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Toxicity assessment of perfluorooctane sulfonate using acute and subchronic male C57BL/6J mouse models☆

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ABSTRACT

Perfluorooctane sulfonate (PFOS) is a principal representative and the final degradation product of several commercially produced perfluorinated compounds. However, PFOS has a high bioaccumulation potential and therefore can exert toxicity on aquatic organisms, animals, and cells. Considering the widespread concern this phenomenon has attracted, we examined the acute and subchronic toxic effects of varying doses of PFOS on adult male C57BL/6 mice. The acute oral LD_{50} value of PFOS in male C57BL/6J mice was 0.579 g/kg body weight (BW). Exposure to the subchronic oral toxicity of PFOS at 2.5, 5, and 10 mg PFOS/kg BW/day for 30 days disrupted the homeostasis of antioxidative systems, induced hepatocellular apoptosis (as revealed by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay), triggered liver injury (as evidenced by the increased serum levels of aspartate aminotransferase, alanine amino transferase, alkaline phosphatase, and gamma-glutamyl transpeptidase and by the altered histology), and ultimately increased the liver size and relative weight of the mice. PFOS treatment caused liver damage but only slightly affected the kidneys and spleen of the mice. This study provided insights into the toxicological effects of PFOS.

1. Introduction

Polyfluoroalkyl substances (PFASs) have been manufactured by 3M Company since 1948 (Buck et al., 2011; Butenhoff et al., 2006; Kissa, 2001). Given their hydrophobic and lipophobic characteristics and their remarkable thermal, biological, and chemical stabilities, PFASs have been utilized in various industrial and consumer products, including protective coatings for carpets, apparel, food packaging, insecticide formulations, and surfactants (Noorlander et al., 2011). The abovementioned products contain compounds that can degrade or metabolize to perfluorooctane sulfonate (PFOS), a principal representative and the final degradation product of several commercially produced PFASs (Hansen et al., 2002; Lau et al., 2007). However, evidence on the metabolic or environmental degradation of PFOS remains lacking to date (Seacat et al., 2002).

PFOS is a predominant perfluorinated pollutant that can cause widespread pollution because it can enter water, atmosphere, soil, sludge, and other environmental media through various means (Giesy and Kannan, 2001). PFOS can be transferred, bioaccumulated, and biomagnified in food chains, and it has been detected in various tissues of many wildlife species and even in the human body (Buck et al., 2011; Houde et al., 2006b). Food, drinking water, indoor environment, and consumer products have been proposed as sources of chronic exposure to PFOS in humans (Domingo, 2012). The United Kingdom Food Standards Agency has estimated that the upper-bound average dietary exposure to PFOS is 0.010 μg/kg body weight (BW)/day, with a 97.5th percentile intake of 0.02 μg/kg BW/day in that country (http://www.food.gov.

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http://dx.doi.org/10.1016/j.envpol.2015.12.008
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uk/multimedia/pdfs/fsis0509.pdf). The daily dietary intake of PFOS in Germany (Fromme et al., 2007), Canada (Tittlemier et al., 2007), and Spain (Ericson et al., 2008) ranges from 0.0006 μg/kg BW to 0.0044 μg/kg BW. PFOS is the predominant PFAS both in human and wildlife blood samples and primarily accumulates in the blood and liver (Houde et al., 2006a; Olsen et al., 2003a).

The widespread distribution and bioaccumulation potential of absorbed PFOS in environment, wildlife, and humans have attracted much concern because of the potential health consequences of chronic exposure to this compound. Numerous mechanistic, toxicological, and human epidemiological studies have investigated the potential health hazards of exposure to PFOS (Lau et al., 2007). Seacat et al. (2002) determined the earliest measurable response of cynomolgus monkeys to low-level PFOS exposure for 182 days. Dong et al. (2009) also studied the chronic immunotoxic effects of PFOS exposure on adult male C57BL/6 mice. A two-year bioassay of PFOS in Sprague–Dawley rats showed an increase in hepatocellular adenoma response to dietary dosing at 20 ppm (Seacat et al., 2003). In addition, Butenhoff et al. (2012) investigated the toxicity and neoplastic potential of PFOS chronically exposed to Sprague–Dawley rats for 104 weeks. PFOS causes hepatotoxic, carcinogenic, adverse reproductive and developmental, and neurotoxic effects on many systems (Organisation for Economic Co-operation and Development , 2002; Dietz et al., 2015; Kennedy, 1987; Lee et al., 2015). Numerous reports have focused on the environmental distribution and toxicity of PFOS. Although the potential health risks of PFOS and related PFASs have been assessed by many studies, research interest on the potential toxicity of PFOS to living organisms is extensive and remains growing (DeWitt, 2015). Therefore, studying the acute and chronic toxic effects of PFOS is necessary to comprehensively characterize this compound.

Considering the widespread occurrence and potential toxicities of PFASs, 3M Co., one of the largest producers of PFASs, ceased the majority of PFOS production in the U.S. in 2000 (3M Company, 2000; U.S. Environmental Protection Agency (EPA), 2006). However, the prevalence of PFOS production has continued to increase in Asia, one of the fastest growing industrial and economic regions in the world, including the major industrialized areas along the Pearl River Delta of Southern China and the major cities of Korea (So et al., 2004). The potential environmental and human health impacts of PFASs have attracted attention and served as an impetus to elucidate their environmental origin, fate, and impact. Further research on PFOS is necessary (Buck et al., 2011). PFOS induces oxidative damage in cultured cells (Krovel et al., 2008; Shi et al., 2008), although the capacity of PFOS to trigger oxidative stress response, cell apoptosis, and organ injury remains to be evaluated. Therefore, this study investigated the acute and subchronic toxic effects of PFOS on adult male C57BL/6 mice. The subchronic toxicity of different PFOS doses to mice after 30 days of exposure was evaluated. Moreover, changes in the activities of antioxidative enzymes and lipid peroxidative products were measured. Hematological, biochemical, histological, and immunohistochemical effects were also examined to evaluate whether or not oxidative stress induces cell apoptosis.

2. Materials and methods

2.1. Animals

The study was approved by the Ethics Committee of Jiangnan University, China (JN No. 20121203-0120). The use and care of animals followed European Community guidelines (Directive 2010/63/EU). Surgical procedures were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. Six-week-old male C57BL/6J mice weighing 16–19 g at the beginning of each experiment were purchased from the Shanghai Laboratory Animal Center (Shanghai, China). Six groups of 10 mice each were used in the acute oral toxicity study, and four groups of 10 mice each were used in the subchronic oral toxicity study. Once distributed into groups on the basis of their weight, the mice were acclimated to the new cage (five per cage) conditions, namely, a temperature of 22 ± 2 °C, a relative humidity of 40%–60%, and an artificial illumination of a 12 h light/dark cycle. Certified laboratory chow and sterilized water were available ad libitum throughout the experiment. Prior to exposure, all mice were acclimatized to the experimental conditions for a week.

2.2. Acute toxicity

PFOS (98% purity; Sigma–Aldrich, St. Louis, MO, USA) was dissolved in deionized water with 2% Tween 80 to serve as the suspensions of the test substance. After 7 days of quarantine and acclimatization, 60 mice (weighing 20–22 g) were randomly divided into six groups of 10 mice each. All control mice received deionized water with 2% Tween 80 only, whereas the test mice were dosed via oral gavage with dosing suspensions (0.3, 0.4, 0.5, 0.6, and 0.7 g PFOS/kg BW) at the start of the study. The general behavior of mice and signs of toxicity were continuously observed for 3 h following oral gavage. Behavioral changes and signs of toxicity and/or death were further observed once daily for up to 14 days. The BW and food intake were monitored on days 0, 3, 7, and 14 in accordance with the method described by Zhang et al. (2015); all remaining mice were euthanized using sodium pentobarbital anesthesia, bled by retro-orbital puncture, sacrificed by cervical dislocation, and then grossly examined for internal organ abnormalities. Histological examinations of tissues of dead mice (those that died during the study) and those of the sacrificed ones on day 14 were taken and placed in formalin and fixed. The median lethal dose (LD_{50}) was estimated using Horn's method (China's Ministry of Health (2003)).

2.3. Subchronic toxicity

The dosing suspensions of the test substance were prepared with 2% Tween 80. The test substance (2.5, 5, or 10 mg PFOS/kg BW/day) was administered to the mice once daily via oral gavage for 30 days (2.5, 5, or 10 mg PFOS/kg BW/day) to achieve a target total administered dose (TAD) of 0.075, 0.15, or 0.3 g PFOS/kg BW. None of these mice died in the subchronic exposure experiment. All control mice received deionized water with 2% Tween 80 via oral gavage. All of the mice were carefully observed for their clinical observations and mortality. The BW, food, and water intake of the mice were also measured on days 0, 4, 8, 12, 16, 20, 24, 28, and 30. The mice were fasted overnight prior to blood collection through retro-orbital injection on day 31 of the study.

2.4. Measurement of organ mass, urine, blood, and tissue samples

Urine, blood, and tissue samples (liver, kidney and spleen) were collected from the animals on day 31 (termination of treatment). All mice were fasted overnight, during which they were placed in metabolism cages individually for urine sample collection prior to blood collection. Sera were obtained by centrifuging the blood samples at 3000 g for 15 min and were subsequently stored at −80 °C until further analyses. The liver, kidney, and spleen tissues were immediately removed, rinsed with cold 0.9% saline, and then weighed for organ mass measurement (liver mass: liver-to-BW ratio; kidney mass: kidney-to-BW ratio; and spleen mass: spleen-to-BW ratio). An approximately 0.5 cm^3 sample was obtained from each tissue and then immediately immersed in a

formaldehyde solution (10%, v/v) (Lutgendorff et al., 2008) for histopathological observation. The remaining liver tissue samples were sectioned into pieces and then stored at −80 °C until further chemical analyses.

2.5. Serum PFOS analysis

PFOS was analyzed as previously described (Hansen et al., 2001; Zheng et al., 2009). In brief, serum (0.5 mL), tetrabutylammonium hydrogen sulfate solution (0.5 M, 1 mL), and sodium carbonate buffer (0.25 M, pH 10, 2 mL) were placed in 15 mL polypropylene tubes and then thoroughly mixed for extraction. Each extracted solution was analyzed using UPLC–MS on a Waters SYNAPT MS system (Waters Co.) equipped with an Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm, Waters Co.) as previously described (Jin et al., 2007). The results obtained were analyzed using MassLynx V4.1 and MassEnt (Waters Co.). Quality control was performed according to the method of Abbott et al. (2009). In brief, check standards were prepared by spiking large volumes of mouse sera at several arbitrary levels and aliquots of frozen sample were analyzed in each analytical set. In addition, duplicate serum samples of the control mice were fortified at two or three levels with known quantities of PFOS during the preparation of each analytical set. The fortified duplicate and several check standards were run in each analytical batch to assess precision and accuracy. On the basis of the precision and accuracy determined from repeat injections of the standard curves, the results were considered quantitative to ±30% and the accuracy of the matrix spiked samples was >95%. The average correlation coefficient of the weighted (1/x) linear regression curve was 0.998. The lower and upper detection limits used for PFOS were 0.1 and 5 ppm, respectively.

2.6. Histological evaluation

The tissue samples were dehydrated in gradient alcohol (75%, 85%, 95%, and 100%) and xylene (100%). The tissues were embedded in paraffin, serially cut into 5 μm sections by using a rotary microtome, and then routinely stained with hematoxylin-E-osin (H&E) for light microscopic examination (DM2000; Leica, Bensheim, Germany) (Zhang et al., 2013).

2.7. Biochemical analyses

Using an automatic biochemistry meter (SELECRTA-E, Vital Scientific), we measured the following blood biochemical parameters: alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine amino transferase (ALT), gamma-glutamyl transpeptidase (γ-GT), and urea nitrogen (BUN). Urinary *N*-acetyl-β–glucosaminidase (NAG) was measured using an ELISA kit (Sigma–Aldrich, St. Louis, MO, USA) in accordance with the manufacturer's instructions.

2.8. Analysis of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), and total antioxidative capacity (T-AOC)

The levels of SOD, CAT, GSH-Px, and T-AOC in liver tissue homogenates were measured using commercial kits (Jiancheng Institute of Biotechnology, Nanjing, China) in accordance with the manufacturer's instructions.

2.9. Lipid peroxidation assay

Using spectrofluorometry as previously described (3M Company, 2000), we determined the lipid peroxidation by reacting malondialdehyde (MDA) with thiobarbituric acid to form a colored complex. MDA level was expressed as nmol/mg protein. Protein concentrations were measured using the 2D Quant Kit (GE Healthcare, Piscataway, NJ, USA).

2.10. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

The TUNEL assay was performed using a commercially available TUNEL kit (Roche) for the in situ detection of apoptotic cells. All sections were counterstained with 0.5 mM 49, 6-diamidineo2-phenylindole (DAPI) for 10 min and then examined under a fluorescence microscope (Nikon, Japan). Each test included negative controls. The apoptotic rate was determined by counting the number of $TUNEL^{+}$ cells/100 cells in four sections from each of the five mice per group.

2.11. Statistical analysis

The mean and standard deviation values were calculated from the data obtained from parallel experiments. All of the data are presented as mean ± standard deviations (SD). One-way analysis of variance was performed using SPSS (Version 13.0, SPSS Inc., Chicago, IL, USA), followed by Fisher's least significant difference test to verify significant differences between the samples. The results were considered to be significant only when $p < 0.05$. The Pearson correlation test was conducted to determine the correlations between the variables.

3. Results

3.1. Acute toxicity of PFOS in mice

In acute PFOS toxicity determination, we found that PFOS (0.3–0.7 g/kg) dose-dependently increased the mortality, general adverse effects (constipation, anorexia, asthenia, and syncope), and weight loss (Table 1). The mice began to die 3 h after receiving 0.4 g of PFOS/kg BW. The dying mice displayed abdominal breathing, hind limb spasticity, tics, and urinary incontinence. Histological investigation also revealed degenerated liver cells, protein-like substances in the glomerulus sac, and edema (data not shown). Constipation was first observed in the 0.7 and 0.6 g/kg groups and then its incidence began to increase. By the end of the respective dosing phases, all of the remaining mice in the 0.4–0.7 g/kg dose groups were constipated, and 1/10 of the mice in the 0.7 g/kg dose group experienced syncope. These observations consistent with the statistically significant decrements in BW gain of the remaining mice (2%–3% in 0.4 g/kg dose, 2%–5% in 0.5 g/kg dose, 2%–8% in 0.6 g/kg dose and 3%–8% in 0.7 g/kg dose). The treatments affected the BW gain and food consumption of the survivors up to 14 days of observation. However, the survivors did not completely recover after 14 days and also exhibited constipation. The LD_{50} of PFOA in mice was 0.579 g/kg BW.

3.2. Subchronic PFOS exposure

3.2.1. Clinical, body and organ weight, food intake and organ mass

No animal-related mortality occurred throughout the experimental period, and the mice in the treatment group displayed rough hair, slow movement, and constipation. Fig. 1 shows the mean BW changes and food consumption of the mice. BW gain and food intake significantly decreased in the PFOS-treated mice compared with the untreated mice ($p < 0.05$). In addition, the BW and food intake changed with PFOS dose. During the 30-day period, significant differences in BW ($p < 0.05$) were observed between the

Table 1
Mortality and toxicity symptoms observed in male C57BL/6 mice exposed to acute oral PFOS.

PFOS (g/kg)	Mortality (D/T)	Mortality (%)	Toxic symptom
0	0	0	None
0.3	0/10	0	None
0.4	2/10	20	Constipation, weight loss
0.5	3/10	30	Constipation, weight loss, anorexia
0.6	5/10	50	Constipation, weight loss, anorexia
0.7	8/10	80	Constipation, weight loss, anorexia, syncope

D/T: dead/treated mice.

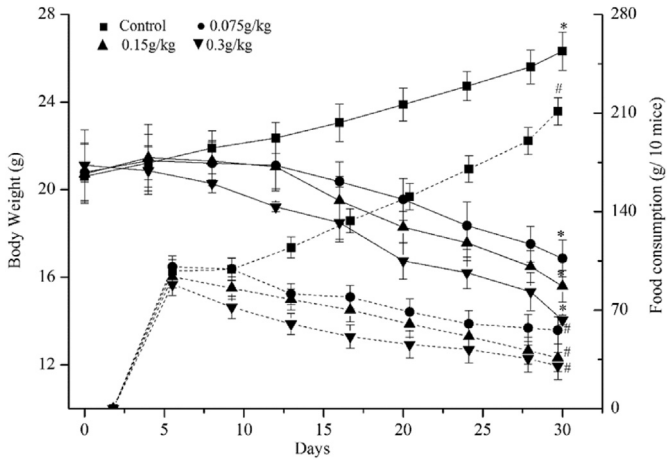

Fig. 1. Changes in mean body weight (BW) and food intake of adult male C57BL/6 mice orally treated with PFOS for 30 days. The values are expressed as mean ± SD (n = 2). BW was measured before mouse treatment; n = 2 in each group. Food intake were measured before mouse treatment and for nine points; n = 2 in each group. $^{*}p < 0.05$, significantly different from the control group (0 ppm PFOS) values in terms of mean BW. $^{\#}p < 0.05$, significantly different from the control group (0 ppm PFOS) in terms of food intake.

Table 2
Correlation between BW and food intake in C57BL/6 mice exposed to subchronic toxicity.

Group	Correlation coefficient
Control	0.936**
0.075 g PFOS/kg BW	0.294
0.15 g PFOS/kg BW	0.234
0.3 g PFOS/kg BW	0.297

$^{**}p < 0.01$.

0.3 g/kg dose group (31.00% weight reduction) and the control group (27.75% weight gain). The food intake also decreased with increasing PFOS dose ($p < 0.05$). In addition, the BW and food intake of the untreated mice increased with time, whereas those of the PFOS-treated mice decreased with time. Table 2 shows the correlation between the BW and food intake in the four exposure groups. The correlation coefficient of the control group was 0.936 ($p < 0.01$), whereas those of the PFOS-treated groups were 0.294, 0.234, and 0.297 ($p > 0.05$).

Liver mass significantly increased by 35.47%, 91.33%, and 154.98% in the groups treated with 0.075, 0.15, or 0.3 g PFOS/kg BW, respectively, compared with the control group. Moreover, kidney and spleen mass slightly decreased in the 0.075 and 0.15 g PFOS/kg BW exposure groups but significantly decreased in the 0.3 g PFOS/kg BW exposure group (Table 3).

3.2.2. Serum PFOS

Serum concentrations of PFOS in the treated mice are shown in Table 3. The serum concentrations of PFOS significantly increased ($p < 0.05$) in a dose-dependent manner in the PFOS-treated mice compared with the control mice.

3.2.3. Histopathological observations

Necropsy revealed no distinguishable morphological differences between the kidney and spleen tissues of the PFOS exposure groups and those of the control mice (data not shown). The liver tissues of the control mice displayed normal color and texture (Fig. 2a). By contrast, the liver tissues of the 0.075 g PFOS/kg BW exposure group exhibited slight tumefaction and dark color (Fig. 2b), whereas obvious tumefaction and discoloration were observed in all the livers obtained from the 0.15 and 0.3 g PFOS/kg BW exposure groups (Fig. 2c and d). These results indicated that PFOS can induce abnormalities related to hepatic injury.

Microscopic observation clearly revealed the presence of normal hepatic sinusoid and hepatic lobules in the control group (Fig. 3a). Moreover, cytoplasmic vacuolation (Fig. 3b), focal or flake-like necrosis (Fig. 3c and d), and hepatocellular hypertrophy (Fig. 3c and d) were universally observed in each treatment group. The manifestations of dose-dependent hepatic toxicity include cytoplasmic vacuolation (Fig. 3b), focal or flake-like necrosis (Fig. 3c and d), and focal hepatocytic degeneration (Fig. 3d).

3.2.4. Effect of PFOS on serum ALP, AST, ALT, and γ-GT levels

The serum AST andγ-GT levels dose-dependently increased in the mice treated with subchronic doses of PFOS for 30 days ($p < 0.05$) (Fig. 4). Compared with the control group, the mice treated with 0.15–0.3 g PFOS/kg BW displayed significantly higher serum ALP and ALT levels. The abovementioned biochemical markers of liver function showed no significant upregulation in the lowest exposure group (0.075 g/kg BW) compared with the control group.

3.2.5. Changes in BUN and NAG activities

Serum BUN and NAG levels were measured to determine the degree of changes in the kidney. In contrast to the remarkable change in the liver of PFOS-treated mice, the BUN and NAG levels only slightly changed in all PFOS-treated groups (Table 4).

3.2.6. Effect of PFOS on liver SOD, CAT, and GSH-Px levels, and T-AOC activities

The levels of SOD, CAT, MDA, and GSH-Px and the activities of T-AOC in the liver tissue homogenates were measured to explore whether or not PFOS exposure leads to oxidative stress in the liver. SOD, CAT, GSH-Px, and T-AOC activities were significantly reduced ($p < 0.05$) in the liver with increasing PFOS dose (Fig. 5). The lowest SOD, CAT, and GSH-Px levels and T-AOC activities were observed in the livers of the 0.3 g PFOS/kg BW exposure group and were lower by 37.75%, 56.46%, 41.98%, and 71.39% than those of the control group ($p < 0.05$), respectively.

Table 3
Serum PFOS and organ mass in adult male C57BL/6 mice orally administered with PFOS for 30 days.

PFOS (g/kg)	n	Serum PFOS (μg/mL)	Organ weight/body weight ratio (g/100 g) for		
			Liver	Kidney	Spleen
0	10	0.02 ± 0.001	5.31 ± 0.42	1.38 ± 0.03	0.54 ± 0.02
0.075	10	70.23 ± 2.35*	8.23 ± 1.26*	1.34 ± 0.05	0.50 ± 0.02
0.15	10	130.56 ± 6.54*	10.16 ± 1.28*	1.30 ± 0.02	0.45 ± 0.02
0.3	10	201.24 ± 8.96*	13.54 ± 1.56*	1.24 ± 0.06*	0.43 ± 0.01*

*Significantly different from respective control ($p < 0.05$).

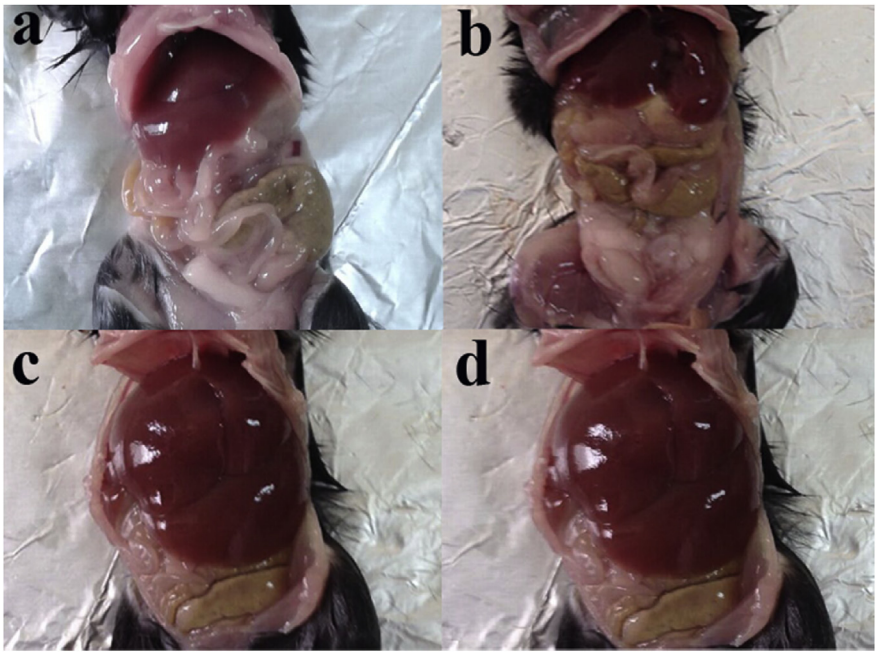

Fig. 2. Observable symptoms in mouse livers exposed to PFOS. All of the mice (n = 10) in the control and three exposure groups were examined. Compared with the normal liver tissues of the control (a), the liver tissues of the mice in the 0.075 g/kg PFOS dose group showed slight tumefaction and dark coloration (b), whereas those in the 0.15 and 0.3 g/kg PFOS dose groups displayed obvious tumefaction and dark coloration (c, d). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

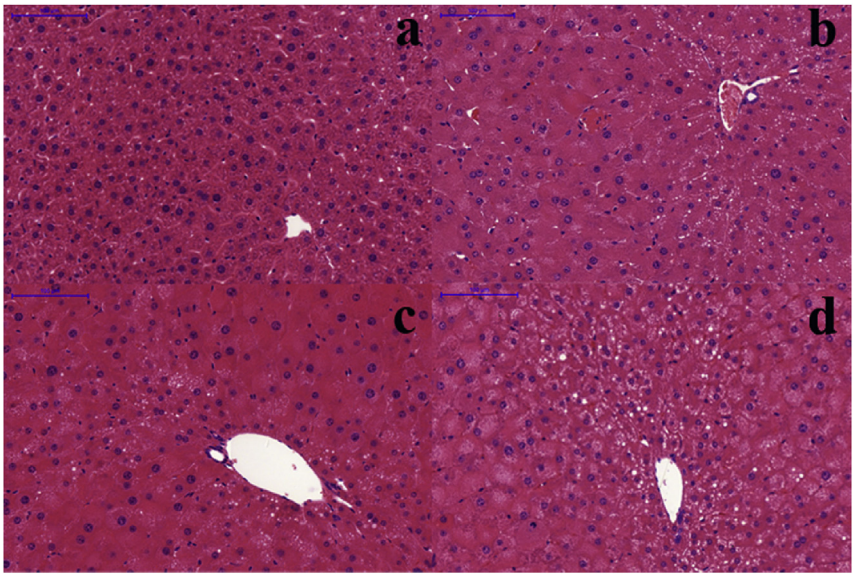

Fig. 3. Histopathological sections of mouse liver of the control and treatment groups. Hematoxylin-E-osin (H&E); (a–d), ×200. (a) control; treated with (b) 0.075 g/kg, (c) treated with 0.15 g/kg, and (d) 0.3 g/kg. The histological sections of each tissue obtained from at least five mice per group were examined.

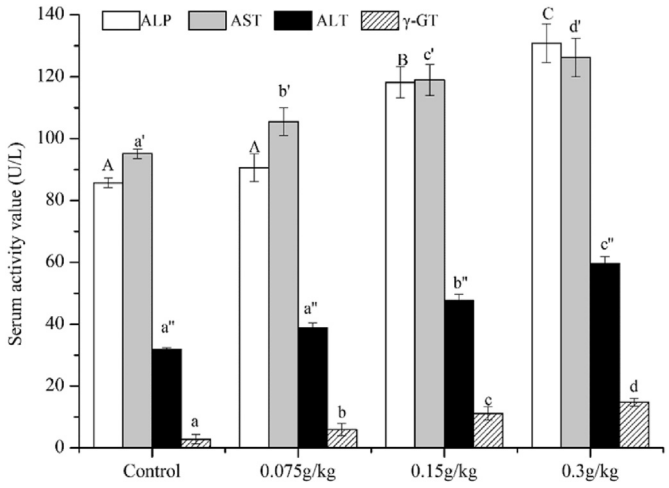

Fig. 4. Serum levels of ALP, AST, ALT, and γ-GT activities after exposure to different PFOS concentrations. Values are expressed as mean ± SD (n = 10). Different letters in the same row indicate significant inter-group differences, $p < 0.05$; same letters in the same row suggest insignificant inter-group differences, $p > 0.05$.

Table 4
Effects of 30-day oral PFOS exposure on BUN and NAG concentrations in male C57BL/6 mice.

PFOS (g/kg TAD)	n	BUN(nmol/L serum)	NAG(U/L urine)
0	10	5.07 ± 0.06	5.32 ± 0.12
0.075	10	5.38 ± 0.15	5.41 ± 0.26
0.15	10	5.76 ± 0.24	5.56 ± 0.28
0.3	10	5.58 ± 0.26	5.64 ± 0.16

*$p < 0.05$, significantly different from the control group (0 ppm PFOS) values.

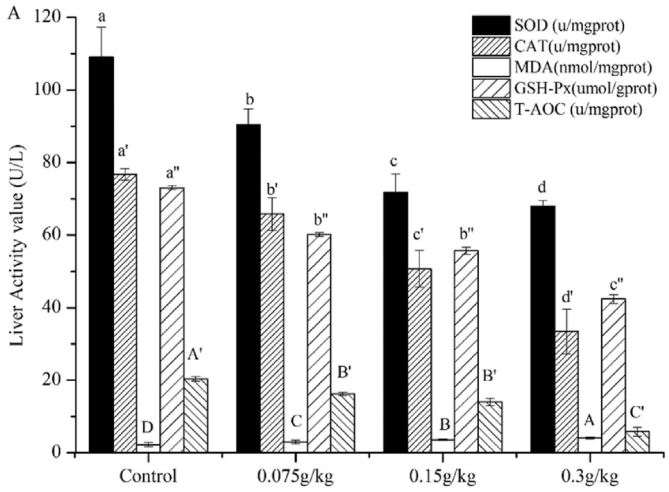

Fig. 5. Hepatic levels of SOD, CAT, MDA, GSH-Px, and T-AOC activities after exposure to different PFOS concentrations. Values are expressed as mean ± SD (n = 10). Different letters in the same row indicate significant inter-group differences, $p < 0.05$; same letters in the same row suggest insignificant inter-group differences, $p > 0.05$.

3.2.7. Lipid peroxidation

MDA was used as an index of lipid peroxidation. The MDA level in the liver tissue was significantly higher in the treated mice than in the control mice ($p < 0.05$). The greatest increase in MDA levels was observed in the liver when a TAD of 0.3 g/kg PFOS was administered (Fig. 5).

3.2.8. TUNEL assay

TUNEL labeling of nuclear DNA fragmentation was performed to detect PFOS-induced hepatocellular apoptosis. The number of $TUNEL^+$ cells in the liver obviously dose-dependently increased in the PFOS-treated group compared with that in the control group (Fig. 6a and b).

4. Discussion

This study evaluated the acute and subchronic toxicities of PFOS in C57BL/6 mice. In the acute toxicity determination, we found that the cause of death or morbidity in the mice became evident at 0.4 g PFOS/kg BW treatment. The dead mice displayed indications of hepatocellular hypertrophy with significant vacuolation, similar to that found in the mice treated with higher doses (0.5, 0.6, and 0.7 g/kg) of PFOS. By contrast, treatment with 0.3 g PFOS/kg BW did not cause obvious adverse clinical signs and mortality. The LD_{50} of PFOS was 0.579 g/kg BW in male C57BL/6 mice. Dean et al. (1978) also obtained an acute oral PFOS LD_{50} value of 251 mg/kg in rats. In addition, Goldenthal et al. (1978) found that the acute oral PFOS LD_{50} of Sherman Wistar albino rats ranges from 50 mg/kg to 1500 mg/kg. To the best of our knowledge, this study is the first to report on the acute effects of PFOS on male C57BL/6 mice.

PFOS caused hepatomegaly as indicated by the increase in liver weight (Fig. 2). PFOS also significantly reduced BW. The BW loss and liver weight increase elevated the liver-to-BW ratio of the 0.3 g/kg PFOS treatment group to 13.34 ± 1.56, which was higher than that of the control (5.31 ± 0.42) (Table 3). The present study also found that the liver mass and BW varied depending on PFOS intake. This result coincided with those of Zheng et al. (2009), wherein male C57BL/6 mice displayed increased liver mass after treatment with 0–40 mg PFOS/kg/day via gavage for 7 days. Dong et al. (2012) also found that the liver mass of male C57BL/6 mice is significantly increased by 5–125 mg PFOS/kg TAD. By contrast, Peden-Adams et al. (2008) found no changes in the liver mass and the BW of B6C3F1 mice treated with 0–5 mg/kg PFOS. These disparities in results can be attributed to differences in dose and mouse strain. Liver mass increased in the PFOS-treated mice; by contrast, the kidney and spleen masses only slightly decreased in the 0.075 and 0.15 g/kg PFOS exposure groups but significantly reduced in the 0.3 g/kg PFOS exposure group (Table 3). These data agree with those of Cui (2008), who observed the following decreasing order of PFOS concentrations in major target organs: liver > heart > kidney > whole blood > lung > testicle, spleen, and brain. The above observations suggest that the increase in liver mass is a sensitive indicator of subchronic exposure to PFOS. Moreover, PFOS exposure significantly increased PFOS concentration in mouse serum, and the lowest serum PFOS concentration we obtained from the mice treated with 0.075 g/kg PFOS is similar to that obtained by Cui (2008) in Sprague–Dawley rats treated with 5 mg/kg/day PFOS. The serum PFOS concentrations in the mice were within the range or significantly exceeded those reported of higher–end human occupational serum PFOS concentrations (Olsen et al., 2003b). At 0.075 g/kg PFOS, serum PFOS concentrations rose and exceeded the highest reported human occupational serum concentration of 12.8 μg/mL (Olsen et al., 1999). This finding supports the hypothesis that subchronic dosing of PFOS in rats results in liver and serum PFOS concentrations that are proportional to external dietary dose and can be used as a measure of internal dose (Seacat et al., 2003). Furthermore, the present study found a significant correlation between BW and food intake in the control mice ($p < 0.01$), verifying that the change in BW resulted from reduced food intake. However, BW showed no correlation with food intake in the PFOS-treated mice. Therefore, the reduction in BW and food intake in the PFOS-treated mice might have

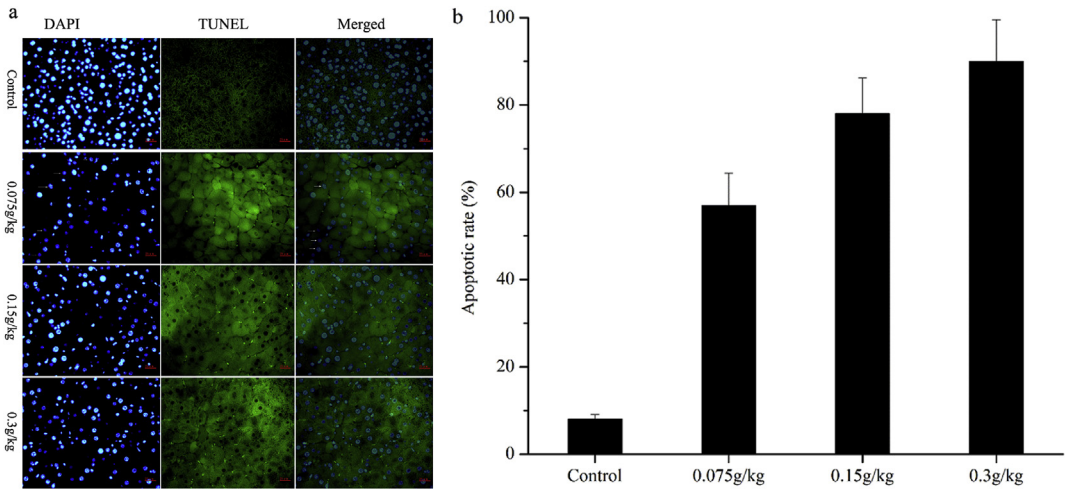

Fig. 6. (a) TUNEL fluorometric staining (counterstained with DAPI) of liver obtained from PFOS- treated mouse. The apoptotic cells are indicated by arrows. Scale bar: 25 μm. (b) Apoptotic rate was determined by counting the number of $TUNEL^{+}$ cells/100 cells in four sections obtained from five mice per group.

resulted from the toxic effects of PFOS.

Studies have shown that exposure to PFOS can increase the size and relative weight of the liver in mice (Elcombe et al., 2012; Kennedy, 1987). The present study revealed that oral exposure to PFOS for 30 consecutive days caused hepatomegaly resulting from the significant increase in liver weight in a dose-dependent manner. In addition, the histopathological evaluation revealed that the livers of the PFOS-treated mice showed morphological changes, including structural damage, hepatocellular necrosis, edema, and inflammatory cell infiltration. Biochemical evaluation also indicated that PFOS treatment significantly increased the concentrations of serum enzymes, including AST, ALT, ALP, and γ-GT. Leakage of large quantities of these serum enzymes into the blood stream is associated with centrilobular necrosis, ballooning degeneration, and cellular infiltration of the liver (Huang et al., 2012). Previous reports have suggested a positive association between PFOS exposure and serum ALT and AST levels (Gallo et al., 2012). Our results have thus confirmed the hepatic toxicity of PFOS in mice. Contrary to the dramatic hepatic changes, only slight changes were observed in the kidneys upon exposure to PFOS. The kidney size, kidney damage biomarkers (BUN and NAG), and histopathological features showed no significant differences between the untreated and PFOS-treated mice. These slight differences may be ascribed to species differences (Son et al., 2008).

Environmental pollutants are well-known inducers of oxidative stress, which can further cause the depletion of antioxidant defenses and mediate other oxido/reduction reactions through different metabolic pathways, such as those mediated by detoxifying enzymes (Livingstone, 2001; Shi and Zhou, 2010). SOD, CAT, and GSH-Px activities are often used as biomarkers to indicate reactive oxygen species (ROS) production (Zelikoff et al., 1996). The present study investigated the effects of PFOS on antioxidant responses in the mouse liver. Results showed that PFOS can induce ROS production during PFOS treatment. The activities of the antioxidant enzymes (i.e., SOD, CAT, GSH-Px, and T-AOC) also dose-dependently reduced in the livers of the PFOS-treated mice. These enzymatic activities are possibly inhibited and inactivated by ROS in the livers of the PFOS-treated mice. Moreover, MDA is an index of lipid peroxidation (Livingstone, 2001). The present study showed that MDA content significantly increased after exposure to PFOS possibly because of ROS generation (Liu et al., 2007). Furthermore, ROS levels are usually in a dynamic balance with antioxidant enzyme levels. However, natural antioxidant defenses may be overwhelmed if the free radicals directly or indirectly induced by xenobiotic pollutants cannot be promptly scavenged. This phenomenon can lead to severe sub-cellular injuries, such as ion loss, protein denaturation, and DNA damage (Nel et al., 2006). The above results suggest that exposure to PFOS may affect cell function and thus impair the mouse liver.

Liver injury is especially sensitive to ROS induced oxidative stress (Cichoż-Lach and Michalak, 2014). ROS production in response to contamination is closely associated with cell apoptosis (Shi and Zhou, 2010). Apoptosis or programmed cell death is induced by stress and during tissue damage under physiological conditions (Zou et al., 2015). TUNEL assay, an immune histochemical approach that uses an in situ apoptosis detection kit, was utilized in the present study to quantify the apoptotic cells in the liver. *In vitro* studies have shown that PFOS and PFOA exert cytotoxic effects on hepatoma HepG2 cells (Florentin et al., 2011). The present study revealed the differential staining of apoptotic cells in the livers of PFOS-treated mice. The evaluated apoptotic rate also significantly increased in a dose-dependent manner after PFOS treatment, indicating the occurrence of PFOS-induced hepatic cell apoptosis.

ROS generation is an important apoptotic signal. ROS induction by PFOS was evident in the present study. The reduced SOD, CAT, GSH-Px, and T-AOC levels and increased MDA level in the subchronic toxicity experiment indicated that oxidative stress was induced by PFOS treatment. The oxidative stimulus may trigger cell apoptosis (as revealed by the TUNEL assay), cause liver injury (as evidenced by the increased serum AST, ALT, ALP, and γ-GT levels and by histopathological changes), and thus increase the size and relative weight of mouse liver.

In conclusion, evaluating the acute and subchronic toxicological effects of PFOS on mice is valuable in assessing the environmental risk and threat to human health posed by PFAS chemicals. The current study found that the acute oral LD50 dose for PFOS is 0.579 g/kg BW in male C57BL/6J mice. A 30-day subchronic oral toxicity experiment demonstrated that PFOS treatment caused ROS generation, disrupted the homeostasis of antioxidative systems, induced hepatocellular apoptosis (as revealed by the TUNEL assay), and ultimately induced liver injury (as evidenced by increased serum AST, ALT, ALP, and γ-GT levels and by histopathological changes). In contrast to the hematological, biochemical,

histological, and immunohistochemical changes in the livers of the PFOS-treated mice, only slight changes were observed in the kidney and spleen in the same group. However, the mechanisms of ROS generation and the effects of PFOS on cell apoptosis remain unknown and require further investigation.

Conflicts of interest

None of the authors has any conflicting interests.

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