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Effects of cyclophosphamide on antioxidative and immune functions of Nile tilapia (*Oreochromis Niloticus*) via the TLR-NF-κB signaling pathway

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ABSTRACT

Intensive aquaculture often results in immunosuppression in fish, which may cause a series of diseases. In this study, to investigate the immunosuppressive mechanisms in fish, tilapia were intrapleural injected cyclophosphamide (CTX) at the doses of 10, 25, 50, 75 and 100 mg·kg^{-1} to induce immunosuppression. We determined the viability of immune cells, the content of lysozyme (LZM) and immunoglobulin M (IgM), the levels of nitric oxide (NO) and antioxidant parameters. Meanwhile, the mRNA levels of complement C3 (*c3*), *igm* and the genes associated with the TLR-NF-κB signaling pathway in the head kidney (HK) and spleen were also determined. The results showed that CTX had a significant cytotoxic effect on peripheral blood leukocytes, HK macrophages and spleen cells in a dose-dependent manner. The protein and mRNA levels of C3 and IgM were down-regulated with the increase of CTX concentrations in serum, HK and/or spleen. The NO and LZM contents decreased significantly in HK and spleen after CTX treatments with 75 and 100 mg·kg^{-1}. CTX treatments with 50, 75 and/or 100 mg·kg^{-1} markedly decreased the antioxidant ability and enhanced lipid peroxidation in HK and spleen. Furthermore, qPCR data showed that CTX treatments with 50-100 mg·kg^{-1} clearly down-regulated the mRNA levels of *tlr2, myd88, irak1, traf6, nfκb1, nfκb2, il-6, il-10* and *tnf-α* in the HK and/or spleen. Overall results suggested that CTX treatment had a cytotoxic effect on immune cells, induced lipid peroxidation, decreased the antioxidant capacity and inhibited immune function. The immunosuppressive mechanisms of CTX may be associated with the TLR-NF-κB signaling pathway.

1. Introduction

The immune system is a major line of defense allowing the body to resist bacterial and viral infections and other stress (Tort et al., 2003). In fish, many factors, such as inappropriate environment, pathogenic bacteria infection and pollutant exposure, can induce immune immunosuppression which increases the risk of disease (Bera et al., 2020; Jia et al., 2016; Qi et al., 2019). It has been reported that improving the immunity of fish is a feasible strategy to defend against diseases (Ahmadniaye Motlagh et al., 2020; Qin et al., 2014). Some studies suggested that diet supplemented with immunopotentiator improved immune parameters and enhanced the ability to resist diseases in fish (Meng et al., 2019). Thus, screening effective immunopotentiator is benefit for aquaculture, which may reduce the use of antibiotics. However, the lack of ideal immunosuppressive models has limited the screening and evaluation of immunopotentiator in fish.

Cyclophosphamide (CTX) is a commonly used alkylating anti-tumor drug. It uses active alkylating groups to interact with the amino groups, sulfhydryl groups, hydroxyl groups, phosphate groups and other nucleophilic groups in the protein and nucleic acid components of cells. The replacement of hydrogen atoms in these groups with alkyl groups enables cross linking of DNA strands, thereby damaging DNA structure and function (Ganesan et al., 2011; Malayappan et al., 2010). CTX mainly acts on the DNA of fast-growing cells, such as the immune cells in the body (Ganesan et al., 2011). Given the cytotoxicity of CTX immune cells (Gilman, 1963), it has been widely used to establish immunosuppressant model in mammals (El-Abasy et al., 2004; Mei et al., 2013). Its immunosuppression mechanism has been widely studied in rats and

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human. For example, CTX mediates cell apoptosis through the Fas pathway(Lee et al., 1997), regulates cytokines through the dendritic-cell-associated C-type lection 1 (Dectin-1), toll-like receptor 2 (TLR2) and TLR4 signaling pathways, and alters the T helper 1 (Th1)/Th2 balance in the body (Elkhalifa and Weiner, 2010; Logani et al., 2012). CTX can also affect redox state, causing lipid peroxidation and cell damage (Singh et al., 2015).

In recent decades, several chemical substances have been commonly detected in the aquatic environment, including CTX with concentrations ranging from 0 ng·L^{-1} - 687.0 μg·L^{-1} (Queirós et al., 2021). The effects of CTX on the immune function of aquatic animals have also been interested. CTX could reduce the white blood cell function and the level of immune parameters including immunoglobulin M (IgM), tumor necrosis factor-α (TNF-α) and lysozyme (LZM) in gibel carp (*carassius auratus gibelio*) (Chen et al., 2011; Chen et al., 2005). Combined treatment with CTX and *Aeromonas hydrophila* inhibited the immune function and decreased the content of TNF-α,while feeding probiotics and polysaccharides improved the adverse effects in *Takifugu puffer* (Hua et al., 2004; Hua et al., 2006). Meanwhile the immunosuppression caused by CTX was found in eel (*Monopterus albus*), but it was recovered by Yupingfeng Powder (a traditional Chinese complex prescription) administration (Yan et al., 2010). These studies indicated that CTX was a potential immunosuppressor for immunosuppressant model establishment in fish that is used in the screening of immune enhancers. However, the molecular mechanism associated with immunosuppression of CTX is rarely studied in fish.

Tilapia (*oreochromis niloticus*) is a global cultured and consumed fish species. It is well-studied as potential animal model to assess toxicology, physiology and pharmacology in fish (Huang et al., 2016). Additionally, in the intensive aquaculture, tilapia was influenced by many risk factors, such as poor environment, high stocking density, and the abuse of antibiotics and pesticides, which caused oxidative stress (Li et al., 2019) and reduction of immune function (Chen et al., 2020). Therefore, it is necessary to establish an immunosuppressive model in fish to screen and evaluate the immunopotentiator. In this study, we used CTX as an immunosuppressor to induce immunosuppressive model, and further evaluated the underlying toxic mechanism for immune cells and tissues in tilapia. Our study provided a potential immunosuppressive model in tilapia, and the results constituted novel insights into CTX immunotoxic mechanism in fish, which might contribute to screening and evaluation for the immunopotentiator in fish.

2. Materials and methods

2.1. Reagents

Cyclophosphamide, L-15 medium, streptomycin/penicillin (S/P), phytohaemagglutinin (Percoll), 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and heparin were purchased from Sigma Company (St. Louis, MO, USA). Fetal bovine serum (FBS) and cell culture plates were ordered from Gibco Company (USA). All other reagents used in the experiment were of analytical grade.

2.2. Fish and treatment

Tilapias with an average weight of 150 ± 5 g were obtained from the Freshwater Fisheries Research Center farm at the Chinese Academy of Fishery Sciences. One week before the experiment, all healthy tilapia were placed in a recirculating aquaculture system under the following conditions: 27 ± 2 °C, pH 6.8~7.6, >5 mg/L dissolved oxygen, <0.05 mg/L NH_3, and <0.01 mg/L H_2S. The fish were fed on a basal diet (28% crude protein and 6% crude fat) twice daily (Tongwei Co., LTD., Chengdu, China). This study was performed in accordance with the World Organization for Animal Health (OIE) laws and regulations on animal welfare, and was approved by the Ethics Committee of the Chinese Academy of Fishery Sciences.

According to our preliminary experiment and previous reports (Chen et al., 2005; Kumari and Sahoo, 2005; Song et al., 2013), the tilapia were randomly divided into six groups and intrapleural injected with CTX at the concentrations of 0 (control), 10, 25, 50, 75 or 100 mg·kg^{-1}, respectively. Each treatment group contained 20 fishes raised in two tanks (ten fishes per tank). Each treatment was administered once every 3 days for consecutive 3 times. The mortality of the tilapia was 0 during the experiment. Samples were collected on the 4th day after the last administration. After MS-222 (Sigma-Aldrich) anesthesia, eight fishes were randomly caught from each treatment group and weighed. Then blood was collected from caudal vein, and centrifuged (5 000 g, 10 min, 4 °C) to collect serum. Meanwhile, head kidney (HK) and spleen tissues were collected aseptically for later analysis.

2.3. Determination of biochemical parameters

Antioxidant parameters including superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), total antioxidant capacity (T-AOC) and malondialdehyde (MDA), were analyzed in accordance with the kit instructions (Jiancheng Institute of Biotechnology, Nanjing, China). SOD activity was determined with WST-method (Peskin and Winterbourn, 2000). GSH level was measured via 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) reaction method (Anderson, 1985). CAT activity was assayed by detecting the decomposition of H_2O_2(Matthews and R., 1987). The thiobarbituric acid (TBA) method was used to measure MDA formation (Ohkawa et al., 1979). T-AOC content was measured according to FRAP (Ferric reducing ability of plasma) method (Szollosi and Varga, 2002).

The levels of LZM in HK, spleen and serum, 8-hydroxydeoxyguanosine (8-OHdG) in the HK and spleen, and IgM were determined with ELISA kits (Mlbio, Shanghai, China). In brief, the purified fish specific antibody was coated on the microplate, and then the sample, standard and horseradish peroxidase (HRP) labeled antibody was added successively. After incubation and washing, the chromogenic agent was added, and the absorbance was measured at 450 nm.

2.4. Detection of immunocyte viability

2.4.1. Detection of peripheral blood leucocytes viability

The peripheral blood leucocytes viability was detected according to previous method (Zheng and Peng, 1992). The sterile whole blood was diluted with L-15 medium (containing 1% treptomycin/penicillin, 0.2% heparin and 0.1% FBS) in 1:1 proportion. Appropriate amount mixture was added slowly into the centrifuge tube filled with 60% percoll separation solution, and then centrifuged at 350 g for 15 min under 4 °C. The white blood cells at the junction of 60% liquid level were gotten. Viable granulocytes were counted by trypan blue exclusion. The cell concentration was adjusted to 10^7 cells·mL^{-1}, and 100 μL·per well was inoculated into 96-well plate. The 96-well plate was centrifuged at 350 g for 10 min, and the supernatant was removed. 100 μL L-15 medium (containing 5% FBS) and 20 μL MTT (5 mg·ml^{-1}) were added to each well, and incubated for 4 h under 27 °C. After dissolution of formazan, the absorbance was determined at the wavelength of 570 nm on microplate reader (Molecular Devices, Sunnyvale, CA).

2.4.2. Detection of HK macrophages viability

The isolation of HK macrophages was determined by the method described by Bayne (Bayne, 1986). Appropriate amount of HK tissues were dissected aseptically and ground. The filtrate was added to the 34% / 51% percoll liquid level, and centrifuged at 350 g for 25 min under 4 °C to obtain macrophages at the interface of the liquid level. The cells were centrifuged at 500 g for 10 min under 4°C and washed twice. Trypan blue method was used to detect the cell activity. The cell concentration was adjusted to 10^7 cells·ml^{-1}. Cells were inoculated on 96-well plate (each well 100μL) for 2 h under 27 °C. MTT assay was used to detect the proliferative activity.

2.4.3. Detection of splenocyte viability

Spleen tissue was collected aseptically, and an appropriate amount of PBS was added for grinding (Nong et al., 2019; Zheng and Ben, 1992). The ground samples were centrifuged at 1500 g for 10 min, and the supernatant was discarded. Then, five to ten times the cell volume of red blood cell lysate was added to re-suspend cells. After 5 min at room temperature, the cells were collected and washed via centrifugation. Collected cells was adjusted to 10^6 cells·mL^{-1}, and seeded in each well of 96-well plate (100 μL per well). 20 μl of ConA with a final concentration of 2 μg·ml^{-1} was added into each well and incubated for 48 h under 27 °C. The supernatant was then discarded. Subsequently, 150 μL DMSO was added into each well to dissolve the crystals. The absorbance was determined at a wavelength of 570 nm on a microplate reader (Molecular Devices), and the results were recorded.

2.5. Nitric Oxide (NO) in the HK and spleen

Appropriate amount of HK or spleen tissues from each group were lysed by NO detection lysate (Biyuntian, S0021). The lysed samples were centrifuged at 10000-14000 g for 5 min, and the supernatant was collected. 75 μL of supernatant was mixed with Griss reagent (1% sulfonamide, 0.1% ethylenediamine dihydrochloride and 2.5% phosphoric acid), and the absorbance was measured at 540 nm.

2.6. Relative mRNA levels of the immune genes in HK and spleen

The total RNA was extracted according to the instructions of RNAiso regent (Takara, Beijin, China). The quality and concentration of the isolated RNA were estimated by measuring the absorbance at 260 and 280 nm on a spectrophotometer (Molecular Devices) and calculating the A260/A280 ratio. Purified RNA (1 μg for each sample) was used to synthesize cDNA by using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara) depending on the manufacturer's instruction. Briefly, 1 μg of total RNA, 2 μL of gDNA Eraser Buffer, 1 μL of gDNA Eraser and 6 μL of RNase-free water were mixed and reacted for 2 min under 42 °C. Then, 10 μL of reaction mixture, 1 μL of PrimeScript RT Enzyme Mix, 1 μL of RT Primer Mix, 4 μL of PrimeScript Buffer and 4 μL of RNase-free water were mixed and reacted at 37 °C for 15 min and 85 °C for 5 s, and the final product is cDNA.

The real-time fluorescence quantitative PCR (qPCR) reaction was carried out on CFX96 Real-time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules Ca, USA) according to a qPCR kit (TB Green™ Premix Ex Taq™ II, Takara). PCR was carried out in the following conditions: pre-denaturation at 95 °C for 30 s, then 40 cycles of 95 °C for 5 s and 57-61 °C for 1 min. The reaction system contained 2 μL of cDNA, 12.5 μL of TB Green Premix Ex Taq II, 1 μLof forward and reverse specific primers, and 8.5 μL of RNase-free water. The relative expression of the target gene was calculated by $2^{-\triangle\triangle Cq}$ (Livak and Schmittgen, 2001) method. The specific primers of target genes and the internal reference *β-actin* primers are shown in Table 1.

2.7. Statistical analysis

SPSS 20.0 software package was used for data analysis, and all values were expressed as mean ± standard error. All data were analyzed by one-way ANOVA with Tukey multiple comparisons. $P < 0.05$ and $P < 0.01$ indicated significant difference between control group and CTX treatment group.

3. Results

3.1. Effects of CTX on the viability of immune cells

Different concentrations of CTX showed varying cytotoxicity in peripheral blood leukocytes, HK macrophages and spleen cells and the cytotoxic effect was in dose-response (Fig. 1). The viability of peripheral blood leukocytes in the 25, 50, 75 and 100 mg·kg^{-1} CTX treatment groups were 96.93%, 92.23%, 92.05% and 88.43% that of the control, respectively ($P < 0.01$ or $P < 0.05$). The viability of HK macrophages in the 10, 25, 50, 75 and 100 mg·kg^{-1} CTX treatment groups were 83.42%, 80.98%, 76.70%, 77.76% and 76.80% that of the control, respectively. When 25, 50, 75 and 100 mg·kg^{-1} of CTX was applied, the viability of spleen cells were 73.10%, 71.07%, 72.16% and 66.90% that of the control, respectively ($P < 0.01$).

Table 1
The primer sequences used in the present study.

Gene	Primer sequence (5′-3′)	GenBank number / references
tlr1	F: CTACAACGCCATCCAAACGC R: ACTGTGGCTGAAATCTCCCG	XM_005460356.3
tlr2	F: AAAAGCATAGATGAGTTCCACATCC R: GTAAGACAAGGCATCACAAACACC	JQ809459.1
tlr5	F: CATTCAGCGGTCTCCCTAACT R: CGACATGGATACTGTTCATGG	XM_019353524.1
myd88	F: CAGGTTCCTGAGGTCGACAG R: CATTTCGTGGACGAACGCAA	KJ130039.1
irak1	F: CCAGTGATCCAGGTCCTTGT R: CGGGCAGGTTGAAGTACAAT	XM_003457627.4
traf6	F: AAGAGCCACCTAGAAGAGCA R: CTGACACTTCACACTGGCAA	XM_005455728.3
Rel	F: GGTCAACAGAAATAGCGGAAGTG R:CCCAGCCATCAGGAGAGAAG	XM_019366581.1
Rela	F; CAGATGAATACAGGCTGAGTGAGAA *R: AGGTGCTGTCTATCTTGTGGAGTG*	XM_005463161.3
rel-b	F: TCACTGCCTCCACCTTTGCT R: ATCCTCATAGTTCCTCTTCCGTTTT	XM_005459330.3
nf-κb1	F: GCAGAAGGAGGCAGTGGAAG R: GACCTGCTGTGTTGGTTTGGT	XM_019363515.1
nf-κb2	F: GAACATCAGACCGACGACCA R: TCTCCGCCAGTTTCTTCCA	XM_003457469.4
il-6	F: TAGAGAAGGAGTACCGCAGCA R: TCTGTGGTAAGGATCTGGGCT	XM_019350387.2
il-8	F: CTGTGAAGGCATGGGTGTGGAG R: TCGCAGTGGGAGTTGGGAAGAA	(Limbu et al., 2018)
il-10	F: CAGCAGCAGGAGCATCAGCATT R: CACAGGAGGACGGTCTGAGAAGT	(Limbu et al., 2018)
il-1β	F: TCAGTTCACCAGCAGGGATG R: GACAGATAGAGGTTTGTGCC	(Ken et al., 2017)
tnf-α	F: AAGCCAAGGCAGCCATCCAT R: TTGACCATTCCTCCACTCCAGA	(Limbu et al., 2018)
c3	F: GGTGTGGATGCACCTGAGAA R: GGGAAATCGGTACTTGGCCT	XM_013274267.2
Igm	F: ACCGAATCGAAAAATGCGGC R: AACACAACCAGGACATTGGTTC	KJ676389.1
β-actin	F: CCTGAGCGTAAATACTCCGTCTG R: AAGCACTTGCGGTGGACGAT	KJ126772.1

3.2. Effects of CTX on immune paramaters

The content of LZM in tilapia serum, HK and spleen tissue decreased with the increase of CTX concentrations (Fig. 2-A, B and C). The LZM content in the groups treated with CTX at 50, 75 and 100 mg·kg^{-1} significantly differed from that in the control ($P < 0.05$). CTX treatment also inhibited the content of IgM in tilapia serum (Figure 2 D). With increasing CTX concentration, a significant difference was observed between the 75 and 100 mg·kg^{-1} CTX treatment groups and the control ($P < 0.05$).

The expression of *c3* and *igm* in the HK and spleen was significantly lower than that in the control with increasing CTX concentration (Fig. 3). In the HK (Fig. 3-A), CTX at 75 and 100 mg·kg^{-1} significantly inhibited the expression of *c3* and *igm* ($P < 0.01$ or $P < 0.05$). CTX at 50, 75 and 100 mg·kg^{-1} in the spleen significantly inhibited the expression of *c3* ($P < 0.01$ or $P < 0.05$; Fig. 3B), and CTX at 75 and 100 mg·kg^{-1} significantly inhibited the expression of *igm* ($P < 0.01$).

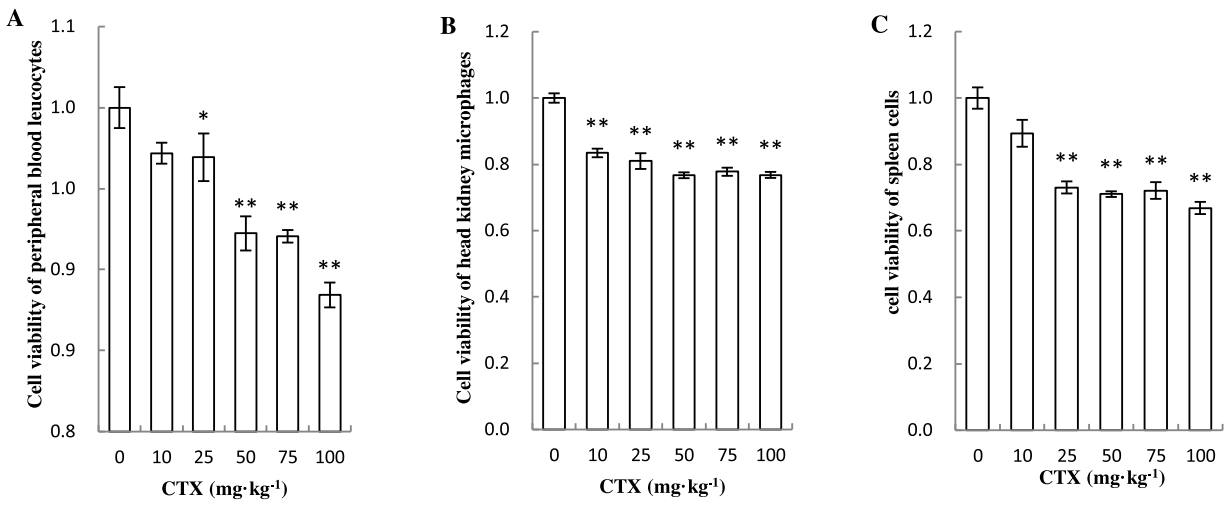

Fig. 1. Effects of CTX on the viability of immune cells. (A) Peripheral blood leukocytes, (B) head kidney macrophages, (C) spleen cells. The values are expressed as means ± SE (n=8). *$P < 0.05$ and **$P < 0.01$ compared with control tilapia. The treatment with 0 mg·kg^{-1} CTX is control.

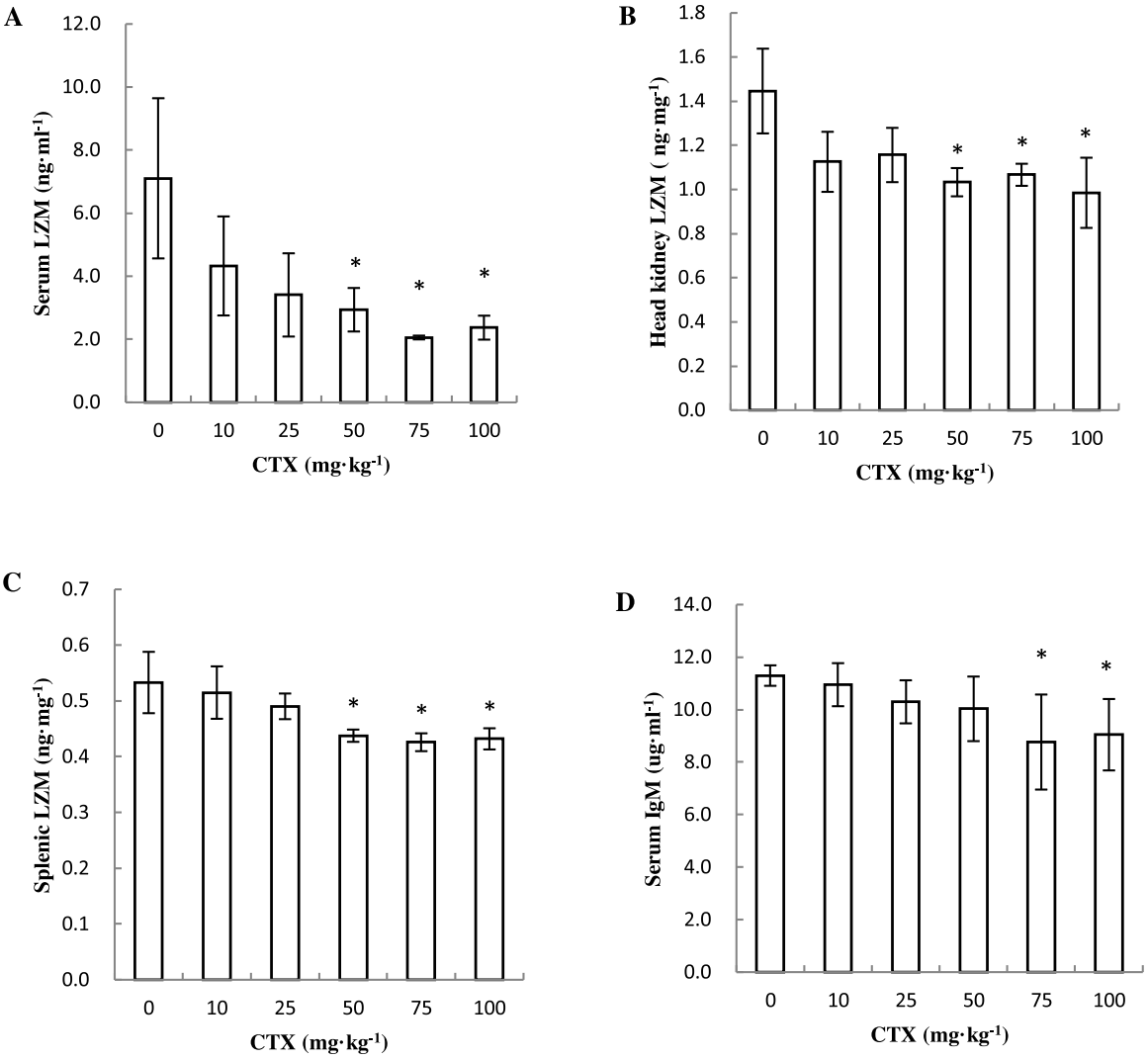

Fig. 2. Effects of CTX on the content of LZM and IgM. (A-C) LZM in serum, head kidney and spleen tissue, (D) IgM in serum. The values are expressed as means ± SE (n=8). *$P < 0.05$ and **$P < 0.01$ compared with control tilapia. The treatment with 0 mg·kg^{-1} CTX is control.

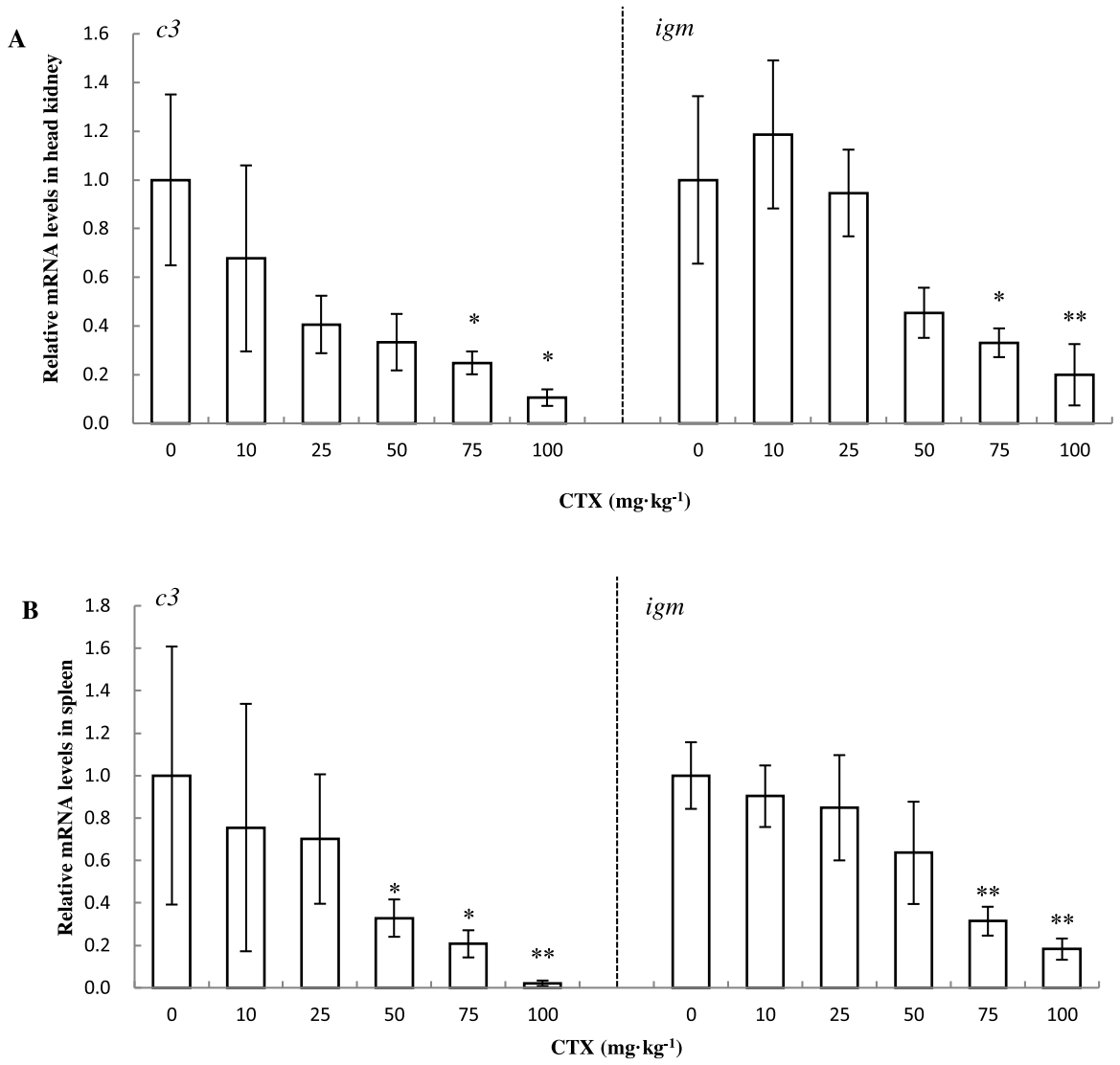

Fig. 3. Effects of CTX on the mRNA levels of *c3* and *igm*. (A) *c3* and *igm* mRNA levels in the head kidney, (B) *c3* and *igm* mRNA mRNA levels in spleen. The values are expressed as means ± SE (n = 8). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control tilapia. The treatment with 0 mg·kg^{-1} CTX is control.

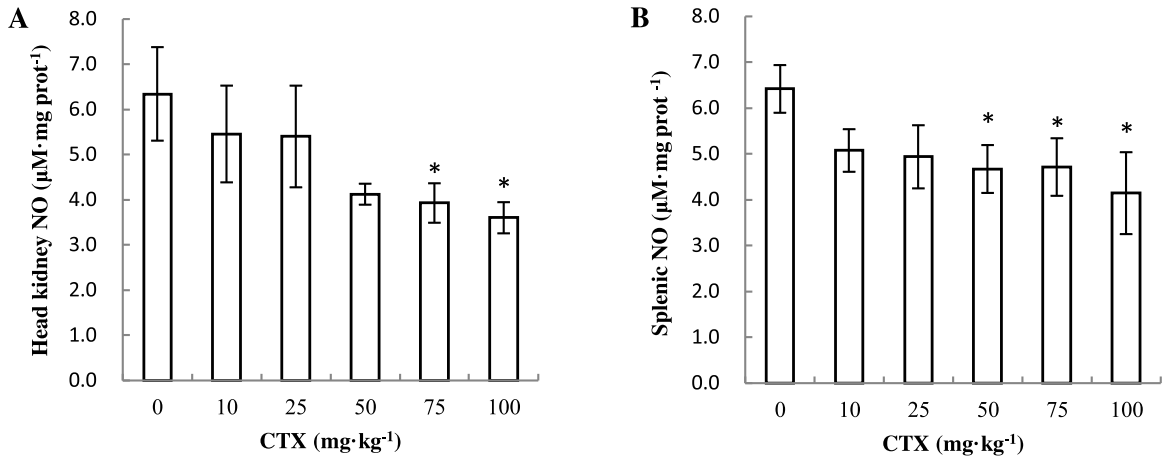

Fig. 4. Effects of CTX on NO contents in the head kidney (A) and spleen (B) The values are expressed as means ± SE (n=8). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control tilapia. The treatment with 0 mg·kg^{-1} CTX is control.

3.3. Effects of CTX on NO content in the HK and spleen

The effects of CTX on NO level in tilapia immune tissue was shown in Fig. 4. The NO content in the HK and spleen decreased significantly with increasing CTX concentration (Fig. 4-A and B). Compared with the control, the NO content was significantly inhibited in the 75 and 100 mg·kg^{-1} treatment groups in the HK (P <0.05), and in 50, 75 and 100 mg·kg^{-1} treatment groups in the spleen (P <0.05).

3.4. Effects of CTX on antioxidant parameters

The effects of CTX on the antioxidant parameters of the HK and spleen were summarized in Table 2. In HK, the content of 8-OHdG increased with increasing CTX concentrations, and markedly increased 8-OHdG was observed in the 50, 75 and 100 mg·kg^{-1} CTX treatment groups ($P < 0.05$). Compared with the control group, the levels of SOD, GSH, T-AOC and CAT were significantly decreased in 75 and/or 100 mg·kg^{-1} CTX-treated groups, while the content of MDA was significantly increased in 75 and 100 mg·kg^{-1} CTX-treated groups ($P < 0.01$ or $P < 0.05$).

In spleen, CTX administration obviously increased the content of 8-OHdG in the 75 and 100 mg·kg^{-1} treatment groups and MDA in 100 mg·kg^{-1} treatment group compared with control group (P <0.05; Table 2). In contrast, CTX administration signally decreased the levels of SOD, CAT, GSH and T-AOC in 50, 75 and/or 100 mg·kg^{-1} treatment groups ($P < 0.01$ or $P < 0.05$; Table 2).

3.5. Effects of CTX on the mRNA levels of immune-associated genes

3.5.1. Effects of CTX on mRNA levels of immune-associated genes in HK

As shown in Fig. 5, the mRNA levels of *tlr2*, myeloid differentiation factor 88 (*myd88*), interleukin-1 receptor-associated kinase 1 (*irak1*), nuclear factor-kB1 (*nfkb1*), *interleukin-6* (*il-6*), *il-10 and tnf-α* were dramatically down-regulated in 50, 75 and 100 mg·kg^{-1} CTX-treated groups compared with control group ($P < 0.01$ or $P < 0.05$). The down-regulation was also observed in the mRNA levels of *myd88, irak1*, and *il-10* after 10 and/or 25 mg·kg^{-1} CTX injection ($P < 0.05$). Similarly, the mRNA levels of tumor necrosis factor receptor-associated factor 6 (*traf-6*) and *nfkb2* were distinctly lower in 75 and 100 mg·kg^{-1} CTX-treated groups than that in control group (P <0.05). Moreover, CTX administration obviously up-regulated the mRNA levels of *tlr-5* at 10 and 25 mg·kg^{-1} and *il-8* and *il-1β* at 100 mg·kg^{-1} ($P < 0.01$ or $P < 0.05$) (Fig. 6.

3.5.2. Effects of CTX on mRNA levels of immune-associated genes in the spleen

Compared with control group, the mRNA levels of *tlr2, tlr5, myd88, traf6, nfkb1* and *il-6* were significantly reduced in treatments with 50, 75 and 100 mg·kg^{-1} CTX ($P < 0.01$ or $P < 0.05$; Fig. 5). Among these genes, the *myd88, traf6 and nfkb1* were also down-regulated by 10 and/or 25 mg·kg^{-1} CTX injection ($P < 0.01$ or $P < 0.05$). CTX administration evidently suppressed the *irak1* and *tnf-α* expression at 100 mg·kg^{-1} and *nfkb2* and *il-10* at 75 and 100 mg·kg^{-1}($P < 0.01$ or $P < 0.05$; Fig. 5). The suppression was also found in *rel* expression at 10-75 mg·kg^{-1} CTX, *rela* expression at 10-50 mg·kg^{-1} CTX, and *relb* expression at 25-75 mg·kg^{-1} CTX ($P < 0.01$ or $P < 0.05$; Fig. 5). Conversely, the *tlr-5* expression in 10 mg·kg^{-1} CTX treatment and *il-8* and *il-1β* expression in 100 mg·kg^{-1} CTX treatment were markedly up-regulated ($P < 0.01$ or $P < 0.05$; Fig. 5).

4. Discussion

This study was the first to report the underlying immunosuppressive mechanism of CTX on immune cells and tissues in tilapia. Herein, we demonstrated the cytotoxic effect of CTX on peripheral blood leukocytes, HK macrophages and spleen cells. Further, we speculated that the imbalanced redox state and TLR-NF-κB signaling pathway were involved in the mechanism of CTX toxicity.

CTX, a commonly used chemotherapeutic drug and immunosuppressant, has been shown to be toxic to immune cells, restricting their transformation into immunoblasts and inhibiting the immune function in animals(Gong et al., 2015; Wang et al., 2017; Zhong and Fang, 2016). Peripheral blood leukocytes, HK macrophages and spleen cells are important immunologically active cells in fish. All such cells play an important role in identifying and phagocytosing foreign pathogenic microorganisms, processing and presenting antigens, activating lymphocytes to initiate specific immune responses and secreting immune factors(Ellis, 1995; Nie, 1997; Secombes, 1986). Their activities and functions are important indicators that can be used to evaluate the

Table 2
Effects of CTX on the antioxidant capacity of the head kidney and spleen in tilapia.

Tissues	Parameters	0 mg·kg^{-1}	10 mg·kg^{-1}	25 mg·kg^{-1}	50 mg·kg^{-1}	75 mg·kg^{-1}	100 mg·kg^{-1}
Head kidney	8-OHdG (ng·mg prot^{-1})	0.66±0.04	0.64±0.06	0.72±0.03	0.76±0.05*	0.77±0.03*	0.81±0.02*
	SOD (U·mg prot^{-1})	22.87±1.04	23.80±1.32	24.65±3.14	20.82±0.78	17.54±0.93*	17.68±0.93*
	CAT (U·mg prot^{-1})	203.89±18.76	204.60±16.38	175.57±29.73	184.95±15.13	151.33±13.46	141.32±16.53*
	MDA (nmol·mg port$^{-1)}$	2.98±0.48	1.83±0.10	3.03±0.62	3.16±0.42	4.41±0.51*	4.70±0.40*
	GSH (μmol ·g prot$^{-1)}$	38.87±5.03	41.82±5.67	36.76±3.27	35.61±5.77	19.14±2.70**	20.26±2.42**
	T-AOC (μmol·g prot$^{-1)}$	79.88±12.57	77.02±7.14	60.67±14.60	50.77±8.62	46.43±8.10*	41.17±11.05*
Spleen	8-OHdG (ng·mg prot^{-1})	0.30±0.01	0.33±0.02	0.33±0.02	0.34±0.02	0.35±0.01*	0.35±0.02*
	SOD (U·mg prot^{-1})	12.88±0.88	12.97±0.25	12.25±1.00	11.94±0.48	10.66±0.47*	10.94±0.28*
	CAT (U·mg prot^{-1})	66.16±5.35	58.51±3.51	59.12±4.38	51.79±3.71*	51.16±7.81*	47.16±2.68**
	MDA (nmol·mg port$^{-1)}$	1.06±0.17	0.68±0.08	0.90±0.10	0.78±0.58	1.24±0.06	1.39±0.11**
	GSH (μmol ·g prot$^{-1)}$	50.75±3.44	38.72±4.78	31.41±3.79**	34.02±2.53**	35.44±2.11**	35.02±2.13**
	T-AOC (μmol·g prot$^{-1)}$	58.32±3.10	59.50±5.98	61.34±3.41	48.60±4.52	40.60±4.40**	37.15±2.30**

Note: The values are expressed as means ± SE (n=8). *$P < 0.05$ and **$P < 0.01$ compared with control tilapia. The treatment with 0 mg·kg^{-1} CTX is control.

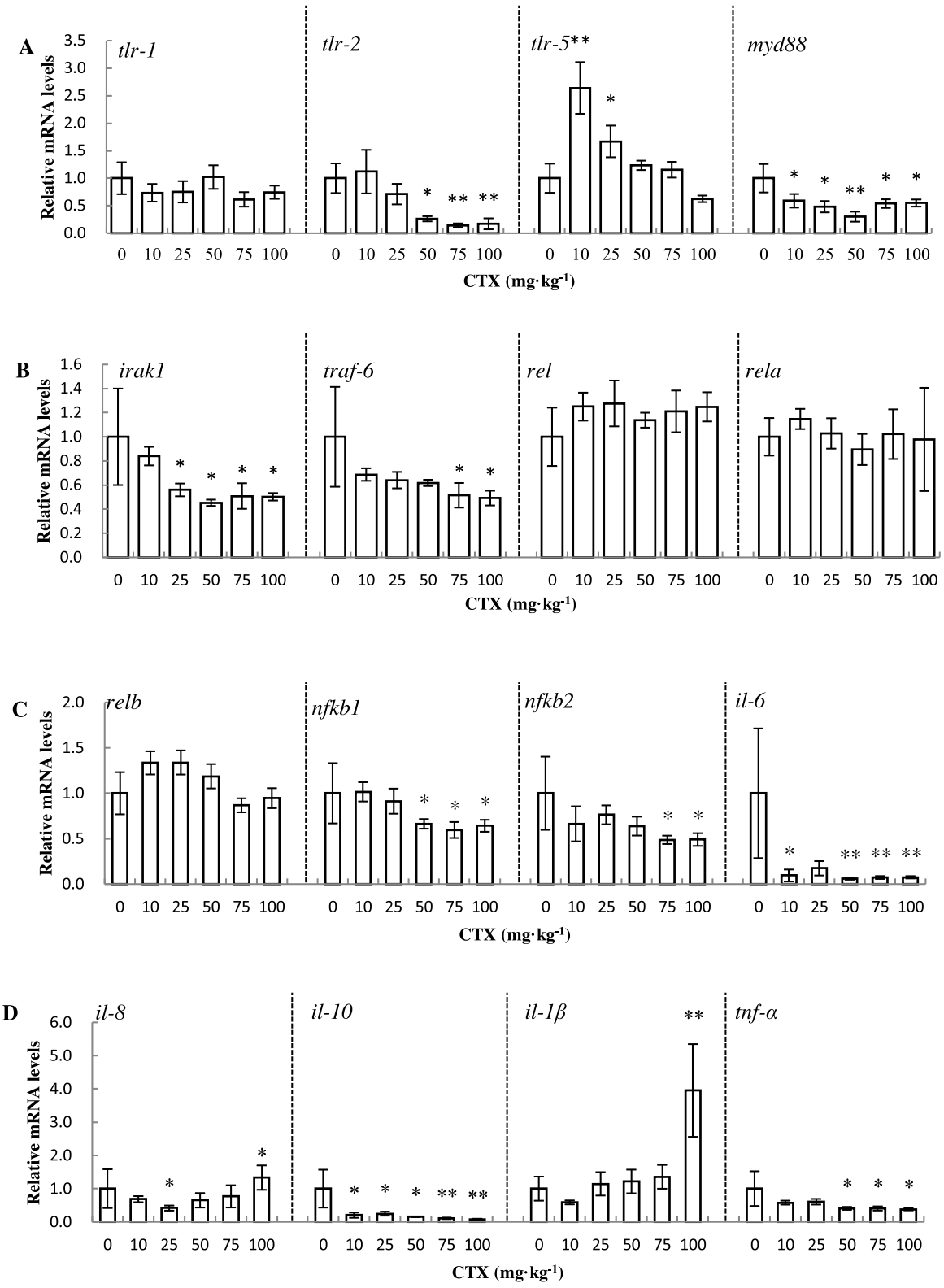

Fig. 5. Effects of CTX on the mRNA levels of genes related to TLRs -NF-kB pathway in the head kidney of tilapia. The values are expressed as means ± SE (n=8). *$P <$ 0.05 and **$P < 0.01$ compared with control tilapia. The treatment with 0 mg·kg^{-1} CTX is control.

immune level of fish.(Haaparanta et al., 1996; Weeks et al., 1986). Early report has shown that CTX decreased the splenic B lymphocyte transformation capacity of *Takifugu obscurus* under LPS stimulation (Hua et al., 2004). The lymphocyte transformation rate of the HK, spleen and peripheral blood in Jian carp was also blocked by CTX injection (Wang et al., 2012). In line with previous studies, our data showed that the viability of peripheral blood leukocytes, HK macrophages and spleen cells in tilapia was decreased under CTX injection, and the cytotoxicity was in a dose-dependent manner. The results suggested that CTX may kill the immune cells or block the proliferation of lymphocytes (Dong

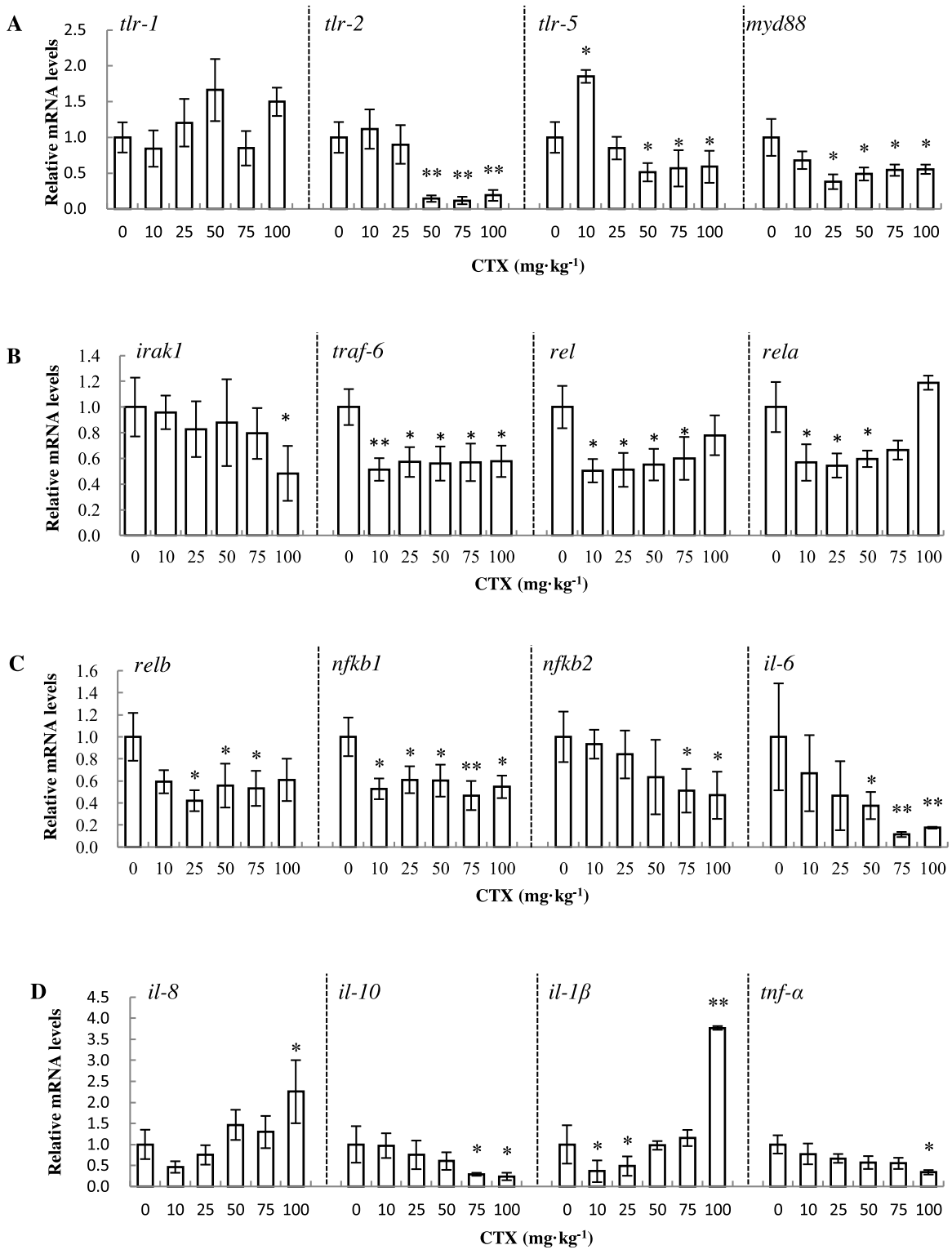

Fig. 6. Effects of CTX on the mRNA levels of genes related to TLRs -NF-kB pathway in the spleen of tilapia. The values are means ± SE (n=8). *$P < 0.05$ and **$P < 0.01$ compared with control tilapia. The treatment with 0 mg·kg^{-1} CTX is control.

et al., 2007) to exert an immunosuppressive effect (Miao et al., 2015).

The immune system in fish includes non-specific immunity and specific immunity. LZM is a hydrolase secreted by mononuclear macrophages, which has a lytic effect by hydrolyzing the peptidoglycan in bacterial cell walls, as well as a non-specific defense effect in the body (Jia et al., 2002). Therefore, LZM is an important non-specific immune factor of fish (Clerton et al., 2001; Hardie et al., 1990). Fish produce specific humoral immunity after antigen stimulation. Immunoglobulin plays an important role in humoral immunity, which is considered as an indicator for assessment of immunity in fish, and IgM is the most common immunoglobulin in teleost (Li, 2009). It has been reported that CTX administration decreased IgM production form B lymphocytes in the peripheral blood, and LZM activity in spleen and HK in crucian carp (*Carassius carassius*). Meanwhile, CTX has been found to decrease serum

LZM activity in Jian carp (Yan et al., 2010), eels (Li, 2009) and *Takifugu obscurus* (Hua et al., 2004). Similarly, our results showed that levels of IgM and LZM in serum, HK and /or spleen were decreased after CTX injection, and the mRNA levels of *c3* and *igm* were also down-regulated in HK and spleen tissue, which indicated that CTX inhibited immune parameters and decreased immune function in tilapia.

NO is the major effector molecule produced by activated macrophages to kill pathogenic microorganisms and tumor cells. It is widely distributed in various organs and tissues in organisms, and plays a dual role in animal physiology and pathology(Zhu et al., 2006). On the one hand, NO inhibits and kills pathogenic microorganisms and participates in the body's anti-infection immunity and defense. On the other hand, high level of NO can produce cytotoxic effects and damage normal cells (Sha et al., 2013). Chen et al.(Chen et al., 2015) have found that intraperitoneal injection of 300 mg·kg^{-1} CTX significantly increases the serum NO level in *Paramisgurnus dabryanus*, which may be associated with increased expression of inducible NO synthase. However, decreased NO level was also reported in HK of *allogynogenetic crucian carp* after CTX injection (Chen et al., 2011). In mice, CTX treatment decreased NO secretion in peritoneal macrophages and splenic lymphocytes (Feng, 2015). In this study, CTX decreased the NO content in the HK and spleen, which corresponded to the inhibition of the proliferation activity of immune cells. These results indicated that the decrease of NO level may be attributed to the toxic effect of CTX on immune cells (Zhao et al., 1998).

The redox imbalance is a major toxicity mechanism of CTX, which is involved in several antioxidant enzymes (e.g. SOD and CAT) and non-enzymatic antioxidants (e.g. GSH). CTX induced oxidative stress and decreased SOD activity and GSH content, resulting in protein, lipid and DNA damage (Singh et al., 2015). CTX treatment with 100 mg·kg^{-1} decreased SOD and GSH levels and promoted MDA formation in mice (Tripathi and Jena, 2009). Similar results were found in hepatopancreas of *Paramisgurnus dabryanus* injected by CTX (Chen et al., 2015). Consistent with previous studies, the decreased antioxidant parameters including T-AOC, SOD, GSH and CAT, and increased MDA in HK and spleen of tilapia revealed that CTX treatment resulted in redox imbalance and lipid peroxidation. In addition, we found the 8-OHdG, a biomarker of the degree of cellular DNA oxidative damage, was increased in CTX-treated group, which further reflected an abnormal redox state in CTX-treated tilapia. The reduced antioxidant capacity may cause accumulation of reactive oxygen radicals (ROS), which leads to cells damage in the HK and spleen and decrease of immune function.

Immune cytokines plays an important role in CTX-induced immunosuppressive mechanism, which is involved in TLRs pathways and NF-κB pathway (An et al., 2002; Fang et al., 2012). TLRs can recruit MyD88 to initiate intracellular signaling cascades including IRAKs and TRAF6 (Fan et al., 2015). The cascades activate NF-κB to promote the production of immune cytokines (Medzhitov, 2009). Rong et al.(Rong et al., 2009) found that the mRNA levels of *tlr2* and *tlr4* were increased first and decreased thereafter after CTX injection in alveolar macrophages of female Kunming mice. The down-regulated genes expression including *tlrs* (*tlr-4, tlr-5* and *tlr-9*) and *myd88* was also found in CTX-damaged small intestinal mucosa of mice, which may be associated with regulation of the TLRs/MyD88 signaling pathway (Gao et al., 2019). In fish, several TLRs have been identified, and they have been confirmed to participate in the immune response (Fan et al., 2015). Knockdown of the *myd88* gene in the zebrafish resulted in diminished resistance to *Salmonella* infection, which reflected that a MyD88-dependent signal transduction mechanism might also exist in fish (van der Sar et al., 2006). Subsequent studies have found that MyD88 activated NF-κb in Atlantic salmon (*Salmo salar* L.) (Rebl et al., 2011). In this study, the mRNA levels of *tlr2* and *tlr5* were down-regulated in HK and/or spleen after CTX injection (50-100 mg·kg^{-1}). Meanwhile, their downstream genes including *myd88, irak1* and *traf6* were also down-regulated. These results indicated that CTX-induced immunosuppression might be related to the TLRs-MyD88 pathway inhibition. In addition, we found the *tlr5* expression was up-regulated after CTX injection at low concentrations (10 and/or 25), which might be an immune response to CTX toxicity in uninjured immune cells. The detailed mechanism is still unclear.

In immune response, activated TLRs-MyD88 pathway further mediate NF-kB to regulate production of cytokines (Akira, 2003; Krishnan et al., 2007). The NF-kb (including five subunits: Rel, Rel-A, Rel-B, NF-κB1 and NF-κB2) plays a major role in the transcriptional activation of cytokines and chemokines, and occupies an important position in the immune response. The effects of CTX on non-specific immune cytokines are extensive and significant. CTX inhibited the expression of *nf-κb* in mouse splenic lymphocytes and the serum cytokine content (Xin, 2017). CTX administration down-regulated the genes expression including *il-2, il-10, il-12, il-4, tnf-α* and *ifn-γ* in the spleen and thymus of mice (Yi et al., 2012). In this study, the mRNA levels of NF-κB including *rel, rela* and *relb nfκb1* and *nfκb2,* and immune cytokines *il-6, il-10* and *tnf-α* in the HK and spleen were decreased, revealing that CTX injection might inhibited NF-kB pathway and block immune response in tilapia. Moreover, in the present study, the mRNA levels of *il-1β* and *il-8* were up-regulated in 100 mg·kg^{-1} CTX treatment. The cause of the up-regulation is still unknown. We speculated that CTX might induce inflammatory response via other mechanism in these tissues.

Conclusion

The present study demonstrated CTX treatment can induce immunosuppression via decreasing the viability of immune cells, disturbing redox state and suppressing immune response. CTX injection decreased the viability of peripheral blood white blood cells, HK macrophages and spleen cells, and the levels of LZM, IgM, C3 and NO in HK and spleen tissue. Meanwhile, CTX injection reduced antioxidant ability and caused lipid peroxidation. Further, CTX administration inhibited the genes expression related to TLRs-NF-kB pathway, which indicated that CTX-induced immunosuppression might be associated with the TLR-NF-κB signaling pathway in tilapia. These data showed a feasibility that CTX was used to establish immunosuppressive model in tilapia, which contribute to screening and evaluation for the immunopotentiator in fish.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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