit the differentiation of osteoclasts (Sakai et al., 2009), while $1,25(OH)_2D_3$ cannot block the activation of IFN-β in human microvascular endothelial cells (HMEC) induced by lipopolysaccharide (LPS) (Equils et al., 2006). Type I interferons (IFN) are a family of cytokines that facilitate communication between cells to trigger the protective defenses of the immune system that help eradicate pathogens (Stetson and Medzhitov, 2006). During the innate

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immune response to infection by microorganisms such as viruses, bacteria and their products for example, type I IFN (IFN-α/β) increase to help deal with the pathogen challenge (Yanai et al., 2018). Interferon regulatory factor-3 (IRF-3) is an activator of type I IFN (Yanai et al., 2018), and when IFN binds to its homologous receptor, downstream activation of the JAK-STAT signaling pathway occurs inducing a panel of interferon-stimulated genes and antiviral proteins, such as IFN-induced gene 56 (*ifi56*) (Grandvaux et al., 2005) and IFN-induced protein IFP35 (Tan et al., 2008), which result in anti-inflammatory status (Schindler and Plumlee, 2008).

In order to distinguish fish type I IFN from mammalian type I IFN, fish type I IFNs were classified as group I- and II-IFNs according to the number of cysteine residues (Zou et al., 2007). Among them, IFN-a and IFN-d belong to group I, and IFN-b and IFN-c belong to group II (Zou et al., 2014). Previous studies have shown that group I-IFNs are present in almost all teleost fish (Aggad et al., 2009), and the action axis of type I IFN in fish is similar to that of mammals (Gan et al., 2019). Whether $1,25(OH)_2D_3$ affects the type I IFN action axis pathway in fish remains unknown. Thus, in this study, the type I IFN axis regulated by $1,25(OH)_2D_3$, named the VD_3/VDR-type I IFN action axis, was validated by *in vivo* and *in vitro* experiments in yellow catfish (*Pelteobagrus fulvidraco*).

Yellow catfish (*Pelteobagrus fulvidraco*) is one of the cultured fish species with high economic and nutritional value. This species is easily infected by different bacteria and viruses in fish farms. *Edwardsiella Ictaluri* is a gram-negative bacterium that causes hole-in-head disease in yellow catfish, and LPS isolated from Gram-negative bacteria can cause systemic inflammation (Beutler and Rietschel, 2003). The current study investigated whether dietary vitamin D_3 and $1,25(OH)_2D_3$ pre-incubation could regulate the immune response *in vivo* after yellow catfish were infected by *E. Ictaluri*, and *in vitro* after head kidney macrophages were challenged with either LPS, or polyinosinic:polycytidylic acid (Poly(I:C)) as an immune-stimulating adjuvant that mimics viral double-stranded RNA (Peine et al., 2013), and whether these immune regulation effects were exerted through the VD_3/VDR-type I IFN axis.

2. Materials and methods

2.1. In vivo experiment

2.1.1. Growth trial

One thousand yellow catfish were purchased from Hubei Zhenghao Fish Fry farm and acclimated for 15 days, during which they were fed a VD_3-free diet. Before the growth trial, fish feeding was terminated for 24 h, and they were randomly weighed into the experimental tanks. The formulas for five different experimental diets are shown in Table 1. Diet I was set as the control group, in which the content of vitamin D_3 met the minimum requirement for yellow catfish based on the growth performance in our previous study (Zhu et al., 2014). Each dietary treatment consisted of four replicate tanks and each tank held 40 fish (initial body weight: 5.0 ± 0.2 g). The growth trial lasted for 12 weeks and fish were fed daily at 9:00 and 15:00 h. During the growth trial, water temperature was 16.0–26.0 °C with a flow velocity of 1.5 L/min, the concentration of dissolved oxygen was > 6.0 mg/L and ammonia-nitrogen was < 0.5 mg/L, and the pH was about 8.0. The growth trial was completed at the College of Fisheries, Huazhong Agricultural University (HZAU) aquaculture center and the protocol of *in vivo* experiment was approved by Animal Use Committee of Huazhong Agricultural University.

2.1.2. E. Ictaluri challenge

After the growth trial, 50 yellow catfish were randomly selected from each experimental diet group and injected with *E. Ictaluri* (kindly provided by Animal Medical Department of Fisheries College at HZAU) that was cultured in BHI medium. Diet I group was set as the positive control (Ctrl+). The bacterial solution was diluted to sublethal concentration (LC50 = 3.14×10^8 CFU/mL) with a sterile saline solution, and each fish was intraperitoneally injected with 0.1 mL of diluted bacterial solution. At the same time, another 50 yellow catfish were taken from the Diet I group without *E. Ictaluri* challenge as the negative control (Ctrl-). During the pathogen challenge, feeding was stopped, and water was oxygenated but not circulated. Mortality was measured every 24 h until 72 h post challenge (hpc). Blood, head kidney, spleen, liver and intestine tissues from six alive yellow catfish from each diet treatment group after *E. Ictaluri* challenge were collected at 72 hpc. These samples were snap frozen in liquid nitrogen and then stored at −80 °C for gene expression studies.

Table 1
Formulation and proximate composition of experimental diets.

Ingredients	I	II	III	IV	V
Ingredients (% dry matter)					
Blood meal	10	10	10	10	10
Skim milk powder	5	5	5	5	5
Corn gluten meal	2	2	2	2	2
Wheat flour	10	10	10	10	10
Casein	30	30	30	30	30
Soybean meal	10	10	10	10	10
Canola meal	10	10	10	10	10
Soybean oil	10	10	10	10	10
Lysine	1	1	1	1	1
Methionine	0.1	0.1	0.1	0.1	0.1
Choline Chloride	0.5	0.5	0.5	0.5	0.5
Vitamin C	0.5	0.5	0.5	0.5	0.5
Yi_2O_3	0.1	0.1	0.1	0.1	0.1
Antioxidant	0.02	0.02	0.02	0.02	0.02
Mineral premix [a]	2	2	2	2	2
Vitamin D-free Vitamin premix [b]	0.5	0.5	0.5	0.5	0.5
Vitamin D_3 premix [c] (IU/kg)	1000	2000	4000	8000	16000
Bran	8.28	8.28	8.28	8.28	8.28
Proximate composition (% dry matter)					
Moisture	5.45	6.00	5.23	5.52	5.55
Crude protein	47.60	47.25	46.44	46.96	46.12
Crude lipid	4.53	4.57	4.56	4.40	4.48
Ash	3.30	3.45	3.47	3.36	3.37
Analytic Dietary Vitamin D_3 (IU/kg)	1120	2260	3950	8030	16600

[a] Mineral premix (mg per kg diet):NaCl 198 mg; $MgSO_4{\cdot}7H_2O$ 2995 mg; Na_2SO_4 4560 mg; K_2SO_4 4098.5 mg; $CaCl_2{\cdot}2H_2O$ 2340 mg; $FeSO_4$ 499.5 mg; Calcium lactate 700.5 mg; $ZnSO_4{\cdot}7H_2O$ 70.6 mg; $MnSO_4{\cdot}4H_2O$ 32.5 mg; $CuSO_4{\cdot}5H_2O$ 6.2 mg; $CoSO_4$ 0.2 mg; KI 0.6 mg; bran 4498.4 mg.

[b] The Vitamin D-free Vitamin premix (per kg diet): was specially made by Nutriera. It contained: vitamin A 2750 IU; Vitamin E 25 IU; Vitamin K_3 15 mg; Vitamin B_1 20 mg; Vitamin B_6 20 mg; Vitamin B_{12} 20 mg; niacin 50 mg; riboflavin 10 mg; D-calcium pantothenate 25 mg; biotin 5 mg; inositol 100 mg; ascorbic acid 500 mg; bran 1757.43 mg.

[c] Vitamin D_3 premix: Coated vitamin D_3 (500000 IU/kg) was provided by Nutriera.

2.1.3. Assessment of serum enzyme activity and lipid peroxidation

The above blood samples were centrifuged at 3500 rpm for 5 min at 4 °C to collect the serum. Analytical kits purchased from the Nanjing Jiancheng Bioengineering Institute (China) were used to measure the activity of catalase (http://www.njjcbio.com/product.asp?commend=1&q=A007-1&sort=hitsorder), superoxide dismutase (SOD) (http://www.njjcbio.com/product.asp?commend=1&q=A001-1&sort=hitsorder), lysozyme (http://www.njjcbio.com/product.asp?commend=1&q=A050&sort=hitsorder), and lipid peroxidation (malondialdehyde, MDA) (http://www.njjcbio.com/product.asp?commend=1&q=A003-1&sort=hitsorder) according to the manuals from the kits.

2.2. In vitro experiments

2.2.1. Experimental animals

Yellow catfish (approximate length of 10 cm and weight of 22.34 ± 1.8 g) were provided by the Fish Breeding Laboratory of the

College of Fisheries, HZAU. Fish were fed with commercial feed, and culture conditions were previously described in section 2.1.1. The fish were acclimated for 15 days prior to the experiments. The protocol of *in vitro* experiment was approved by Animal Use Committee of Huazhong Agricultural University.

2.2.2. Isolation and culture of macrophages with $1,25(OH)_2D_3$

Isolation and culture of macrophages were performed as previously described (Shim et al., 2002). Briefly, 36 healthy yellow catfish were randomly divided into 12 groups, with six groups for subsequent LPS and Poly(I:C) immune challenge, respectively. Fish were anaesthetized and head kidneys were dissected on ice. The samples of head kidney were soaked in autoinduction medium (AIM) for 20 min on ice and then placed in a disposable cell sieve (100 μm) with addition of 4 mL L-15 medium (Gibco, USA). The samples were mushed into cell suspensions using the blunt end of a 2.5 mL syringe and collected in a petri dish. Percoll cell separation solution (4 mL of 34%, GE, Hangzhou, China) was added in a 15 mL centrifuge tube, and then 4 mL of 51% Percoll cell separation solution was slowly added from the bottom of the same tube with a long syringe needle. In order to demarcate the layer between the two densities of Percoll solutions, 100 μL of 1M HCl was added per 50 mL of 51% Percoll solution before it was added to the above-mentioned tube. The head kidney cells were slowly added over the Percoll gradient and then the tube was centrifuged at 4 °C, 400 × *g* for 30 min (Laborzentrifugen, Sigma, USA). The white blood cell mixture between 34% and 51% Percoll cell separation solutions was aspirated with a pipette and washed with L-15 medium. The isolated cell mixture was cultured for 24 h in L-15 medium containing 5% fetal bovine serum (FBS, Si Jiqing, Hangzhou, China), 100 U/mL anti-penicillin and 100 μg/mL anti-streptomycin (Gibco, USA) at 28 °C and 5% CO_2, after which non-adhesive cells were removed and the adherent macrophages were retained for further use.

The isolated macrophages in 24-well plates were cultured with $1,25(OH)_2D_3$ (Selleck, USA) for 72 h, and then challenged for 6 h with 10 mg/L LPS (from *Escherichia coli*, Sigma, USA), or 100 mg/L Poly(I:C) (Sigma, USA) at 28 °C with 5% CO_2. There were six treatment groups for LPS and each treatment had three replicates: Ctrl + Ctrl ($1,25(OH)_2D_3$ and LPS free), Ctrl + LPS ($1,25(OH)_2D_3$ free + LPS), 1pM $1,25(OH)_2D_3$ + LPS, 10 pM $1,25(OH)_2D_3$ + LPS, 100 pM $1,25(OH)_2D_3$ + LPS and 200 pM $1,25(OH)_2D_3$ + LPS. There were also six treatment groups for Poly(I:C) and each treatment had three replicates: Ctrl + Ctrl ($1,25(OH)_2D_3$ and Poly(I:C) free), Ctrl + Poly(I:C) ($1,25(OH)_2D_3$ free + Poly(I:C)), 1 pM $1,25(OH)_2D_3$ + Poly(I:C), 10 pM $1,25(OH)_2D_3$ + Poly(I:C), 100 pM $1,25(OH)_2D_3$ + Poly(I:C) and 200 pM $1,25(OH)_2D_3$ + Poly(I:C).

All the experiments with macrophage culture were repeated twice.

2.2.3. Detection of macrophage apoptosis

After pathogen challenge for 6h, apoptosis of macrophages was detected by Annexin V-FITC/PI kit (http://www.njjcbio.com/product.asp?commend=1&q=G003&sort=hitsorder) (Jiancheng Bioengineering Institute, Nanjing, China). Control and immune-challenged macrophages were detected using EDTA-free trypsin and concentrated by centrifuged at 250 × *g* for 5 min. The pelleted cells were resuspended in 500 μL of binding solution, mixed with 5 μL Annexin V and 5 μL PI, and incubated at room temperature for 15 min in the dark. The detection of macrophage apoptosis was performed by flow cytometry (CytoFLEX S, Xitogen Biotechnology, China).

2.2.4. Detection of macrophage phagocytic activity

Analysis of macrophage phagocytic activity was carried out following pathogen challenge using the BSA-FITC kit (http://www.solarbio.com) (Solarbio, Beijing, China). After LPS and Poly(I:C) challenge, 1 μL of BSA-FITC was added to each well and mixed with the medium, incubated for 3 h in the incubator. Macrophages were then collected with EDTA-free trypsin. After digestion, the incubation mixture was washed three times with 500 μL PBS and resuspended in 500 μL of PBS. The phagocytic activity of the macrophages was then analyzed by flow cytometry (CytoFLEX S, Xitogen Biotechnology, China) based on fluorescence intensity of macrophages labeled by BSA-FITC.

2.2.5. Production of reactive oxygen species (ROS) by macrophages

The DCFH-DA kit (http://www.fanbobiochemicals.com.cn/product_view.asp?id=77) (Fanbo Biochemical, Beijing, China) was used to detect the macrophage production of ROS after pathogen challenge. Macrophages were collected with EDTA-free trypsin. After digestion, the incubation mixture was washed three times with 500 μL PBS, resuspended in 500 μL PBS and then mixed with 1 μL of DCFH-DA at room temperature under dark conditions for 15 min. ROS production by macrophages was then analyzed by flow cytometry (CytoFLEX S, Xitogen Biotechnology, China) based on the fluorescence intensity of the active oxygen fluorescent probe DCFH-DA.

2.3. Total RNA extraction and gene expression analysis by real-time quantitative PCR (qPCR)

Total RNA was extracted from the collected tissues as described under 2.1.2. and from the cultured macrophages as described under 2.2.2 by TRIzol (Invitrogen, USA). Prior to RNA extraction, 1 mL TRIzol reagent was added to the tube containing tissue and fully ground in the homogenizer. Meanwhile, added 1 mL TRIzol reagent to each cell culture well to completely lyse macrophages. Concentration and integrity of total RNA were quantified respectively by Nanodrop spectrophotometer and agarose gel electrophoresis. The ratio of OD_{260}/OD_{280} from 1.9 to 2.1 was chosen for synthetic cDNA synthesis using the Prime Script™ RT reagent kit gDNA Eraser (Perfect Real Time) (TaKaRa, Dalian, China). Primers for the genes *vdr, irf-3, ifn-a, jak1, stat1, ifi56* and *ifp35* were designed using the Primer Premier software according to *Pelteobagrus fulvidraco* sequences (Table 2), with *β-actin* being used as a house-keeping gene. The qPCR mixtures included 2 μL of cDNA, 0.4 μL of each forward and reverse primers from 10 μM stocks, 7.2 μL of RNase-free dH_2O and 10 μL of Hieff ™ qPCR SYBR Green Master Mix (Yeasen, Shanghai, China). The qPCR cycle conditions were: 95 °C for 5 min for pre-denaturation, 40 cycles of denaturation at 95 °C for 10 s, annealing at different temperatures (Table 2) for 20 s, and extending at 72 °C for 20 s in the qPCR instrument QuantStudio™ 7 Flex Real-Time PCR System (ThermoFisher Scientific, USA). The results were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 2
Primers and annealing temperature used for in real-time qPCR.

Name	Sequence (5′-3′)	Annealing temperature (°C)
vdr,qPCR forward	GATGAGGCGAAGAAGGAAG	60.56
vdr,qPCR reverse	CAGACAGTGAGTGTAGAGAC	61.07
irf-3,qPCR forward	ATCAACAGACTGCCACTCAACCAC	60.45
irf-3,qPCR reverse	CGTTCTCCACCAACTGCTCCATC	60.24
ifn-a,qPCR forward	AGGAGGAATACAAGAGGCGACAGG	60.78
ifn-a,qPCR reverse	CACATGACGGAGGTTGAGCAGATG	60.28
jak1,qPCR forward	GCACGCTGGTGATTCCTGACTG	61.51
jak1,qPCR reverse	CTCGCACTATGGCTCTGAAGAACG	60.15
stat1,qPCR forward	GCAGTTCTCCTCCATCACCAAGC	60.89
stat1,qPCR reverse	CCAACACTCCTTCGATCCACAACC	60.49
ifi56,qPCR forward	GGCACAGCTAAGGAGTGTCATGG	60.66
ifi56,qPCR reverse	GGCCAGCTCAATCACAGACAGAG	60.61
ifp35,qPCR forward	TCCGAGGCTGGAGGAACAAGG	62.32
ifp35,qPCR reverse	GCACCAGTCGCTTCGCAGATAC	61.41
β-actin,qPCR forward	GGATTCGCTGGAGATGATG	54.96
β-actin,qPCR reverse	TCGTTGTAGAAGGTGTGATG	53.23

2.4. Calculations and statistical analysis

Weight gain (WG) was calculated as: WG (%) = 100*(FBW-IBW) / IBW

where FBW represents the final body weight, and IBW represents the initial body weight.

Immune protective rate (%) = 100*[1-(EG $_{\text{Cumulative mortality}}$ − PCG $_{\text{Cumulative mortality}}$)]

where EG represents the experimental group, and PCG represents the positive control group.

Raw data were analyzed by one-way ANOVA using the SPSS 20.0 software after normality and homogeneity of variance was confirmed. Multiple comparisons were conducted with Tukey's multiple-range tests. Data were expressed as mean ± SE and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Growth, pathogen resistance and antioxidant ability in the growth trial

As shown in Fig. 1, the weight gains of the yellow catfish were significantly different between the VD_3 16600 IU/kg and VD_3 2260 IU/kg treatment groups ($p < 0.05$), but there were no significant difference among other treatment groups ($p > 0.05$).

Resistance to *E. Ictaluri* challenge is shown in Table 3. No mortalities were found in non-challenged fish in the control group during the challenge period. After challenge, the cumulative mortality of yellow catfish decreased and immune protective rate increased with the increasing concentration of dietary VD_3.

Serum antioxidant indicators after challenge with *E. Ictaluri* were shown in Table 4. The activities of catalase and lysozyme increased with increasing level of dietary VD_3 ($p < 0.05$), and lysozyme activity peaked at the 8030 IU/kg VD_3 diet group. The SOD content decreased in the 2260 IU/kg VD_3 diet group ($p < 0.05$), but increased from 2260 IU/kg to 16600 IU/kg VD_3 diet group as the VD_3 level increased. MDA content had no significant correlation with dietary vitamin D_3 content ($p > 0.05$).

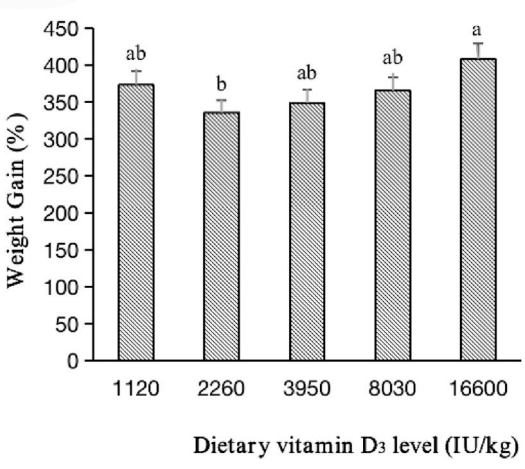

Fig. 1. Weight gain of yellow catfish fed experimental diets containing different vitamin D_3 concentrations. Yellow catfish (initial body weight: 5.0 ± 0.2 g) were weighed after 12 weeks of feeding with diet containing 1120, 2260, 3950, 8030, 16600 IU/kg vitamin D_3 (n = 160/treatment). Error bars represent the 95% confidence intervals and means with different superscript letters are significantly different ($p < 0.05$).

Table 3
Effect of different dietary vitamin D_3 levels on survival of juvenile yellow catfish in response to *E. Ictaluri* challenge.

	Time (hpc)	Ctrl- [a]	Dietary vitamin D_3 level (IU/kg)				
			Ctrl + [b]	2260	3950	8030	16600
Initial fish number for challenge	0	50	55	52	48	50	52
Death number after challenge	24	0	12	10	9	8	9
	48	0	5	5	5	4	3
	72	0	2	2	1	2	2
Cumulative death number		0	19	17	15	14	14
Cumulative mortality (%)		0	34.55	32.69	31.25	28.00	26.92
Immune protective rate (%)				5.38	9.55	18.96	22.08

[a] Ctrl-: negative control, 1120 IU/kg Vitamin D_3 + E. *Ictaluri* free.
[b] Ctrl +: positive control, 1120 IU/kg Vitamin D_3 + E. *Ictaluri*.

3.2. Effect of $1,25(OH)_2D_3$ on macrophage apoptosis

The apoptosis scatter plot (Fig. 2) was obtained by Annexin V-FITC/PI double standard detection. Compared with the Ctrl + Ctrl, the apoptosis rate increased in Ctrl + LPS ($p < 0.05$). As compared to the Ctrl + LPS, apoptosis of macrophages was significantly reduced with increasing content of $1,25(OH)_2D_3$ ($p < 0.01$, Fig. 2A). Similar results were found in macrophages stimulated with Poly(I:C) that apoptosis of macrophages significantly increased after challenged ($p < 0.05$). However, macrophages pre-incubated with $1,25(OH)_2D_3$ decreased the apoptosis in a dose-response manner ($p < 0.05$) (Fig. 2B).

3.3. Effect of $1,25(OH)_2D_3$ on phagocytic activity of macrophages

LPS significantly induced macrophage phagocytosis of BSA-FITC ($p < 0.01$). The phagocytic activity in the $1,25(OH)_2D_3$ treatment groups was higher than that in the Ctrl + LPS, and peaked at 100 pM-$1,25(OH)_2D_3$-LPS ($p < 0.01$) (Fig. 3A). Similar to the LPS treatment group, Poly(I:C) stimulated macrophage phagocytosis of BSA-FITC ($p < 0.01$). Compared with Ctrl + Poly(I:C), the $1,25(OH)_2D_3$ at concentrations between 1 pM and 100 pM significantly enhanced the phagocytic activity of macrophages ($p < 0.01$), which peaked at 10 pM-$1,25(OH)_2D_3$ + Poly(I:C) ($p < 0.01$) (Fig. 3B).

3.4. Effect of $1,25(OH)_2D_3$ on macrophage ROS production

LPS and Poly(I:C) stimulated the production of ROS in macrophages ($p < 0.05$), while $1,25(OH)_2D_3$ dose-dependently attenuated the production of ROS induced by these two immune stimulants. The lowest ROS level was found in the 200 pM-$1,25(OH)_2D_3$ group in both LPS and Poly(I:C) treatments (Fig. 4).

3.5. Effect of vitamin D_3 on expression of type I interferon-related axis genes

3.5.1. In vivo experiment

The expression of key genes in the type I IFN-related axis were measured in the head kidney, spleen, liver, and small intestine of the fish infected by *E. Ictaluri* (Fig. 5). In the head kidney, the expression of *irf3*, *ifi56* and *ifp35* increased with the increasing of dietary VD_3 content, peaked at VD_3 3950 IU/kg, and subsequently decreased at higher VD_3 levels. The expression of *vdr* peaked at 2260 IU/kg VD_3, and then decreased at higher VD_3 levels. The expression of *ifn-a* increased until it peaked at VD_3 8030 IU/kg and then decreased at higher VD_3 levels. The highest expression of *jak1* occurred at VD_3 16600 IU/kg. The expression of *stat1* initially decreased, then peaked at VD_3 3950 IU/kg, and subsequently decreased to a stable level.

Table 4
Effect of dietary vitamin D_3 levels on serum antioxidant indicators of juvenile yellow catfish after *E. Ictaluri* challenge.

Dietary vitamin D_3 level (IU/kg)	1120	2260	3950	8030	16600
Catalase (U/ml)	97.56 ± 3.50[a]	125.89 ± 4.85[b]	133.02 ± 2.73[b]	138.71 ± 7.07[b]	146.08 ± 8.28[b]
Superoxide Dismutase(SOD)(U/ml)	103.49 ± 2.55[a]	86.89 ± 3.21[b]	90.76 ± 3.05 [ab]	101.33 ± 2.75[a]	98.03 ± 3.39 [ab]
Malondialdehyde (MDA)(nmol/ml)	6.54 ± 0.32	7.61 ± 0.37	6.36 ± 0.38	7.75 ± 0.49	6.82 ± 0.28
Lysozyme (U/ml)	262.82 ± 9.22[a]	276.92 ± 7.55 [ab]	278.20 ± 11.73 [ab]	320.52 ± 12.82[b]	306.41 ± 8.21 [ab]

Note:Means with different superscript letters are significantly different ($p < 0.05$).

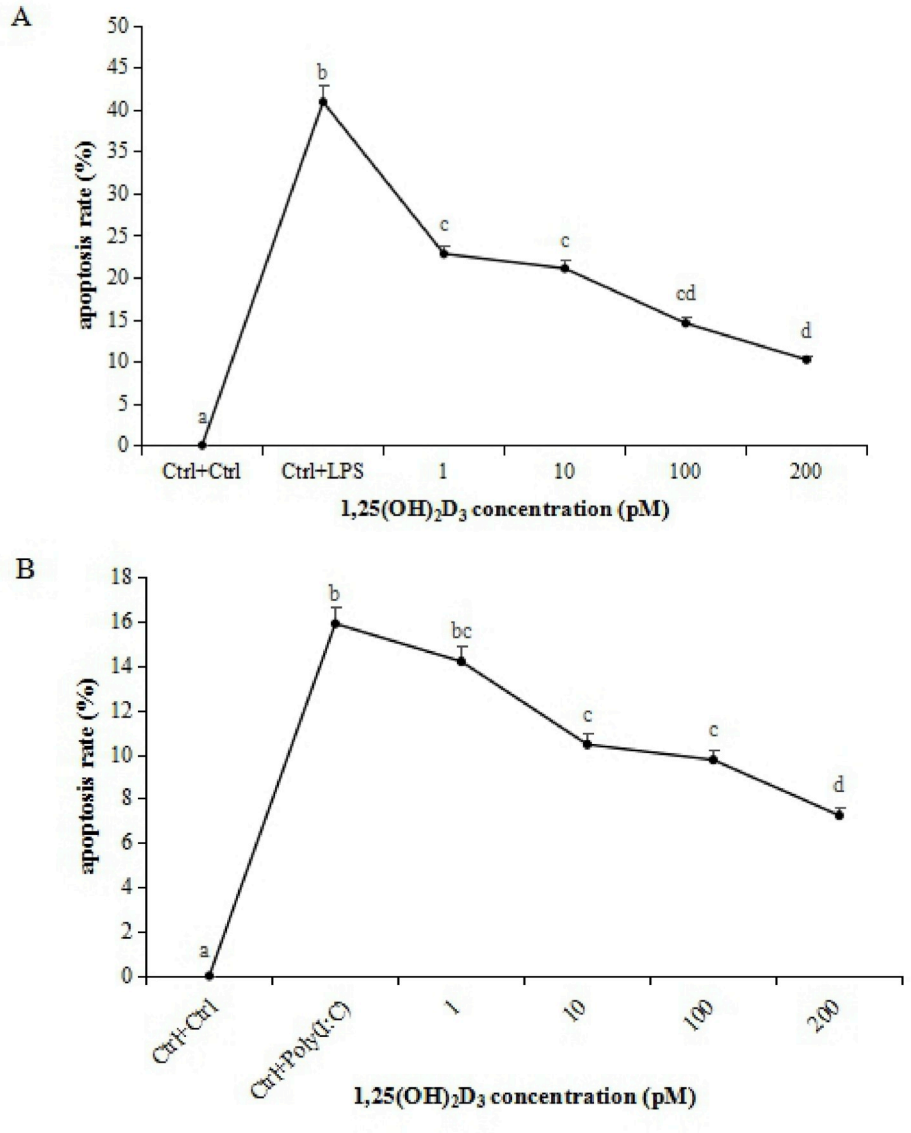

Fig. 2. Apoptosis rate of macrophages after challenge with LPS (A) and Poly(I:C) (B). The macrophages were pre-incubated with different concentrations (0, 1, 10, 100, 200 pM) of $1,25(OH)_2D_3$ for 72 h before challenge with 10 mg/L LPS or 100 mg/L Poly(I:C) for 6 h. Details of the experimental steps were shown in Table S1. Values are means (n = 9). Error bars represent the 95% confidence intervals and means with different superscript letters are significantly different ($p < 0.05$).

In the spleen, the expression of *vdr*, *irf3*, *ifn-a*, *ifi56* and *ifp35* increased with the increasing of the dietary VD_3 level, peaked at VD_3 3950 IU/kg, and subsequently decreased at higher VD_3 levels ($p < 0.05$). The expression of *jak1* and *stat1* were different from the above genes. The expression of *jak1* initially decreased, then increased, and finally peaked at VD_3 8030 and VD_3 16600 IU/kg ($p < 0.05$). The expression of *stat1* decreased and maintained at the same level in the range of VD_3 3950–16600 IU/kg ($p < 0.05$).

In the liver, the expression of *vdr*, *ifn-a*, *jak1* and *ifi56* peaked at VD_3 2260 IU/kg ($p < 0.05$), while *irf3*, *stat1* and *ifp35* peaked at VD_3 3950 IU/kg ($p < 0.05$).

In the small intestine, the expression of *vdr* and *ifn-a* peaked at VD_3 3950 IU/kg ($p < 0.05$). The expression of *stat1* decreased with dietary VD_3 level until it reached at the content of VD_3 16600 IU/kg. The expression level at VD_3 16600 IU/kg increased to the same level as that of VD_3 1120 IU/kg. The expression of other genes increased with the increasing of the dietary VD_3 level.

3.5.2. *In vitro experiment*

The current results showed that $1,25(OH)_2D_3$ enhanced the

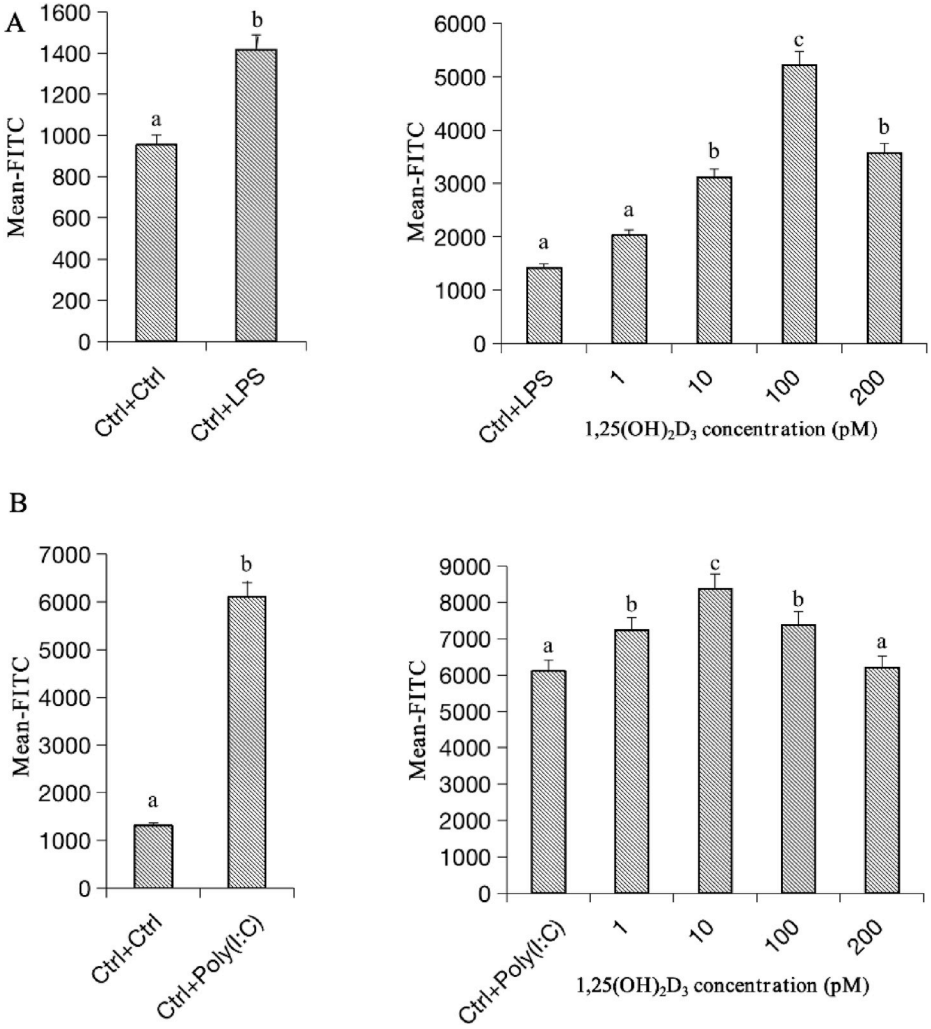

Fig. 3. Phagocytic activity of macrophages after challenge with LPS (A) and Poly(I:C) (B). The macrophages were pre-incubated with different concentrations (0, 1, 10, 100, 200 pM) of 1,25$(OH)_2D_3$ for 72 h before challenge with 10 mg/L LPS, or 100 mg/L Poly(I:C) for 6 h. Details of the experimental steps were shown in Table S1. Mean-FITC indicated fluorescence intensity, and strong fluorescence intensity represented strong phagocytosis of macrophages. Values are means (n = 9). Error bars represent 95% confidence intervals and means with different superscript letters are significantly different ($p < 0.05$).

expression of macrophage IFN-related axis genes after LPS, or Poly(I:C) stimulation (Fig. 6). As shown in Fig. 6A in the LPS treatment groups, the expression of *vdr, irf-3, ifi56* and *ifp35* were dose-dependently up-regulated with the addition of 1,25$(OH)_2D_3$ ($p < 0.05$). The expression of *ifn-a* and *jak1* were also up-regulated as compared to the Ctrl + Ctrl group, and peaked at 100 pM-1,25$(OH)_2D_3$ + LPS ($p < 0.05$) and 10 pM-1,25$(OH)_2D_3$ + LPS ($p < 0.01$), respectively. The expression of *stat1* peaked at the concentration of 10 pM-1,25$(OH)_2D_3$ + LPS ($p < 0.05$) and was down-regulated at 200 pM-1,25$(OH)_2D_3$ + LPS as compared to the Ctrl + Ctrl ($p < 0.05$).

In the Poly(I:C) treatment groups, the expression of *vdr* and *ifp35* were 1,25$(OH)_2D_3$ dose-dependently up-regulated, *irf-3, ifn-a* and *ifi56* peaked at 100pM-1,25$(OH)_2D_3$ + Poly(I:C), and the level of *jak1* and *stat1* were consistent with that of the LPS group (Fig. 6B).

4. Discussion

In mammals, vitamin D can interact with the immune system, and vitamin D deficiency is a risk factor for a number of cancers and autoimmune diseases (Sun, 2010). Previous research has shown that 1,25$(OH)_2D_3$ has anti-inflammatory function (Kamen and Tangpricha, 2010; Lim et al., 2005). In the study of human diseases, a high dose of 1,25$(OH)_2D_3$ can dampen the inflammatory response to bacterial or viral infection through down-regulating the expression of *ifn-β* (Hansdottir et al., 2010; Bange et al., 1994). However, there is very limited evidence of this occurring in fish species, and these studies have not explored mechanisms of action. For example, in rainbow trout (*Oncorhynchus mykiss*), increasing dietary vitamin D_3 content increased vitamin D levels in plasma and increased the number of white blood cells and immunoglobulins (Dehghanizadeh et al., 2014). Vitamin D_3 has also been shown to inhibit the production of pro-inflammatory cytokines by juvenile carp (*Cyprinus carpio*) intestinal cells elicited in response to LPS (Jiang et al., 2015). Although these studies suggest the possible immunomodulatory function by vitamin D_3 in fish, the mechanisms of action remain unclear.

The current study was designed to investigate immunomodulatory properties of vitamin D_3 in juvenile yellow catfish and to explore mechanisms of action; specifically, involvement of the VD_3/VDR-type I interferon axis. To our knowledge, this study is the first to demonstrate that vitamin D_3 can regulate fish innate immune response following both *in vivo* and *in vitro* immune challenge, and that the VD_3/VDR-type I interferon axis is involved.

For the *in vivo* study, fish were fed different levels of dietary vitamin D_3 for 12 weeks, then challenged by *E. Ictaluri*. The immune responses of the fish in different treatment groups were detected after pathogen challenge. This *in vivo* study showed that increasing dietary vitamin D_3

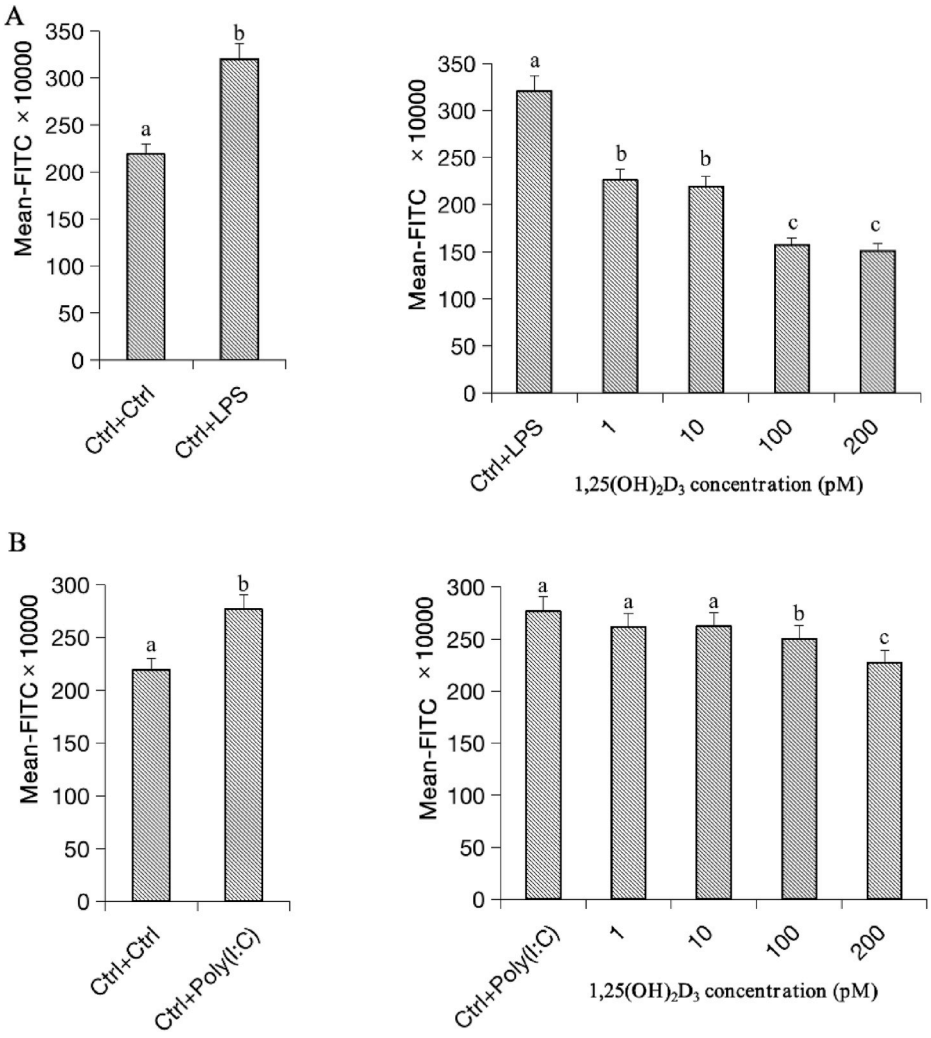

Fig. 4. Macrophage ROS production after challenge with LPS (A), or Poly(I:C) (B). The macrophages were pre-incubated with different concentrations (0, 1, 10, 100, 200 pM) of $1,25(OH)_2D_3$ for 72 h before challenge with 10 mg/L LPS, or 100 mg/L Poly(I:C) for 6 h. Details of the experimental steps were shown in Table S1. Mean-FITC indicated fluorescence intensity, and the high fluorescence intensity represented the high level of ROS. Values are means (n = 9). Error bars represent 95% confidence intervals and means with different superscript letters are significantly different ($p < 0.05$).

levels did not diminish the growth of juvenile yellow catfish, which is consistent with an earlier growth study performed by our group (Zhu et al., 2015). Increasing dietary vitamin D_3 content also increased serum lysozyme, catalase and SOD activities after pathogen challenge, indicating that dietary vitamin D_3 content may help yellow catfish resist possible oxidation and inflammation caused by the infecting pathogen. This immune protective function of dietary vitamin D_3 was also found in sea bream (*Sparus aurata* L.) (Cerezuela et al., 2009) and European sea bass (*Dicentrarchus labrax* L.) (Dioguardi et al., 2017), in which dietary vitamin D_3 significantly increased the serum peroxidase and phagocytic activity of leucocytes. Previous studies in mammals also found that vitamin D_3 increased the content of serum lysozyme activity and SOD in weanling pigs (Li et al., 2001), and SOD activity in the kidney of rats, which improved the total antioxidant capacity and reduced the MDA content (Sezgin et al., 2013).

Since the macrophage is one of the main phagocytic cells in the inflammatory phase, which is responsible for removing pathogens from tissues and cells at the site of injury, we measured phagocytic activity, apoptosis and ROS content to evaluate the function of macrophages. For this *in vitro* study, LPS and Poly(I:C) were used as fish macrophage immune stimulants and apoptosis, phagocytic activity, ROS production and the expression of key genes in the type I IFN action axis were assessed as indicators of immune modulation by vitamin D_3. Our results showed that LPS and Poly (I:C) can stimulate macrophage apoptosis and enhance phagocytosis and ROS production. In general, lower vertebrates have strong resistance to LPS (Berczi et al., 1966), but in recent years, studies have also found that LPS induces apoptosis in the trout ovary (MacKenzie et al., 2006) and salmon lymphocytes (Xiang et al., 2008). LPS stimulation increased the phagocytic activity of Atlantic salmon (*Salmo salar* L.) macrophages and increased the production of intracellular ROS (Dalmo and Seljelid, 1995). Poly(I:C), as a viral mimic, induced fish cell apoptosis (Lockhart et al., 2004) and upregulated the expression of apoptotic factors in fish (Miest et al., 2012). Poly (I:C) also stimulation increased ROS production in rainbow trout head kidney macrophages (Fierro-Castro et al., 2012). Our results showed that $1,25(OH)_2D_3$ enhanced macrophage phagocytosis and clearance of ROS induced by LPS and Poly (I:C) challenge, while protecting against apoptosis in yellow catfish. The results from our *in vitro* study were consistent with several previous mammalian studies. Human myeloid leukemic cells pre-incubated with $1,25(OH)_2D_3$ reduced apoptosis rate after stimulation with idarubicin (Ketley et al., 1997). Vitamin D_3 at the concentration of 100 nM significantly enhanced the phagocytosis of live *Mycobacterium tuberculosis* by macrophages in normal human subjects (Chandra et al., 2004). Vitamin D_3 deficiency has also been shown to reduce the phagocytic activity of macrophages in mice, and phagocytic activity could be restored after pro-incubation with $1,25(OH)_2D_3$ (Bar-

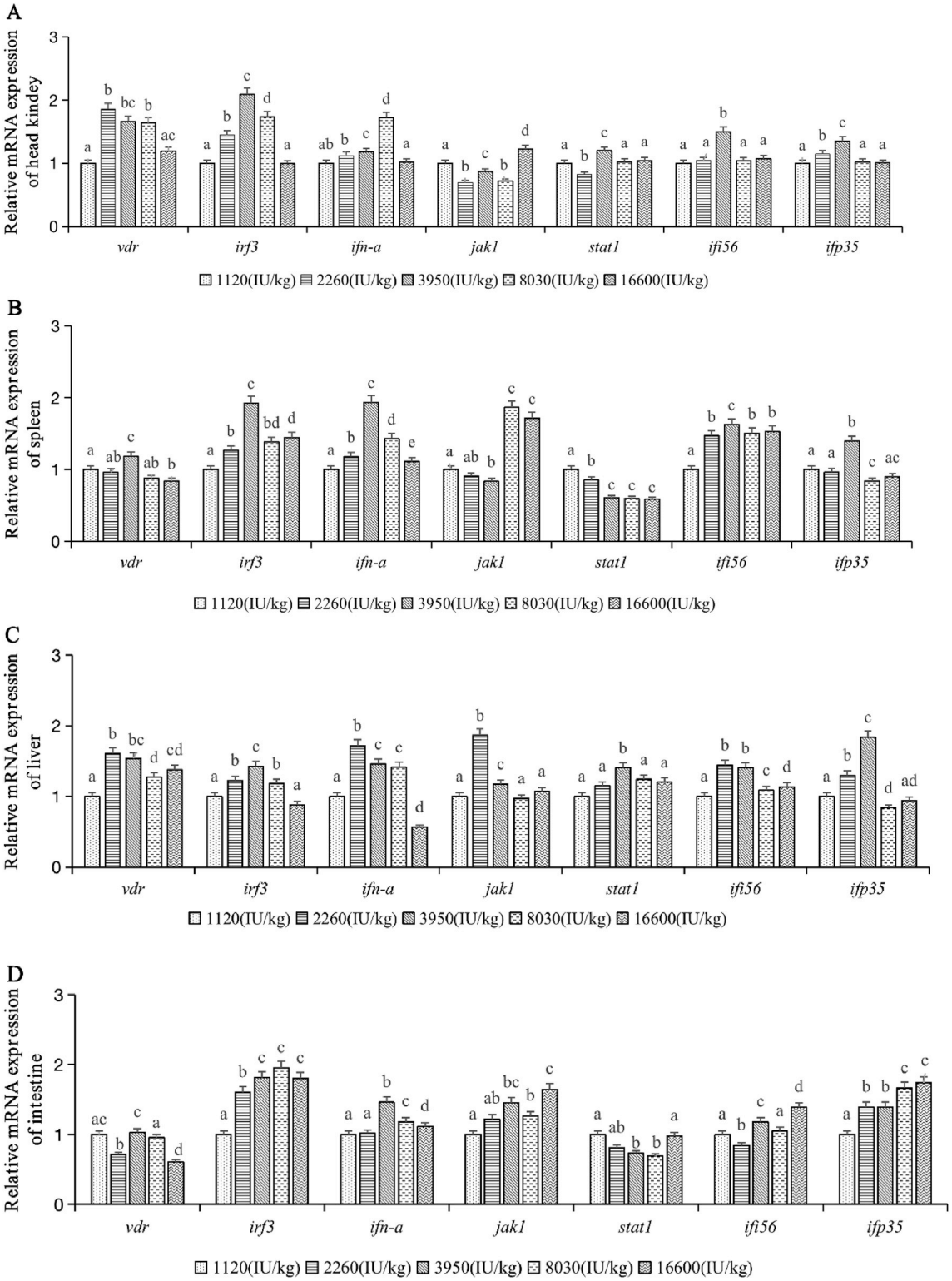

Fig. 5. Expression of type I interferon action axis genes in the head kidney (A), spleen (B), liver (C) and intestine (D) of yellow catfish after challenged with *E. Ictaluri*. Yellow catfish were fed for 12 weeks with diets containing 1120, 2260, 3950, 8030 and 16600 IU/kg of vitamin D_3. The head kidney, spleen, liver and intestine were collected for qPCR validation after 72 h of intraperitoneal injection of $3.14*10^8$ CFU/mL *E. Ictaluri* (Table S1). Values are means (n = 9). Error bars represent 95% confidence intervals and means with different superscript letters are significantly different ($p < 0.05$).

Shavit et al., 1981). Vitamin D_3 up-regulated the level of antioxidant enzyme Glutathione (GSH) and thus, decreased the production of ROS in human monocyte cells (https://www.sciencedirect.com/science/article/pii/S0006291X13009662, Jain and Micinski, 2013).

Previous studies have shown that both LPS and Poly (I:C) can induce type I IFN responses, leading to anti-inflammatory and antiviral responses (Reimer et al., 2008). After pathogen infection, toll-like receptors (TLRs) on host cell membranes and cytoplasmic receptors RIG-1 or MDA-5 are activated to induce expression of the interferon genes (Sakaguchi et al., 2003). The JAK-STAT pathway is the key pathway for

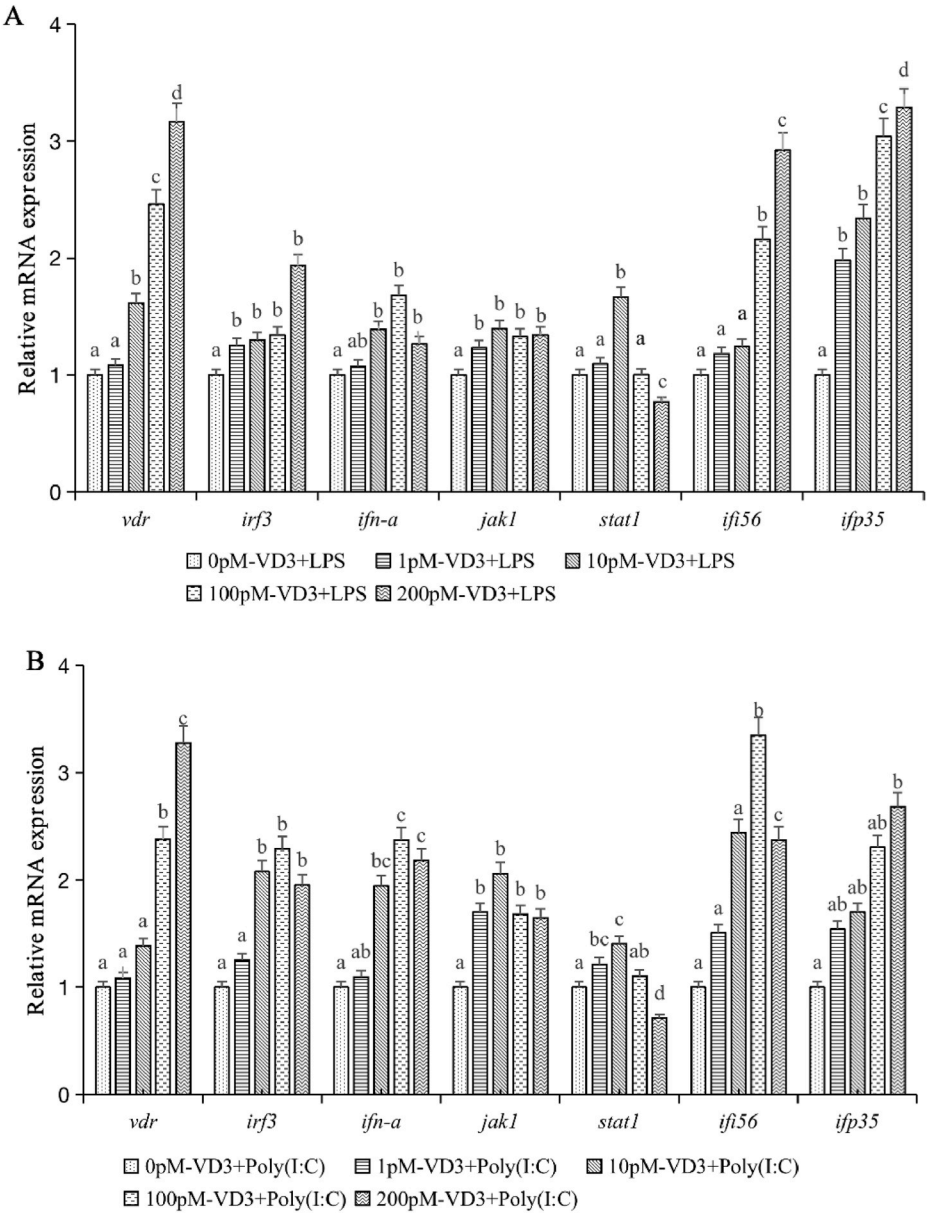

Fig. 6. Expression of macrophage type I interferon action axis genes after exposure to LPS (A), or Poly(I:C) (B). Different concentrations of $1,25(OH)_2D_3$ (0, 1, 10, 100, 200 pM) were added to the medium and macrophages were cultured for 72 h, followed by exposure to LPS (10 mg/L), or Poly(I:C) (100 mg/L) for 6 h. Macrophages were collected for qPCR validation, and the details of experimental steps were shown in Table S1. Values are means (n = 9). Error bars represent 95% confidence intervals and means with different superscript letters are significantly different ($p < 0.05$).

IFN stimulated immune function against host pathogen infection and effective clearance of invading pathogens (Jin et al., 2018; Guo et al., 2009; Li et al., 2009). This pathway also mediates the inflammatory process by activating the respiratory burst and expression of pro-inflammatory cytokines (Olavarría et al., 2010). At the same time, the production of type I IFN will activate caspase-1 and caspase-3 which biologically actives pro-inflammatory cytokines such as IL-18, leading to the occurrence of inflammation (Rintahaka et al., 2008). As previous reported, the anti-inflammatory function of vitamin D was associated with VDR (Sun, 2010). VDR is expressed in almost all kinds of immune cells and can respond to $1,25(OH)_2D_3$ (Calton et al., 2015; Harvey et al., 2013; Han et al., 2016). Several anti-inflammation and anti-infection signaling pathways were reported to be mediated through vitamin D/VDR action (Sun, 2010; Wu et al., 2010). The current study investigated the function of $1,25(OH)_2D_3$ on the gene expression along the type I IFN action axis and attempted to verify the hypothesis that $1,25(OH)_2D_3$ can regulate the immune response after pathogen infection through VD_3/VDR-type I IFN action axis.

Our *in vivo* experiment showed that expression of *vdr, irf-3, ifn-a, jak1, stat1, ifi56* and *ifp35* varied in different tissues under different dietary vitamin D_3 concentrations but were all dose-dependent. The *in vitro* experiment showed that the expression of *vdr* dose-dependently increased in macrophages pre-incubated with different doses of $1,25(OH)_2D_3$. After *in vitro* challenge with LPS and Ploy(I:C), the levels of *irf-3, ifn-a, jak1, ifi56* and *ifp35* in all $1,25(OH)_2D_3$ pre-incubation groups were up-regulated to varying degrees as compared to the Ctrl + Ctrl group indicating that $1,25(OH)_2D_3$ pre-incubation had a positive effect on the anti-inflammatory response to LPS or Poly(I:C), and this effect was exerted through the VD_3/VDR-type I IFN action axis. Meanwhile, the expression of *ifn-a, jak1* and *stat1* increased at a lower dose of $1,25(OH)_2D_3$, but decreased at a higher dose of $1,25(OH)_2D_3$. Similarly, the *in vivo* study showed that the expression of *vdr, irf-3, ifn-a, stat1, ifi56* and *ifp35* was significantly up-regulated in both head kidney and spleen in response to the dietary vitamin D_3 contents of 2260–8030

IU/kg and decreased at 16600 IU/kg. This could be explained by the fact that *ifp35* and *ifi56* are inhibitors of viral replication and function to clear cells infected with pathogens (Hwang et al., 2017), and over-expression of these genes could inhibit viral replication (Tan et al., 2008). This result is similar to previous studies in humans. After stimulation of human tracheobronchial epithelial cells with respiratory syncytial virus (RSV) pre-incubated with $1,25(OH)_2D_3$, the release of IFN-β was inhibited due to the decreased expression of STAT1 protein and inhibited translocation of STAT1 to the nucleus (Telcian et al., 2017). Another study on human respiratory viruses found that high concentration of $1,25(OH)_2D_3$ decreased the expression of *ifn-β* and *stat1* (Bange et al., 1994). Thus, the results from the current study can be extrapolated that antiviral proteins *ifi56* and *ifp35* inhibits transcription of viral genes, but overexpression of these antiviral proteins at higher dose of $1,25(OH)_2D_3$ has a negative regulatory effect on *ifn* gene and possibly inhibits the activation of the promoter of *ifn* gene.

In summary, the current study demonstrated that increasing dietary vitamin D_3 decreased mortality, enhanced the immune protective rate, and also increased serum lysozyme, catalase and SOD activities after fish were pathogen challenged *in vivo*. Moreover, $1,25(OH)_2D_3$ decreased apoptosis, enhanced phagocytic activity and decreased production of ROS in macrophages challenged with LPS and Poly(I:C). Additionally, increasing dietary vitamin D_3 content *in vivo* and $1,25(OH)_2D_3$ pre-incubation *in vitro* appeared to trigger the innate immune response through regulating the expression of *vdr*, *irf-3, jak1, stat1, ifn-a*, *ifi56,* and *ifp35,* which are key genes in the VD_3/VDR-type I IFN action axis. These results indicated that vitamin D_3 may helped yellow catfish resist possible oxidation and inflammation caused by pathogen infection and enhanced their immunity against the pathogen.

Declaration of competing interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2020.103644.

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