Chronic Effects of Waterborne PFOS Exposure on Growth, Development, Reproduction and Hepatotoxicity in Zebrafish

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Abstract—To evaluate the potential effects on growth and reproduction of perfluorinated compounds (PFCs), the zebrafish fry (F0, 14 d post-fertilization, dpf) were chronically exposed to various concentrations of PFOS (0, 10, 50 and 250 µg l–1) until sex mature (70 d) and effects on reproductive capacity and endocrine disrupted potential were assessed. No significant adverse effects on survival were observed in developing zebrafish exposed to PFOS for 70 d under all tested concentrations. Reduction of growth (weight and length) and histological alternations most prominently with lipid droplets accumulation in the liver were only observed in the male fish. Whole body triiodothyronine (T3) levels were not significantly changed in both male and female. Gonadal somatic index (GSI) of the females was significantly reduced in the exposure groups. Despite hepatic vitellogenin (VTG) gene expression was significantly up-regulated, no intersex was found in testes as well as the sex ratio was not changed. After 70 d exposure, remaining fish were placed to clean water for 30 d to assess recovery of hepatotoxicity, but histological alternations in the male fish liver were not reversible. To determine possible developmental effects of PFOS exposure on the F1 generation, a subset of exposed females was paired with unexposed males. The results showed that hatching rates were not affected. However, embryo malformations largely with curved spines were observed in 50 and 250 µg l–1 of PFOS treated groups and resulted in mortality. Taken together, the study suggested that long-term maternal exposure zebrafish to lower concentration of PFOS could impair development in the F1 generation, and the risk assessment of estrogenic endocrine disruption of PFOS in the aquatic environment is likely small.

Keywords: PFOS, development, vitellogenin, hepatotoxicity, thyroid hormone, zebrafish

INTRODUCTION

The uses of perfluorinated chemicals (PFCs) as surfactants for commercial and industry applications during several decades have resulted in a global distribution of stable precursors/metabolites, such as perfluorooctanesulfonate (PFOS), perfluorooctanoic acid (PFOA) in the aquatic environment, wildlife and human (e.g., Giesy and Kannan, 2001; Kannan et al., 2002; Hoff et al., 2003, 2005; Interdisciplinary Studies on Environmental Chemistry—Biological Responses to Chemical Pollutants, Eds., Y. Murakami, K. Nakayama, S.-I. Kitamura, H. Iwata and S. Tanabe, pp. 37–54. © by TERRAPUB, 2008.)
Martin et al., 2004; Dai et al., 2006; So et al., 2007; reviewed by Lau et al., 2004, 2007; Houde et al., 2006a, b). PFOS is believed to be an end-stage metabolite and environmental degradation product of N-alkyl-perfluorooctanesulfonamide (PFSM) chemistries used in a wide range of commercial products (3M, 2003). PFOS is resistant to biotic or abiotic degradation and thus stable in the environment, furthermore, it can be bioaccumulated through food chain, which is most abundant and detected in blood and liver samples from various aquatic mammals, birds, fish and humans and thus regarded emerging persistent organic pollutants (POPs). The persistence of PFOS in wildlife and aquatic environment has caused great concerns over its toxicity.

In the aquatic environment, detected PFOS concentrations in surface water are generally low (<0.1 µg l⁻¹). However, higher concentrations of PFOS have been detected in fish. For instance, the measured PFOS in the liver was up to 7,760 µg kg⁻¹ wet weight from plaice (Pleuronectes platessa) (Hoff et al., 2003), and 9,031 µg kg⁻¹ wet weight from feral gibel carp (Carassius auratus gibelio) in Belgium (Hoff et al., 2005). It is worth noting that high concentrations of PFOS were also detected in fish eggs (145–381 µg kg⁻¹) in lake whitefish (Coregonus clupeaformis) from Michigan waters, USA (Kannan et al., 2005), suggesting oviportal transfer of this compound. Laboratory study also demonstrated that water-borne exposure PFOS can be bioaccumulated in rainbow trout (Oncorhynchus mykiss) (Martin et al., 2003), and oviportal transfer in fathead minnow (Pimephales promelas) (Ankley et al., 2005). For example, the mean concentrations of PFOS in ovary were 64,600 µg kg⁻¹ after fathead minnows exposure to 30 µg l⁻¹ PFOS for 21 d.

Based on existing data, PFOS has been shown to influence membrane function and structure of hepatocytes, as assessed by increase in serum alanine aminotransferase (ALT) activity in carp (Cyprinus carpio) (Hoff et al., 2003) and hepatic PFOS concentration was significantly and positively related to serum ALT activity in both feral carp (C. carpio) and eel (Anguilla anguilla) (Hoff et al., 2005). In reproductive and developmental toxicity, it appeared that plasma androgens and estrogens can be affected after fathead minnow (Pimephales promelas) exposed to PFOS (Oakes et al., 2004, 2005; Ankley et al., 2005). PFOS exposure to zebrafish embryos resulted in developmental toxicity and altered certain gene expression (Shi et al., 2008). Recently, several studies showed that estrogenic properties of PFOA (induction of vitellogenin, VTG) in rare minnow (Gobiocypris rarus) (Wei et al., 2007), induction of VTG in cultured male tilapia hepatocytes (Liu et al., 2007) and in male medaka (Oryzias latipes) treated with fluorotelomer alcohol (FTOHs) (Ishibashi et al., 2008).

Despite the widespread of PFOS detected in the environment as well as several studies of toxicity in fish, compared to other POPs, relatively little is known concerning the long-term toxicity of PFOS in fish. Present available ecotoxicological information has been derived from short-term test and ecotoxicological consequence in aquatic environment has been largely unknown (Oakes et al., 2004). For environmental risk assessment, a comprehensive understanding on the temporal responses (i.e. the time for initial induction,
maximum induction, adaptation and recovery) of stress in fish at various biological hierarchies is essential (Wu et al., 2005). Therefore, the aims of the present study were (1) to investigate the effects of PFOS on growth and reproductive toxicity in the zebrafish under chronic exposure basis for estimating ecological risk; (2) to study the reversibility of the morphological changes in fish liver after the cessation of PFOS exposure; (3) to select VTG gene expression as biomarker and evaluate whether PFOS exposure could impair fish reproduction through endocrine disrupted activities. The developing zebrafish fry (14 dpf) were chosen, since it is well-known that early life stage is more sensitive stage to toxicant stress. The zebrafish were exposed to various concentrations of PFOS until sex mature and the eggs from the maternal exposure were fertilized with those unexposed male, which may be useful for evaluation of the effects of maternal derived PFOS on the offspring.

MATERIALS AND METHODS

Chemicals

Heptadecafluorooctanesulfonic acid potassium salt (PFOS, >99%) was obtained from Tokyo Kasei Kogyo Co. Ltd (Tokyo, Japan) and stock solution was prepared by dissolving crystal in HPLC-grade dimethyl sulfoxide (DMSO) and stored at 4°C. MS-222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt) was obtained from Sigma (St. Louis, MO, USA). All other chemicals used in the present study were analytical grade.

Fish

Adult zebrafish (Danio rerio) (AB strain) were maintained in charcoal-filtered recalculating aerated tap water. The light period was 12h:12 h light: dark cycle and the temperature was controlled at 27 ± 0.5°C. Fish were fed twice with freshly hatched Artemia nauplii and once flake food daily (Tetra, Germany). Fertilized eggs were collected and examined under stereomicroscope. The fry were maintained until 14 d post-fertilization (dpf) for subsequent experiments, since this is the time when the fry are stably survival.

Experimental design

The fry were randomly distributed into 20L glass tank container for control and exposure groups. There have three replicates in each group and contained about 140 fry. The fry were exposed in a semi-static system and the water was renewed half every other day. Both the control and exposure groups received DMSO (0.002%, v/v), respectively. From our primary experiment, nominal concentrations of PFOS were 10, 50 and 250 µg l⁻¹, in which fry could survive under these concentrations. The exposure regime included PFOS exposure period (70 d) and recovery in clean water (30 d). At 40 and 70 d exposure, about 30 fish from each treatment group were randomly selected for measurement of length, weight, meanwhile, fish were sampled for histological examination of the gonads.
and liver, VTG gene expression and T1 measurement. Remaining fish were transferred to 20 L glass tank and reared in dechlorinated municipal tap water to allow for 30 d recovery. The fish were anesthetized in 0.03% MS-222. The body weight, length and gonad weight were recorded. The gonadal-somatic index (GSI = gonad weight × 100/body weight-gonad weight) and condition factor (K = weight (g) × 100/length (cm²)) were calculated. The gonads of adult fish were examined under a dissecting microscope to evaluate the sex. On the other hand, after 70 d exposure, the exposed female fish were removed and placed into clean water and were paired with unexposed male fish. The hatching rates, malformation, survival and development in the F1 embryo-larvae were assessed.

Whole fish and liver were frozen in liquid nitrogen and stored at −80°C for thyroid hormone analysis and VTG gene expression, respectively. During the experimental period, residual food and faces in the test chamber were removed every day and 50% of the exposure water was renewed once in 3 d. The test chambers were cleaned once every two week.

**Histology**

For histological examination, tissue of liver and the middle portion of testis (40, 70 d exposure and 30 d recovery) were fixed in Bouine fixative solution. Tissues were dehydrated in ethanol, embedded in paraffin wax and sectioned at 4–5 µm. The sections were stained with haematoxylin and eosin (H and E) and examined under light microscope.

**RNA extraction and Quantitative real-time PCR assay**

Total RNA was isolated from liver using 1 ml Trizol (Invitrogen Inc., USA) following the manufacture’s protocol. The purity of extracted total RNA was determined by UV spectrophotometry (260 nm reading and 260/280 ratio). All RNA samples were DNase treated prior to be converted to cDNA. A 2 µg total RNA was converted to cDNA using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) with random primers, according to the manufacturer’s instructions. Gene specific primer pairs were based on the cDNA sequences of zebrafish VTG and β-actin (GenBank accession numbers NM001044897 and AF057040). The primers for VTG gene were 5′-AGCTGCTGAGAGGCTTGTTA-3′ and 5′-GTCCAGGATTTCCCTCAGT-3′. Forward and reverse for β-actin were 5′-CGAGCAGGAGATGGGAACC-3′ and 5′-CAACGGAAACGCTCATTGC-3′. Quantitative PCR was carried out using the SYBR® Green PCR kit (Toyobo, Tokyo, Japan) and analyzed on an ABI PRISM 7000 Sequence Detector System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The thermal cycle was as follows: 10 min at 95°C, followed by 40 cycles at 95°C for 30 s and one cycle at 60°C for 20 s and 72°C for 1 min. For VTG gene, PCR reactions were performed on three replicate samples. The expression level of VTG was normalized to its β-actin mRNA content. Fold change was used for evaluating gene expression according to the methods of Ding *et al.* (2007).
**Table 1. Effect of PFOS exposure on growth of female zebrafish.** The 14 dpf zebrafish were exposed to 0 (control), 10, 50 and 250 µg PFOS L⁻¹ for 40 and 70 d and after recovery for 30 d. Differences in each parameter were assessed using one-way analysis of variance and Tukey’s multiple range tests. Values represent means ± SEM. *P < 0.05; **P < 0.01. n = 15–20.

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**Table 2. Effect of PFOS exposure on growth of male zebrafish.** The 14 dpf zebrafish were exposed to 0 (control), 10, 50 and 250 µg PFOS L⁻¹ for 40 and 70 d and after recovery for 30 d. Differences in each parameter were assessed using one-way analysis of variance and Tukey’s multiple range tests. Values represent means ± SEM. *P < 0.05; **P < 0.01. n = 15–20.

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<td>0.36 ± 0.01**</td>
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Thyroid hormone extraction and radioimmunoassay

Thyroid hormone (TH) extraction procedure was followed the method developed by Crane et al. (2004). Fish were homogenized in 2 ml methanol containing 1 mM 6-N-propylthiouracil (PTU) and 0.1M NaOH, then the homogenates were sonicated, vortexed and centrifuged with 3,700 g for 10 min at 4°C. The supernatants were removed to clean tube and a 2 ml methanol containing 1 mM PTU was added, vortexed and centrifuged. The supernatants were removed, a 5 ml chloroform and 0.5 ml NH₄OH (2 M) were added and centrifuged. The supernatants were transferred to new tube. Finally, a 0.5 ml NH₄OH (2 M) were added and repeated the above step. The supernatants that collected were evaporated to dryness with nitrogen and stored at –80°C. All extracts were re-suspended in 400 µl NaOH (0.1 M) for T₃ RIA analysis. The efficiency of the thyroid hormone extraction protocol was determined by adding 100 µl of ¹²⁵I radiolabelled T₃ to each adult fish homogenates prior to extraction. Minimum detectable levels of T₃ in the RIA were 0.5 ng ml⁻¹.

Statistical analysis

Normality of data was verified using the Kolmogorov-Smirnov test, and homogeneity of variances checked by Levene’s test. One-way analysis of variance (ANOVA) and Tukey’s multiple range tests were used to determine significant difference between control and exposure groups. Chi-Square test was used for analyzing the data of sex ratio and malformation. All statistical analysis was performed using SPSS 13.0 soft wear (SPSS, Chicago, IL, USA) and P < 0.05 was considered statistically significant.

RESULTS

Survival, growth and condition factor

There were no significant effects of exposure to PFOS on fish survival for any of the exposure regimes for all experiments. No mortality was observed during the exposure period. PFOS did not affect body length and weight in the females in all PFOS-treated groups after 40 and 70 d exposure (Table 1), while reduced body weight and length were observed after 30 d recovery in the 50 and 250 µg l⁻¹ of PFOS-treated groups compared to control fish (Table 1). No significant differences were observed in the condition factor (K) between the solvent control and treatment groups. In the male fish, a small but significantly increased in the weight was observed in the 50 and 250 µg l⁻¹ PFOS-treated groups for 40 d (Table 2). After 70 d exposure, the body weight and length was significantly reduced in the 250 µg l⁻¹ PFOS-treated group. The decreased body weight and length were further recorded in the 50 and 250 µg l⁻¹ PFOS-treated groups after 30 d recovery (Table 2). Condition factor (K) of the males from this experiment in the 50 and 250 µg l⁻¹ group was significantly higher compared to the solvent group (Table 2).
Fig. 1. Mean (±S.E.M.) gonadosomatic index (GSI; [testes weight/body weight]×100) of the female fish after exposure to 0, 10, 50 and 250 µg l⁻¹ PFOS for 70 d and recovery for 30 d. ANOVA and Tukey’s multiple range tests were used to determine significant difference between control and exposure group. *Indicates significantly different (P < 0.05) of GSI compared to the control group. N = 10–20.

Fig. 2. Sex ratio of zebrafish (Danio rerio) treated with PFOS from 14 dpf until sexual mature with PFOS concentrations of 0, 10, 50 and 250 µg l⁻¹, respectively. Black ( ■ ) represents percentage of males, white (□) fish percentage of females. A χ²-test indicated no statistically significant effects of the PFOS treatment compared with the unexposed control group.
After 70 d exposure, the gonads of the female fish were removed and the GSI was calculated. The average GSI in the 50 and 250 µg l⁻¹ of PFOS treated groups was significantly (P < 0.05) lower than the control fish (Fig. 1). Lower GSI was further observed in the 50 and 250 µg l⁻¹ exposure groups after 30 d recovery (Fig. 1). In the present study, testes were also examined and morphological inter-sex was not observed in all the exposure groups in the male fish. A male to female gonadal sex ratio of 72:28 was present in the solvent-control group. The recorded of females was 34.1%, 36.4% and 40.1% in the 10, 50 and 250 µg l⁻¹ PFOS treated groups, respectively (Fig. 2). The results of Chi-square analysis for trends (P > 0.05) and for sex ratio was not show significantly changed in the exposure groups compared with the control (P > 0.05).

Liver histology

For histological examination, 4–5 male and female from each group were sampled. In the control fish, the liver of zebrafish appeared soft and the color is sanguine. In the male fish, after 40 d exposure, there were no obvious morphological alternations in the lower concentration (10, 50 µg l⁻¹) exposure groups compared with the control. However, exposure of 250 µg l⁻¹ of PFOS for 40 d, the liver

Fig. 3. Light micrographs of liver from zebrafish. (A) Male fish of control group, X400; (B) male fish exposed to PFOS for 40 d at 250 µg l⁻¹; (C) Control female; (D) exposed female for 40 d at 250 µg l⁻¹ PFOS. Accumulation of lipid droplets (arrow B) can be observed. X400.
seemed brittle and pale in all the male fish. By histological examination of liver structure, the homogeneous parenchyma of zebrafish liver from control fish was composed of hepatocytes arranged in a typically three-dimensional architecture (Fig. 3A). After 40 d exposure to 250 µg l−1 of PFOS, the most marked change was vacuolization mainly due to accumulation of lipid droplets (Fig. 3B). Likewise, similar alternation could be observed in the 250 µg l−1 PFOS treated male fish and in the group after 40 d exposure. Furthermore, the same symptoms were still observed in the 250 µg l−1 PFOS-treated males after recovery by 30 d. In the female fish, the control structure is shown in Fig. 3C, no obvious treatment related morphological differences were observed in all the female fish during the exposure and recovery period (Fig. 3D).
After 40 days exposure, all sampled fish were sexually differentiated. The testis of the mature zebrafish contained all spermatogenetic cells, such as spermatogonia, spermatocyte and spermatid. In the solvent control group, fewer spermatogonia could be observed and the structure of cysts major containing spermatocytes and spermatid (Fig. 4A). However, the testis contained mainly early spermatogenetic stages including spermatogonia and primary spermatocyte in 250 µg l\(^{-1}\) treated group after 70 d exposure, suggesting developmental delay.
Chronic Effects of Waterborne PFOS Exposure

There were no obvious histological alternations in the exposure groups, suggesting the lack of fundamental changes in testicular morphology in this study supports the absence of reproductive impairment in response to PFOS exposure under these concentrations.

VTG gene expression

The mRNA expression of VTG was detected at 40, 70 d exposure and 30 d recovery in both male and female fish. In male fish, VTG was significant up-regulated by 1.84-fold and 3.73-fold in 50 and 250 $\mu$g l$^{-1}$ PFOS treated group after 40 d exposure; A significant induction of VTG could further be observed in 10 $\mu$g l$^{-1}$ PFOS (3.13-fold) and 50 (5.09-fold) $\mu$g l$^{-1}$ after 70 d PFOS exposure; After 30 d recovery, A strong up-regulation ($p < 0.01$) of VTG gene expression was detected in 50 $\mu$g l$^{-1}$ (16.08-fold) PFOS exposure group (Fig. 5A). In female fish, similar induction ($p < 0.01$) of VTG transcript levels was also observed in 250 $\mu$g l$^{-1}$ treated group. No significant differences were found between control and treated groups after 70 d exposure. After recovery, VTG transcript level were significantly decreased ($P < 0.01$) in the 250 $\mu$g l$^{-1}$ PFOS treated group (Fig. 5B).

Thyroid hormone (T$_3$) concentration

The total T$_3$ level (TT$_3$) from the whole body homogenate showed no significant difference between the control and treatment group after 40 and 70 d exposure and after 30 d recovery (Table 3).

Hatching, fecundity, deformation and survival in the F1 generation

After 70 d exposure, the exposed females were removed into clean water and were paired with unexposed males. No significant differences in the total number of eggs per breeding trial were observed between the solvent control group and the treatment groups (data not shown). The hatching rates were 74.3%, 78.3%, 81.25% and 82.2% in the control, 10, 50 and 250 $\mu$g l$^{-1}$ of PFOS-treated groups, respectively, and showed no significant difference. The malformation was not

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Table 3. Effect of PFOS exposure on T3 concentration of the zebrafish (ng g$^{-1}$). The 14 dpf zebrafish were exposed to 0 (control), 10, 50 and 250 µg PFOS L$^{-1}$ for 40 and 70 d and after recovery for 30 d. Differences were assessed using one-way analysis of variance. Values represent means ± SEM, n = 5–6.
observed in the control and 0.01 µg l⁻¹ PFOS-treated fish. However, the observed percentage of malformations was significantly higher in the 50 and 250 µg l⁻¹ of PFOS-treated groups with 37.5% and 100% and all the deformed larvae died after 96h hatching, while those larvae appeared normal could survive. The most pronounced malformation was skeletal deformities with curved spines.

DISCUSSION

Zebrafish fry (14 dpf) were selected for exposure, since early life stage is more sensitive to toxicants stress. In addition, we conducted partial life-cycle test and studied the effects of PFOS on the deformation and survival of F1 generation. Compared with previous studies in fish, the present study employed lower concentration of PFOS, which detected in fish. In the present study, we were unable to determine actual test PFOS concentrations during exposures, and thus we reported nominal concentrations. However, we employed a static-renewal exposure regime with 50% test chemical replacement every 3 d and thus consistent PFOS concentrations would be expected. There was no mortality in all the treated groups (10, 50, 250 µg l⁻¹) in all the exposure regimes. The results are consistent with a previous study using adult fathead minnow, which showed no mortality after exposure to 300 µg l⁻¹ PFOS, but exposure to 1,000 µg l⁻¹ for 21 d reduced survival of the fathead minnow (Ankley et al., 2005). Significant reduction of total body length and weight was only observed for male fish raised under continuous exposure to 250 µg l⁻¹ PFOS, whereas no effects were observed in female fish, suggesting the inhibition of growth may be gender-specific. Ankely et al. (2005) reported that no significant adverse effects on growth were observed in developing fathead minnows held for 24 d at PFOS concentrations up to 300 µg l⁻¹. In mammals, several sub-chronic exposures of rats, mice and monkeys to PFOS have resulted in effects on body weight gain in females and in males (Seacat et al., 2002, 2003; Thibodeaux et al., 2003; Luebker et al., 2005a, b). Overall, the results from the mammalian and our study indicate that chronic PFOS exposure has similar effects on growth. However, PFOS exposure does not appear to affect condition factor (K) in the male and female fish. In contrast, Oakes et al. (2004) showed that the condition factor of fathead minnow in the female was significantly decreased, while no difference was observed in the male fish after exposure to 300 µg l⁻¹ for 39 d. Condition factor is an easily measured parameter and has been shown to be a useful indicator of toxic stress to organic pollutants in fish (Anderson et al., 2003). Furthermore, it is reflective of changes in food intake, lipid deposition and protein budgets (Smolders et al., 2003). The unchanged condition factors in the female fish in the present study may suggest that the overall health and somatic fitness of zebrafish was not compromised. The increased K in the male fish could be related to the accumulation of lipid droplets in the liver, since study has shown that PFOS exposure caused increased intracellular free fatty acids and free cholesterol in the liver (Seacat et al., 2002).

In order to evaluate the effects of PFOS exposure on the F1 generation, the exposed female fish were paired with unexposed males. A significant malformation as well as mortality was observed in 50 and 250 µg l⁻¹ PFOS treated-groups.
Unfortunately, we were unable to measure PFOS concentrations in the eggs. The results may suggest that oviparous transfer of the compound from adult to offspring and adult exposure (F0) could significantly affect F1 generation survival. In contrast, a previous study showed that PFOS could be transferred to gonads in fathead minnows (Ankley et al., 2005), however, Ankley and co-authors did not show any abnormal change and survival of the embryos and larvae derived from adults exposed to 300 µg l⁻¹ PFOS. In mammals, PFOS has been shown in rats and mice throughout pregnancy, to severely affect postnatal survivor of neonatal rats and mice (Thibodeaux et al., 2003; Luëber et al., 2005a, b; Fuentes et al., 2006, 2007).

Relative gonad size (GSI) was significantly smaller in the female fish after exposure to 250 µg l⁻¹, suggesting PFOS could reduce fish reproductive potential. The quality of eggs as well as the offspring viability would depend on GSI and the levels of sex hormones produced during gametogenesis (Kime, 1998). Ankely et al. (2005) reported that adult fathead minnow exposed to 300 µg l⁻¹ PFOS for 21 d exhibited decreased aromatase activity and elevated concentrations of plasma 11-ketotestosterone and testosterone. Oakes et al. (2004) reported that exposure of fathead minnows to PFOA, did not find any significant changes in female GSI, but altered plasma concentrations of both steroidal androgens and estrogens and they suggested that as reduced circulation steroid hormones are associated with reduced GSI, reduction of GSI might be expected given the observed reduction of 17β-estradiol.

The results presented confirm the estrogenic activities of PFOS in zebrafish. VTG is synthesized in the liver of female fish in response to estradiol-17β (E2)-induced estrogen receptor (ER) activation. Measurement of vitellogenin (VTG) mRNA or VTG levels in male fish has been one of the most commonly used biomarkers for exposure to estrogenic activities of chemicals in the aquatic environment (Denslow et al., 1999; Jobling et al., 2003). No sex chromosomes have been identified in zebrafish, thus environmental factors (e.g., sex steroids and xeno-estrogens) could affect on the processes of sex differentiation. In general, the sex differentiation fish around 45 dpf in zebrafish. For example, changes in the proportions of males and females were observed in populations of juvenile zebrafish exposed, during the critical period of gonadal development, to xenoestrogens (Örn et al., 2003; 2006; Drastichova et al., 2005). Circulation of testosterone was reduced while E2 was evaluated after exposure to 1000 µg l⁻¹ PFOA in fathead minnow (Oakes et al., 2004). Although several studies as well as our study showed estrogenic activities of PFOS, PFOA in fish or cultured tilapia hepatocytes (Oakes et al., 2005; Wei et al., 2007; Liu et al., 2007), sex ratio was not changed as well as intersex was not observed. This result could be explained as the weak estrogenic activity of PFOS. Our results suggest that gene expression may be more sensitive than gross morphological endpoints (e.g., GSI, intersex, sex ratio) and different endpoints should be employed when evaluating environmental estrogenic activities of chemicals in the aquatic environment.

Histological examination revealed that the most pronounced morphological alternation is accumulation of lipid in the liver of male fish, suggesting the
hepatic toxicity is gender specific. Moreover, such a “recovery/adaptive” response was not observed after recovery, as indicated by lipid droplets accumulation in the hepatocytes. Liver is a major target organ for PFOS accumulation. PFOS has also been shown to accumulate in wildlife by binding to proteins in blood and liver tissues (Giesy and Kannan, 2001). Seacat et al. (2002) showed that PFOS accumulates in liver and induces hepatocellular hypertrophy, centrilobular vacuolation, and mild bile stasis, lipid-droplet accumulation in both male and female rats. It is possible that these affects may be directly attributed to PFOS binding in the hepatocytes. The gender-specific toxicity may be due to the elimination of PFOS and interaction between PFOS and endogenous steroid hormones. Study has shown that the elimination of PFOA is related to testosterone and cortisol. Since PFOS is metabolically inert, sex-related difference observed in the biological effects of PFOS is not due to a difference in metabolism of PFOS. By using treatment of estradiol or testosterone in rats, Kudo et al. (2002) showed that PFOA is transported into urine by a several organic anion transporters and sex hormones regulated the transporters gene expression differentially in kidney of male and female adult rats. So far the gender difference in elimination of PFOS in fish is not well known. Future research on pharmacokinetics of PFOS in fish may be warranty.

Thyroid hormones are known to regulate growth and development in fish. In fish, thyroid hormones have a major role on regulation of growth and development that can be affected as a result of xenobiotic exposure (Arcand-Hoy and Benson, 1998; Power et al., 2001). In zebrafish, thyroid hormones are necessary for embryonic to larval transition and larval to juvenile transition (Liu and Chan, 2002). Hence, if thyroid disruption occurs during either transition, growth retardation might occur (Brown, 1997). In the present study, total T3 levels remained unchanged after PFOS exposure for 40 and 70 d as well as after recovery. The results suggested that chronic PFOS exposure did not arrested thyroid hormone production. In contrast, significantly reduced in the levels of serum thyroxine (T4) and triiodothyronine (T3) have been measured in rats and mice after exposure to PFOS (Seacat et al., 2002; Thibodeaux et al., 2003; Lau et al., 2003; Luebker et al., 2005a, b; Chang et al., 2008), whereas changes in thyroid gland histology or clinically significant elevations of thyroid stimulating hormone (TSH) did not occur. Recently, Chang et al. (2008) studied PFOS exposure to rats and hypothesized that exposure to PFOS may increase free thyroxine (FT4) in the rat serum due to the ability of PFOS to compete with thyroxine for binding proteins and increase in FT4 would increase the availability of the thyroid hormone to peripheral tissues for utilization, metabolic conversation, and therefore increase T3. Since thyroid hormones play important roles in development, further investigations are necessary to elucidate the changes on thyroid hormone due to PFOS exposure.

In summary, results from the present study indicated that long-term PFOS exposure could result in significant mortality and impair fish reproduction in the offspring. Although hepatic VTG gene expression was significantly up-regulated and showed estrogenic activities in zebrafish, sex ratio was not changed, suggesting
Chronic Effects of Waterborne PFOS Exposure

that lower concentrations of PFOS does not affect fish due to endocrine disruption, but may be through bioaccumulation through food web and impairment on the offspring in the aquatic environment. Although the concentration of PFOS was general low (0.1 µg l⁻¹) in surface water measured from North America and Asia (Hansen et al., 2002; Taniyasu et al., 2003; Boulanger et al., 2004; Tseng et al., 2006; So et al., 2007; Zhao et al., 2007; Becker et al., 2008) and the exposure concentration of PFOS was relatively higher than those detected in surface water in the present study, it should be noted that PFOS can be bioaccumulated through food chain and can be oviparous transferred to offspring. Therefore, in the aquatic environment, PFOS can be magnified and bioaccumulated in fish and exhibited reproductive toxicity in the offspring and may lead to ecological consequence.

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