Fish and Shellfish Immunology 86 (2019) 239–245

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Impaired immune function and structural integrity in the gills of common carp (*Cyprinus carpio* L.) caused by chlorpyrifos exposure: Through oxidative stress and apoptosis

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ARTICLE INFO

Keywords:
Chlorpyrifos
Common carp
Oxidative stress
Apoptosis
Environmental pollutants
Immune

ABSTRACT

As one of the mucosal lymphatic tissues, the gill is an important immune organ in fish. Water environmental pollutants enter fish body through the gill. Therefore, the gill is the initial site where pollutants produce toxic effects in water. Chlorpyrifos (CPF), a broad-spectrum organophosphate insecticide, is widely used for agricultural pests and causes river pollution. In the present study, we investigated histopathological effect, oxidative stress indexes (SOD, GSH, T-AOC, and MDA), and apoptosis-related genes (P53, PUMA, Bax, Bcl-2, Apaf-1, Caspase-9, and Caspase-3) in the gills of common carp exposed to CPF. The results indicated that CPF exposure decreased SOD, T-AOC, and GSH; increased MDA; decreased Bcl-2 mRNA expression; and increased P53, PUMA, Bax, Apaf-1, Caspase-9, and Caspase-3 mRNA expressions in common carp gills. Our results proved that CPF exposure caused oxidative stress and apoptosis in common carp gills; CPF exposure destroyed the structural integrity and affected the immune function through oxidative stress and apoptosis in common carp gills. These will provide evidence for the toxic effects of water environmental pollutants on immune function and structural integrity in fish gills.

1. Introduction

Fish immunology has been paid extensive attention because it plays an important role in the evolution of immune system. As one of mucosal lymphatic tissues, the gill is an important immune organ in fish. Its immune function and structural integrity are necessary for normal growth and disease resistance [1]. Aquatic system is exposed to a large number of pollutants, including pesticides [2–4]. These pollutants enter fish body through gills. Therefore, the gill is the initial site where pollutants produce toxic effects in water.

Chlorpyrifos (CPF), an organophosphate insecticide, is widely used in prevention and control of agricultural pests. However, CPF can pollute rivers, cause its accumulation in fish, and affect neurodevelopment in humans. CPF was detected in two rivers (Strymonas and Nestos) in northern Greece [5]. There was high concentration of CPF in Qaraoun Lake and the Hasbani River in Lebanon [6]. Sun and Chen found CPF residue in one hundred thirty-seven samples of fish bought on the market in Taiwan [2]. Prenatal exposure to CPF affected neurodevelopment in 3-year-old children [7]. Sun et al. found that $CuSO_4$ and As_2O_3 can decrease superoxide dismutase (SOD) activity and cause oxidative stress in chicken brains [8]. CPF exposure can decrease total antioxidant capacity (T-AOC) in Nile tilapia gonads [9]; decrease glutathione (GSH) content and increase malondialdehyde (MDA) content in guinea-pig gills [10]; and can cause oxidative stress. Oxidative stress can induce apoptosis [11]. P53, Bax, Bcl-2, and Apaf-1 genes play important roles in regulating apoptosis [12–14]. Increased Apaf-1 mRNA expression was found during apoptosis in zebrafish larvae treated by Carbendazim [15]. PUMA, a pro-apoptosis gene, can cause rapid cell death through a p53-dependent mechanism [16]. Caspase-9 and Caspase-3 are the executioners of apoptosis [17]. Relative mRNA expressions of PUMA and caspase-9 increased when Phorbol 12-myristate 13-acetate induced apoptosis in zebrafish embryos [18]. Some studies found that toxic chemicals can cause apoptosis through increasing P53, PUMA, Bax, Apaf-1, Caspase-9, and Caspase-3 mRNA expressions; decreasing Bcl-2 mRNA expression. CPF caused the apoptosis of SH-SY5Y cells [19], QSG7701 cells [20], and common carp brains [21] through analyzing P53, Bax, Bcl-2, Caspase-9, and Caspase-3 expressions.

Structural integrity and immune function of fish gills may be affected through apoptosis and oxidative damage [22]. However, relationship between CPF-induced apoptosis and oxidative stress, and

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https://doi.org/10.1016/j.fsi.2018.08.060
Received 2 August 2018; Received in revised form 24 August 2018; Accepted 30 August 2018
Available online 31 August 2018
1050-4648/

structural integrity and immune function of gills is unknown in fish. Therefore, we investigated histopathological effect, oxidative stress indexes (SOD, GSH, T-AOC, and MDA), and apoptosis-related genes (P53, PUMA, Bax, Bcl-2, Apaf-1, Caspase-9, and Caspase-3) in the gills of common carp exposed to CPF, which will provide evidence for toxic effect of water environmental pollutants on immune function and structural integrity in fish gills.

2. Materials and methods

2.1. Animal models and tissue samples

All procedures used in this study were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University under the approved protocol number SRM-06. Sixty juvenile common carp (average body length 14.18 ± 1.55 cm, average body weight 95.72 ± 9.34 g) purchased from a freshwater fish farm were randomly distributed in six laboratory tanks (1.0 × 0.5 × 0.8 m) (10 fish per tank), and were fed with feed. After 15 days of adaptation, the fish in three tanks (the CPF group) were added CPF with 14.5 μg CPF · L^{-1} water. The toxic concentration was determined based on 1/50 of the 96-h LC_{50} value of CPF in carp [23]. One-third of water in the tank was replaced daily. Water quality parameters including temperature, pH, and dissolved oxygen were measured before and after each water change. During the tests, water at 20 ± 1 °C, pH 7.4 ± 0.2, and dissolved oxygen above 8 mg L^{-1} was maintained. Photoperiod was 12-h light/12-h dark. The fish were fed with a commercial diet (containing protein 47.7% and lipid 10.7%) twice daily (08:00 and 16:00) to apparent satiation. During the experimental period, no fish died in the control group and two fish died in the CPF group.

On the 15th, 30th, and 45th days of the experiment, twelve common carp were randomly selected (two fish each tank) and were sacrificed. Fish gills were immediately removed. Then the tissues were put on ice plate and were rinsed in saline. Each sample was divided into five parts. The first part was fixed with 2.5% glutaraldehyde for ultrastructural examination. The second part was fixed in 10% formaldehyde solution for histological examination and TdT-mediated dUTP nick end labeling (TUNEL) assay. The third part was homogenized for the determination of antioxidant indices (T-AOC; SOD, GPx, GST, and CAT activities; and GSH content) and oxidative stress indices (iNOS activity; and NO, H_2O_2, and MDA contents). The last part was immediately placed in liquid nitrogen, and then was stored in a −80 °C freezer for the determination of relative mRNA expressions of apoptosis factors (P53, PUMA, Bax, Bcl-2, Apaf-1, Caspase-9, and Caspase-3).

2.2. Ultrastructural observation

On the 45th day of the experiment, tissue samples not exceeding 1 mm^3 in size were placed in 2.5% glutaraldehyde at 4 °C for 3 h. The samples were washed in 0.1 M sodium phosphate buffer at 4 °C for 1 h and were stained in 1% tetral sodium phosphonium phosphate buffer at 4 °C for 1 h. And then the tissue was dehydrated in a gradient series of ethanol (50, 70, 90, and 100% ethanol for 10 min, respectively). The samples were washed using pure acetone and were soaked into embedding solution overnight. The tissue specimens were embedded in epoxy resin and were cut into ultra-thin sections. Ultrathin sections were stained with uranyl acetate and lead citrate for the observation of the ultrastructure of the gills using transmission electron microscope (TEM, model JEM-1200EX, JEOL JEM, Japan).

2.3. Histopathological observation

For histological examination, gill tissues fixed in 10% formaldehyde solution were dehydrated through a graded series of ethanol, were cleared in xylene, and were sembedded in paraffin. Sections that were 5–6 mm thick were prepared from paraffin blocks using a Reichert microtome. These sections were then stained with hematoxylin-eosin. The sections were examined under a Leica DME 100 light microscope.

2.4. TUNEL assay

The gill tissues were taken from the 10% formaldehyde solution, were dehydrated in a graded series of ethano (70, 95, and 100%), and were embedded in paraffin wax. Paraffin-embedded gill tissues were sectioned and were mounted between two glass slides. The slides were dewaxed with different gradients of alcohol (100, 95, 90, 80, and 70%) and xylene at room temperature. And then the slides were washed with distilled water (dH_2O, 2 × 3 min). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide at room temperature for 10 min, and then the slides were rinsed with dH_2O. In Situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, USA) was used to identify apoptosis according to the manufacturer's instructions. The slides were incubated with a terminal TdT/nucleotide mixture at 37 °C for 1 h and were rinsed with phosphate-buffered saline with 0.05% Triton X-100 (PBST, 3 × 5 min). The slides were incubated with horseradish peroxidase at 37 °C for 30 min, and were rinsed with PBST (3 × 5 min). The slides were counterstained with hematoxylin. The slides were rinsed in tap water for 10 min, and the slides were rinsed once with dH_2O. The slides were dehydrated with different gradients of alcohol (100, 95, 90, 80, and 70%) and were deetherified with xylene. Finally, the slides were observed in microscope (Nikon eclipse 80i, Japan).

2.5. Oxidative stress indices

The kits manufactured by Nanjing Jiancheng Bioengineering Institute (China) were used to detect T-AOC; SOD, GPx, GST, CAT, and iNOS activities; and NO, H_2O_2, MDA, and GSH contents. The detection was performed according to the instructions given by the reagent company.

2.6. Relative mRNA expressions

Primer sequences (Table 1) of apoptosis-related genes (P53, PUMA, Bcl-2, Bax, Apaf-1, Caspase-9, and Caspase-3) and β-actin published in GenBank were synthesized by Invitrogen Biotechnology Co. Ltd. (Shanghai, China).

Total RNA was isolated from carp gills using TRIzol reagent (Takara Biochemicals, Dalian, China) following the manufacturer's protocol. RNA concentration and purity were examined spectrophotometrically on the ratio of absorbance at 260 nm and 280 nm (Healthcare Bio-Sciences AB, Sweden). Complementary DNA (cDNA) was synthesized using PrimeScript™ RT reagent Kit (TaKaRa, Japan) in a volume of 60 μL (containing 5 μg of the total RNA) following the manufacturer's instructions. The synthesized cDNA was diluted 5-fold with sterile water and was stored at −20 °C for RT-qPCR.

RT-qPCR was performed with FastStart Universal SYBR Green Master (ROX) (Roche, Switzerland) using LightCycler® 96 Real-PCR System (Roche, Switzerland). Reaction mixture (10 μL) contained 5 μL of 2 × SYBR Green PCR Master Mix, 0.3 μL each of forward and reverse primers, 3.4 μL of sterile dH_2O, and 1 μL of template cDNA. RT-qPCR cycle parameters are as follows: 52 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min, 95 °C for 15 s, and 60 °C for 20 s. Melting curve analysis showed only one peak for each RT-qPCR product. There were three duplications for each sample. Relative mRNA expressions were calculated according to the Pfaffl method [24].

2.7. Statistical analysis

SPSS 22.0 software was used to perform all statistical analyses in one-way analysis of variance (ANOVA). Statistical differences between the groups were determined by nonparametric Kruskal-Wallis ANOVA test and Mann-Whitney U test. $P < 0.05$ was considered significant.

Table 1
Specific primers of apoptosis-related genes used in RT-qPCR.

Gene	Accession number	Primer (5′→3′)	Product size, bp
P53	AF365873	Forward: GGG CAA TCA GCG AGC AAA	18
		Reverse: ACT GAC CTT CCT GAG TCT CCA	21
PUMA	NM001045472	Forward: TGG AAA GCA GAG TGG ACG AA	20
		Reverse: GAT GGC AGG GCT GGA TGA	18
Bax	AF231015	Forward: GGC TAT TTC AAC CAG GGT TCC	21
		Reverse: TGC GAA TCA CCA ATG CTG T	19
Bcl-2	NM001030253	Forward: TCA CTC GTT CAG ACC CTC AT	20
		Reverse: ACG CTT TCC ACG CAC AT	17
Apaf-1	JQ743659	Forward: AGG TTC TCC TCT GGT GGT T	19
		Reverse: TAG CAG GCA CTT TGA TGT CT	20
Caspase-9	NM152884	Forward: AAA TAC ATA GCA AGG CAA CC	20
		Reverse: CAC AGG GAA TCA AGA AAG G	19
Caspase-3	NM131877	Forward: CCG CTG CCC ATC ACT A	16
		Reverse: ATC CTT TCA CGA CCA TCT	18
β-actin	L08165	Forward: GAT GGA CTC TGG TGA TGG TGT GAC	24
		Reverse: TTT CTC TTT CGG CTG TGG TGG TG	23

3. Results

3.1. Ultrastructural observation

Ultrastructural results in common carp gills were recorded and were shown in Fig. 1. The cells in the control group (Fig. 1(a)) showed clear cell nuclei, homogeneous cytoplasm, and intact mitochondria. The cells in the CPF group showed the shrinking of cytoplasm and chromatin (Fig. 1(b1)), nuclear chromatin agglutination (Fig. 1(b2)), and mitochondria swelling (Fig. 1(b3)).

3.2. Microstructural observation

Microstructure results of gills were shown in Fig. 2. The cells in the control group (Fig. 2(a)) showed normal structure with the secondary gill lamellae and pavement cells. The cells in the CPF group showed the gill lamella epithelia hyperplasia and lamellae fusion (Fig. 2(b1)); mucous cells hypertrophy and hyperplasia, and mucous cells

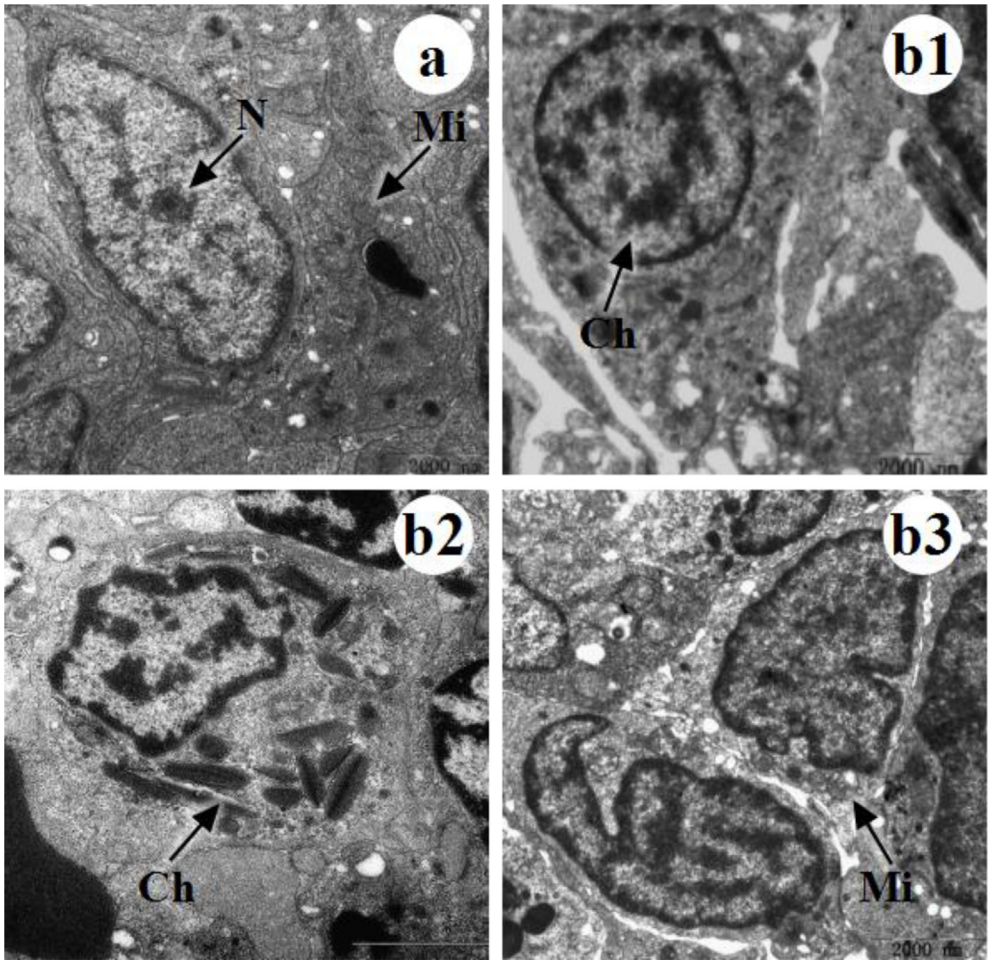

Fig. 1. The ultrastructure of common carp gills for 45 days. a: the control group; b1-b3: the CPF group. a × 10,000; b1 × 10,000; b2 × 15,000; b3 × 10,000. N: nucleus, Ch: chromatin, Mi: mitochondria.

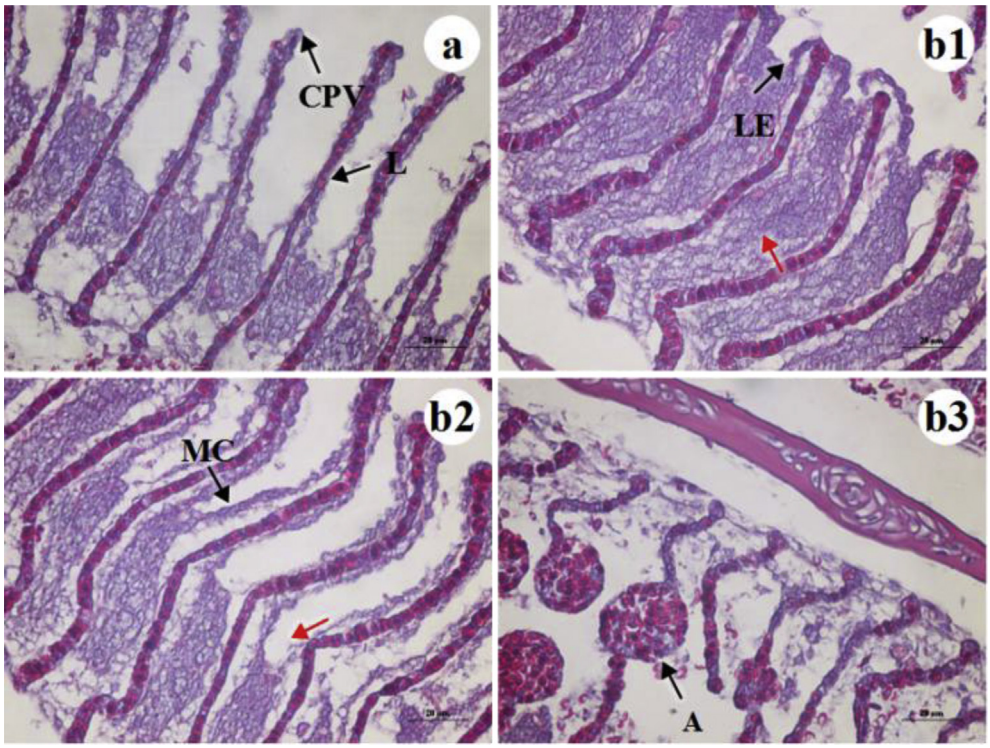

Fig. 2. Histology (hematoxylin and eosin staining, × 400) of common carp gills on the 45th day. a: the control group; b1-b3: the CPF group. b1: black arrow indicated gill lamellar epithelia hyperplasia and red arrow indicated several lamellae fusion; b2: black arrow indicated mucous cells hypertrophy and hyperplasia and red arrow indicated mucous cells vacuolation; b3: black arrow indicated aneurysm. CPV: pavement cells, L: lamellae, LE: lamellar epithelia, MC: mucous cells, A: aneurysm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

vacuolation (Fig. 2(b2)); and several aneurysms (Fig. 2(b3)).

3.3. TUNEL assay

TUNEL assay results of common carp gills were shown in Fig. 3. The number of TUNEL-positive apoptotic cells (showed yellow brown) in the CPF group was higher than that in the control group in the gill filament and lamellar epithelia. Apical aneurysms were observed in the CPF group.

3.4. Oxidative stress indices

Oxidative stress indices in carp gills on the 15th, 30th, and 45th days were presented in Fig. 4. SOD and T-AOC activities; and GSH content of the CPF group were significantly lower ($P < 0.05$) than those of the control group at all the time points. MDA content of the CPF group was

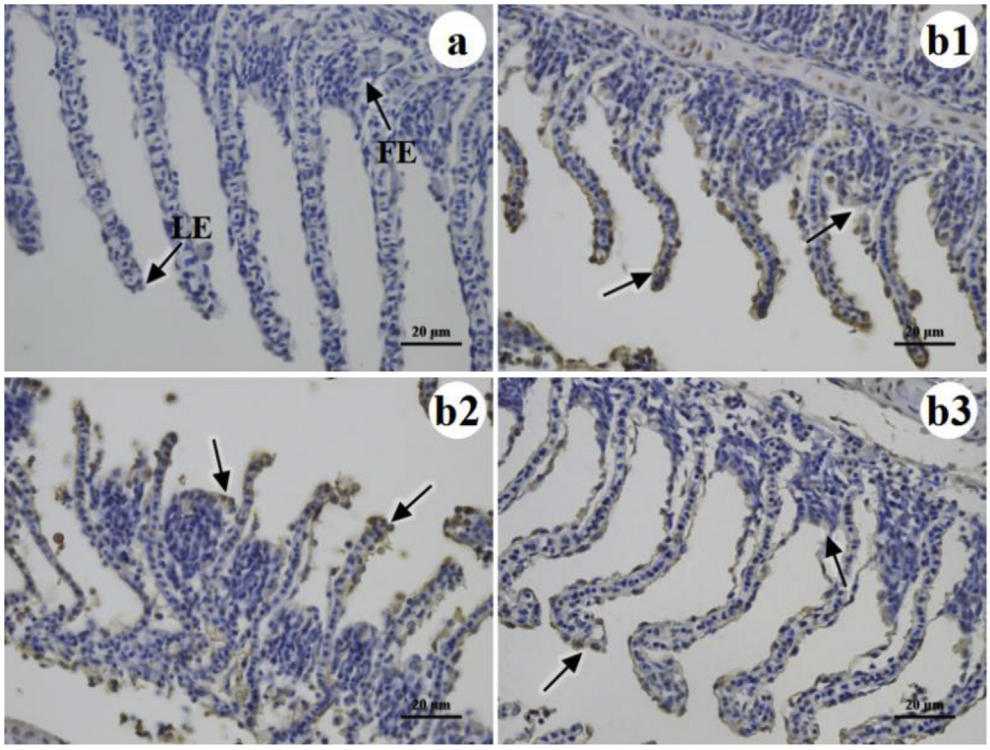

Fig. 3. TUNEL detection of apoptotic cells of common carp gills on the 45th day. a: the control group; b1-b3: the CPF group. Scale bar represents 20 μm.

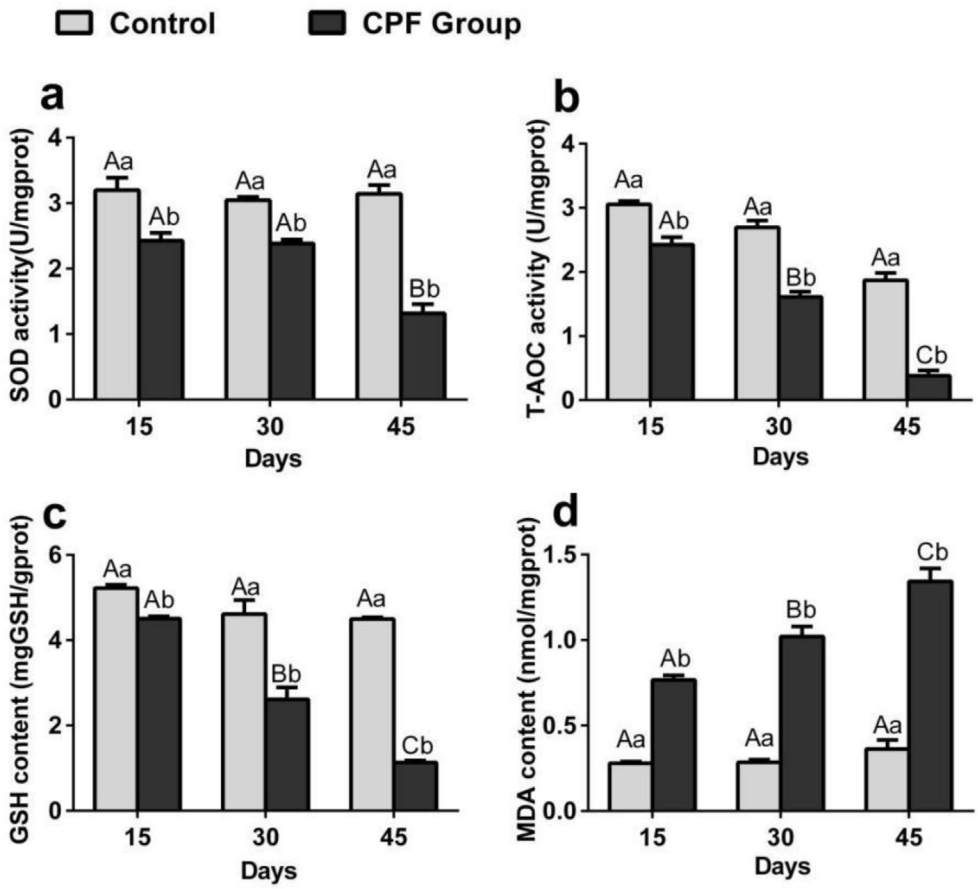

Fig. 4. Determination of SOD, T-AOC, GSH, and MDA in common carp gills on the15th, 30th, and 45th days. Statistically significant differences: data with different uppercase letters within the same group at different time points are significantly different ($P < 0.05$), and data with different lowercase letters in different groups at the same time point are significantly different ($P < 0.05$). Data represented mean ± SD.

significantly higher ($P < 0.05$) than that of the control group at all the time points. SOD activity, T-AOC, and GSH content decreased significantly ($P < 0.05$) with extension of CPF exposure time, and reduced to the minimum level on the 45th day. MDA content increased significantly ($P < 0.05$) with the increase of CPF exposure time and reached the maximum level on the 45th day.

3.5. Relative mRNA expressions

The effects of CPF on mRNA expressions of apoptosis-related genes on the 15th, 30th, and 45th days were shown in Fig. 5. Relative mRNA expressions of P53, PUMA, Bax, Apaf-1, Caspase-9, and Caspase-3 in the CPF group increased significantly ($P < 0.05$) compared with those in the control group at each time point. A significant decrease ($P < 0.05$) for Bcl-2 mRNA expression in the CPF group was found compared with that in the control group at each time point. After CPF exposure, mRNA expressions of P53, Caspase-9, and Caspase-3 increased significantly ($P < 0.05$) with the increase of exposure time; and reached the highest levels on the 45th day. PUMA and Apaf-1 mRNA expressions increased significantly ($P < 0.05$) on the 45th day compared with those on the 15th and 30th days. Bax mRNA expression increased significantly ($P < 0.05$) on the 30th and 45th days compared with that on the 15th day. Bcl-2 mRNA expression decreased significantly ($P < 0.05$) with the increase of CPF exposure time and reached the lowest level on the 45th day (see Fig. 6).

4. Discussion

Fish is considered an excellent biological indicator for assessing environmental pollutants, since they are organisms on the top of the aquatic food chain, long living, easy to collect, and of optimum size for analyses [25]. Gill is extremely important for respiration, acid-base balance, and nitrogenous waste excretion of fish. Here, we investigated toxic effect of CPF on common carp gills. In our experiment of ultrastructure and TUNEL assay, we found chromatin shrinking, nuclear chromatin agglutination, and mitochondria swelling in CPF-treated carp gills; and the number of TUNEL-positive apoptotic cells in the gill of common carp exposed to CPF increased. Our results indicated that CPF caused apoptosis in common carp gills. Previous study also found chromatin shrinking appeared in CPF-treated placental explants [26], and TUNEL-positive cells increased in Cu-treated zebrafish gills [27].

Studies have shown that environmental pollutants can cause oxidative stress by decreasing SOD, T-AOC, and GSH; and increasing MDA in fish livers, kidneys, and gills. Excess Cd, Mn, Fe, Co, Ni, Cu, and Zn can reduce GSH content in fish livers and kidneys [28]. CPF poisoning can cause the decrease of SOD and T-AOC, and the increase of MDA in goldfish livers [29]; and the decrease of GSH and the increase of MDA in guppies gills [10]. In our study, similar results were also found. CPF treatment decreased SOD, T-AOC, and GSH; and increased MDA content in the common carp gills, indicating that CPF caused oxidative stress in the common carp gills. It may be due to following mechanisms. SOD [30], T-AOC [31], and GSH [32] can protect against oxidative stress. MDA can induce oxidative stress [33]. In addition, T-AOC, GSH, and MDA in CPF-treated group showed a time-dependent effect at all time points in common carp gills. A time-dependent effect was also found in MDA content in the spleens of pigeons treated by avermectin [34].

Mitochondria are the centre of apoptosis regulation. Oxidative stress can induce apoptosis [35]. Oxidative stress can activate P53 and cause apoptosis [36]. Activated P53 can up-regulate PUMA which is localized in mitochondrial membrane [37]. PUMA can activate Bax [16]. Bax interacts with antiapoptotic gene Bcl-2 [38], which plays important roles in apoptosis by proapoptotic and antiapoptotic effects [39]. Bcl-2 is integrated within the outer mitochondrial membrane [40]. Down-regulated Bcl-2 led to the up-regulation of Caspase-9 [41]. Then

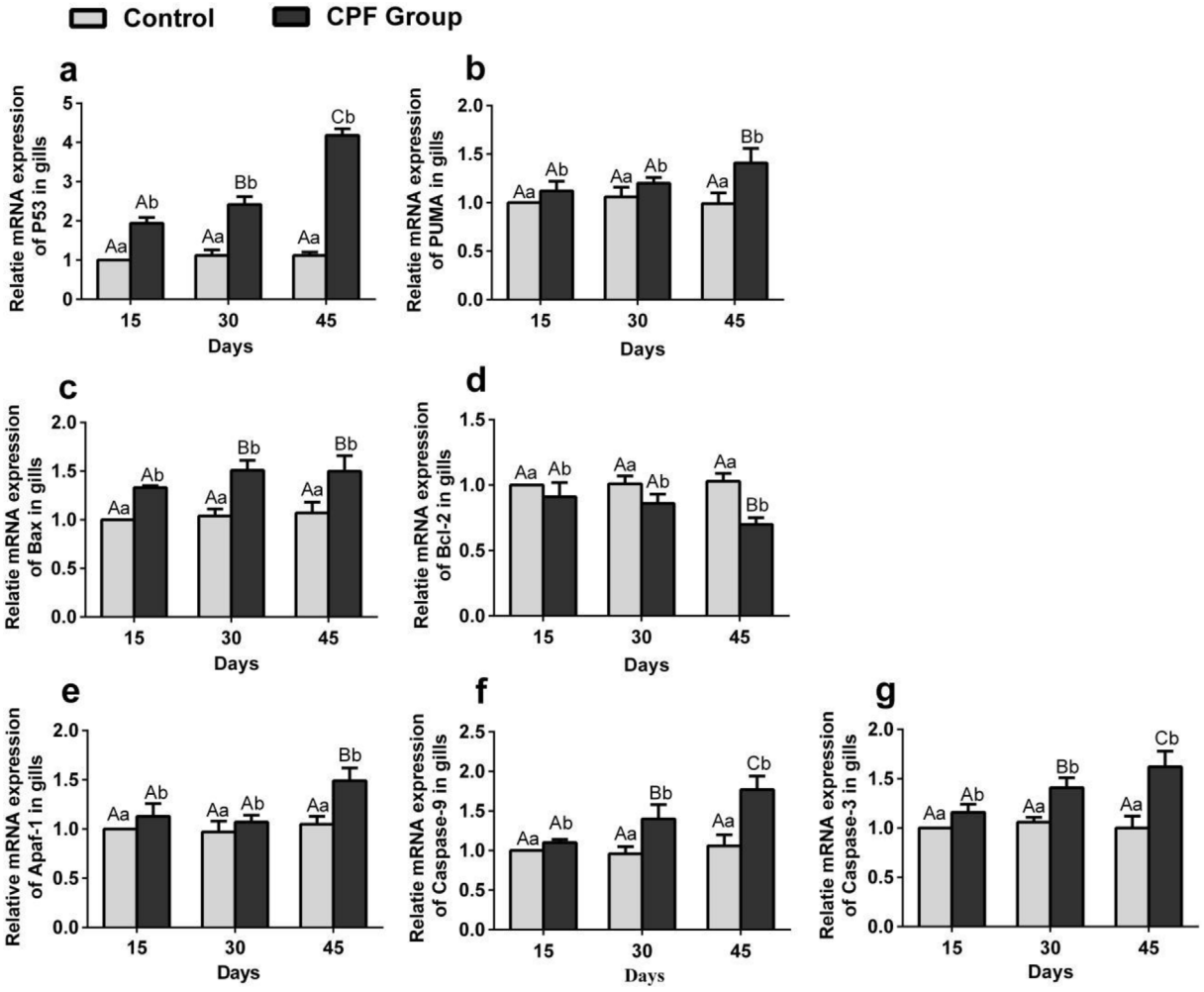

Fig. 5. Relative mRNA expressions of P53, PUMA, Bcl-2, Bax, Apaf-1, Caspase-9, and Caspase-3 in common carp gills on the15th, 30th, and 45th days. Statistically significant differences: data with different uppercase letters within the same group at different time points are significantly different ($P < 0.05$), and data with different lowercase letters in different groups at the same time point are significantly different ($P < 0.05$). Data represented mean ± SD.

activated Caspase-9 and Apaf-1 form apoptotic complex in the cytosol [42], and the apoptotic complex activates Caspase-3, and eventually Caspase-3 executes apoptosis [20]. In our study, CPF up-regulated P53, PUMA, Bax, Apaf-1, Caspase-9, and Caspase-3 mRNA expressions; and down-regulated Bcl-2 mRNA expression in common carp gills, indicating apoptosis caused by CPF in common carp gills through P53-mitochondria-Caspase-3 pathway. Similar results were obtained in other studies. P53, Bax, Caspase-9, and Caspase-3 mRNA expressions increased; Bcl-2 mRNA expression decreased; and apoptosis occurred in Cu-treated chicken hearts [43] and monocrotophos-treated PC12 cells [36]. Liu et al. found that 3-propyl nitrate increased PUMA mRNA expression, and resulted in apoptosis of mouse ovarian cells [44]. Salinomycin increased Bax and Apaf-1 mRNA expressions, decreased Bcl-2 mRNA expression, and induced apoptosis in primary chicken cardiomyocytes [42]. Carbendazim up-regulated Apaf-1 mRNA expression and induced apoptosis in zebrafish larva [15]. Cypermethrin increased Caspase-9 and Caspase-3 mRNA expressions, and led to apoptosis in zebrafish livers [45]. Altun et al. found that CPF caused an increase in mRNA expression of Caspase-3 and apoptosis in common carp brains [21]. In addition, we also found that CPF induced apoptosis in a time-dependent effect on mRNA expressions of P53, Caspase-9, and Caspase-3 in common carp gills. Qi et al., found that homoharringtonine treatment up-regulated Caspase-9 mRNA expression in a time-dependent effect in MCF7 cells [46].

Pathological changes in fish are powerful indicators of exposure to environmental stressors. Gill plays an important role in fish immunity. However, the effects of CPF exposure on histopathology have rarely been studied. A previous study showed that gills appear as epithelial hypertrophy, telangiectasis, oedema with epithelial separation from basement membranes, and epithelial desquamation in carp exposed to CPF [47]. This is consistent with our results. These findings suggested that CPF exposure destroyed structural integrity of the gills, which ultimately would influence normal physiological activities.

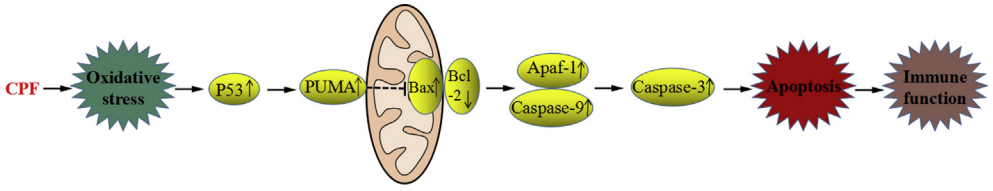

Fig. 6. Effects of CPF exposure on immune function through oxidative stress and apoptosis in common carp gills.

5. Conclusion

CPF exposure can cause oxidative stress and apoptosis in common carp gills. Oxidative stress was involved in CPF-caused apoptosis via P53-mitochondria-Caspase-3 pathway in common carp gills. CPF exposure destroyed structural integrity and affected immune function through oxidative stress and apoptosis in common carp gills.

Conflicts of interest

None of the authors have any financial or personal relationships that could inappropriately influence or bias the content of the paper.

Acknowledgments

This work was supported by grants from the Scientific Research Projects of Education Department of Heilongjiang Province (No.12541006).

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