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Role of the Nrf2-ARE pathway in perfluorooctanoic acid (PFOA)-induced hepatotoxicity in *Rana nigromaculata*☆

Juan Tang [a], Xiuying Jia [a], Nana Gao [a], Yingzhu Wu [a], Zhengquan Liu [a], Xiangjun Lu [a], Qiongxia Du [a], Jianbo He [a], Ning Li [a], Bin Chen [a], Jinxiao Jiang [a], Wenli Liu [a], Ying Ding [a, c], Weiqin Zhu [a, c], Hangjun Zhang [a, b, c, *]

[a] College of Life and Environmental Sciences, Hangzhou Normal University, Xuelin Road 16#, Xiasha Gaojiao Dongqu, Hangzhou, Zhejiang Province, 310036, China
[b] Guangzhou Key Laboratory of Environmental Exposure and Health, School of Environment, Jinan University, Guangzhou, 510632, China
[c] Key Laboratory of Hangzhou City for Ecosystem Protection and Restoration, Hangzhou Normal University, Hangzhou, 310036, China

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ABSTRACT

Perfluorooctanoic acid (PFOA) is widely distributed in various environmental media and is toxic to organisms. This study demonstrated that PFOA induces hepatotoxicity in the frog and evaluated the role of CYP3A and the Nrf2-ARE signaling pathway in regulating responses to PFOA-induced hepatotoxicity. *Rana nigromaculata* were exposed to 0, 0.01, 0.1, 0.5, or 1 mg/L PFOA solutions in a static-renewal system for 14 days. Liver tissue samples were collected 24 h after the last treatment. Hepatic histology was observed by HE staining and transmission electron microscopy. The oxidative stress levels in the liver were measured. The expression levels of CYP3A, Nrf2, NQO1, and HO-1 mRNA were measured by quantitative reverse transcription–polymerase chain reaction. PFOA-treated frog liver tissue exhibited diffuse cell borders, cytoplasmic vacuolization, broken nuclei, nuclear chromatin margination, and swollen mitochondria. In addition, the livers of PFOA-treated frogs showed a significantly elevated content of reactive oxygen species, malondialdehyde, glutathione and glutathione *S*-transferase activity compared to the livers of control frogs. However, the glutathione peroxidase activities concomitantly decreased in PFOA-treated frogs compared to those in the control group. Furthermore, compared with control frogs, the expression levels of CYP3A, Nrf2, and NQO1 mRNA significantly increased in PFOA-treated frogs. HO-1 mRNA expression remarkably increased only in groups treated with 0.5 or 1 mg/L PFOA. Our results indicate that PFOA induces hepatotoxicity in a dose-dependent manner. Furthermore, the results of the comparison analysis between different gender groups illustrated that PFOA is more toxic to female frogs than male frogs. Our results demonstrated that PFOA causes liver damage and that CYP3A enhances PFOA-induced female frogs hepatotoxicity are more virulent than male through biotransformation, and the activation of the Nrf2-ARE pathway is induced to protect against hepatotoxicity in *Rana nigromaculata*, all of which provide the scientific basis for the protection of amphibians against environmental contaminants.

1. Introduction

Since the 1950s, polyfluoroalkyl chemicals (PFCs) have been extensively used in industrial and consumer products, such as plating, firefighting foams, and food packaging (Lewandowski et al., 2006). Notably, PFCs are characterized by their high chemical and thermal stabilities, surface activity, and repellence to water and oil. Currently, PFCs are globally present in various environmental media, including water and soil, and also accumulate in the blood, liver, muscles, lungs, and kidneys of organisms (Ahrens et al., 2009). PFCs can lead to multiple toxicity effects, including hepatotoxicity, immunotoxicity, and reproductive toxicity (Cui et al., 2009; Lau et al., 2006; Zhang et al., 2014). One of these PFCs, perfluorooctanoic acid (PFOA), is frequently detected in humans and the environment (Ahrens, 2011; Houde et al., 2011; Wang et al.,

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* Corresponding author. College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou, Zhejiang, 310036, China.
E-mail address: 13819172516@163.com (H. Zhang).

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2013). PFOA have been found at relatively high concentrations near fluorochemical manufacturing facilities, up to ~56 μg/L in the River Alz (near a fluorochemical factory) in Germany (Loos et al., 2008), ~500 ng/L in the Tennessee River, USA (near fluorochemical manufacturing facilities) (Hansen et al., 2002), and 0.85–260 ng/L in the Pearl River and Yangtze River, China (So et al., 2007). In addition, studies showed that PFOA also has been found in drinking water and ground water in Germany (Schaefer, 2006) and USA (Davis et al., 2007). This chemical potentially causes hepatomegaly, hepatic peroxisome proliferation, and compromised postnatal survival and growth deficits in mammals (Berthiaume and Wallace, 2002; Cui et al., 2009). Moreover, PFOA was shown to increase hepatic fatty acyl-CoA oxidase activity, produce oxidative stress, and modulate the expression of inflammatory cytokines in fish that were exposed to 10, 50, 100 mg/L PFOA for 7 d (Yang, 2010).

Amphibians play an important role in the ecosystem and are utilized as indicators of ecological security (Niemi and McDonald, 2004). Global amphibian populations have dramatically declined over the past several decades (Muths and Fisher, 2017). The International Union for Conservation of Nature's Red List of Threatened Species (2008) indicated that a considerable proportion of amphibians face extinction. Reports in the literature similarly indicate that the decline in amphibian populations is cause for increased concern worldwide (Hof et al., 2011; Houlahan et al., 2000; Wake, 1991). A previous study reported that environmental pollutants are one of the primary reasons for the attenuation of the global amphibian population (Hayes et al., 2010). Amphibians commonly inhabit ponds, ditches, and paddy fields where PFCs are also commonly detected (Boone and James, 2003). In previous studies, hepatohistological impairment is characterized by hepatocyte degeneration and hypertrophy in PFC-exposed *Xenopus laevis* (Lou et al., 2013). Because the liver is well-known as the primary target organ of PFOA, the hepatotoxicity of PFOA in amphibians should be investigated. However, whether PFOA causes liver damage during metabolism in amphibians remains uncertain.

PFCs exert morphological and biochemical effects through the transactivation of the nuclear receptor peroxisome proliferator activated receptor alpha (PPARα), which regulates physiological processes such as lipid metabolism, inflammation, and cell proliferation (Rosen et al., 2009). A causal relationship between liver damage and increased expression levels of PPARα was identified in rats after dietary exposure to ammonium PFOA (Elcombe et al., 2010). PFOA activates PPARα in the rat liver and alters the messenger RNA (mRNA) levels of several cytochrome P450 (CYP) enzymes and CAR-regulated enzymes involved in the phase I detoxification of environmental xenobiotics (Ren et al., 2009). In addition, nuclear factor E2-related factor 2 (Nrf2), the master regulator of phase II detoxification enzymes, is involved in responses to cellular stress from electrophilic and oxidative toxicants produced by phase I metabolism and environmental xenobiotics (Cavin et al., 2008). The activation of Nrf2 protects against hepatotoxicity in mice (Liu et al., 2008). Therefore, the effect of PFOA on the regulatory pathway of liver detoxification in amphibians should be further investigated.

However, it remains unknown whether PFOA also causes liver oxidative damage in frogs, whether there is a difference between PFOA toxicity in the female frogs and male frogs, and what other effects this treatment may have on frogs. We hypothesized that PFOA could induced hepatotoxicity by the Nrf2-ARE pathway in the liver tissue of amphibians. Thus, the current study evaluated hepatoxicity in *Rana nigromaculata* exposed to different doses of PFOA. Furthermore, the possible underlying molecular mechanism of hepatotoxicity in amphibians was investigated with a specific focus on detoxification enzymes, including CYPs and the Nrf2 pathway. The results of this study may provide a theoretical basis for the protection of amphibians.

2. Materials and methods

2.1. Chemicals

PFOA white crystals ($C_8HF_{15}O_2$, >96% purity, CAS: 335-67-1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Malondialdehyde (MDA), glutathione (GSH), glutathione *S*-transferase (GST), and glutathione peroxidase (GPx) assay kits were purchased from Jiancheng Bioengineering, Inc., Nanjing, China.

2.2. Animals

Adult male and female frogs (*R. nigromaculata;* 3 years old, 52 ± 4 g, 7 ± 1 cm) were purchased from Zhejiang ChangXing Creative Ecological Agriculture Development Co., LTD (Huzhou, Zhejiang, China). The frogs adapted to the new surroundings for 1 week and were fed *Eisenia fetida* twice a day before the formal experiment. All experimental protocols were approved by the local government and all animals underwent procedures performed in accordance with the experimentation ethics review committee. The animals are described in detail in the Supplemental Material (S1).

2.3. Animal treatment and tissue preparation

Two-hundred healthy frogs (100 male, 100 female) were selected and randomly divided into five groups (n = 20 per group): 0, 0.01, 0.1, 0.5, or 1 mg/L PFOA group. PFOA dose selection was based on environmentally relevant concentrations previously reported about PFOA exposure and equivalent to 0.2-, 5-, 10- and 20-fold of the relatively high concentration of 56 μg/L in *R. nigromaculata* (Loos et al., 2008). After 14 days of exposure, all frogs were sacrificed by pithing. Livers were quickly removed, cleared of any adhering tissues, weighed, and then stored at −80 °C with liquid nitrogen until assayed. Animal treatment conditions are described in detail in the Supplemental Material (S2).

2.4. Morphology observation by transmission electron microscopy

The fresh liver samples were fixed in 2.5% glutaraldehyde and kept at 4 °C overnight. Tissue samples were post-fixed in 1.5% osmic acid for 1 h, washed with 0.1 M phosphate buffered saline, and sequentially dehydrated in a gradient series of 50%–100% alcohol. Next, the samples were embedded in pure acetone, sliced into 70 nm thin sections, and then stained with uranyl acetate and lead citrate. Finally, the sections were examined using transmission electron microscopy (TEM, H-7650, Hitachi, Japan).

2.5. Histomorphometric analysis

For histological evaluation, the tissue sections were stained with hematoxylin and eosin. The stained sections were observed using a BX20 fluorescence microscope (OLYMPUS, Japan). Specific details are described in the Supplemental Material (S3).

2.6. Biochemical evaluation

Tissue ROS content was determined using a commercially available enzyme-linked immunosorbent assay (ELISA, enzyme-linked immunosorbent assay, R&D Systems, Shanghai, China). Excitation light was set at 485 nm, and fluorescence intensity values were measured at 538 nm. MDA content was detected using a spectrophotometer at an absorbance of 532 nm, and the results were expressed as nmol·mg^{-1} protein. The activities of GSH, GST,

and GPx were assayed using commercial reagent kits obtained from the Institute of Biological Engineering of Nanjing Jiancheng (Nanjing, China) following the manufacturer's instructions. All specific details are described in the Supplemental Material (S4).

2.7. Quantitative reverse transcription–polymerase chain reaction

Total RNA was extracted using TRI reagent. RNA content, quality, and purity were verified by monitoring 260/280 nm absorbance ratios through 1% agarose-formaldehyde gel electrophoresis with ethidium bromide staining. Then, complementary DNA (cDNA) was synthesized using SuperScript™ III First-Strand Synthesis SuperMix for quantitative reverse transcription–polymerase chain reaction (qRT-PCR; Life Technologies, Milwaukee, WI, USA) in accordance with the manufacturer's instructions. Primers were designed using Primer Premier 6.0 and Beacon Designer 7.8 (Table 1). PCR amplification was performed by two-step qRT-PCR with a CFX384 TouthTM Real-Time PCR Detection System (BioRad, Hercules, CA, USA). Gene expression levels were subjected to statistical analysis using the comparative Ct ($\triangle\triangle$Ct) method, in which relative expression is calculated as $2^{-\triangle\triangle Ct}$ and Ct represents the threshold cycle. The RT-PCR program was reported by our previous studies (Jia et al., 2014; Tang et al., 2016), and more detailed steps are available in the Supplementary Materials (S5).

2.8. Statistical analysis

Experimental data were subjected to statistical analysis using Origin 8.0, and all the data were presented as the means ± standard deviation. Sample data were analyzed by one-way analysis of variance, and a value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Ultrastructural observations of the liver

The morphology of liver cells from male frogs exposed to 0.01, 0.1, 0.5, or 1 mg/L PFOA for 14 days are shown in Fig. 1. Livers from the control group exhibited normal cell nuclei and mitochondria (Fig. 1 A and F). However, after 14 days of exposure to 0.01 mg/L PFOA, the nuclei displayed nuclear chromatin margination and the mitochondria appeared slightly swollen (Fig. 1 B and G). After 14 days of exposure to 0.1 mg/L PFOA, nuclear chromatin margination and swollen mitochondria intensified. In addition, mitochondria exhibited slight crest expansion (Fig. 1 C and H). Nuclear chromatin margination, swollen mitochondria, and crest expansion were also observed in frogs exposed to 0.5 mg/L PFOA for 14 days (Fig. 1 D and I). Visible nuclear chromatin margination, swollen mitochondria, crest expansion, and increased mitochondrial matrix were observed in male frogs exposed to 1 mg/L PFOA for 14 days (Fig. 1 E and J).

The morphology of liver cells from female frogs exposed to 0.01, 0.1, 0.5, and 1 mg/L PFOA for 14 days are shown in Fig. 2. Liver cells from the control group exhibited normal cell nuclei and mitochondria (Fig. 2 A and F). After 14 days of exposure to 0.01 mg/L PFOA, nuclear chromatin margination and slightly swollen mitochondria were observed (Fig. 2 B and G). The liver cells of frogs exposed to 0.1 mg/L PFOA for 14 days exhibited intense nuclear chromatin margination, broken nuclei, and swollen mitochondria (Fig. 2 C and H). Fig. 2 D and I show the nuclear chromatin margination, broken nuclei, swollen mitochondria, and crest expansion that were observed in frog livers after 14 days of exposure to 0.5 mg/L PFOA. Fig. 2 E and J show the nuclear chromatin margination, broken nuclei, swollen mitochondria, and crest expansion that were observed in frog livers after 14 days of exposure to 1 mg/L PFOA.

3.2. Histopathological evaluation

Images of HE-stained liver sections from female frogs are presented in Fig. 3 A–E. Fig. 3 A shows the normal histomorphology of livers from the control group. Fig. 3 B shows that liver cells from frogs exposed to 0.01 mg/L PFOA for 14 days had diffuse borders. These borders were also observed in liver cells from female frogs exposed to 0.1 mg/L PFOA for 14 days (Fig. 3 C). Cell borders diffusion and cytoplasmic vacuolization intensified in female frogs exposed to 0.5 or 1 mg/L PFOA for 14 days (Fig. 3 D and E).

Images of HE-stained liver sections from male frogs are shown in Fig. 3 F–J. Fig. 3 F shows an image of normal liver tissue from the control group. Fig. 3 G shows diffuse cell borders of liver tissue from the 0.01 mg/L PFOA exposure group. Similar results were observed for the group exposed to 0.1 mg/L PFOA for 14 days (Fig. 3 H). The group exposed to 0.5 mg/L PFOA for 14 days exhibited extremely diffuse cell borders and intense cytoplasmic vacuolization (Fig. 3 I). Cell border diffusion and cytoplasmic vacuolization intensified further in the group exposed to 1 mg/L PFOA for 14 days (Fig. 3 J).

3.3. Analysis of ROS content

The ROS contents of liver tissues from male and female frogs are shown in Fig. 4. Compared with the corresponding control group, the ROS contents of liver tissues from male and female frogs in the 0.01, 0.1, 0.5 and 1 mg/L PFOA-treated groups significantly increased in a dose-dependent manner ($P < 0.01$). In addition, the ROS contents of liver tissues from female frogs in the 0.01, 0.1, and 0.5 mg/L PFOA-treated groups were significantly increased compared with those from male frogs in each corresponding group ($P < 0.01$).

3.4. Analysis of MDA, GSH, GST, and GPx activities

Relative to that in the control, MDA of liver tissues from male frogs was significantly increased after exposure to 0.01–1 mg/L

Table 1
Primer sequences for Real-time PCR.

Gene	Accession No.	Primer sequence (5′ to 3′)	Product Size (bp)
CYP3A	NM_001015786.1	F: 5′- GTGCTTTCACCTACATTCACCAGT-3′ R: 5′-GGCTCATCTTTCTCCACATAACCCT-3′	113
Nrf-2	NM_001007489.2	F: 5′- GATGAATGCTTGAAGATTTTGGGTG-3′ R: 5′-GACTGGAGGAAGGTCTGGTTTGT-3′	125
NQO-1	NM_001016468.2	F: 5′-CACTGAAAATGAATGGCTGGGATG-3′ R: 5′-CCTCTGCTGGATAACTAAAGTGGTCT-3′	129
HO-1	XM_002934720.3	F: 5′-CTAGAGGAGTTCAAGCTGGTCATG-3′ R: 5′-CTCAAGGTCTTCTTCTAGGGCAT-3′	153

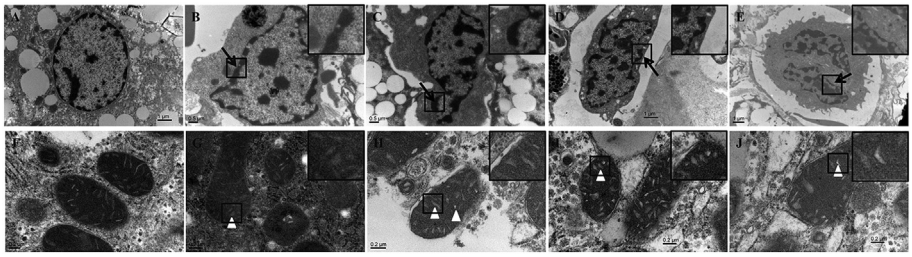

Fig. 1. The morphology of liver cells from male frogs exposed to 0, 0.01, 0.1, 0.5 or 1 mg/L PFOA for 14 days. (A) Normal cell nucleus of the control liver (12,000 ×). (B) Nuclear chromatin margination after exposure to 0.01 mg/L PFOA for 14 days (6000 ×) (black arrow). (C) Intense nuclear chromatin margination after exposure to 0.1 mg/L PFOA for 14 days (6000 ×) (black arrow). (D) Nuclear chromatin margination intensified after exposure to 0.5 mg/L PFOA for 14 days (6000 ×) (black arrow). (E) Nuclear chromatin margination occurred after exposure to 1 mg/L PFOA for 14 days (6000 ×) (black arrow). (F) Normal mitochondria of the control liver (60,000 ×). (G) Slight mitochondrial swelling in the group exposed to 0.01 mg/L PFOA for 14 days (60,000 ×) (white triangle). (H) Swollen mitochondriaand slight crest expansion in the group exposed to 0.1 mg/L PFOA for 14 days (white triangle). (I) Swollen mitochondria and crest expansion in the group exposed to 0.5 mg/L PFOA for 14 days (60,000 ×) (white triangle). (J) Swollen mitochondria, crest expansion, and increased mitochondrial matrix in the group exposed to 1 mg/L PFOA for 14 days (60,000 ×) (white triangle).

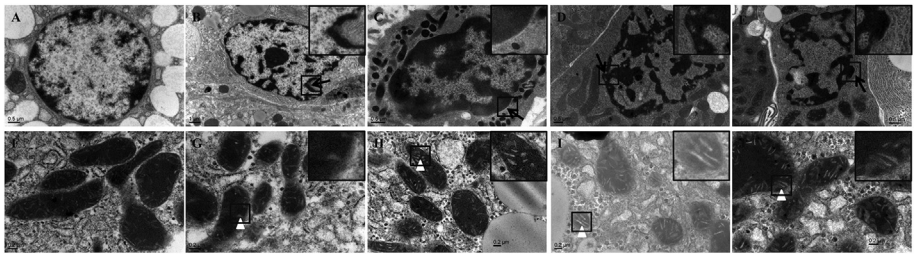

Fig. 2. The morphology of liver cells from female frogs exposed to 0, 0.01, 0.1, 0.5 or 1 mg/L PFOA for 14 days. (A) Normal cell nucleus of the control liver (12,000 ×). (B) Nuclear chromatin margination was observed after exposure to 0.01 mg/L PFOA for 14 days (6000 ×) (black arrow). (C) Intense nuclear chromatin margination and broken nuclei after exposure to 0.1 mg/L PFOA for 14 days (6000 ×) (black arrow). (D) Nuclear chromatin margination and broken nuclei after exposure to 0.5 mg/L PFOA for 14 days (6000 ×) (black arrow). (E) Nuclear chromatin margination and broken nucleus were also observed after exposure to 1 mg/L PFOA for 14 days (6000 ×) (black arrow). (F) Normal mitochondria of the control liver (60,000 ×). (G) Slightly swollen mitochondria in the group exposed to 0.01 mg/L PFOA for 14 days (60,000 ×) (white triangle). (H) Swollen mitochondria in the group exposed to 0.1 mg/L PFOA for 14 days (white triangle). (I) and (J) Swollen mitochondriaand crest expansion in the group exposed to 0.5 and 1 mg/L PFOA for 14 days (60,000 ×) (white triangle).

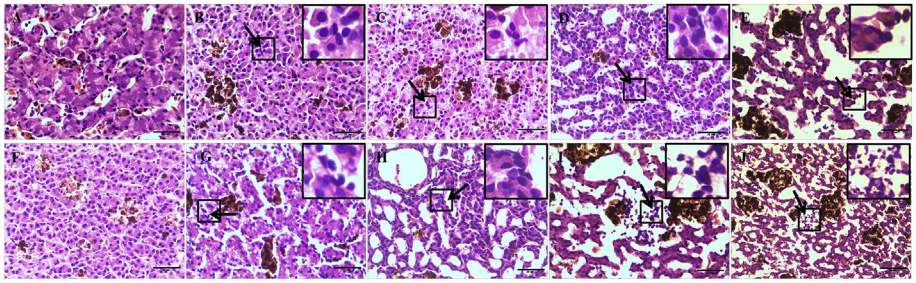

Fig. 3. HE staining of liver tissue from male and female frogs. A to E are male and F to J are female. (A) Normal control group. (B) Diffuse cell borders in the group exposed to 0.01 mg/L PFOA for 14 days (black arrow). (C) Diffuse cell borders were also observed in the group exposed to 0.1 mg/L PFOA for 14 days (black arrow). (D and E) Intense border diffusion and cytoplasmic vacuolization after exposure to 0.5 and 1 mg/L PFOA for 14 days (black arrow). (F) Normal control group. (G) Diffuse borders of liver cells in the group exposed to 0.01 mg/L PFOA for 14 days (black arrow). (H) Diffuse cell borders and cytoplasmic vacuolization in the group exposed to 0.1 mg/L PFOA for 14 days (black arrow). (I) Intense hepatocyte borders diffusion and cytoplasmic vacuolization in the group exposed to 0.5 mg/L PFOA for 14 days (black arrow). (J) Hepatocyte border diffusionand cytoplasmic vacuolization intensified after exposure to 1 mg/L PFOA (black arrow).

PFOA ($P < 0.01$), while that from female frogs was significantly increased in the 0.1, 0.5, and 1 mg/L PFOA-treated groups ($P < 0.05$; $P < 0.01$). In addition, the liver tissues of the 0.1, 0.5, and 1 mg/L PFOA-treated female frog groups had significantly less MDA than those of the corresponding male frog groups ($P < 0.01$) (Fig. 5A). These results indicated that low doses of PFOA may cause liver lipid peroxidation in *R. nigromaculata* with gender differences.

Compared with those of the corresponding control group, the liver tissues of both sexes had significantly higher GSH after exposure to 0.01, 0.1, 0.5, and 1 mg/L PFOA ($P < 0.05$; $P < 0.01$). In addition, the liver tissues of the 0.01, 0.1, and 0.5 mg/L PFOA-treated female frog groups had significantly higher GSH than those of the corresponding male frog groups ($P < 0.05$; $P < 0.01$) (Fig. 5 B).

As shown in Fig. 5C, all PFOA-treated frogs showed significantly higher hepatic GST activity in the liver than the control frogs ($P < 0.01$). In addition, the liver tissues of female frogs exposed to 0.01, 0.1, 0.5, and 1 mg/L PFOA contained higher GST activity than those of the corresponding male frog groups ($P < 0.05$; $P < 0.01$).

After exposure to 0.01, 0.1, 0.5, and 1 mg/L PFOA for 14 days, both male and female frogs showed significantly less GPx in the liver

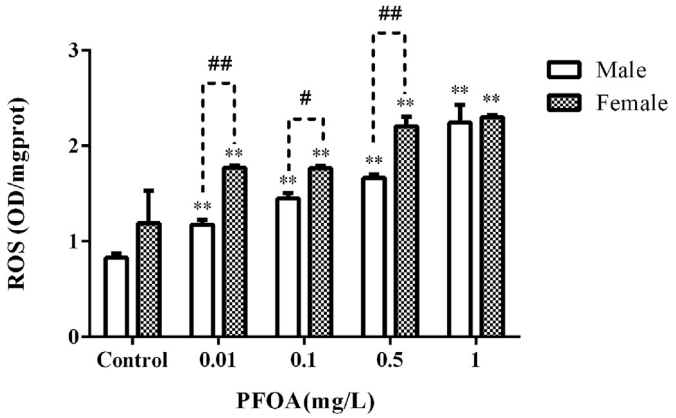

Fig. 4. Effect of PFOA on ROS in the liver of frogs exposed to 0, 0.01, 0.1, 0.5, or 1 mg/L PFOA for 14 days. Data are represented as the means ± standard deviation. $^{**}P < 0.01$ compared to the corresponding control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared to the corresponding male frog group.

than the corresponding control groups ($P < 0.05$). Compared with the corresponding male frog groups, female frogs showed a significant increase in liver GPx in the 0.01 mg/L PFOA-treated group and a significant decrease in liver GPx in the 1 mg/L PFOA-treated group ($P < 0.01$) (Fig. 5 D).

3.5. The mRNA expression of CYP3A, Nrf2, NQO1, and HO-1 in the liver

The relative mRNA levels of CYP3A in frogs exposed to 0.01, 0.1, 0.5, and 1 mg/L PFOA for 14 days were significantly upregulated compared with that in the corresponding control group ($P < 0.01$). All PFOA-treated female groups showed significantly lower CYP3A mRNA expression in the liver than the corresponding male frog groups ($P < 0.01$) (Fig. 6A). This result indicates a concentration-dependent relationship of PFOA on CYP3A mRNA expression in frogs (see Fig. 7).

Hepatic Nrf2 mRNA expression was significantly increased in the 0.01, 0.1, 0.5, and 1 mg/L PFOA-treated female groups and in the 0.1, 0.5, and 1 mg/L PFOA-treated male groups, compared with the corresponding control groups ($P < 0.05$; $P < 0.01$). In addition, the 0.5 and 1 mg/L PFOA-treated female groups showed significantly higher hepatic Nrf2 mRNA expression than the corresponding male groups ($P < 0.01$) (Fig. 6B).

Hepatic NQO1 mRNA expression was significantly increased in the 0.01, 0.1, 0.5, and 1 mg/L PFOA-treated female groups and in the 0.1, 0.5, and 1 mg/L PFOA-treated male groups, compared with the corresponding control groups ($P < 0.01$). In addition, this expression significantly increased in the 0.1, 0.5, and 1 mg/L PFOA-treated female groups compared with the corresponding control groups ($P < 0.05$; $P < 0.01$). Furthermore, the 1 mg/L PFOA-treated female group showed significantly higher NQO1 mRNA expression in the liver than the corresponding male group ($P < 0.01$) (Fig. 6C).

We also observed a significant increase in hepatic HO-1 mRNA expression in the 0.5 and 1 mg/L PFOA-treated male and female groups compared with the corresponding control groups ($P < 0.05$; $P < 0.01$). The 0.5 mg/L PFOA-treated female group showed significantly higher HO-1 mRNA expression in the liver than the corresponding male group ($P < 0.01$) (Fig. 6D).

4. Discussion

In the current study, we investigated the effect of the Nrf2-ARE pathway in the PFOA-stimulated *R. nigromaculata* liver and the role of the Nrf2-ARE pathway in this process. Our results showed that PFOA induces hepatic oxidative damage and that this damage is more serious in female frogs. The upregulation of CYP3A may

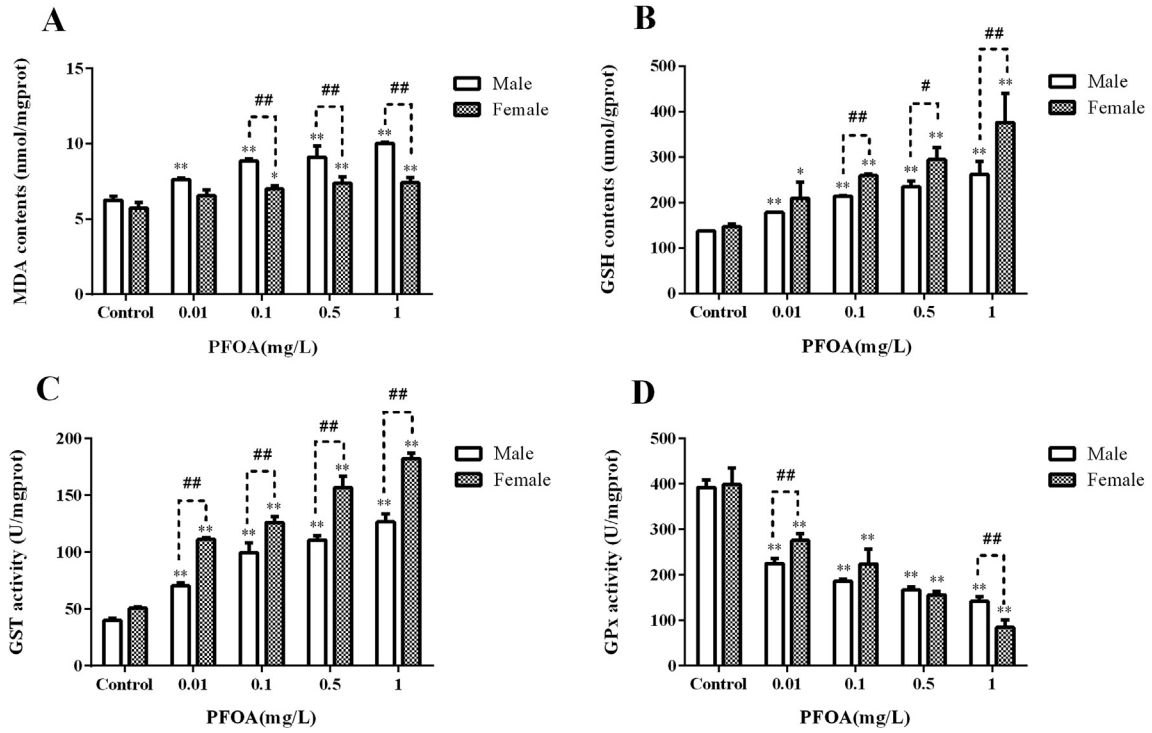

Fig. 5. Effect of PFOA on MDA (A), GSH (B), GST (C), and GPx (D) in the liver of frogs exposed to 0, 0.01, 0.1, 0.5, or 1 mg/L PFOA for 14 days. Data are represented as the mean ± standard deviation. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the corresponding control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared to the corresponding male frog group.

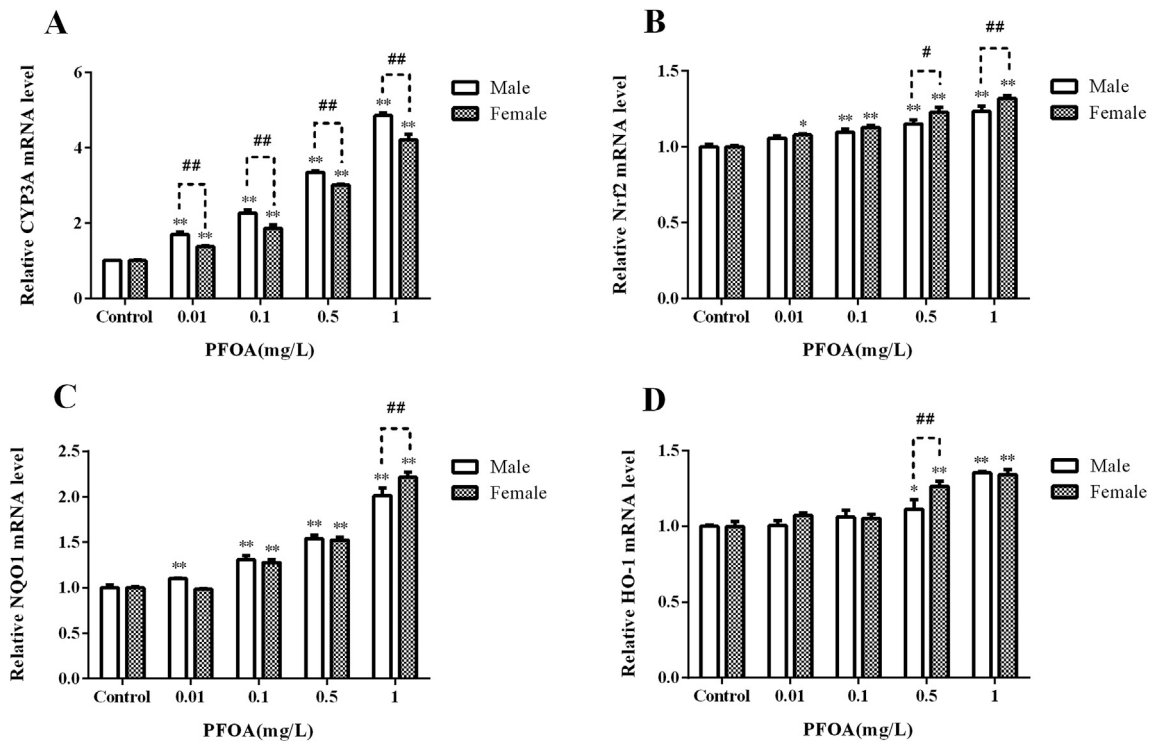

Fig. 6. Effect of PFOA on CYP3A (A), Nrf-2 (B), NQO-1 (C), and HO-1 (D) mRNA levels in frogs exposed to 0, 0.01, 0.1, 0.5, or 1 mg/L PFOA for 14 days. Data are represented as the means ± standard deviation. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the corresponding control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared to the corresponding male frog group.

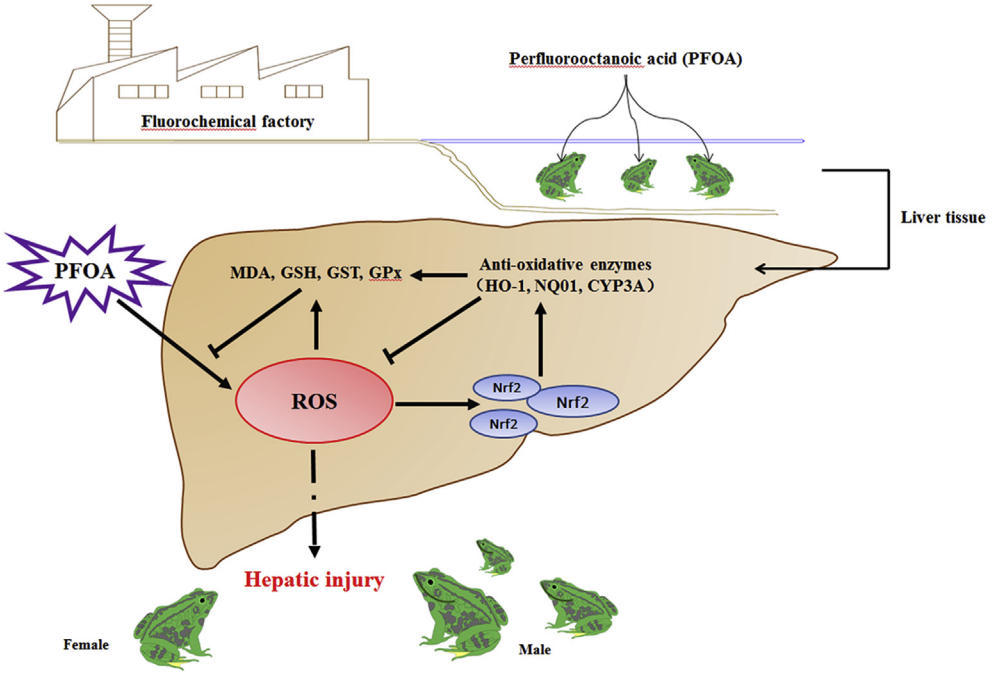

Fig. 7. The proposed signaling pathway of regulating hepatotoxicity related to gene expression associated with the PFOA-induced Nrf2-ARE pathway in the liver of *Rana nigromaculata*. Right arrow, activation; down tack, inhibition.

enhance PFOA-induced hepatotoxicity through biotransformation. In addition, the upregulation of NQO1, HO-1 and Nrf2 may suppress PFOA-induced hepatotoxicity. Furthermore, the Nrf2-ARE pathway is involved in the regulation of hepatotoxicity in PFOA-stimulated frogs.

The hepatotoxicity of PFCs has been extensively studied in a wide range of animals, including mammals and lower vertebrates. Exposure to PFOA/PFOS induces hepatocytic hypertrophy and cytoplasmic vacuolation in male Sprague-Dawley rats (Cui et al., 2009). In addition, during exposure, the absolute and relative

liver weights of the rat increases, and hepatocyte hypertrophy and apoptosis occurs (Perkins et al., 2004). PFOA increases liver weight and causes hepatocytes to swell, and numerous peroxisomes and lipids accumulates in hepatocytes (Wolf et al., 2008). Furthermore, cytoplasmic vacuolization occurs in the livers of male and female zebrafish and their offspring (Keiter, 2013). Additional studies in PFOA-treated female zebrafish have reported that dose-dependent hepatic cell damage is characterized by diffuse cell borders, vacuolization, nuclear enlargement and pycnosis, and collapsed cellular structures (Liu et al., 2008). Although the livers of rats exposed to low doses of PFOA exhibited no drastic histological changes, male rats exposed to 5 mg PFOA/kg/D exhibited diffuse hepatocyte borders and cell vacuolar degeneration (Fang et al., 2012). The ultramicrostructure of livers from male rats exposed to 0, 1, 5, or 10 mg PFOA/kg/day for 14 days exhibited swollen mitochondria and irregular nuclei with condensed chromatin (Zhang et al., 2008). In the current study, our results revealed that PFOA exposure causes hepatic damage, including cytoplasmic vacuolization, cellular border diffusion, nuclear breakage, nuclear chromatin margination, and mitochondrial swelling in hepatocytes. No sex-specific hepatohistological impairment was observed. These results support the conclusion that PFOA induces histological damage in the livers of *R. nigromaculata*. In addition, our previous study showed that histological damage caused by environmental toxicant exposure can be restored in rats (Tang et al., 2016). Therefore, we suspect this restoration occurs after termination of PFOA exposure and plan to use a recovery test group in future studies.

Oxidative stress is responsible for primary sclerosing cholangitis–induced liver damage because direct structural damage and indirect damage are caused by ROS-induced activation of multiple intracellular signaling pathways, including Nrf2 (Circu and Aw, 2010). PFOA-treated frogs exhibited significantly increased ROS levels and MDA contents, suggesting that excessive ROS generation causes oxidative stress in frog livers (Liu et al., 2007). Thus, in frogs, PFOA activates Nrf2 indirectly through oxidative stress. To activate phase II genes for detoxification, dimers bind to ARE to activate antioxidative genes that are involved in antioxidant systems. These systems consist of enzymes and nonenzyme proteins, such as GSH, GPx, and GST, and the expression levels of these proteins are related to increased ROS levels (Kobayashi and Yamamoto, 2006). Research has shown that PFOA exposure decreases GSH content and GPx and GST activities in hepatocytes and produces oxidative stress by altering antioxidant parameters (Liu et al., 2007, 2008). In this study, GSH and GST activities significantly increased, whereas GPx activity significantly decreased in PFOA-treated female and male groups compared with the corresponding control groups., suggesting that PFOA induces oxidative stress in the frog liver by altering cellular oxidative homeostasis. In addition, PFOA-treated female frogs showed significantly higher GSH, GST, and GPx and lower MDA content in the liver tissue than the corresponding male frogs groups. These results indicated that PFOA induces excessive ROS generation and oxidative stress and that female frogs experience greater PFOA toxicity than male frogs. The Nrf2-ARE pathway plays a protective role in the PFOA-induced liver damage of frogs. However, liver damage was not completely eliminated, a result that may be related to the induction of Nrf2 signal pathways by PFOA (Ren et al., 2009).

PFOA indirectly activates Nrf2 through oxidative stress, further activating phase II genes that exert protective effects (Shi and Zhou, 2010). Nrf2 knockout mice exhibit weakened repair processes and severe inflammation, indicating a causal relationship between decreased Nrf2 expression and liver injury (Xu et al., 2008). These studies provide evidence that Nrf2, a sensor of exogenous toxins, has a key role in the activation of defense systems by exogenous toxins (Klaassen and Reisman, 2010). Thus, hepatic Nrf2 expression at the mRNA level significantly increased in a dose-dependent manner in male and female frogs exposed to 0.01, 0.1, 0.5, and 1 mg/L PFOA for 14 days. Once activated, Nrf2 dissociates from a heterodimer that consists of Keap1 and transfers into the nucleus, where it forms a heterodimer with the Maf protein; the heterodimer then identifies and binds to ARE and initiates the transcription of genes involved in phase II detoxification (Kobayashi and Yamamoto, 2005). Studies have reported that PFOA and PFOS upregulate the expression of Nrf2 and phase II genes NQO-1 and GST (Ren et al., 2009) and that Nrf2 and its target gene HO-1 protect zebrafish against PFOS-induced damage (Shi and Zhou, 2010). The present study found that hepatic NQO-1 and HO-1 mRNA levels significantly increase in male and female frogs exposed to PFOA and further activates Nrf2-ARE expression at the enzyme level to protect frogs from PFOA-induced liver damage.

CYPs may play a protective role in organisms against the damage induced by environmental toxins by catalyzing the oxidative and reductive metabolism of environmental xenobiotics and endogenous compounds. CYPs comprise a superfamily of heme-containing monooxygenases, and the CYP1A and CYP3A subfamilies are frequently used as biomarkers in toxicological studies given that their expressions significantly affect potential toxicity risks (Guengerich, 1991; Uno et al., 2012). The hepatic activity of ethoxyresorufin *O*-deethylase (EROD) is used as an index for assessing the enzyme levels of CYPs and is associated with halogenated organic contaminants (Braune et al., 2011). Exposure to PFOA or PFOS significantly induces EROD activity in a dose-dependent manner (Han et al., 2012). Using microarray techniques, PFOA induces CYP expression in the liver (Guruge et al., 2006). PFOA significantly increases the mRNA levels of CYP3A and CYP1A in the livers of male and female Atlantic salmon, suggesting that detoxification is involved in PFOA-induced toxicity (Mortensen et al., 2011). In the present study, CYP3A expression significantly increased in the livers of male and female frogs exposed to PFOA. In addition, the PFOA-treated female groups showed significantly lower CYP3A mRNA expression than the corresponding male groups. Therefore, PFOA affects the detoxification systems of amphibians by inducing the expression of CYPs at the enzyme and mRNA levels. However, the induction of CYP expressions may consequently enhance the toxicity of PFOA through the biotransformation of CYPs (Souidi et al., 2005).

Taken together, our findings in *Rana nigromaculata* demonstrate that PFOA induces hepatic injury and that female frogs are more sensitive to this toxicity. This damage may be mediated by CYP3A and genes associated with the Nrf2 signaling pathway.

5. Conclusion

Liver cells from male and female frogs exposed to PFOA showed obvious structural damage, which was characterized by cell border diffusion, cytoplasmic vacuolization, broken nuclei, nuclear chromatin margination, and mitochondrial swelling. In addition, PFOA caused excessive ROS generation, an increased MDA content, and disrupted antioxidant systems, thus significantly increasing the GSH content and GST activity. By contrast, PFOA significantly inhibited GPx activity. The sensitivity of male frogs to this damage was weaker than that of the female frogs. Furthermore, the Nrf2-ARE pathway was indirectly activated by oxidative stress. PFOA-induced liver damage is related to the induced expression of genes associated with phase I and phase II detoxification. Expression of CYP3A, which is involved in phase I detoxification, was significantly increased at the mRNA level. Upregulated CYP3A mRNA expression may enhance PFOA-induced hepatotoxicity through biotransformation. The expression levels of the phase II

detoxification genes NQO1 and HO-1 and the HO-1 regulator Nrf2 significantly increased at the mRNA level to exert protective effects against PFOA-induced hepatotoxicity. In conclusion, these results collectively indicate that PFOA exposure induces oxidative stress in frog livers and further activates Nrf2-ARE genes to protect against PFOA-induce hepatotoxicity.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.02.037.

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