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Induction of oxidative stress and apoptosis by PFOS and PFOA in primary cultured hepatocytes of freshwater tilapia (*Oreochromis niloticus*)

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Abstract

Perfluorinated organic compounds (PFOCs) are emerging persistent organic pollutants (POPs) widely present in the environment, wildlife and human. We studied the cellular toxicology of perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) on oxidative stress and induction of apoptosis in primary cultured hepatocytes of freshwater tilapia (*Oreochromis niloticus*). Cultured hepatocytes were exposed to PFOS or PFOA (0, 1, 5, 15 and 30 mg L\(^{-1}\)) for 24 h, and a dose-dependent decrease in cell viability was determined using trypan blue exclusion method. Significant induction of reactive oxygen species (ROS) accompanied by increases in activities of superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) were found, while activities of glutathione peroxidase (GPx) and glutathione-S-transferase (GST) were decreased. Glutathione (GSH) content was reduced following treatment of PFOA and PFOS. A dose-dependent increase in the lipid peroxidation (LPO) level (measured as maleic dialdehyde, MDA) was observed only in the PFOA exposure groups, whereas LPO remained unchanged in the PFOS exposure groups. Furthermore, a significant activation of caspase-3, -8, -9 activities was evident in both PFOS and PFOA exposure groups. Typical DNA fragmentation (DNA laddering) was further characterized by agarose gel electrophoresis. The overall results demonstrated that PFOS and PFOA are able to produce oxidative stress and induce apoptosis with involvement of caspases in primary cultured tilapia hepatocytes.

**Key words:** Perfluorinated organic compounds; primary cultured hepatocytes; reactive oxygen species; oxidative stress; caspases; apoptosis; tilapia
1. Introduction

Perfluorinated organic compounds (PFOCs) have been used extensively in a variety of industry and consumer products, such as surfactants, lubricants, adhesives, refrigerants, paper coating, fire retardants, propellants and insecticides over the last 50 years (Key et al., 1997). The strong C-F bonds of these chemicals make them resistant to hydrolysis, photolysis, microbial degradation and metabolism. Recently, these chemicals have been found to have a global distribution in the environment and wildlife (Giesy and Kannan, 2001; Kannan et al., 2001, 2002; Martin et al., 2004; So et al., 2004). Among these compounds, perfluorooctane sulfonate (PFOS) has been found to be the predominant perfluorinated pollutant, which has been detected in livers of mink (e.g., 0.97-3.68 µg g⁻¹ wet weight) (Giesy and Kannan, 2001), in blood plasma of bald eagles (e.g., 13-2220 ng mL⁻¹) (Kannan et al., 2001). In humans, PFOS and perfluorooctanoic acid (PFOA) have also been detected in serum samples from the general population of USA (Hansen et al., 2001; Olsen et al., 2003, 2004) and in human milk and blood samples in China (So et al., 2006; Yeung et al., 2006) (e.g., PFOS, 45-360 ng L⁻¹, PFOA, 47-210 ng L⁻¹ in milk; PFOS, 52.7 ng mL⁻¹, PFOA, 1.59 ng mL⁻¹ in blood samples) and in human serum in Japan (Harada et al., 2006). Among these measured PFOCs, PFOS and PFOA were the two dominant chemicals detected in all the milk and serum samples. Furthermore, liver and blood are the major sites of accumulation for these chemicals (Seacat et al., 2002), implicating that liver is a major target organ.

High concentrations of PFOS have been detected in the liver (7760 ng g⁻¹ wet weight) from plaice (Pleuronectes platessa) (Hoff et al., 2003a), and feral gibel carp (Carassius auratus gibelio) (up to 9031 ng g⁻¹ wet weight) in Belgium (Hoff et al., 2005). Likewise, high contents of PFOS and PFOA were also detected in tilapia (Oreochromis niloticus) (1100 ng g⁻¹ wet weight) in Taiwan (Tseng et al., 2006) and mullet (Mugil incilis) bile (up to 3673 µg L⁻¹) in the north coast of Colombia, South America (Olivero-Verbel et al., 2006). The widespread distribution and environmental persistence of these compounds has generated great concerns regarding their potential environmental impact.
Although there are numerous reports regarding the environmental distribution of these compounds, information on their toxicity, especially toxic mechanisms, is not well known. At present, knowledge of the toxicological effects regarding these compounds are largely based on in vivo and in vitro mammalian studies under laboratory conditions. For example, exposure to PFOS could induce hepatocellular hypertrophy and lipid vacuolation and loss of body weight in rat (Seacat et al., 2002). In addition, hepatic peroxisome proliferation has been observed in both rodents and monkeys (Berthiaume and Wallace, 2002; Hu et al., 2002; Starkov and Wallace, 2002). The most pronounced toxic effects of PFOA are interference with lipid metabolism, such as increasing peroxisomal fatty acid β-oxidation and inducing acyl-CoA oxidase activity, which catalyzes the first and limiting-step in fatty acid oxidation (Kawashima et al., 1989) and other related metabolism. PFOS can also increase membrane fluidity (Hu et al., 2002) and inhibit gap junction intracellular communication (Hu et al., 2002). Recently, Guruge et al. (2006) showed that gene expression was significantly altered in the liver of Sprague-Dawley rats treated with PFOA, and most of these genes were involved in the transport and metabolism of fatty acids and lipids, cell communication, adhesion, growth and apoptosis.

Bioaccumulation of PFOS in fish has been demonstrated in laboratory experiments (Martin et al., 2003). In freshwater fish, Hoff et al. (2003a) showed that PFOS could influence the average DNA basepair length in carp (Cyprinus carpio), suggesting that PFOS might interfere with homeostasis of DNA metabolism. In addition, PFOS also caused liver damage, as assessed by increase in serum alanine aminotransferase (ALT) activity, while peroxisomal β-oxidation was not affected. In marine fish, Hoff et al (2003b) reported that serum ALT was positively correlated with liver concentration of PFOS in feral bib (Trisopterus luscus). Another study by Hoff and coauthors (2005) demonstrated that hepatic PFOS concentration was significantly and positively related to serum ALT activity in both feral carp (Cyprinus carpio) and eel (Anguilla anguilla), suggesting that PFOS might influence membrane function and structure of hepatocytes. A recent study showed that exposure of fathead minnow (Pimephales promelas) and rainbow trout (Oncorhynchus mykiss) to PFOS
resulted in an increase in hepatic fatty acyl-CoA oxidase activity (Oakes et al., 2005).

In this study, we employed primary cultured hepatocytes from freshwater tilapia (*O. niloticus*) as an *in vitro* model to investigate the toxicity and toxic mechanisms of PFOS and PFOA. Primary cultured cells maintain most of their original *in vivo* characteristics and therefore may facilitate the extrapolation of the results to *in vivo* situations. The main purposes of this study were to (1) examine cellular toxicology of PFOS and PFOA on oxidative stress by measuring catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx) activities, reactive oxygen species (ROS), glutathione (GSH) and lipid peroxidation; (2) investigate whether apoptosis may be induced; and (3) determine DNA fragmentation in PFOS and PFOA-treated tilapia hepatocytes.
2. Materials and Methods

2.1. Fish

Freshwater tilapia (*Oreochromis niloticus*) of 50-100 g each were purchased from a local fish farm remote from industrial and agricultural areas. They were kept in a 300 L fiber-glass tank with re-circulating water for 3 weeks (22 ± 0.5 °C, 12 h light:12 h dark cycles). The fish were fed once every other day with commercial fish food (J.W. Vitra, 35% protein).

2.2. Cell culture medium and chemicals

Dulbecco’s Modified Eagle’s Medium/Nutrient Mixture F-12 Ham (DMEM/F12) culture medium (with L-glutamine and 15 mM HEPES, without phenol and sodium bicarbonate), 2’,7’-Dichlorofluorescin diacetate (DCFH-DA) were obtained from Sigma (St. Louis, USA). Heptadecafluoroocanesulfonic acid potassium salt (PFOS) and pentadecafluoroocanoic acid (PFOA) were obtained from Tokyo Kasei Kogyo CO., LTD (Tokyo, Japan) and ACROS (New Jersey, USA), respectively. Antioxidative enzyme detection kits (SOD, CAT, GPx, GR, GST, GSH) and LPO detection kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Caspase detection kits were obtained from Beyotime Institute of Biotechnology (Nanjing, China). All chemicals used were of analytical grade.

2.3. Preparation of cultured hepatocytes

The isolation and primary culture of tilapia hepatocytes were carried out in accordance with the method developed by Kim and Takemura (2003) and modified by Zhou et al. (2006). Briefly, fish were stunned by a blow to the head and then decapitated. The liver was carefully excised and transferred onto a glass petri dish, and rinsed twice with phosphate buffered saline (PBS: 136.9 mM NaCl; 5.4 mM KCl; 0.81 mM MgSO₄; 0.44 mM KH₂PO₄; 0.33 mM Na₂HPO₄; 5.0 mM NaHCO₃, pH 7.6) without Ca²⁺. The liver was dissected into small pieces and the tissue was digested for 20 min at room temperature with PBS containing 0.1% collagenase (Sigma) on a
shaker. The softened liver tissue was agitated and filtered through 70-µm nylon mesh (Falcon, NJ, USA). The resulting cell suspension was transferred to a sterilized centrifuge tube (50 ml, Falcon, NJ, USA) and then centrifuged 3 times at 90×g for 3 mins in the buffer containing 1.5 mM CaCl\textsubscript{2} at 10°C. After the last wash, the cell pellet was re-suspended in DMEM/F12 medium (Sigma, St. Louis, USA). Cells were counted using a haemocytometer (Reichert, Buffalo, NY, USA), and those with more than 90% viability by the trypan blue exclusion method were used for the experiment. The isolated hepatocytes were seeded at a density of 60×10\textsuperscript{4} ml\textsuperscript{-1} (200 µl: 120000/per well) in a 96 well “Primaria” plate (Falcon, NJ, USA). The culture medium contained DMEM/F12, 100 i.u. ml\textsuperscript{-1} penicillin, 100 µg ml\textsuperscript{-1} streptomycin, and 0.5% ITS (insulin-transferrin-selenium, Gibco). After 24 h culture, the medium was removed and replaced by an appropriate exposure medium. For each batch of experiments, hepatocytes were prepared from four individual fish.

2.4. Hepatocyte viability assay

The trypan blue exclusion assay was used to estimate cell viability. Briefly, at the end of the experiments, the hepatocytes were harvested and an aliquot of the cell suspension was diluted 1:1 with 0.4% trypan blue solution. Cell number was determined using haemocytometer. Greater than 100 cells per field were examined and cell viability was expressed as a percentage of the control value.

2.5. Detection of reactive oxygen species (ROS)

ROS levels were determined using 2’,7’-dichlorofluorescein deacetate (DCFH-DA), which is de-esterified to 2’,7’- dichlorodihydrofluorescein (DCFH\textsubscript{2}) by cellular esterases, and then DCFH\textsubscript{2} is oxidized by ROS to form fluorescent 2’,7’-dichlorofluorescein (DCF) (LeBel et al., 1992). An increase in fluorescence intensity is used to quantify the generation of net intracellular ROS. Similarly, various concentrations of PFOA and PFOS (0, 1, 5, 15, 30 mg L\textsuperscript{-1}, respectively) were added to each well. After 23.5 h exposure, 25 µl DCFH-DA stock solution (200 µM) was added
to each well and the hepatocytes were incubated for 30 mins in the dark, then the culture medium was removed and 100 µl DMSO was added to each well. After incubation for another 20 mins, the fluorescence intensity was measured immediately using a microplate reader (Molecular Device, M2, Union City, CA, USA). Excitation and emission wavelengths were 490 and 530 nm, respectively. Control groups received 0.1% DMSO. Five replicates were used in each experiment.

2.6. Activities of antioxidant enzymes and GSH content

After PFOS or PFOA treatment (0, 1, 5, 15, 30 mg L⁻¹) for 24 h, the culture medium was removed, and the hepatocytes were rinsed twice with cold phosphate buffered saline (PBS), then lysed by sonication using microson ultrasonic cell disruptor (XL 2000, Misonix incorporated) at 5 W for 5 seconds on ice. The homogenate was centrifuged at 10,000 g for 10 mins at 4°C to precipitate insoluble material. The supernatant was removed and assayed for CAT, GR, SOD, GST, GPx activities and GSH content using kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), following the manufacturers specifications. Control groups and all exposure groups received 0.1% DMSO. The CAT, SOD, GPx and GST activities were expressed as units mg⁻¹ protein, GR activity was expressed as units g⁻¹ protein. GSH content was expressed as mg GSH g⁻¹ protein. The units of the antioxidant enzyme activities were defined as follows: One unit of CAT activity was defined as the amount of enzyme decomposing 1 µmol H₂O₂ in 1 second; One unit of GR activity was defined as the amount of enzyme depleting 1 mmol NADPH in 1 min; For SOD, One unit of activity was defined as the amount of protein necessary to decrease the reference rate to 50% of maximum inhibition; One unit of GST activity was defined as the amount of enzyme depleting 1 µmol GSH in 1 min; One unit of GPx activity was defined as the amount of enzyme depleting 1 µmol GSH in 1 min. Five replicates were used in each experiment.

2.7. Lipid peroxidation
For the determination of lipid peroxidation, a 200 µl culture medium that contained a specific concentration of PFOS or PFOA (0, 1, 5, 15, 30 mg L\(^{-1}\)) was added to each well. After exposure for 24 h, the medium was removed and hepatocytes were rinsed twice with cold PBS, then lysed by sonication using microson ultrasonic cell disruptor (XL 2000, Misonix incorporated) at 5 W for 5 seconds on ice. The homogenate was centrifuged at 10,000 g for 10 min at 4°C to precipitate insoluble material. The supernatants were removed for lipid peroxidation (measured as MDA) content determination by the kit (Nanjing Jiancheng Bioengineering Institute Nanjing, China). The MDA concentration was expressed as nmol mg\(^{-1}\) protein. Control groups received 0.1% DMSO. Five replicates were used in each experiment.

2.8. Caspase activity assay

Caspase activities were determined by a colorimetric assay based on the ability of caspase-3, -8, -9 to change acetyl-Asp-Glu-Val-Asp \(p\)-nitroanilide (Ac-DEVD-\(p\)NA), acetyl-Ile-Glu-Thr-Asp \(p\)-nitroanilide (Ac-IETD-\(p\)NA) and acetyl-Leu-Glu-His-Asp \(p\)-nitroanilide (Ac-LEHD-\(p\)NA) into a yellow formazan product (\(p\)-nitroaniline (\(p\)NA)), respectively. An increase in absorbance at 405 nm was used to quantify the activation of caspases activities. After 24 h exposure, hepatocytes from thirty-two wells were collected and rinsed with cold PBS, and then lysed by lysis buffer (40 µl) for 15 mins on ice. Cell lysates were centrifuged at 18,000 g for 10 mins at 4°C. Caspase-3, -8, -9- activities in the supernatant were assayed using the kit. The caspase activities were expressed as percentage of enzyme activity compared to control. Control groups received 0.1% DMSO. All the experiments were carried out in triplicates.

2.9. DNA fragmentation

After exposure to different concentrations of PFOS or PFOA (0, 15, 30 mg L\(^{-1}\)) and time, cells (5×10\(^6\) cell density) from sixteen wells were collected at 24 and 48 h
and rinsed with cold PBS. Hepatocytes were lysed in 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 mM NaCl, 0.5% proteinase and 0.5% sodium dodecyl sulfate (SDS) at 50°C for 10 h. This lysate was then extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged at 12,000 g for 5 mins at 4°C, then DNA was precipitated with 3 M Sodium Acetate (NaAc) (pH 5.2) and 100% cold ethanol and deposited at -20°C for 10 h. The precipitated DNA was centrifuged at 12,000 g for 10 mins at 4°C and the DNA pellets were washed with 70% ethanol, air-dried, and dissolved in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8.0), then 5 mg L-1 RNase was add to each sample to remove RNA. DNA samples (5 µl) were electrophoresed on a 2% agarose gel in a buffer (4.84 g L-1 Tris base, 1.14 ml L-1 glacial acetic acid, 1 mM EDTA) for 6 h at 46 V. Control group received 0.1% DMSO. Standard DNA markers were also electrophoresed. After electrophoresis, the gel was stained in 0.5 mg L-1 ethidium bromide solution and DNA laddering was visualized by Biospectrum Imaging System (America, UVP).

2.10. Protein assay

Protein content was assayed by the Bradford method, using bovine serum albumin (Sigma) as a standard.

2.11. Statistical analysis

For each experiment, replicate wells from four different fish were used. 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 test were used to determine significant differences between control and exposure groups. The criterion for statistical difference was p<0.05. All statistical analysis was performed using SPSS 13.0 (SPSS, Chicago, IL, USA).
3. Results

3.1. Hepatocyte viability

A dose-dependent decrease in the cell viability was observed following treatment of PFOS or PFOA (Fig. 1). There was no significant reduction in cell viability in the lowest exposure group (1 mg L\(^{-1}\)) compared with the control. With increasing concentrations, cell viability was decreased to 89.8±1.9, 79.7±1.5, and 68.9±1.6 at 5, 15 and 30 mg L\(^{-1}\) of PFOS treatment, respectively (Fig. 1A). Likewise, cell viability was also decreased to 88.9±1.7, 77.1±1.8, and 73.6±1.4 upon exposure to 5, 15, and 30 mg L\(^{-1}\) of PFOA, respectively (Fig. 1B). By using protein content as an endpoint for cell viability, it is noteworthy that protein content was significantly reduced in the present study in both PFOS and PFOA exposure groups, with PFOA showing a greater effect than PFOS. The two assays give the same result for PFOA exposure, however, protein assay is less sensitive than trypan blue for PFOS, so we only present trypan blue results.

3.2. Reactive oxygen species (ROS)

In PFOS treated cells, ROS concentrations were significantly increased (52.7%) in the 15 mg L\(^{-1}\) exposure group compared with the control, while ROS levels remained unchanged in other exposure groups (Fig. 2A). In PFOA treated cells, a dose-dependent generation of ROS was measured with an increase of 43.0% in the 15 mg L\(^{-1}\) exposure group and 60.0% in the 30 mg L\(^{-1}\) exposure group, respectively (Fig. 2B).

3.3. Activities of antioxidative enzyme and GSH content

In PFOS treated cells, SOD activity was significantly increased (56.6%) in the 15 mg L\(^{-1}\) treated group compared with the control. However, SOD activity remained unchanged when the hepatocytes were expose to lower concentrations (1 and 5 mg L\(^{-1}\)) and higher concentrations (30 mg L\(^{-1}\)) (Table 1). For PFOA treatment, SOD
activity was significantly increased by 18.4% and 23.0% in the 5 and 15 mg L\(^{-1}\) exposure groups, respectively, while SOD activity remained unchanged in other exposure groups (Table 1).

The CAT activity was only significantly increased at the highest exposure concentration (30 mg L\(^{-1}\)) with an increase of 37.5% in the PFOS treatment group and 19.0% in the PFOA treatment group compared with the control. There were no significant differences in other treatments (1, 5, 15 mg L\(^{-1}\)) (Table 1).

The GPx activity was significantly reduced in a concentration-dependent manner in both PFOS and PFOA exposure groups (Table 1). In PFOS exposure hepatocytes, an inhibition of 58.6% and 72.1% of the enzyme activities was observed in 15 mg L\(^{-1}\) and 30 mg L\(^{-1}\) treated groups, respectively. The GPx activity remained unchanged in the other treatment groups. Under PFOA exposure, the enzyme activities were inhibited by 43.2%, 50.0% and 48.6% in 5, 15 and 30 mg L\(^{-1}\) treatment, respectively. There was no significant difference in 1 mg L\(^{-1}\) treatment group compared with the control (Table 1).

Under PFOS exposure, GR activity was significantly induced by 100% at 30 mg L\(^{-1}\) compared with the control (Table 1). PFOA caused approximately 91.7%, 91.7% and 125.7% increase in the enzyme activities in 5, 15 and 30 mg L\(^{-1}\) treated cells, respectively (Table 1).

GST activity was significantly decreased at 30 mg L\(^{-1}\), with a 74.3% and 24.0% inhibition of PFOS and PFOA exposure, respectively (Table 1), while there was no significant change in other exposure groups.

Under PFOS exposure, there was no significant difference in the GSH content at 1 and 5 mg L\(^{-1}\) of PFOS exposure compared with the control, while approximately 36.6% and 56.4% reduction was observed at 15 and 30 mg L\(^{-1}\) treated hepatocytes (Table 1). Under PFOA exposure, GSH content showed a concentration-dependent reduction of 20.0%, 25.9% and 28.2% following treatment with 5, 15 and 30 mg L\(^{-1}\) compared with the control (Table 1).

3.4. Lipid peroxidation
MDA is indicative of lipid peroxidation. Levels of MDA remained unchanged at all concentrations of PFOS exposure (Fig. 3A). Under PFOA exposure, a good dose-dependent increase in the MDA content was observed (53.8%, 59.6% and 90.5% at 5, 15 and 30 mg L\(^{-1}\), respectively) (Fig. 3B).

3.5. Caspases activity

Under PFOS exposure, caspase-3, -8, -9 activities significantly increased at the highest concentration (30 mg L\(^{-1}\)) with an increase of 73.3%, 181.3%, 171.6% compared with the control, while these enzymes activities showed no significant difference when the hepatocytes were exposed to 15 mg L\(^{-1}\)PFOS (Fig. 4A). Under PFOA exposure, a slight but significantly increase in caspase-3, -8, -9 activities was observed at 15 mg L\(^{-1}\) exposure with an increase of 11.0%, 20.0%, 23.0%, respectively, and caspase-3, -8, -9 activities were further increased by 173.5%, 258.1%, 189.0% in the 30 mg L\(^{-1}\)PFOA exposure group (Fig. 4B).

3.6. DNA fragmentation

After the hepatocytes were exposed to PFOS or PFOA for 24 h, there was no distinct DNA laddering (not shown). However, typical DNA laddering was observed after 48 h exposure (Fig. 5) when the hepatocytes were exposed to 15 and 30 mg L\(^{-1}\), indicating the occurrence of apoptosis at these concentrations.
4. Discussion

Fish liver is a major target organ for PFOS and PFOA accumulation. As such, primary cultured hepatocytes could serve as a model for studying the toxicity and toxic mechanisms of these emerging chemicals of concern. Our results clearly showed that PFOS and PFOA could induce reactive oxygen species (ROS) production and correspondingly antioxidative responses in hepatocytes, resulting in oxidative damages, as exemplified by lipid peroxidation and DNA damages. These two chemicals also activate caspases, leading to apoptosis.

Both PFOS and PFOA can decrease cell viability in cultured hepatocytes in a dose-dependent manner, and the loss of cell viability may be mediated through ROS induction. This result is consistent with the study of Panaretakis et al. (2001), in which a dramatic increase in cellular content of superoxide anions and hydrogen peroxide was observed after treatment with 200 μM or 400 μM PFOA for 3 h in the human hepatoma cell line (HepG2). ROS can be generated at many different organelles including mitochondrion, which is responsible for aerobic respiration, the most common source of ROS generation (Kelly et al., 1998). The mitochondrial toxicity of PFOCs has been suggested as one of their mechanisms of toxicity, suggested as a subcellular target potentially mediating cytotoxicity. For example, the structure of PFOA is similar to a long-chain fatty acid, and therefore is capable of inducing change of the mitochondrial permeability transition (MPT) (Panaretakis et al., 2001). However, a recent study showed that neither PFOA nor PFOS could induce alternation of MPT in freshly isolated mitochondria from rat liver, while MPT induction is specific to N-acetyl perfluorooctane sulfonamide, which causes the release of cytochrome c, leading to inhibition of respiration and ROS generation (O’Brien and Wallace, 2004).

In the present study, SOD activity was significantly increased with enhanced ROS production, and presumably to counteract oxidative damage. However, higher concentration of PFOS and PFOA (30 mg L⁻¹) appeared to induce no significant
change in SOD activity (Table 1). This may be explained by the excess production of superoxide radicals, which, after their transformation to H$_2$O$_2$, cause an oxidation of the cysteine in the enzyme and deactivate SOD activity. A significant CAT induction was observed at 30 mg L$^{-1}$ of PFOS and PFOA exposure. CAT is mainly located in peroxisome and is responsible for the reduction of hydrogen peroxide and protection from the oxidation of unsaturated fatty acids in cell membrane (Livingstone, 2001). Therefore, the higher activity of CAT can be attributed to the increase of pro-oxidants.

GST was significantly reduced and strongly inhibited by high concentration (30 mg L$^{-1}$) of PFOS and PFOA. A decrease in GST may suggest a failure in detoxification and occurrence of oxidative stress in the cells (Santos et al., 2004). In the present study, the decrease in GST activity was concomitant to the decrease in GSH content in cultured hepatocytes. Similarly, increase in GSH content can be viewed as an adaptation to low level of oxidative stress or failure of adaptation due to a severe oxidative stress, leading to suppressed GSH content and detoxification of oxidized GSH (GSSG) to GSH. ROS could be reduced by oxidizing GSH to GSSG, which is catalyzed by GPx. GPx catalyzes the reduction of hydrogen peroxides and is considered as an efficient protective enzyme against lipid peroxidation (Winston and Di Giulio, 1991). Indeed, a dose-dependent reduction of GPx activity was observed in both PFOS and PFOA exposure, suggesting an adaptive response to maintain the homeostasis of reduced GSH. GR activity significantly increased in the present study. GR is supposed to maintain cytosolic concentration of GSH by reducing GSSG to GSH. Hence, an increase in GR activity indicates compensation of cellular GSH depletion. In the present study, however, higher activity of GR was not associated with a high activity of GPx, suggesting a depletion of GSH content and failure to maintain homeostasis of GSH/GSSG. Indeed, significant reduction of GSH content was observed under both PFOS and PFOA exposure.

The induction of ROS could enhance oxidation of polyunsaturated fatty acids leading to lipid peroxidation. In the present study, a significant increase in MDA content was found after PFOA exposure. The increase in MDA content could be attributed to the generation of ROS. Despite a marked increase of ROS generation
observed in 15 mg L\(^{-1}\) of PFOS exposure, MDA level remained unchanged. The reasons are unknown. A previous study also showed that although a significant increase in ROS generation of the common carp spermatozoa after exposure to duroquinone was observed, LPO (measure as thiobarbituric acid reactive substances, TBARS) was not induced (Zhou et al., 2006).

In order to assess whether PFOS and PFOA could induce apoptosis via caspase pathway, we examined caspase-3, -8, -9 activities and analyzed possible DNA fragmentations. Caspases are key regulating enzymes in apoptosis in a variety of cells. In mammals, caspase-3 has been identified as key executors of apoptosis and is one of the most important caspases activated downstream of apoptosis pathways (Cohen, 1997). There are two pathways of caspase activation during apoptosis. The first one is mediated by death receptors, such as Fas, and controlled by caspase-8, which in turn activates downstream effector caspases (Nagata, 2000). In the second pathway, diverse apoptotic signals converge at the mitochondrial level, causing the release of certain apoptosis-inducing proteins (e.g., cytochrome c and apoptosis-inducing factor) leading to activation of the effector caspases via caspase-9 (Desagher and Martinou, 2000). These two apoptotic pathways could be interconnected by the caspase-8-mediated cleavage of Bid, which triggers the activation of the mitochondrial pathway (Desagher and Martinou, 2000). In the present study, we demonstrated that PFOS and PFOA could activate caspase-3, 8 and 9 and induce apoptosis in cultured fish hepatocytes. The occurrence of visible DNA laddering further confirms apoptotic development in the exposed cells. However, a distinct DNA laddering was not observed after 24 h exposure to both PFOS and PFOA, whereas a clear DNA fragmentation was evident after longer time exposure (48 h), suggesting a time-lag before DNA fragmentation would occur. A time lag in DNA fragmentation has also been described with other toxicants, such as toosendanin (Tang et al., 2004) and flavanones (Shen et al., 2004).

The generation of reactive oxygen species (ROS) has been shown to be an important apoptotic signal and indeed, induction of ROS by PFOS and PFOA was clearly evident in the present study. On the basis of results obtained in the present
study, it may be hypothesized that, in cells exposed to PFOS and PFOA, ROS induce oxidative stress and cause damage to cell membrane (as evident from increased LPO level) and subsequently, the oxidative stimulus may trigger through cytosolic signaling pathway of the cell and disrupts the mitochondria (as evident by MTT assay), which might result in formation of a complex with procaspase-9, leading to activation of initiator caspase-9 and cascade of effector caspases such as 3, and 8, which initiate apoptosis.

In summary, the present study demonstrated that PFOS and PFOA could result in generation of ROS, and may overwhelm the homeostasis of antioxidative systems. Activation of caspase-3, 8 and 9, DNA laddering and apoptosis were clearly evident. However, the mechanisms of ROS generation, and the effects of these compounds on mitochondria permeability remain unknown. In addition, whether the activation of caspases is mediated through cytochrome c-dependent apoptosis pathway and release of cytochrome c, needs further investigation.
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Figure legends

Fig. 1. Viability of cultured hepatocytes after exposure to various concentrations of (A) PFOS (0, 1, 5, 15 and 30 mg L^{-1}) and (B) PFOA (0, 1, 5, 15 and 30 mg L^{-1}) for 24 h. Value represents the mean±S.E.M. of eight replicate wells from four individual fish. Significance between control and exposure groups are indicated by *$P<0.05$, **$P<0.01$.

Fig. 2. Dose-dependent induction of reactive oxygen species (ROS) in the cultured hepatocytes after various concentrations of (A) PFOS (0, 1, 5, 15 and 30 mg L^{-1}) and (B) PFOA (0, 1, 5, 15 and 30 mg L^{-1}) for 24 h. Value represents the mean±S.E.M. of eight replicate wells from four individual fish. Significance between control and exposure groups are indicated by *$P<0.05$, **$P<0.01$.

Fig. 3. Dose-dependent lipid peroxidation (LPO) formation in the cultured hepatocytes after exposure to various concentrations of (A) PFOS (0, 1, 5, 15 and 30 mg L^{-1}); (B) PFOA (0, 1, 5, 15 and 30 mg L^{-1}) for 24 h. Value represents the mean±S.E.M. of five replicate samples from four individual fish. Significance between control and exposure groups are indicated by *$P<0.05$, **$P<0.01$.

Fig. 4. Dose-dependent induction of caspase-3, -8, 9 activities in the cultured hepatocytes after exposure to various concentrations of (A) PFOS (0, 15 and 30 mg L^{-1}); (B) PFOA (0, 15 and 30 mg L^{-1}) for 24 h. Value represents the mean±S.E.M. of three replicate samples from four individual fish. Significance between control and exposure groups are indicated by *$P<0.05$, **$P<0.01$.

Fig. 5. DNA laddering induced after exposure to various concentrations of PFOS (0, 15 and 30 mg L^{-1}) and PFOA (0, 15 and 30 mg L^{-1}) in the cultured hepatocytes for 48 h measured on agarose gel electrophoresis. L-1, 30 mg L^{-1} PFOA; L-2, 15 mg L^{-1} PFOA; L-3, 30 mg L^{-1} PFOS; L-4, 15 mg L^{-1} PFOS; L-5, 0.1% DMSO; L-6, DNA marker.
Table 1. Dose-dependent antioxidative enzymes activity and GSH content in the cultured hepatocytes after exposure to various concentrations of PFOS (0, 1, 5, 15 and 30 mg L\(^{-1}\)) and PFOA (0, 1, 5, 15 and 30 mg L\(^{-1}\)) for 24 h. Value represents the mean±S.E.M. of five replicate samples from four different fish. Significance between control and exposure groups are indicated by *\(P<0.05\), **\(P<0.01\). The CAT, SOD, and GST activities were expressed as units mg\(^{-1}\) protein, and GR activity was expressed as units g\(^{-1}\) protein, and the GPx activity was expressed as units mg\(^{-1}\) protein. GSH content was expressed as mg GSH g\(^{-1}\) protein.
Fig. 1

(A) PFOS

Viability (% control)

Concentration (mg L\(^{-1}\))

(B) PFOA

Viability (% control)

Concentration (mg L\(^{-1}\))
Fig. 2
Fig. 3

(A) PFOS

(B) PFOA
Fig. 4
Table 1

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