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The PFOS disturbed immunomodulatory functions via nuclear Factor-κB signaling in liver of zebrafish (*Danio rerio*)

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ABSTRACT

Excessive perfluorooctane sulfonate (PFOS) in natural water ecosystem has the potential to detrimentally affect immune system, but little is known of such effects or underlying mechanisms in fish. In the present study, we evaluated the effects of PFOS on growth performance, organizational microstructure, activities of immune-related enzymes and expressions of immune-related genes in male zebrafish (*Danio rerio*) exposed to different concentrations of 0, 0.02, 0.04 and 0.08 mg/L of PFOS for 7, 14, and 21 days or cotreatment with PFOS and PDTC to investigate the effects of PFOS on immune system and the potential toxic mechanisms caused by PFOS. The results indicated that PFOS accumulated in livers after exposure, and remarkably elevations were found in three exposure groups compared with the control group at three stages. The growth of the adult zebrafish in the experiments was significantly inhibited, the microstructures of liver were serious damaged. The ROS levels were remarkably increased. The activities of ACP, AKP, and lysozyme were obviously decreased, while the activities of MPO and NF-κB were significantly increased. The expressions of immune-related mRNA were significantly affected. After co-treatment with PFOS and PDTC, the growth inhibition, the morphological damage, the ROS induction, and the expressions of immune-related mRNA were reversed. Taken together, the results indicated that PFOS can significantly inhibit the growth, disturb the immune system by changing the normal structure of liver, the activities of immune-related enzymes, and a series of gene transcriptions involved in immune regulation in liver of male zebrafish. PFOS-induced pro-inflammatory effect of hepatocytes was observed, and the involvement of NF-κB signaling pathway was participated in its action mechanism. These findings provide further evidence that PFOS interferes with the immune regulation of liver of male zebrafish under in vivo conditions.

1. Introduction

Perfluorooctane sulfonate (PFOS), a fluorinated organic compound, has been widely used in a range of industrial and commercial applications for many years, such as wetting agents, fire retardants, stain resistant treatments for leather and carpets, cosmetics, components of pharmaceuticals and insecticides [1,2]. Due to the properties of global distribution, persistence, high bioaccumulation and negligible elimination, PFOS has been widely recognized as a man-made persistent organic pollutant and been found in the organisms and environment in the world [3–5]. PFOS is mostly found in the environment in relatively low concentrations (around the ng/L range) [6]. For example, PFOS concentrations ranged from below 10 pg/L to 703 ng/L in surface water of ocean, coastal areas, and river estuaries [7,8]. However, the concentration of PFOS can up to 2260 μg/L after an accidental release of fire fighting foam [9] and the levels in waste water and nearby river system of a semiconductor fabrication plant could reach 12,566 mg/L and 5.4 mg/L, respectively [10]. Meanwhile, PFOS has been demonstrated to accumulate in aquatic organisms [11,12]. The maximum concentrations of PFOS detected in the liver were up to 1.8–9.03 μg/g and 72.9 μg/g in some fish species from Tokyo bay, Flanders and an accidental spill site [9,11,13]. The PFOS levels in various edible fish muscles ranged from 0.27 ng/g to 5.98 ng/g wet weight [14,15].

PFOS could result in a series of adverse effects in aquatic organisms,

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such as hepatotoxicity and disturbance of DNA metabolism homeostasis, reproductive and developmental toxicity, disturbed lipid metabolism, induction of oxidative stress and apoptosis, the opening of the tight junction [16–21]. Despite the toxicity and biological effects on aquatic organisms, very few studies have been investigated on the toxicity of PFOS on immune system and the underlying molecular mechanisms involved in its toxicity.

It is widely accepted that PFOS has estrogenic and endocrine disrupting effects [22,23]. PFOS could cause estrogenic effects that could disturb the expressions of estrogenic-related genes, and thus result in the developmental and reproductive abnormalities in some teleost fish [22,24,25]. PFOS had disruptive endocrine effects through the peroxisome proliferators-activated receptors (PPARs) in fish [26], which could regulate the energy homeostasis, cell proliferation, differentiation and survival. The immune system serves as a potential target for endocrine disrupters, especially environmental estrogens [27]. More and more studies suggested that endocrine disrupting chemicals could interfere with the gene expressions related to the innate immune system during the early developmental stages of zebrafish (*Danio rerio*) and that the increased levels of estrogen could contribute to a decreased anti-viral immune response, causing pregnancy-associated morbidities in mice [28]. It is generally recognized that PFOS exposure could change the immune process in some experimental biological models [16,29].

Inflammation is one of the first responses of the immune system after being exposed to exogenous chemicals, which might play a very important role in the diseases' development [30]. More and more evidence indicated that expressions of the inflammatory related genes play an important role in the functional capacity of the immune system, such as the pro-inflammatory cytokines interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α), which both increased in the brains of rat pups after prenatal exposure to PFOS [31]. However, few studies have investigated the toxic effects of PFOS on immune toxicity in gene expression profiles in aquatic organisms, and the underlying molecular mechanisms involved have not been clarified.

Zebrafish (*Danio retrio*) are widely used as an important ecological toxicological model to measure the environmental pollutants based on its advantages, including small size, high reproductive performance, rapid organogenesis, morphological and physiological similarities to mammals, high sensitivity to the harmful substances [32]. Previous studies have showed that zebrafish were used to evaluate the immunization toxicity of chemicals in the laboratory [33–36]. In the present study, to remove sex as a factor/variable, the effects of waterborne PFOS on the growth performance, histological changes, the levels of ROS, the activities of immune-related enzymes and the transcriptional profiles of immune-related genes were examined in the liver of male zebrafish exposed to different concentrations of 0, 0.02, 0.04 and 0.08 mg/L of PFOS or cotreatment with PFOS and PDTC to investigate the effects of PFOS on immune system and the underlying toxic mechanisms caused by PFOS.

2. Materials and methods

2.1. Test animals and chemicals

Adult male zebrafish three months old (*Danio rerio*) (AB strain), with mean body weight and body length of 0.19 ± 0.03 g and 2.5 ± 0.3 cm, were obtained from Taiyuan fish hatcheries in Shanxi Province, PR China. They were kept in a flow-through system with dechlorinated tap water (pH, 7.0–7.4; water temperature, 28 ± 1 °C; light regime, 14-h light and 10-h dark) for 15 days to acclimate to laboratory conditions before exposure, and the death rate is less than 1%. Zebrafish were fed with commercially available adult zebrafish compound feed during the period of acclimation and exposure.

PFOS solution (~40% in H_2O) and NF-κB antagonist pyrrodiline ditriocarbamate (PDTC) were purchased from Sigma-Aldrich.

2.2. PFOS exposure

Six hundred and sixty male zebrafish were randomly divided into four groups and exposed to different concentrations of 0 (control), 0.02, 0.04, 0.08 mg/L of PFOS dissolved in dechlorinated-tap water for 21 days, respectively. Each group has three replicates (n = 55 for each replicate). PFOS concentration used in the experiment was based on the 96 h LD_{50} (2.58 mg/L) detected in the pre-experiment and the actual concentrations in natural freshwater. During the experiment, the exposure conditions were maintained as follows: water temperature, 28 ± 1 °C; light regime, 14-h light and 10-h dark; pH, 7.0–7.4; dissolved oxygen, 5.0–6.0 mg/L; hardness, 20.0 mg/L (as $CaCO_3$). The concentrations of heavy metals were nearly zero or hardly detected. Thus, the water quality could satisfy the national aquaculture standard. Exposure media was completely changed every 3 d and the aquariums were completely cleaned. All animal work was authorized by the Institutional Animal Care and Use Committee of Shanxi Agricultural University.

After exposure for 7, 14 and 21 d, fifty zebrafish from each group were dried on filter paper, anesthetized with MS 222 (Sigma, USA) and dissected on ice to collect the liver. The body weight and body length of 30 fish were measured to evaluate their growth performance. Ten livers were collected and rinsed in physiological saline (0.85% NaCl) and immediately fixed in Bouin's fixative solution for histopathological observation. Liver tissue samples of 20 zebrafish were collected for determination of enzyme activity levels and ROS using the specific ELISA kits (n = 10, 2 livers were mixed to one sample). Another 20 tissues were snap frozen by liquid nitrogen and then stored at −80 °C for real-time fluorescent quantitative PCR (QRT-PCR) analysis of related mRNA expression and determination of PFOS concentration in livers.

2.3. Co-treatment of PFOS and PTDC

In order to determine the role NF-κB signaling in liver of zebrafish, another animal experiment was carried out. One hundred and eighty male zebrafish were randomly divided into three groups (control, PFOS, PFOS + PDTC), and each group has three replicates (n = 20 for each replicate). Control and PFOS groups were exposed to 0 and 0.08 mg/L of PFOS dissolved in dechlorinated-tap water and injected with 120 mg/kg/d saline by i.p., respectively. PFOS + PDTC group was exposed to 0.08 mg/L of PFOS dissolved in dechlorinated-tap water and injected with 120 mg/kg/d PTDC by i.p. After exposure for 21 d, 20 male zebrafish from each group were chosen to determine the body weight and body length. Ten livers in each group were used to fixed in Bouin's fixatve solution for histopathological observation. Ten livers in each group were collected t determine ROS. Another 10 livers in each group were snap frozen by liquid nitrogen and then stored at −80 °C for QRT-PCR analysis of related mRNA expression.

2.4. Determination of PFOS concentration in liver

The PFOS concentration in livers was determined by high pressure liquid chromatography (Waters, Milford, MA, USA) in conjunction with tandem mass spectrometry (Micromass, Manchester, UK). After aliquots of 5 μL were loaded on an Optiguard C18 pre-column (10 mm × 1 m inner diameter, Alltech, Deerfield, IL, USA), the analysis was carried out in a Fluophase column (50 mm × 1 mm inner diameter, Sercolab, Antwerp, Belgium). The flow rate of mobile phase (4 mM NH_4OAc/CH_3OH) was 40 μL/min. A gradient elution was increased from 45% to 90% CH_3OH in 3 min before reverting to the initial conditions after 5 min. PFOS was determined under negative electrospray ionization by signal reactant monitoring (m/z 499 → 99). The internal standard (1H, 1H, 2H, 2H-perfluorooctane sulfonic acid) was measured under the same conditions (m/z 427 → 81) with 0.1 s dwell time. Three independent replicates for each sample were prepared and analyzed. The

calibration curves of PFOS were linear in the concentration ranging from 0 to 400 ng/mL, and the correlation coefficient was 0.9998. The extraction recovery rate of PFOS was determined using deionized water which included known amounts of PFOS. The PFOS concentration was calculated from the standard curves and the mean extraction recovery of PFOS through the entire analytical procedure ranged from 86% to 105%. The limit of detection for PFOS was 0.172 μg/L.

2.5. Histological analysis

After being fixed for 24 h, the liver samples were rinsed by current water, subsequently dehydrated in a gradient alcohol, cleared in xylene, and then embedded in paraffin. Section of 5 μm were cut serially on a rotary microtome (Paraffin machine, Leica RM 2245, Germany), and collected on glass microscope slides. Then the sections were stained with Delafield's hematoxylin and alcoholic Eosin and mounted in neutral balsam. At last, the histological variations were observed under the optical microscope (Olympus, Japan) and quantified by Image Pro Plus (version 6.0, Media Cybemetics, USA).

2.6. Biochemical assays

The protein level of each sample was measured using BCA Protein Assay Kit (Jiangsu KeyGEN BioTECH Corp., Ltd, China) at the wavelength of 562 nm. Lysozyme content was determined using the turbidity method following the protocols of commercially available kits (Nanjing Jiancheng Bioengineering Institute, China) by transmittance at 530 nm. The activities of myeloperoxidase (MPO), acid phosphatase (ACP) and alkaline phosphatase (AKP) were determined using the colorimetric method, and the micro enzyme linked immunosorbent assay (ELISA) following the protocols of commercially available kits (Nanjing Jiancheng Bioengineering Institute, China) by absorbance at the wavelength of 460 nm, 520 nm and 520 nm, respectively. The ROS levels were determined using Chemical fluorescence method by measuring the oxidative conversion of 2′,7′-dichlorofluorescin diacetate (DCFH-DA, Nanjing Jiancheng Bioengineering Institute, China) to fluorescent dichlorofluorescein at the optimal excitation wavelength of 500 and 485 nm, and the optimal emission wavelength of 525 nm. NF-κB levels in liver samples were measured using commercially available a zebrafish NF-κB ELISA kits (Qiaodu biotechnology company, Shanghai, China) employing the quantitative sandwich enzyme immunoassay technique. Antibody specific for zebrafish NF-κB was precoated onto a 96-well microplate. A series of gradient dilutions of NF-κB standard and the concentrations of NF-κB in the samples were then interpolated from the standard curve.

2.7. RNA isolation and quantitative real-time PCR

Total RNAs were extracted from liver tissues using Trizol Reagent (Takara Biological Engineering Company, Dalian China) according to the manufacturer's instructions and digested by RNase-free DNase I (Promega Madison, WI, USA) to remove genomic DNA contamination. After being purified, the RNA samples were measured by absorbance at 260 nm and 280 nm with a NanoDrop Spectrophotometer (NanoDrop, USA). The RNA quality in each sample was verified by 1% agarose formaldehyde gel electrophoresis by visual inspection of 18S and 28S ribosomal RNA bands and by determining the A260 nm/A280 nm ratios. Then reverse-transcriptase reactions were performed with 500 ng total RNA using PrimeScript® RT Master Mix (Takara Biological Engineering Company, Dalian, China). Specific primers of IRF-1, IRF-7, IKBA,TGF-B,IL-1B,TNF-a, IRF-3, IL-6, IL-15, RAG-1, RAG-2, P65, and β-actin genes were designed with Beacon Designer 8, and all pairs of primers were listed in Table 1. Before quantitative analyses, the amplification efficiencies between target genes and internal control genes were compared, and the results showed that the amplification efficiencies between target genes and internal control genes were between 100% and 105%, and the differences were less than 5%. So the comparative Ct method was used to determine mRNA levels by real-time fluorescent quantitative PCR (QRT-PCR) in the study. QRT-PCR was performed using the two-Step QRT-PCR kit (Takara Biotechnology Company, Dalian, China) and FTC2000 QRT-PCR system (Canada). Melting curve analysis and agarose gel electrophoresis were performed to verify the specificity of PCR amplicons. Negative control without template was used to eliminate contamination. QRT-PCR was conducted in three replicate samples for each selected gene. The β-actin was selected as reference gene to normalize the mRNA levels of each target gene. A Ct-based relative quantification with efficiency correction normalized to β-actin was calculated using the $2^{-\Delta\Delta Ct}$ method [37].

2.8. Statistical analysis

The experimental data were presented as the means ± standard deviations (SD) and statistically analyzed using SPSS 20.0 software (IBM company). Before analysis for difference, the normality and equal variance for the data were checked by Levene's test. No violation of the assumptions for analysis of variance was detected. One-way analysis of variance (ANOVA) was used to detect the significant differences among treatments followed by Tukey test for multiple comparisons between treatment group and control group. The correlation analysis between PFOS dose and enzymes activities or the mRNA expressions, were detected with Pearson correlation coefficient [38]. Values were considered to be statistically significant when $p < 0.05$.

3. Results

3.1. PFOS concentration in livers

PFOS concentrations in livers were below determination limit in the control group during the exposure. PFOS accumulated in livers after exposure, and remarkably elevations were found in three exposure groups compared with the control group at three stages. After 7 d of exposure, PFOS levels in livers reached 2.40 ± 0.18, 3.72 ± 0.41, 5.07 ± 0.24 μg/g wet weight for the exposure groups of 0.02, 0.04, 0.08 mg/L, respectively. After exposure for 14 and 21 d, the levels were 7.19 ± 0.28, 13.58 ± 0.87, 22.84 ± 1.06 and 23.46 ± 1.65, 38.48 ± 2.71, 59.61 ± 3.18 μg/g wet weight, which were significantly higher than those at the respective concentrations after 7 d of exposure.

3.2. The effects of PFOS on growth performance in zebrafish

As shown in Fig. 1, the body weight and body length in zebrafish were significantly affected by PFOS. No mortality occurred during the 21-d exposure. No significant differences were found between groups in the initial body weight and body length of zebrafish ($p > 0.05$ among all treatments) prior to exposure.

After exposure for 7 d, no significant differences were observed in the body length between the control and treatment groups. However, body weight was significantly decreased by 18.9% ($p < 0.01$) and 17.35% ($p < 0.01$) in zebrafish exposed to 0.04 and 0.08 mg/L of PFOS compared to 0.370 g in the control group. After exposure for 14 and 21 d, body length were significantly decreased by 38.39% ($p < 0.01$) and 11.24% ($p < 0.01$) in 0.08 mg/L of PFOS group compared to 2.953 and 3.050 cm in the control group. The body weight was 0.378 and 0.383 g in the control groups after exposure for 14 and 21 days. Zebrafish exposed to 0.04 and 0.08 mg/L of PFOS had significantly decreases by 24.92% ($p < 0.05$) and 24.53% ($p < 0.05$), and 26.17% ($p < 0.01$) and 24.4% ($p < 0.01$) in body weight.

Table 1
Nucleotide sequences of primers used for quantitative real-time RT-PCR and product sizes.

	Name	Sequence(5′-3′)	Sequence(5′-3′)	Genbank accession No.
1	actin	F:CAAAGCCAACAGAGAGAAG	R:CATCACCAGAGTCCATCA	AY222742.1
2	ikbaA	F:CACCTTGCCATCATTCAC	R:CTGGTTGTTCTGTCTGTTC	AY163840.1
3	1L_1B	F:CCTGAACAGAATGAAGCAC	R:TAAGACGGCACTGAATCC	NM_212844.2
4	IL_6	F:GTGCTATTCCTGTCTGCAT	R:ACATCCTGAACTTCGTCTC	NM_001079833.1
5	IL_15	F:CTTCATCGCAGGATTGTG	R:GCAACAGAGCACTTAGAC	NM_001039565.1
6	IRF_1	F:GACTTACTCACGCCTTCA	R:TCTTCTCCTCCTTGTTGAC	NM_001040352.1
7	IRF_3	F:CTGCCACTCAACCACATT	R:CAACTGCTCCATCACCAT	NM_001143904.1
8	IRF_7	F:TGCTGAGGTCTACAATGC	R:CGTCTGAATAGCTGGTCTC	BC065902.1
9	P65	F:GGCTACTATGAGGCAGAT	R:CCACATCCTTCTTCTTCAC	AY163839.1
10	RAG1	F:CTCGTCAGAATCCTCAAGA	R:GCTTAGTGCTCAGATGGT	NM_131389.1
11	RAG2	F:GAAGGAACAAGATGGAGAAG	R:AGTAGCCTGTCTGAGATTC	NM_131385.3
12	SPI_1	F:CGGCTTAGTCCCTTTATTG	R:GTTGTTGTGCTCCTCTTG	NM_001045416.1
13	TGFB1	F:GAGGTTATACTGCGACTGT	R:CGGCTTCACCATCATATCT	NM_182873.1
14	TNF_a	F:TGGTGATGGTGTCTAGGA	R:GGTCTTATGGAGCGTGAA	NM_212859.2
15	IL-10	F:CATTTGTGGAGGGCTTTC	R: GGTTCCAAGTCATCGTTG	NM_001020785.2

Abbreviations: IL-1β, Interleukin 1 beta; IL-6, Interleukin 6; IL-15, Interleukin 15; IRF-1, Interferon regulatory factor 1; IRF-3, Interferon regulatory factor 3; IRF-7, Interferon regulatory factor 7; P65, Transcription factor p65; RAG-1, Recombination activating gene 1; RAG-2, Recombination activating gene 2; TGF-1, Transforming growth factor beta 1; TNF-a, Tumor necrosis factor alpha; IκBα, NF-kappaB inhibitor alpha-like protein A.

3.3. The effects of PFOS on the microstructure of the liver tissues in zebrafish

Histopathological changes were examined in section of liver tissues after exposure to PFOS for 21 d under the light microscope (Fig. 2).

As shown in Fig. 2, normal histological structure was observed in the control group. The liver cells with regular shape and clear cell boundary were nearly round and arranged closely. The nucleus, which was large and clear, was located in the center of the cells (Fig. 2A, E, I). After 7 d of exposure to PFOS, the nucleus became shrinking in the liver cells exposed to 0.08 mg/L of PFOS (Fig. 2D). After 14 d of exposure, serious damages such as shrinkage of nuclear and cell volume, dissolved cell membranes and disappeared cell structure were found in liver cells of the fish exposed to 0.08 mg/L of PFOS (Fig. 2H). After 21 d of exposure, more severe deformation and disorder were appeared, cell membrane dissolution increased, cell boundary was fuzzy, even some liver nuclei disappeared, number of vacuoles was increased obviously after exposed to 0.04 mg/L and 0.08 mg/L of PFOS (Fig. 2J, K, L).

3.4. Effects of PFOS on ROS content and NF-κB expressions in livers of zebrafish

After 7 d of PFOS exposure, the level of ROS was 3426.076 mg/pro in the control group. The ROS content was significantly increased in fish exposed to 0.08 mg/L of PFOS compared with that in the control group ($p < 0.05$) (Fig. 3B). NF-κB concentrations were remarkably elevated in zebrafish exposed to 0.04 and 0.08 mg/L of PFOS ($p < 0.05$) compared to 2022.303 pg/ml of NF-κB in the control group (Fig. 3A). After exposure for 14 and 21 days, the levels of NF-κB and ROS in all PFOS-exposed fish were significantly higher than those of the control group (Fig. 3A, B).

3.5. Effects of PFOS on activities of immune-related enzymes in livers of zebrafish

The effects of PFOS on levels of immune-related enzymes were shown in Fig. 3. After 7 d of PFOS exposure, the activities of MPO and LSZ were significantly increased by 25.20% and 64.93% in fish exposed to 0.08 mg/L of PFOS compared with 5.084 U/g tissue and 17.488 U/mgprot in the control group ($p < 0.05$), leading to a significant positive correlation between PFOS dose and the activities of MPO and LSZ ($r = 0.717$, $r = 0.805$, $p < 0.01$) (Fig. 3E, F). However, the activities of ACP and AKP were significantly decreased by 7.885%, 17.95%, 18.370% and 39.538% in zebrafish exposed to 0.04 and 0.08 mg/L of PFOS compared with 100.492 and 89.421 King unit/prot in the control group, respectively (Fig. 3C, D). A negative correlation was found between FPOS concentration and the activities of ACP and AKP ($r = -0.740$, $r = -0.918$, $p < 0.01$).

After exposure for 14 and 21 d, the MPO activities were notably elevated in fish exposed to 0.04 and 0.08 mg/L of PFOS after exposure for 14 d and in all PFOS-exposed fish after exposure for 21 d (Fig. 3E), resulting in a significant positive correlation between PFOS dose and MPO activity ($r = 0.914$, $r = 0.878$, $p < 0.01$). On the contrary, the activities of ACP and AKP were significantly reduced in all PFOS-exposed zebrafish compared with the control (Fig. 3C, D), causing a negative correlation between PFOS dose and the activities of ACP and AKP ($r = -0.868$, $r = -0.796$; $r = -0.825$, $r = -0.780$, $p < 0.01$). The fish had significantly decreased LSZ levels in 0.08 mg/L of PFOS group

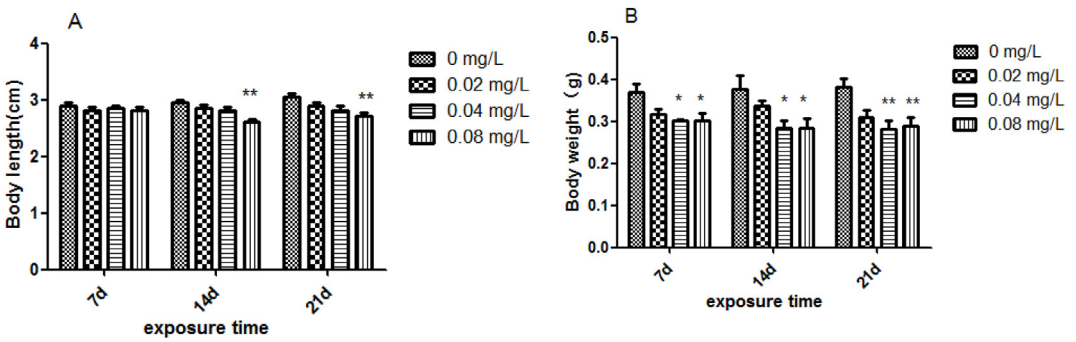

Fig. 1. Effects of PFOS on growth parameters in male zebrafish exposed to different concentrations of PFOS for 7, 14 and 21 d. A: Body length; B: Body weight. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

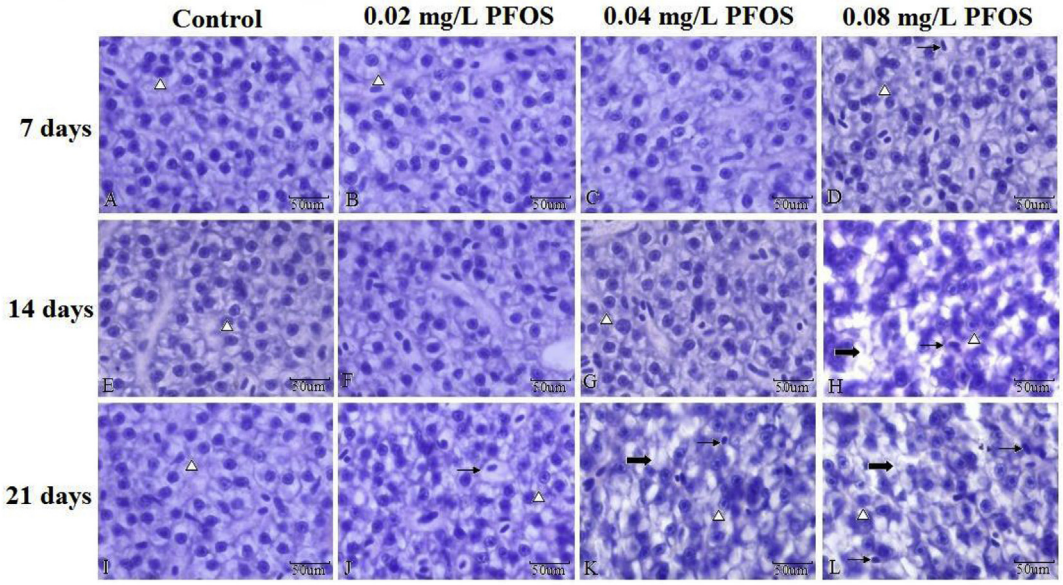

Fig. 2. Effects of PFOS on the microstructure of the liver tissues. A, E, I: the liver of control group for 7, 14 and 21 d; B, F, J: the liver exposed to 0.02 mg/L of PFOS for 7, 14 and 21 d; C, G, K: the liver exposed to 0.04 mg/L of PFOS for 7, 14, and 21 d; D, H, L: the livers exposed to 0.08 mg/L of PFOS for 7, 14, and 21 d. The scale was 50 μm. Δ For Hepatic cells, ⇒ for vacuolus, → for nuclear atrophy.

after exposure for 14 d and in zebrafish exposed to 0.04 and 0.08 mg/L of PFOS after exposure for 21 d (Fig. 3F). There was a negative correlation between the exposure concentration of PFOS and LSZ activities ($r = -0.634$, $r = -0.792$, $p < 0.01$) .

3.6. Effects of PFOS on immune-related gene expressions in male zebrafish

The expression profiles of immune-related genes in the liver of the zebrafish after exposure to PFOS were shown in Fig. 4. After 7 d of exposure, a strong down-regulation of IkBa gene expression was detected in all PFOS exposure group (Fig. 4B). The mRNA levels of RAG-1 and RAG-2 were significantly elevated in zebrafish exposed to 0.08 mg/L of PFOS (Fig. 4K, L). The mRNA expression levels of p65, IL-15, TGF-β, IRF-1, IRF-3, and IRF-7 were significantly increased in zebrafish exposed to 0.04 and 0.08 mg/L of PFOS (Fig. 4A, E, G, H, I, J). The zebrafish had higher mRNA levels of IL-6 and TNF-α in all PFOS exposure group (Fig. 4D, F). However, the IL-1β mRNA levels were relatively unchangeable (Fig. 4C).

After exposure for 14 and 21 d, similar down-regulation of IkBa expressions was observed in all PFOS exposure groups (Fig. 4B). But the mRNA expression levels of IL-1β, TNF-α, TGF-β, IRF-1, IRF-7, RAG-1 and RAG-2 were significantly enhanced in fish exposed to 0.04 and 0.08 mg/L of PFOS after exposure for 14 d and in all PFOS exposure groups after exposure for 21 d (Fig. 4C, F, G, H, J, K, L). The mRNA expression levels of p65, IL-6, IL-15 and IRF-3 were significantly increased in all PFOS exposure zebrafish (Fig. 4A, D, E, I). The correlation analysis showed that there was a negative correlation between PFOS concentration and IkBa leves ($r = -0.675$, $p < 0.01$), while a positive correlation between PFOS dose and the expressions of IL-1β, TNF-α, TGF-β, IRF-1, IRF-3, IRF-7, RAG-1, RAG-2, p65, IL-6, and IL-15 ($r = 0.935$, $r = 0.352$, $r = 0.599$, $r = 0.863$, $r = 0.905$, $r = 0.724$, $r = 0.941$, $r = 0.762$, $r = 0.740$, $r = 0.709$, $r = 0.698$).

3.7. The response of liver in male zebrafish after co-treatment of PFOS and PDTC

After exposure for 21 d, PFOS significantly inhibited the growth including the body length and body weight. However, the inhibition was obviously relieved after coexposure to PFOS and NF-κB antagonist compared with the PFOS group (Fig. 5A and B). The morphology of livers was impaired after PFOS exposure, including disordered microstructure, fuzzy cell boundary, disappeared liver neclei, increased vacuoles compared with the control group (Fig. 6B). After cotreatment with FPOS and PDTC, the impairment was relieved (Fig. 6C). The ROS content was significantly increased after exposure to PFOS for 21 d. However, after coexposure to PFOS and PDTC, the induction level of ROS was remarkably inhibited by the antagonist effects of NF-κB inhibitor (Fig. 7A). Similar to the results of ROS level, the mRNA expressions of P65, IL-1B, IL-6, IL-15, TNF-α, TGF-β, IRF-1, IRF-3, IRF-7, RAG-1, and RAG-2 were significantly increased after PFOS exposure for 21 d, while the elevation of mRNA expressions were all significantly decreased by co-treatment with PDTC (Fig.7B, 7D-7M). However, the expressions of IkBa and IL-10 were remarkably reduced after PFOS exposure. Co-treatment with PDTC reversed the expressions of IkBa and IL-10, and the expressions of IkBa and IL-10 mRNA were significantly increased compared with the PFOS-exposed group (Fig. 7C, N).

4. Discussion

The immune system is a host defense system that protects against many physical and chemical factors even disease. Usually, the changes induced by chemical toxic agent occur earlier than other toxic symptoms. The liver of fish is the most important detoxification organ, and the most important target organ when the fish is infected by the external poison. Many studies have shown that PFOS has an adverse effect on liver of fish [22,39–41]. As a result, we continuously exposed zebrafish to different doses PFOS (0, 0.02, 0.04 and 0.08 mg/L) for multiple end-points (7, 14 and 21 d) to investigate effects of PFOS on immune system and the underlying toxic mechanisms in the liver of zebrafish in this study.

The persistence and bioaccumulation of PFOS in biota have caused scientific and societal concern [42]. This concern has been confirmed because PFOS was measured at detectable concentrations and in several organisms including wildlife and fish [43]. PFOS has been determined at high concentrations in livers of many fish species, such as plaice *Pleuronectes platessa* (7760 ng/g ww) and feral carp *Carassius auratus* (9031 ng/g ww) from Belgium [11]. A noticeable absorption of PFOS was found in the marine medaka embryo from 2 to 10 dpf, and the PFOS concentration was up to 12.1–69.7 μg/g ww at the end of exposure [23]. In this study, PFOS accumulated in livers after exposure,

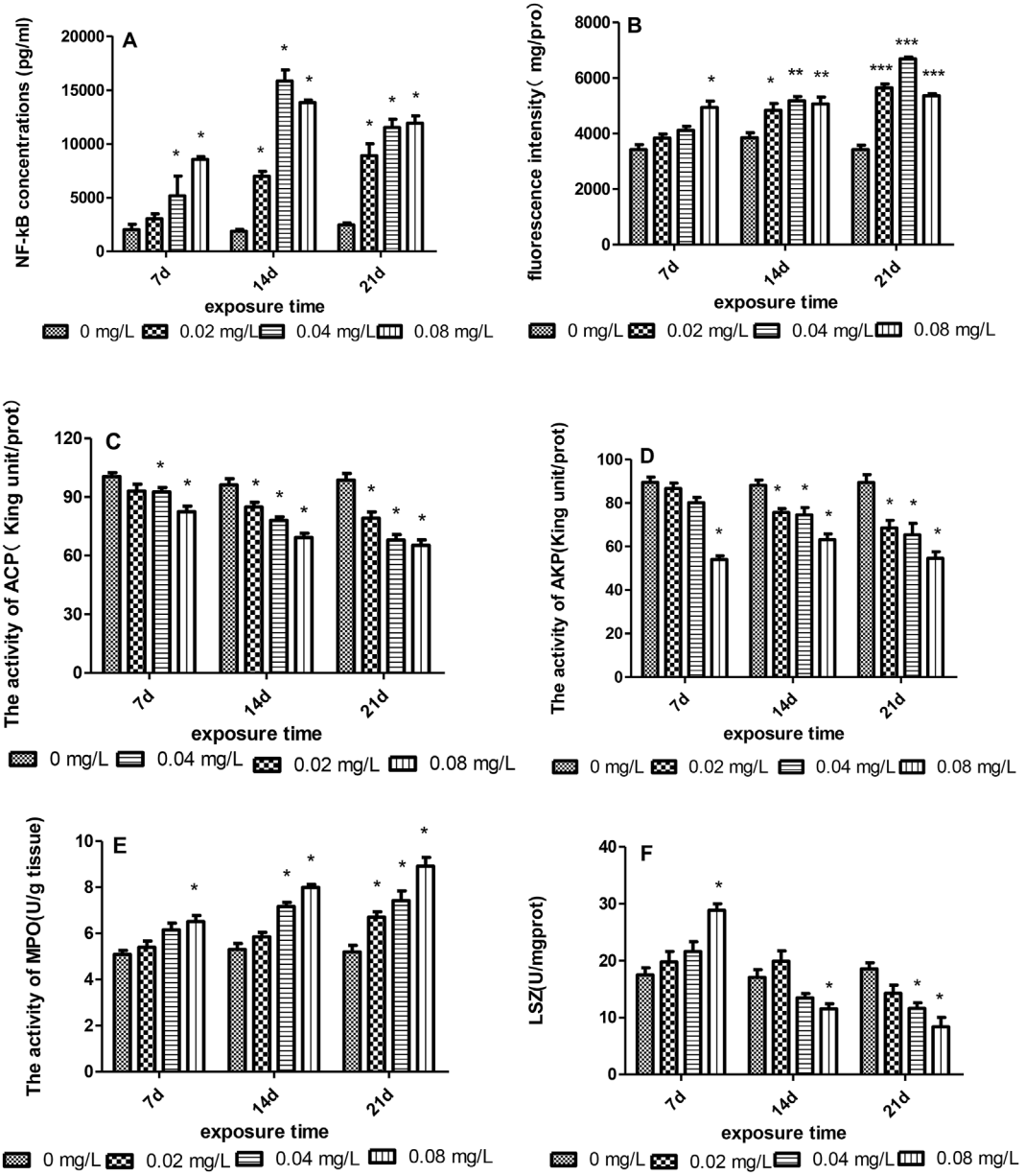

Fig. 3. Effects of PFOS on the activities of immune-related enzymes and ROS levels in the livers of zebrafish exposed to different concentrations of PFOS for 7, 14 and 21 d. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

and remarkably elevations were found in three exposure groups compared with the control group at three stages. These results indicated that PFOS had a high bioaccumulation potential in fish.

Growth is the net result of many essential processes, such as assimilation, respiration, and excretion, which is one of the most important indices to evaluate the effects of environmental stressors on animals at the population level [44]. Some studies demonstrated PFOS could inhibit the growth of fish [45]. In the present study, we measured the growth performance of zebrafish by body weight and length. We found that the PFOS exposure significantly reduced the body weight and length in male zebrafish. Similar results were obtained in previous reports [22,46]. Therefore, it is indicated that chronic PFOS exposure could inhibit the growth of fish.

Histopathology is considered to be a biomarker for analyzing the ultra-structural changes of tissues. It has been proved that PFOS could cause the damage of the liver [47,48]. In this study, the histopathological changes were observed in the liver tissues of zebrafish after PFOS exposure. With the increasing of exposure concentration and time, the phenomenon of atrophy or deviation from the center of the cell and the loosening of cytoplasm increased. The number of vacuoles increases obviously, and the irregular condition of hepatocytes and the damage of liver tissue structure became serious. Our results were consistent with previous studies [40,46]. These results demonstrated that PFOS obviously led to histopathological damages of liver in zebrafish.

Lysosomes are usually regarded as biomarkers for functional defense and the health status in aquatic animals because they can eliminate bacteria by hydrolyzing components of the bacterial cell walls [49]. Moreover, lysosomes are sensitive to ROS triggered by immune stress or stressors [50]. Once the ROS is overproduced, which can increases the permeabilization of lysosomal membranes and the release of lysosomal proteolytic enzymes to the cytosol, such as lysozyme (LSZ), acid phosphatase (ACP), alkaline phosphatase (AKP) [51]. Therefore, the stability of lysosomal membranes and the lysosomal proteolytic enzymes are widely considered to be immune biomarkers under stress conditions. ACP and AKP are the main enzymes in the lysosomes and are regarded as a reliable indicator for toxicological evaluation of

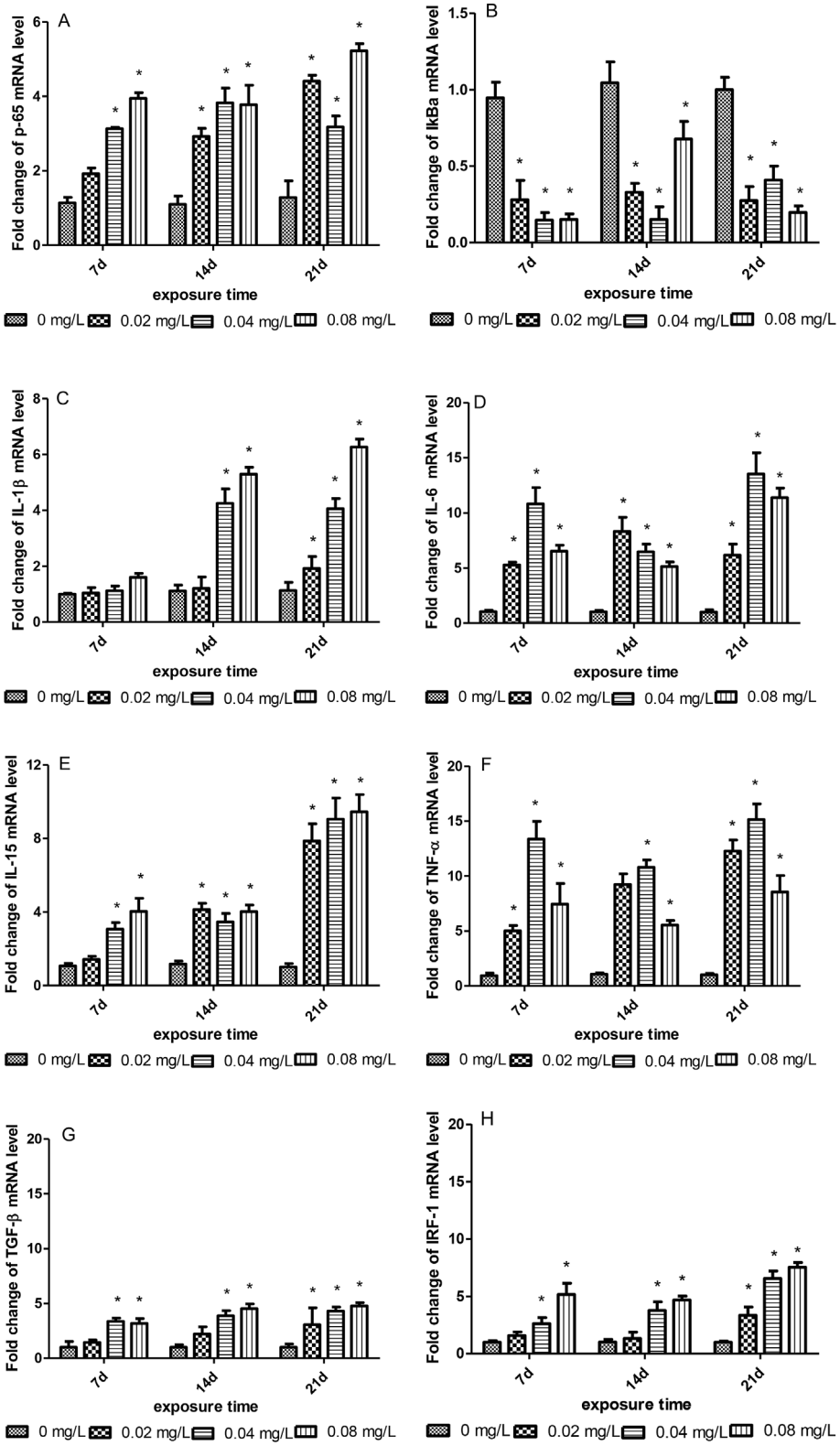

Fig. 4. Effects of PFOS on the expressions of immune-related genes in the liver of male zebrafish exposed to different concentrations of PFOS for 7, 14 and 21d. A: P65; B: IKBa; C: IL-1β; D: IL-6; E: IL-15; F: TNF-α; G: TGF-β; H: IRF-1; I: IRF-3; J: IRF-7; K: RAG-1; L: RAG-2. n = 10. Asterisks represent significantly different when compared with controls, respectively:*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

xenobiotics [52]. In this study, the activities of ACP and AKP were inhibited in liver of male zebrafish after PFOS exposure. The activities of ACP and AKP were decreased with the increase of PFOS concentration and exposure time compared with the controls, which was agreement with the activities of AKP and ACP by PFOS exposure [53].

Lysozymes (LSZ), as one of the primary enzymes in the lysosomes, are vital defense factors for fish innate immune system, which can protect the body from the invasion of foreign pathogens by their

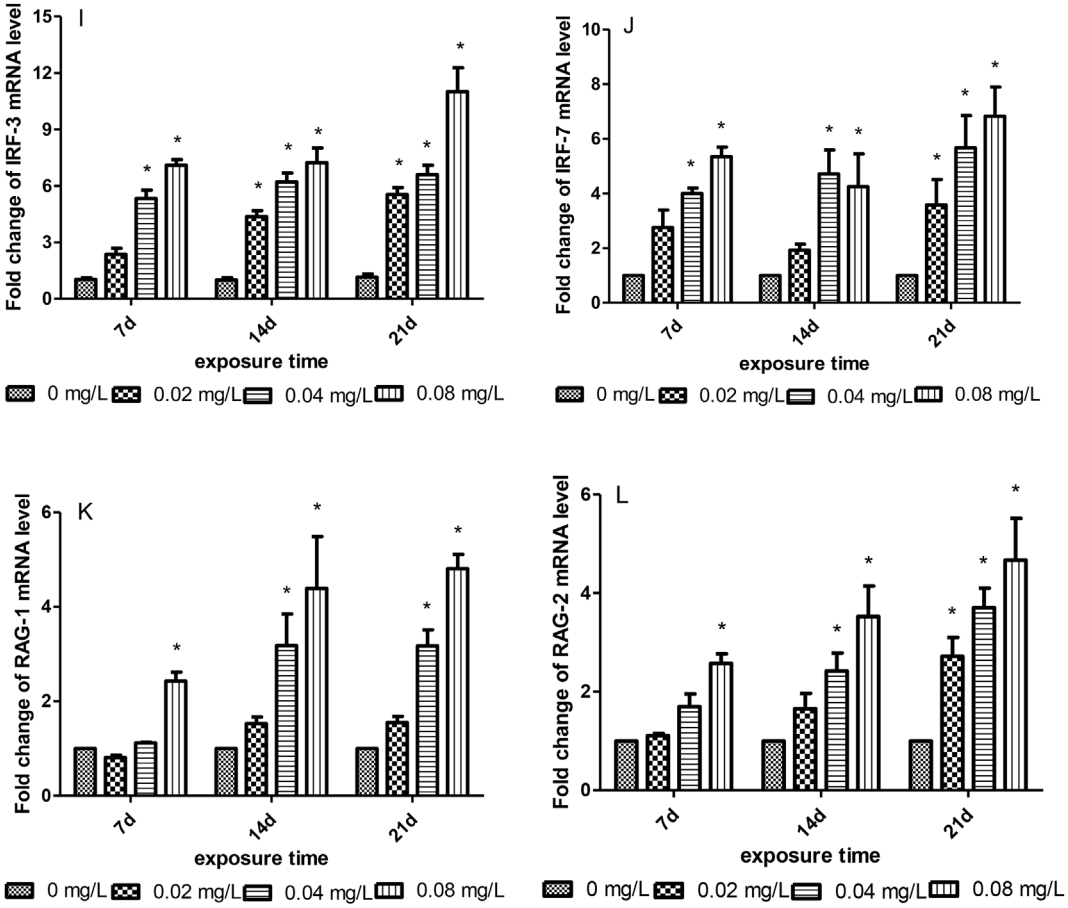

Fig. 4. (*continued*)

antibacterial, anti-inflammatory, anti-virus and other functions [54,55]. Therefore, LSZ levels were usually used as the most important innate immune indexes in aquatic organisms, which directly reflected the level of immunity [56]. The present study showed that the activity of LSZ was increased after 7 d of exposure to PFOS, however, with the increase of exposure time and concentrations, the activity of LSZ was decreased in 0.04 and 0.08 mg/L of PFOS groups compared with the control group. It might be because that low dose of PFOS at the short exposure time could stimulate fish immunity to produce compensatory stress, while high dose of PFOS at the long time exposure exceeded the ability of physiological regulation in liver, and then produced immune toxicity.

Myeloperoxidase (MPO) is one of the key enzymes involved in the inflammatory response, and is a potent inflammatory medium for many activated leukocytes [57,58]. Based on hydrogen peroxide produced by chloride ion and neutrophils, MPO catalyzes the production of hypochlorous acid and a variety of free radicals. If the body can not effectively remove these oxidants and free radicals, it will cause a variety of pathological processes and tissue damage. Thus, MPO can cause tissue damage through the production of toxic free radicals and oxides. MPO regulates inflammatory response in a variety of ways, and its content can reflect the inflammatory response and injury of the tissues [59]. In this study, the content of MPO was significantly decreased after PFOS exposure at different exposure time and concentration. Thus, we assumed that PFOS exposure make liver cells of zebrafish not effectively eliminate the oxidant and free radicals, which cause a certain degree of

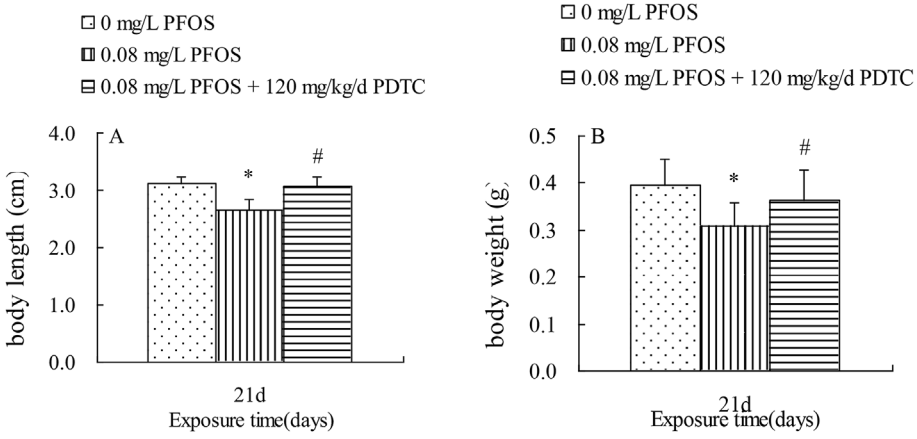

Fig. 5. Effects of PFOS and PDTC on the growth parameters in male zebrafish cotreatment with PFOS and PDTC for 21 d. A: body length; B: body weight. Asterisks represent significantly different when compared with controls, respectively. Hashtags represent significantly different between PFOS and PDTC groups. *or #$p < 0.05$, **or ##$p < 0.01$, *** or ###$p < 0.001$.

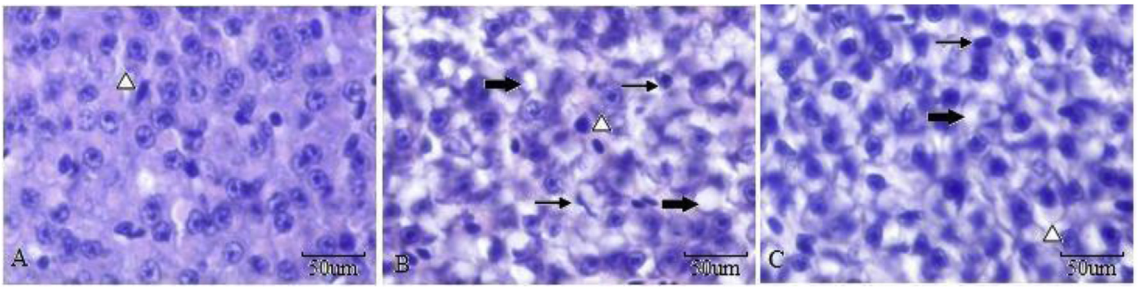

Fig. 6. Effects of PFOS and PDTC on the microstructures of the liver tissues after exposure for 21 d. A: the liver of control group; B: the liver exposed to PFOS; C: the liver after cotreatment with PFOS and PDTC. The scale was 50 μm. Δ For Hepatic cells, ⇒ for vacuolus, → for nuclear atrophy.

injury and pathological changes.

ROS is a major factor in regulating apoptotic processes. Increased accumulation of ROS is involved in induction of apoptotic death in many types of cells [60,61]. Previous studies have shown that PFOS exposure can result in oxidative stress by increasing the generation of free oxygen radicals and interfering with the antioxidant defense systems [62]. In the present study, a significantly dose-dependent production of ROS was observed in liver after PFOS exposure, indicating that PFOS accelerated the formation of ROS and induced the occurrence of potential oxidative stress in the liver of males. Similar results were also shown in previous studies [57,58,63,64]. This result further supported evidence for histological changes of liver caused by PFOS. These results show that ROS plays important proapoptotic roles in PFOS-induced apoptosis in liver.

Cytokines are regarded as the reliable biomarkers for disease determination because of their susceptibility to environmental toxicants [65]. With the increased PFOS exposure, the immune expressions of IL-1β, IL-6, IL-15, TNF-α, TGF-β, IRF-1, IRF-3, IRF-7, RAG-1, and RAG-2 mRNA were significantly elevated in a concentration- and time-dependent manner, consistent with previous observations [66,67]. Moreover, the changes of most mRNA expressions were significantly correlated with the PFOS concentrations, which demonstrated a coordinated modulation process in liver responded to increased PFOS exposure. PFOS increased the expression and secretion of IL-1β in dose- and time dependent manners in astrocytes [66]. Also, the production of TNF-α and IL-6 were promoted in mixed spleen cell cultures after exposure to 50 mg/kg and 125 mg/kg PFOS, indicating that PFOS promoted pro-inflammatory effects on peripheral immune organs [68]. The released pro-inflammatory productions during the inflammatory process result in the pathogenesis of degenerative and inflammatory disease [69], in accordance with the histopathological observation in our study.

Inflammatory cytokines are modulated at multiple levels: transcriptional, post-transcriptional and post-translational levels. The transcriptional adjustment of inflammatory cytokines are very complicated including a lot of transcriptional factors, such as Nuclear factor-κB (NF-κB) and many members of the C/EBP, ATF/CREB and STAT family [67]. NF-κB is considered be an important regulator of pro-inflammatory gene expressions, which can be activated and transferred from the cytoplasm to the nucleus to promote the transcription of pro-inflammatory genes by various inflammatory stimuli and environmental stressors, including IL-1β [70]. In most cell types, NF-κB complexes (composed of P65 and P50 subunits) are retained in the cytoplasm by IκB [71]. Under stress, the phosphorylation of IκB by IκB kinase complex expropriated the subunits of p65 and p50 and triggered the NF-κB activation, which causes the degradation of IκB [72]. Previous studies indicated that PFOS could result in differential regulation of NF-κB. PFOS caused translocation of NF-κB from the cytoplasm to the nucleus and activated the NF-κB signaling pathway in C6 glioma cell lines [66]. Moreover, it has been reported that PFOS affects the regulation of the immune system by simultaneously increasing tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) expression via the induction of NF-κB transactivation [67]. Pro-inflammatory IL-1β is an NF-κB-drived gene [73]. NF-κB is also the first transcription factor proved to be redox-regulated, and the generation of ROS is closely related to the NF-κB signaling pathway [74]. In this study, ROS level was significantly increased, and the phosphorylation and degradation of IκBα were induced, which was accompanied with the increase of nuclear p65 NF-κB. Similar results were obtained in a study by Singh et al. (2012), which found that PFCs caused IκB degradation in HMC-1 cells, which was followed by the elevation of p65 NF-κB in nucleus [75]. Furthermore, we found that PFOS exposure elevated the levels of ROS, the expressions of IL-1β, IL-6, TNF-α mRNA, and the activities of NF-κB in livers, which indicated that PFOS could facilitate the secretion of pro-inflammatory cytokines through activating the NF-κB signaling pathway, consistent with the positive correlation between the NF-κB activity or p65 expression and the expressions of pro-inflammatory cytokines. Study on PFOA-exposed zebrafish also indicated that NF-κB pathway is the important pathway to mediate inflammatory cytokine in zebrafish spleen [76]. In addition, pretreatment with AKT inhibitor (LY294002) could significantly weaken PFOS-induced NF-κB activation and IL-1β secretion [66]. From our results, after co-treat the liver using PFOS and NF-κB inhibitor, the growth inhibition, the morphological damage, the induction of ROS levels, and the expressions of immune-related mRNA were reversed, suggesting a potential role of NF-κB in immunomodulation upon PFOS treatment. Similar results were obtained after coexposure of macrophages to BPA with NF-κB inhibitor [77]. PDTC treatment also significantly attenuated NF-κB activation and inhibited the increase in PFOS-induced TNF-α and IL-6 in hepatocyte [63,64]. Moreover, study on RAW264.7 cells also showed that treatment with PDTC inhibited the morphologic changes, reversed the expressions of immune-related genes and key membrane molecules, and the translocation of p65 from cytosol to nucleus [57,58]. The suppression of NF-κB signaling restrained the immune-related parameters, which demonstrated the role of NF-κB in the immune modulation effect of PFOS on liver of zebrafish. Thus, all these confirmed that the NF-κB signaling pathway was involved in PFOS-mediated immune and inflammatory reaction of liver. The NF-κB signaling pathway underlined in the immunomodulatory mechanism of PFOS exposure in liver, this remains elusive and need to be further investigated.

5. Conclusions

In summary, this study explored the effects of PFOS on growth performance, histopathology, immune-related enzymes and the expressions of immune-related genes in liver. Our results clearly demonstrated FPOS exposure could significantly inhibit the growth of male zebrafish. Moreover, FPOS could disturb immunomodulatory functions on liver of male zebrafish by changing the normal structure of liver, disturbing the activities of immune-related enzymes, and a series of gene transcriptions involved in immune regulation in a concentration-dependent manner within a relatively low and environmentally

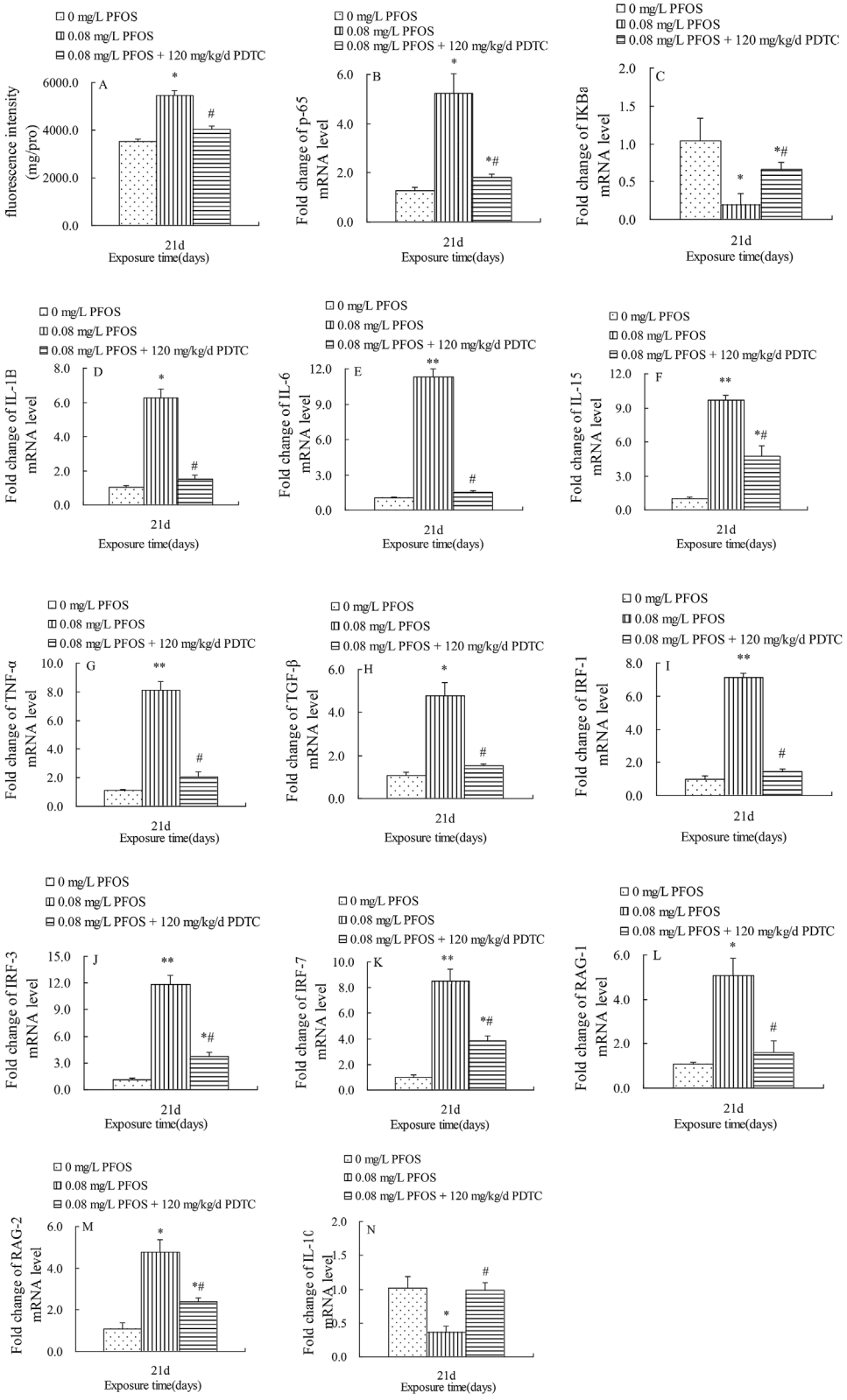

Fig. 7. Effects of PFOS and PDTC on the ROS content and the expressions of immune-related genes in the liver of male zebrafish cotreatment with PFOS and PDTC for 21 d. A: ROS; B: P65; C: IKBa; D: IL-1B; E: IL-6; F: IL-15; G: TNF-α; H: TGF-β; I: IRF-1; J: IRF-3; K: IRF-7; L: RAG-1; M: RAG-2; N: IL-10. n = 10. Asterisks represent significantly different when compared with controls, respectively. Hashtags represent significantly different between PFOS and PDTC groups. *or $\#p < 0.05$, **or $\#\#p < 0.01$, *** or $\#\#\#p < 0.001$.

relevant concentration. PFOS-induced pro-inflammatory effect of hepatocytes was observed, and the involvement of NF-κB signaling pathway was participated in its action mechanism. These findings provide further evidence that PFOS interferes with the immune regulation of liver under in vivo conditions. The data provided in this study shed new light on the understanding of PFOS immunotoxicity in fish.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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