JOURNAL OF ENVIRONMENTAL SCIENCES XX (2015) XXX–XXX

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Toxic effects of perfluorononanoic acid on the development of Zebrafish (*Danio rerio*) embryos

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ARTICLE INFO

Article history:
Received 23 August 2014
Revised 13 November 2014
Accepted 15 November 2014
Available online xxxx

Keywords:
Perfluorononanoic acid
Zebrafish embryo
Danio rerio
Developmental toxicity

ABSTRACT

Perfluorononanoic acid (PFNA) is a nine-carbon perfluoroalkyl acid widely used in industrial and domestic products. It is a persistent organic pollutant found in the environment as well as in the tissues of humans and wildlife. There is a concern that this chemical might be a developmental toxicant and teratogen in various ecosystems. In the present study, the toxic effects of PFNA were evaluated in zebrafish (*Danio rerio*) embryos. One hour post-fertilization embryos were treated with 0, 25, 50, 100, 200, 300, 350, and 400 μmol/L PFNA for 96 hr in 6-well plates. Developmental phenotypes and hatching rates were observed and recorded. Nineteen genes related to oxidative stress and lipid metabolism were examined using Quantitative RT-PCR and confirmed by whole mount *in situ* hybridization (WISH). Results showed that PFNA delayed the development of zebrafish embryos, reduced the hatching rate, and caused ventricular edema and malformation of the spine. In addition, the amount of reactive oxygen species in the embryo bodies increased significantly after exposure to PFNA compared with that of the control group. The Quantitative RT-PCR and WISH experiments demonstrated that mRNA expression of the *lfabp* and *ucp2* genes increased significantly while that of *sod1* and *mt-nd1* decreased significantly after PFNA exposure. The mRNA expression levels of *gpx1* and *mt-atp6* decreased significantly in the high concentration group. However, the mRNA expression levels of both *ppara* and *pparg* did not show any significant variation after exposure. These findings suggest that PFNA affected the development of zebrafish embryos at relatively low concentrations.

Introduction

Perfluoroalkyl acids (PFAAs) are a family of perfluorinated compounds (PFCs) consisting of high-energy carbon–fluorine (C–F) bonds. Perfluoroalkyl acids include perfluorooctanoic acid (PFOA) and perfluorooctanesulfonate (PFOS), which have both been widely used in commercial and consumer applications due to their unique hydrophilic and lipophobic physicochemical characteristics. While these characteristics are desirable in industrial applications, they also increase resistance to degradation by natural processes, such as metabolism, hydrolysis, photolysis, and biodegradation (Kudo and Kawashima, 2003), and increase persistence in the environment (Renner, 2009). Today, they are found throughout the global environment and have been detected in the tissues of wildlife and humans. Recently, additional regulatory exposure-reduction control measures from the United States Environmental Protection Agency (US EPA) have led the fluoropolymer industry to work toward

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http://dx.doi.org/10.1016/j.jes.2014.11.008
1001-0742/

Please cite this article as: Liu, H., et al., Toxic effects of perfluorononanoic acid on the development of Zebrafish (*Danio rerio*) embryos, J. Environ. Sci. (2015), http://dx.doi.org/10.1016/j.jes.2014.11.008

phasing out PFOA by 2015. Although the manufacture of PFOA is being phased out, and the manufacture of PFOS has already been stopped in the US, alternative PFAAs, such as perfluorononanoic acid (PFNA), continue to be used in certain products.

PFNA is a nine-carbon member of the PFAA family, and has been found in the environment and in human serum at a level much lower than that of PFOA or PFOS; however, the levels in aquatic environments and organisms are higher than those of PFOA or PFOS (Quakenbush and Citta, 2008). It has also been reported that the concentration of PFNA in some wildlife, such as Chinese sturgeon (*Acipenser sinensis*), is much higher than that of PFOA (Peng et al., 2010). Levels of PFNA in human serum have risen in recent years, ranging from 2.15×10^{-4} to 2.47×10^{-2} μmol/L (Calafat et al., 2007a, 2007b), with its presence correlated to PFNA ingested with food and water (Karrman et al., 2009; Weihe et al., 2008). Only a few studies have investigated its toxicity, however, which have indicated that nine-carbon PFNA is an immune system toxicant (Fang et al., 2008) and can induce developmental toxicity in mice when administered throughout the gestational period (Wolf et al., 2010).

Zebrafish (*Danio rerio*) are a tropical freshwater fish belonging to the minnow family (*Cyprinidae*) of order *Cypriniformes*. Zebrafish are useful model organisms for vertebrate development and gene function studies, and their use in drug discovery and safety assessment of pharmaceutical agents and other chemicals has been extensively pursued (Hill et al., 2005; Sipes et al., 2011). As a toxicological model species, zebrafish have advantages such as small body size, ease of husbandry and breeding, high fecundity (a single spawning produces 100–200 eggs each week), *in vitro* fertilization, development, and transparent embryos and early stage larvae. Our previous study on adult zebrafish exposed to PFASs indicated that fatty acid β-oxidation and oxidative stress responses in the liver were disturbed by PFDoA (Liu et al., 2008). Whether PFASs cause similar toxic effects in the early stages of zebrafish development remains unclear. In this study, we explored the effect of PFNA on the early stages of zebrafish development.

1. Materials and methods

1.1. Chemicals

Perfluorononanoic acid (PFNA, CAS number 375-95-1, 97% purity) was purchased from Sigma-Aldrich (St. Louis, USA). Stock solutions of PFNA (0.01 mol/L) were prepared by stirring to dissolve the chemicals in water. Working solutions were prepared by serial dilution with fish water (3.5 g/L NaCl, 0.05 g/L KCl, 0.1 g/L $CaCl_2$, 0.025 g/L $NaHCO_3$ with pH of 6.8–7.2).

1.2. Zebrafish embryos and larvae

Adult wild-type zebrafish (Tuebingen strain) were provided by Peking University, a sub-center of the National Zebrafish Resources of China, and were kept in an automatic zebrafish housing system (ESEN, EnvironScience, China) at (28 ± 0.5)°C in a 14-hr light/10-hr dark cycle. The fish water was prepared by the system at a pH and conductivity range of 7.2–8.0 and 500–580 μS, respectively. Zebrafish embryos were obtained by natural spawning of adult zebrafish. Embryos were raised and maintained at (28 ± 0.5)°C in fish water (Westerfield, 2000). This study was approved by the Ethical Review Committee of the Institute of Zoology, Chinese Academy of Sciences.

1.3. Chemical treatment and phenotype observation in toxicity tests

Spawning and fertilization took place within 30 min after the lights were turned on in the morning. Eggs were transferred to a 10 cm Petri dish. Clean embryos were cultured in 6-well plates with 3 mL of fish water in each well, with three replicates for the control group and each treatment group. Fifty normally shaped fertilized embryos were assigned to the control and each treatment group. The exposure experiment was initiated at one hour post-fertilization (hpf). Exposure concentrations of PFNA were set at 0, 25, 50, 100, 200, 300, 350, and 400 μmol/L. Embryos were cultured in an incubator at (28 ± 0.5)°C during the 96 hr exposure experiment. The embryo test procedure, as described previously (Nagel, 2002), was prolonged from 48 to 72 hpf in order to evaluate hatching rate. The selected endpoints of this study are shown in Appendix A Table S1. The opaque embryo rate at 8 and 24 hpf, failed gastrulation at 8 hpf, hatching rate and ventricular edema rate were recorded. Photographs at each stage of development were taken using a microscope.

1.4. Reactive oxygen species assay of whole zebrafish embryos

We characterized reactive oxygen species (ROS) production in whole zebrafish embryos during PFNA exposure at 24, 48, 72, and 96 hpf. ROS production was detected in live zebrafish embryos and larvae using a free permeable radical sensor (H2DCFDA, Molecular Probes, Life Technologies, USA) as described previously (Goody et al., 2013). A non-fluorescent form of fluorescein was converted into the highly fluorescent 2′,7′-dichlorofluorescein (DCF) molecule upon cleavage of the acetate group through oxidation. Anesthetized zebrafish were distributed in each well of a 96-well plate and incubated with H2DCFDA at 10 μmol/L for 30 min. Positive groups were treated with H_2O_2 at 25 μmol/L for 30 min. The ROS removing group was treated with N-acetylcysteine (NAC) at 100 μmol/L. Before the assay, the plate was rinsed three times using fish water. The photographs were taken with a Nikon Eclipse Ti–S microscope under a ~580 nm wavelength filter and Nis-elements F package software with identical parameters used. Fluorescence intensity was measured with a microplate reader (BioTek Gen5 1.11, USA) using excitation and emission filters of 488 nm and 525 nm, respectively.

1.5. Quantitative real-time PCR

We used 96 hpf larvae for RNA extraction and subsequent qPCR assays. Total RNA of the zebrafish larvae was isolated using a Trizol reagent (Ambion, Life Technologies, USA) and the isolation process strictly followed the manufacturer's instructions.

Quantitative real-time PCRs were performed on a LightCycler®480 qPCR system (Roche Diagnostics GmbH, Switzerland) using a SYBR Green Real Master Mix without Rox (Tiangen, China). Primers for nineteen genes involved in

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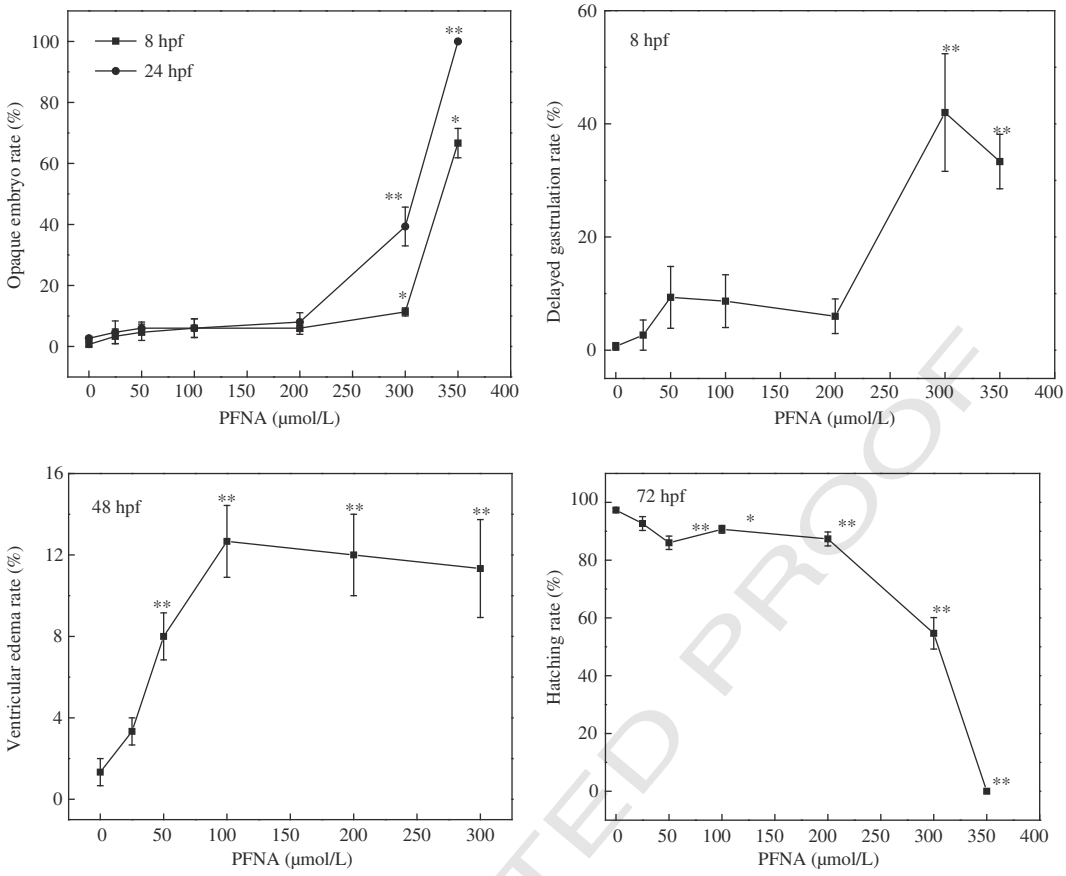

Fig. 1 – Developmental toxic effects in zebrafish (*Danio rerio*) embryos and larvae after embryonic exposure to various concentrations of PFNA from 8 to 72 hpf. (a) opaque embryo rates at 8 and 24 hpf caused by PFNA at various concentrations; (b) rate of delayed gastrulation of embryos at 8 hpf; (c) rate of ventricular edema at 48 hpf; (d) hatching rate at 72 hpf. Mean ± SEM; $n = 50$, repeat for three times; $^{*}p < 0.05$; $^{**}p < 0.01$ (control group vs. PFNA treated groups).

oxidative stress and lipid metabolism (Appendix A Table S2) were designed to investigate mRNA expression. The housekeeping gene β-actin was used as an internal control. The relative quantification of target genes was calculated based on the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

1.6. Whole mount in situ hybridization (WISH)

Whole mount *in situ* hybridization was performed as depicted previously (Thisse and Thisse, 2008). In brief, once embryos reached the 96 hpf stage, they were fixed at 4°C overnight in a 4% (W/V) PFA solution, then dehydrated by adding methanol and stored at −20°C. The 3′ end of the genes, including their 3′ UTR, was used to generate the antisense digoxigenin-labeled RNA probe used for WISH (Appendix A Table S3). Each *in situ* antisense probe for each gene of interest was individually optimized and a final probe concentration of 1 ng/mL served as a working solution. After hybridization, alkaline phosphatase conjugated anti-digoxigenin-AP Fab fragments (Roche) with BM purple (Roche) were used for the detection of the WISH probe via the production of a purple precipitate (catalyzed by the alkaline phosphatase). To avoid the formation of non-specific purple precipitates, the antibody was equilibrated adequately in appropriate alkaline (pH 9.5) Tris buffer before the addition of the substrate reagent.

1.7. Photography

Images of live embryos were captured using a Nikon SMZ 1500 dissecting microscope (Nikon, Japan) (×1 HR Plan Apo objective; numeric aperture, 0.13) with a Nikon DXM1200 digital camera driven by Nikon Act-1 version 2.12 software (Nikon, Japan). The fluorescent images of zebrafish stained by H2DCFDA probes were taken with a Nikon Eclipse Ti-S microscope under a ~580 nm wavelength filter and processed using Nis-elements F package software (Nikon, Japan).

1.8. Statistical analyses

Data were analyzed using SPSS for Windows 17.0 Software (SPSS, Inc., Chicago, USA) and presented as means with standard errors (mean ± SE). Differences between the control and treatment groups were determined using one-way analysis of variance (ANOVA). A p value of <0.05 was considered statistically significant. Origin 8.5.0 SR1 software with a nonlinear curve

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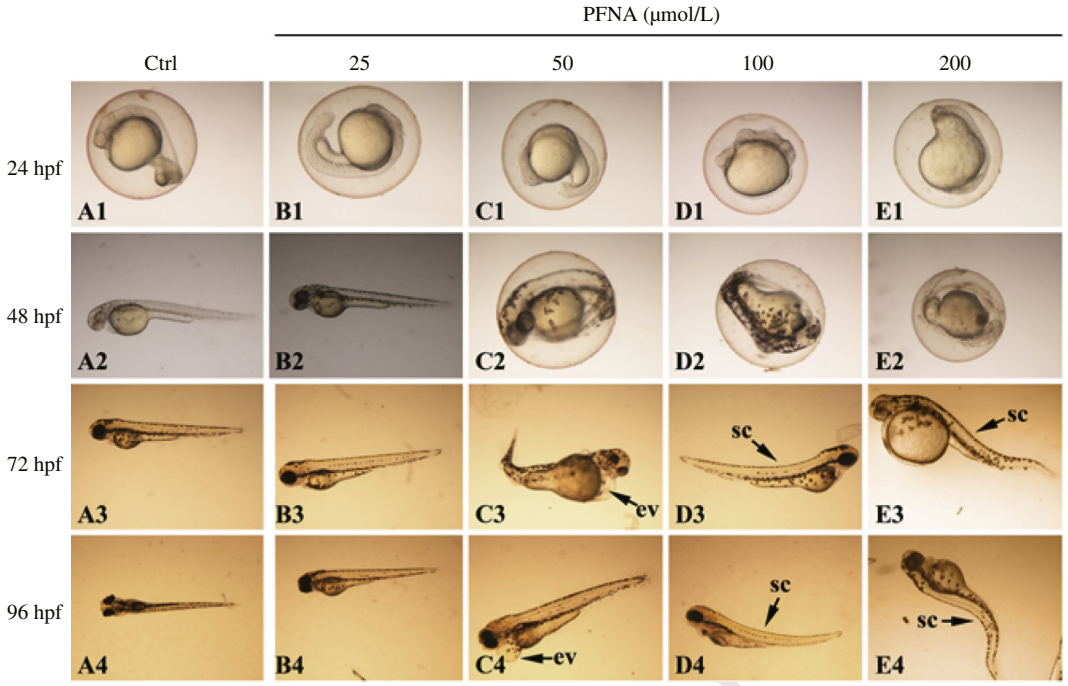

Fig. 2 – Toxic effects of PFNA on the development of zebrafish (*Danio rerio*) embryos. (A1–A4) Each stage of the normal development process of embryos in the control group. (B1–B4; C1–C4; D1–D4; E1–E4) Typical pictures of each stage of the development process of embryos at various PFNA concentrations (25, 50, 100 and 200 μmol/L).ev: ventricular edema; sc: malformation of the spine.

fit was used to calculate LC_{50} and also develop graphs (Origin Lab Corporation, USA).

2. Results

2.1. Developmental toxicity of PFNA

PFNA was acutely toxic to zebrafish embryos, with half-lethal concentration (LC_{50}) values of 342 and 302 μmol/L PFNA at 8 and 24 hpf, respectively. All embryos in the 400 μmol/L PFNA group turned opaque at 8 hpf. The number of opaque embryos was positively correlated with the concentration of PFNA. The number of opaque embryos did not increase at 8 or 24 hpf at low PFNA concentrations (lower than 200 μmol/L). However, the number of opaque embryos increased markedly when the PFNA concentration increased from 200 to 400 μmol/L (Fig. 1).

The rate of gastrulation was not significantly influenced at low PFNA concentrations (lower than 200 μmol/L), but increased significantly at higher concentrations (300 and 400 μmol/L). At 48 hpf, the ventricular edema rate increased significantly with PFNA concentration, and this trend even followed for lower PFNA concentrations (25 and 50 μmol/L). At 72 hpf, the hatching rate dropped markedly with increasing PFNA concentration.

The embryos of the control group developed normally in regular fish water. Hatching began at 48 hpf and finished at 72 hpf (Fig. 2A1–A4). The embryos of PFNA-treated groups showed hatching delay and serious malformations at each stage of development (Fig. 2B1–E4).

2.2. ROS assay

Compared with the control group, significant ROS production in zebrafish (*D. rerio*) larvae was detected in PFNA-treated groups at each concentration and time point (Fig. 3). Also, compared with the control group, nonanoic acid (NA, 100 μmol/L) did not cause significant ROS production in zebrafish larvae (Appendix A Figs. S1 and S2). These results implied that it was PFNA in total that caused ROS production rather than its acidity. The positive groups were treated with H_2O_2 at 25 μmol/L for 30 min. The ROS-removing group was treated with N-acetylcysteine (NAC) at 100 μmol/L. ROS production caused by PFNA treatment was attenuated by adding NAC, further proving that PFNA treatment can cause ROS production in the zebrafish body.

2.3. Gene expression assayed by qRT PCR

To investigate the toxic effects of PFNA on zebrafish embryo development, we surveyed the transcription levels of nineteen genes related to oxidative stress and lipid metabolism using qRT PCR.

Compared with that in the control group, the mRNA expression levels of uncoupling protein 2 (*ucp2*) and fatty acid binding protein 1 liver (*lfabp*) were significantly upregulated in the PFNA-treated groups in a dose-dependent manner (Fig. 4). mRNA expression levels of both NADH dehydrogenase subunit 1 (*mt-nd1*) and superoxide dismutase 1 (*sod1*) were significantly downregulated in all PFNA-treated groups, while mRNA expression levels of ATP synthase F0 subunit 6

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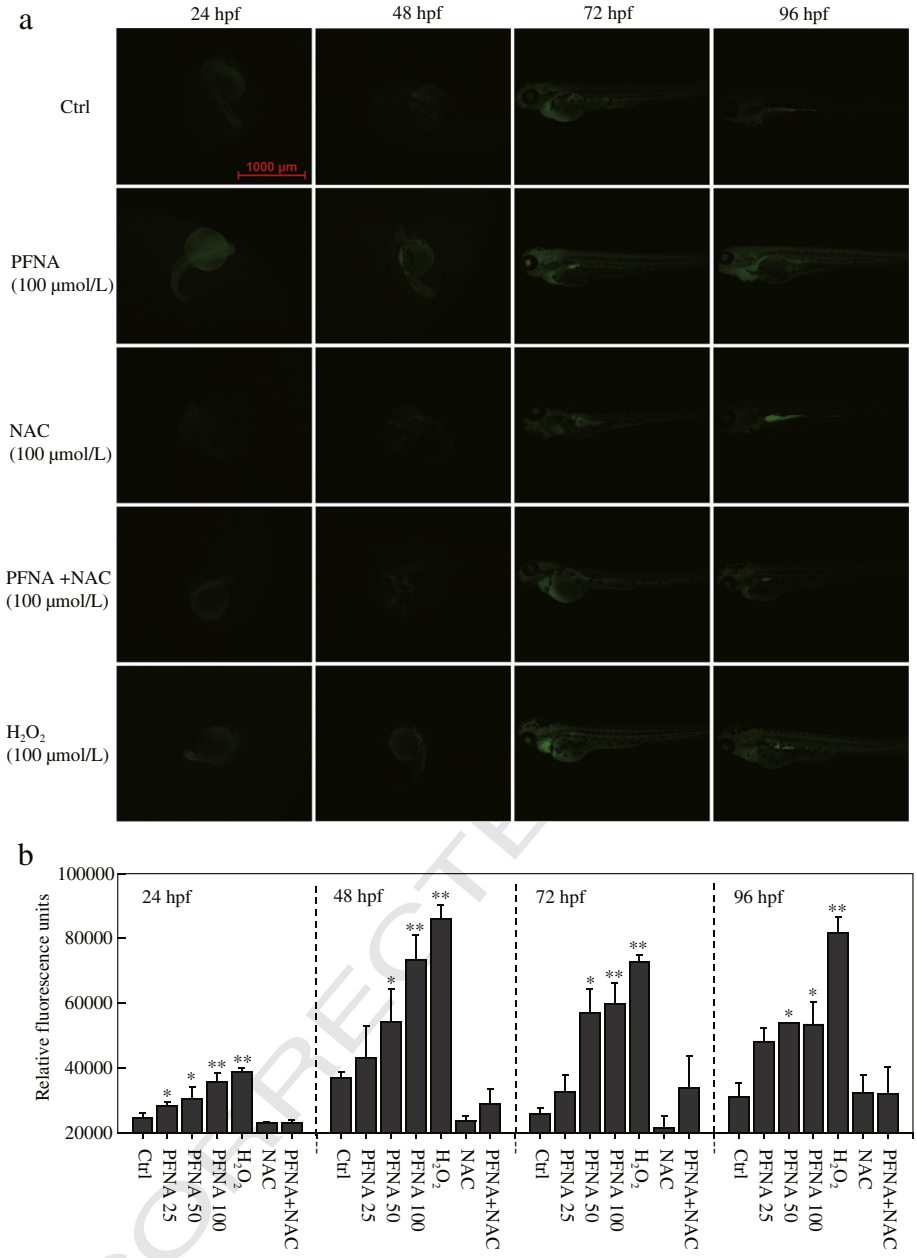

Fig. 3 – PFNA can cause ROS production in zebrafish (*Danio rerio*) larvae in a time- and dose-dependent manner compared with the control group. ROS were detected with a fluorescent probe (H2DCFDA) staining at 10 μmol/L. (a) significant ROS were detected in PFNA treated groups at each concentration and time compared with the control group. Positive group were treated with H_2O_2 at 25 μmol/L for 30 min. ROS remove group were treated with N-acetylcysteine (NAC) at 100 μmol/L. ROS production caused by PFNA treatment can attenuate by adding NAC. Scale bar = 1000 μm. (b) Fluorescence intensity was measured by a microplate reader, using excitation and emission filters of 488 and 525 nm, respectively. Mean ± SEM; $n = 8$ $^{*}p < 0.05$; $^{**}p < 0.01$ (control group vs. treated groups).

(*mt-atp6*) and cytochrome c oxidase subunit I (*cox1*) significantly decreased in only the 100 μmol/L and 50 μmol/L PFNA treated groups, respectively. The transcriptional levels of peroxisome proliferator-activated receptor alpha a, b (*pparaa*, *pparab*) and peroxisome proliferator-activated receptor gamma (*pparg*), as well as other genes, remained unchanged (Appendix A Fig. S3).

2.4. mRNA distribution in tissue tested by WISH

We performed whole mount *in situ* hybridization to further confirm the tissue distribution of the genes (*ucp2*, *lfabp*, *ppara*, and *pparg*). Results showed that the variations were consistent with the qRT PCR results (Fig. 5). *Lfabp*, *ppara* and *pparg* were mainly distributed in the liver, and *ucp2* was distributed

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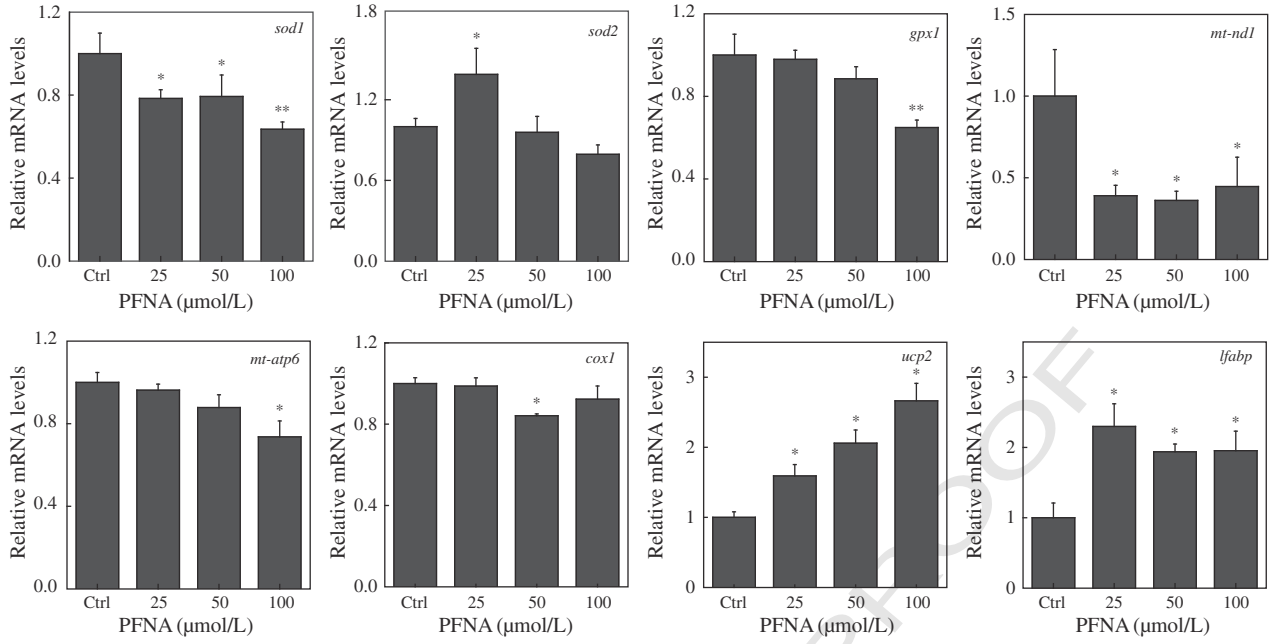

Fig. 4 – Quantitative RT-PCR analysis of zebrafish (*Danio rerio*) larvae mRNA expression levels of control and PFNA treated groups at various concentrations. Mean ± SEM; n = 6 *$p < 0.05$; **$p < 0.01$ (control group vs. PFNA treated groups). sod1: superoxide dismutase 1; SOD2: superoxide dismutase 2; gpx1: glutathione peroxidase 1; mt-nd1: NADH dehydrogenase 1, mitochondrial; mt-atp6: ATP synthase 6, mitochondrial; cox1: cytochrome c oxidase I, mitochondrial; ucp2: uncoupling protein 2; lfabp: fatty acid binding protein 1a, liver.

in the liver as well as other tissues. The mRNA transcription levels of *ucp2* increased after PFNA treatment, *lfabp* did not increase significantly compared with the qPCR results, and the mRNA transcription levels of *ppara* and *pparg* did not change significantly.

3. Discussion

Perfluoroalkyl acids have been detected in mammalian tissues, even in remote areas such as in humans and polar bears living in the arctic (Olsen et al., 2000; Smithwick et al., 2006). Many studies have examined the effects of PFAAs (especially PFOA or PFOS) in laboratory animals including rodents, birds, fish, and amphibians (Abbott et al., 2007; Ankley et al., 2004; Ankley et al., 2005; Cheng et al., 2011; Shi et al., 2008). In this study, we assessed the developmental toxicity of PFNA using zebrafish as a model. Results showed that PFNA was acutely toxic to zebrafish embryos at higher concentrations and it had obvious adverse effects on embryonic development. Developmental abnormalities caused by PFNA included delay in hatching and tail malformation. These phenomena were also reported by Zheng et al. (2012), who investigated the effects of several PFCs on the developmental toxicity of zebrafish embryos. Hatching

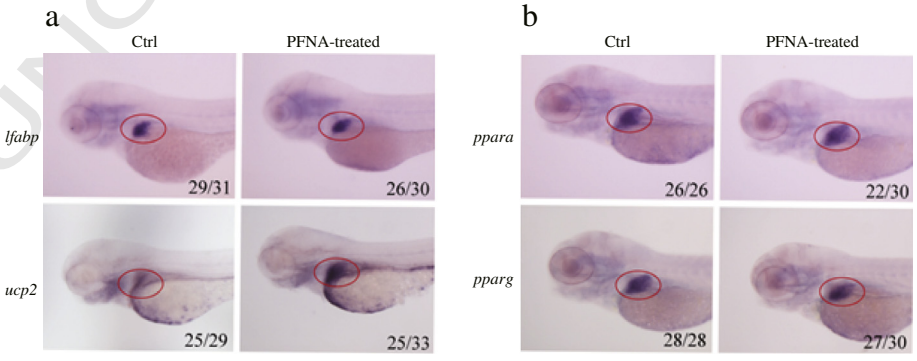

Fig. 5 – Expression of the genes of interest in the liver of zebrafish (*Danio rerio*) larvae assayed by WISH. (a) Expression levels of *ucp2* upregulated in the PFNA treated groups (PFNA 100 μmol/L) vs. the control group. Expression levels of *lfabp* turned to be up regulation tendency. (b) Expression levels of *ppara* and *pparg* unchanged in the PFNA treated groups (PFNA 100 μmol/L) vs. the control group. Anterior to the left and dorsal to the top. Red circles mark the liver area. The number in the lower right corner of the picture is the incidence.

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rate is a toxicological endpoint for evaluating the teratogenicity of chemicals. In this study, the hatching rate dropped considerably with increasing PFNA concentrations, even at relatively low concentrations. Ventricular edema was another significant toxic effect caused by PFNA exposure, indicating that PFNA might cause injury or inflammation to the cardiovascular system. However, the mechanism of toxicity needs further study.

Previous studies have shown that PFCs can increase ROS content in mammalian liver cells (Hu and Hu, 2009; O'Brien and Wallace, 2004; Panaretakis et al., 2001) and cerebellar granule cells (Reistad et al., 2013). In this study, we assayed the ROS levels in zebrafish larvae at a series of embryo development stages and found that PFNA caused significant ROS production as early as 24 hpf, and sustainable levels of ROS were produced during the entire PFNA treatment period. ROS are highly reactive and can attack biomolecules such as proteins, lipids, and DNA, causing damage to living cells. The excess amount of ROS production during the development stage may be a key explanation for the toxicity effect of PFNA (Shi and Zhou, 2010). Four genes (*lfabp*, *ucp2*, *mt-nd1*, and *sod1*) were shown to have significant changes in expression levels after PFNA exposure. Compared with that of the control group, the transcriptional levels of *lfabp* and *ucp2* were significantly increased in the PFNA treated groups, while the transcriptional levels of *mt-nd1* and *sod1* were significantly downregulated. *Ucp2* is a member of the mitochondrial anion carrier proteins (MACP) family (Millet et al., 1997). It plays an important role in the use of energetic substrates (glucose and fatty acids) and the production of ROS. Several investigators reported that *ucp2* is important for reducing ROS formation and protecting cells from their damaging effects (Brand, 2000). *Ucp2* was significantly upregulated in the PFNA-treated groups in our study, which might be related to PFNA inducing excess ROS in the mitochondria of zebrafish embryos. *Lfabp* was also upregulated in the treated groups, consistent with our previous findings in adult zebrafish (Zhang et al., 2012b); however, whether the overexpression of *lfabp* in zebrafish embryos was the cause of PFNA toxicity, or simply the result of PFNA toxicity, remains to be determined. Previous evidence shows that FABPs play an important role in the uptake, sequestering, and transport of fatty acids, and interact with other transport and enzyme systems (Atshaves et al., 2010). With a structure similar to fatty acids, PFAAs might successfully compete with these natural ligands for FABP binding (Luebker et al., 2002). PFAAs are known to activate and upregulate the expression of nuclear receptors, such as *ppara* and *pparg* in rodents (Shipley et al., 2004; Wolf et al., 2008; Wolf et al., 2010), which, once activated, form heterodimers with retinoic acid receptors (RARs) or retinoid X receptors (RXRs). These, in turn, bind to response elements such as FABP genes and stimulate their transcription. Zhang et al. (2012a) found that the transcription levels of *ppara* and *pparg* in male zebrafish livers increased after PFNA treatment. In the present study, however, *ppara* and *pparg* showed no significant change after PFNA treatment. This phenomenon for activation of *ppara* and *pparg* by PFCs indicates that rodents and non-rodents react differently to PPARs; however, the exact mechanism remains unclear. In this study, *mt-nd1* was significantly downregulated in the PFNA-treated groups. *Mt-nd1* encodes NADH dehydrogenase 1 in the inner membrane of mitochondria, which plays an important role in electron transportation in the respiratory chain. The downregulation of *mt-nd1* may reduce electron transfer and induce excessive cations to accumulate in the mitochondrial matrix, thereby causing damage to the developing zebrafish embryos. Superoxide dismutase 1 (*sod1*), also known as Cu/Zn superoxide dismutase, binds copper and zinc ions and is one of the two isozymes responsible for destroying free superoxide radicals in the body (Valentine et al., 2005; Zelko et al., 2002). In this study, we found that PFNA exposure resulted in hypergeneration of ROS in zebrafish embryos and reduction in *sod1* enzyme activities, which plays a protective role in antioxidization. Glutathione peroxidase 1 (*gpx1*) encodes a member of the glutathione peroxidase family, which is a family of proteins functioning in the detoxification of hydrogen peroxide, making it one of the most important antioxidant enzymes in humans (Lei et al., 2007; Lubos et al., 2011). In this study, the mRNA level of *gpx1* was decreased in treated groups under high PFNA concentration. From these mRNA changes, we found that after PFNA treatment, the genes related to ROS production were upregulated while the genes related to ROS removal were downregulated. Thus, ROS was accumulated in the body of the zebrafish embryos.

4. Conclusions

PFNA was toxic to the development of zebrafish embryos and caused a significant increase in ROS content in the zebrafish embryo body. The mRNA expression levels of *lfabp* and *ucp2* were significantly increased in the PFNA-treated groups, while the levels of *mt-nd1* and *sod1* were significantly decreased. These gene expression variations were consistent with the increase in ROS content in the zebrafish body. However, the mRNA expression levels of *ppara* and *pparg* did not change significantly. These findings were confirmed by the WISH experiment: the expression of *ucp2* was upregulated, *lfabp* showed an upregulation tendency, and the expression levels of *ppara* and *pparg* did not change significantly.

Acknowledgments

This work was supported by the National Basic Research Program (973) of China (No. 2013CB945204) and the National Natural Science Foundation of China (Nos. 31320103915 and 21377128).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2014.11.008.

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Please cite this article as: Liu, H., et al., Toxic effects of perfluorononanoic acid on the development of Zebrafish (*Danio rerio*) embryos, J. Environ. Sci. (2015), http://dx.doi.org/10.1016/j.jes.2014.11.008

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Please cite this article as: Liu, H., et al., Toxic effects of perfluorononanoic acid on the development of Zebrafish (*Danio rerio*) embryos, J. Environ. Sci. (2015), http://dx.doi.org/10.1016/j.jes.2014.11.008