Perfluorooctane sulfonate induces heart toxicity involving cardiac apoptosis and inflammation in rats

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Abstract. Perfluorooctane sulfonate (PFOS) is a persistent pollutant that exerts toxicity and induces cardiogenesis in humans and animals. Yet, the effect of PFOS exposure on cardiac toxicity in adult rats has, to our knowledge, not been reported and the mechanism still remains unknown. The present study aimed to investigate the toxicity of PFOS on rat hearts and any associated mechanisms. Rats were exposed to 0 (control), 1 and 10 mg/kg PFOS every other day for 14 days. Body weight and heart weight were recorded. The serum levels of lactate dehydrogenase (LDH), creatine kinase (CK), creatine kinase-isoenzyme-MB (CK-MB) and cardiac troponin-T (cTn-T) in heart tissues were measured using biochemical assays. TUNEL staining and western blotting were applied to analyze levels of apoptosis in rat hearts. Pathological assessment and immunohistochemistry analysis of heart tissues were used to evaluate the levels of PFOS-induced cardiotoxicity and inflammatory infiltration. PFOS exposure at the dosage of 10 mg/kg significantly increased the percentage of heart to body weight; however, it did not alter the body weight. At 10 mg/kg, PFOS significantly increased expression levels of myocardial injury markers, such as cTn-T, LDH, CK and CK-MB, while 1 mg/kg PFOS upregulated the expression level of cTn-T in rats. Notably, cardiac fibrosis and myocardial hypertrophy appeared in the 10 mg/kg PFOS group. In addition, TUNEL-positive cells were significantly increased by exposure to 10 mg/kg PFOS in rat heart tissues. The protein expressions profiles of p53 and Bax were also significantly upregulated in the 10 mg/kg PFOS group. Inflammatory infiltration, detected by analyzing expression levels of IL-1β and TNF-α, was significantly raised by 10 mg/kg PFOS exposure. In conclusion, these results demonstrated that 10 mg/kg PFOS-induced cardiac toxicity in rats, which was associated with an increase in apoptosis and the expression of proinflammatory cytokines.

Introduction

Perfluorooctane sulfonate (PFOS) is a degradation product of perfluorinated compounds and is characterized by widespread use and environmental stability (1). This chemical compound has been used in a large variety of industrial and commercial material, such as plastic packaging bags, cosmetics and textiles (2). It is globally distributed and can be detected in soil, air, water, wildlife and in humans (1,2). It can accumulate biologically through the food chain, having a half-life of ~5 years in human serum (3). Therefore, the potential toxicity of PFOS is concerning. The chief manufacturer of PFOS in the United States, The 3M Company, is to halt production (4), and a series of international regulations were set out to restrict usage of polyfluoroalkyl substances in 2016 (5). However, emission of PFOS still persists in Asian markets (6,7). A recent study reported that PFOS concentrations in serum are significantly increased with age in the general Chinese population, suggesting that they may have common exposure sources (8).

As previously reported, PFOS involves several toxic effects in the cardiovascular (9) and reproductive systems (10), effecting immunological (11) and hepatic functions (12). Notably, the heart exhibits the second greatest bioaccumulation of PFOS after the liver, and it is also a target organ for PFOS. Our previous study indicated that several marker proteins involved in cardiovascular development, such as Brachyury, GATA4, myocyte enhancer factor 2C and α-actinin, were downregulated when exposed to PFOS (13). In a marine medaka model and in embryonic stem cell (ESC)-derived cardiomyocytes, prenatal PFOS exposure disrupts the expression of genes associated with cardiac development, and affects the function of the heart (9,14). PFOS has been considered to induce cardiac mitochondrial damage and gene transcript disorder (15), which may be one possible toxicity mechanism. Nevertheless, to our knowledge, the possible impact of PFOS on cardiac dysfunction in adult rats has not been investigated, and the underlying toxicity mechanism has not yet been fully elucidated.
Apoptosis is a type of programmed cell death that participates in various pathological events (16). It has been widely reported that excessive apoptosis is responsible for structural abnormality and dysfunction of the heart (17,18). During all stages of heart development, PFOS possesses the potential to alter key genes, reduce ATP production, stimulate reactive oxygen species (ROS) generation and induce apoptosis (19). In a zebrafish embryo model, PFOS exposure induced apoptosis and upregulated gene expression levels of P53 and Bax, which are associated with apoptosis. These genes are also closely associated with the JNK and p38 signaling pathway (20,21). In addition, a potential association between PFOS and inflammation has been revealed in a previous study (22); however, the specific mechanism is unclear. PFOS can modulate the inflammatory factors, such as TNF-α and IL-6, in vivo and in vitro (22). Therefore, it was hypothesized that PFOS toxicity in the cardiac tissue of adult rats might be associated with inflammation and apoptosis.

The current study aimed to explore whether PFOS exposure would induce heart impairment and a degree of pathological change in rats. Moreover, the level of apoptosis and inflammatory infiltration in cardiomyocytes was investigated to provide evidence for further research on PFOS-induced cardiac toxicity.

Materials and methods

Ethics statement. Animal experiments in the current study were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (23). Procedures of animal experiments were approved by The Ethics Committee of the Laboratory Animal Care and Welfare, Zhejiang Academy of Medical Sciences (Zhejiang, China).

Animals. A total of 48 male Sprague Dawley (SD) rats (220±5 g) in 8-week old were purchased from the Laboratory Animal Center of Zhejiang Province (Hangzhou, China) and bred in house. All rats were housed in a specific pathogen free facility under a 12-h light/dark cycle in an ambient temperature of 22-26˚C and relative humidity of ~55%. Animals were fed standard laboratory rat chow. Animals were provided food and water ad libitum.

Experimental protocols. SD rats were divided into three groups (each, n=6) that received 0 (control), 1 and 10 mg/kg PFOS (Sigma Aldrich; Merck KGaA). The rats were intraperitoneally (I.P) injected at 1 cm to the left of the midline of the lower abdomen with PFOS every other day for 14 days. The dose, period and drug-delivery way of PFOS was selected based on previous reports (24,25). On day 14, all animals were placed on the operating table and immobilized with ketamine (40 mg/kg; intramuscular injection in the front of the thigh) and xylazine (5 ml/kg; I.P) anesthesia, after which 5 ml blood was obtained from the aorta abdominals. After blood and heart tissues were obtained, rats were sacrificed by exsanguination from the carotid artery. Rat hearts and blood were used for biochemical and pathological assays (Fig. 1A). One sample from each group was used for preliminary experiments.

Body weight and hearts weight determinations. Changes of body weight in each group were measured every other day until the 14th day. At the end of the PFOS treatment, the rats were sacrificed and hearts were quickly moved, carefully blotted dry and weighed. The percentage of heart weight to body weight was calculated as follow: (heart weight/body weight) x100%.

Heart tissues and blood biochemical assays. Blood samples were obtained from the abdominal aorta and left to stand at room temperature for 1 h, and then at 4˚C for 2 h, followed by 3,000 x g centrifugation for 10 min at 4˚C. The supernatant was used for serum lactic dehydrogenase (cat. no. 201902; LDH), creatine kinase (cat. no. 201823; CK) and creatine kinase-isoenzyme-MB (cat. no. 201811; CK-MB) measurements. The collected heart tissues were homogenized and centrifuged at 3,200 x g for 30 min at 4˚C. The supernatant was harvested for cardiac troponin-T (cat. no. 201904; cTn-T) measurement. All biomarkers were determined using commercial ELISA kits (BD Biosciences).

Histological analysis. The isolated hearts were fixed in 4% paraformaldehyde for 24 h at room temperature and embedded in paraffin for histological analysis. Next, samples were cut into 5-µm sections and heated at 65˚C for 20 min. Slides were then deparaffinized with xylene and dehydrated in a grade series of ethanol through 70, 80, 90, 95 and 100%. The slices were stained with Masson for 10 min at room temperature to evaluate fibrosis and stained with wheat germ agglutinin (WGA) for 15 min at 37˚C to analyze myocardial hypertrophy. Images were captured (magnification, x200 or x400) under a light microscope (Leica Microsystems GmbH). The fibrosis area of heart tissues and the cardiomyocyte cross-sectional area were measured using Image-Pro Plus software version 6.0 (Media Cybernetics, Inc.). The percentage of fibrotic areas were calculated.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. For all groups, 5-µm sections were stained using the In Situ Cell Death kit-TMR (cat. no. MK1012; Wuhan Boster Biological Technology, Ltd.) overnight at 4˚C. The process of TUNEL staining were performed according to the manufacturer’s instructions. The slides were incubated with TUNEL reaction mixture for 1 h at 37˚C before being rinsed 3 times with PBS. The nucleus was stained by DAPI (1 µg/ml; cat. no. C1002; Beyotime Institute of Biotechnology) for 10 min at room temperature. A drop of antifade mounting medium (cat. no. P0098; Beyotime Institute of Biotechnology) was added for 5 min at room temperature. Images were taken with a fluorescence microscope and the number of TUNEL-positive cells in five random views were selected in each sample. Cells were counted by ImageJ analysis software (version 1.51; National Institutes of Health).

Immunohistochemical analysis. For immunohistochemical assay, heart tissues were fixed in 4% paraformaldehyde for 24 h at room temperature and embedded in paraffin for immunohistochemical analysis. Sections were blocked using 5% goat serum (cat. no. G1209; Wuhan Boster Biological Technology, Ltd.) and incubated with specific primary antibodies overnight.
at 4˚C, such as IL-1β Rabbit mAb (1:800; cat. no. SRP8033; Sigma-Aldrich; Merck KGaA) and TNF-α Rabbit mAb (1:500; cat. no. ab6671, Abcam). The sections were washed with PBS three times, followed by staining with horseradish peroxidase-conjugated secondary anti-rabbit IgG (1:5,000; cat. no. GB23303; Wuhan Boster Biological Technology, Ltd.) for 2 h at room temperature and visualizing with substrate DAB. Images were obtained and captured (magnification, x200) using a fluorescent microscope (Leica Microsystems GmbH). The number of positive cells was analyzed by ImageJ analysis software (version 1.51; National Institutes of Health).

**Western blotting.** Protein from myocardial tissue was extracted using the cell and tissue total protein extraction kit (cat. no. KC415; Shanghai Kang Cheng Bioengineering Co., Ltd.). Protein concentration was quantified using the bicinchoninic acid (BCA) protein assay kit (cat. no. P0010; Beyotime Institute of Biotechnology) and was diluted to the same concentration with 5X loading buffer (cat. no. P0015L; Beyotime Institute of Biotechnology). A total of 50 µg protein was separated using 15% SDS-PAGE. Protein was transferred to polyvinylidene fluoride membrane and blocked in 5% skimmed milk in Tris-buffered saline for 90 min at room temperature. After blocking, the membranes were incubated overnight with primary antibodies against p53 mouse mAb (1:1,000; cat. no. 2524; Cell Signaling Technology, Inc.), anti-Bax antibody (1:800; cat. no. A00183; Wuhan Sanying Biotechnology) and GAPDH mouse mAb (1:3,000; cat. no. G3214; Bioworld Technology, Inc.) overnight at 4˚C. Following washing with Tris-buffered saline containing 1% Tween, membranes were incubated for 2 h with horseradish peroxidase-conjugated IgG (1:5,000, cat. no. GB23303; Wuhan Boster Biological Technology, Ltd.) secondary antibodies at room temperature for 2 h. The specific protein bands were visualized using an enhanced chemiluminescence detection kit (cat. no. 33021; Boster Biological Technology, Ltd.). The intensity of each band was quantified using Quantity One software version 6 (Bio-Rad Laboratories, Inc.).

**Statistical analyses.** Data are presented as mean ± standard deviation. Values from three groups were analyzed using one-way ANOVA followed by Student-Newman-Keuls multiple comparison post hoc test. Statistical analysis was performed in GraphPad Software (Prism Version 8.01; GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Effect of PFOS on body weight and associated heart weight in rats.** Body weight in 0 (control), 1 and 10 mg/kg PFOS groups were recorded every two days for 14 days. There was no significant difference in the body weight of rats among the three groups, though a slight reduction was observed between control and 10 mg/kg PFOS group (Fig. 1A). As presented in Fig. 1C, the percentages ratio of heart weight to body weight demonstrated no significant increase in 1 mg/kg group (P>0.05), but were significantly increased in the 10 mg/kg PFOS group compared with control group (0.44±0.03% vs. 0.37±0.02%; P<0.05; Fig. 1C).

**PFOS-induced myocardial injury in rats.** In order to detect the toxic effect of PFOS on the heart, cTn-T, LDH, CK and CK-MB, which are markers for myocardial injury, were measured in rats. In the 1 mg/kg PFOS group, only the level of cTn-T in heart tissues was significantly elevated compared with control group (P<0.05; Fig. 2A). Whereas in the 10 mg/kg PFOS group, cTn-T, LDH, CK and CK-MB levels were all significantly increased when compared with the control group (P<0.05; Fig. 2A-D). These results suggested that 10 mg/kg
PFOS could induce significant myocardial injury in rats. In addition, gavage administration was selected to test whether PFOS could elevate myocardial injury in rats. There were no significant differences between gavage administration and intraperitoneal injection administration on cTn‑T, LDH, CK and CK‑MB expression levels among the three groups (P>0.05; Fig. S1).

To determine the toxicological effect of PFOS on rat hearts over a longer duration, PFOS was administrated every other day for 28 days to model sub‑chronic exposure. The levels of cTn‑T, LDH, CK and CK‑MB were examined after 28 days of PFOS exposure. The 10 mg/kg PFOS group demonstrated significantly elevated levels of cTn‑T, LDH, CK and CK‑MB compared with the 0 mg/kg PFOS group (P<0.05; Fig. S2). The expression level of cTn‑T was also significantly increased in the 1 mg/kg PFOS group compared with the control group (P<0.05; Fig. S2A). However, there were no significant differences in expression levels of LDH, CK and CK‑MB between the 1 mg/kg PFOS group and 0 mg/kg PFOS group (P>0.05, Fig. S2B-D).

PFOS is associated with cardiac fibrosis and myocardiac hypertrophy in rats. To further determine the toxic influence of PFOS on heart tissues in rats, Masson and WGA staining were performed. The rats in the 1 mg/kg PFOS group exhibited no significant increase in cardiac fibrosis and hypertrophy when compared with the control group (P>0.05; Fig. 3A and B). However, the fibrosis area and the myocyte cross‑sectional area were markedly upregulated in rat hearts exposed to 10 mg/kg PFOS compared with the control group (fibrosis area, 7.2±1.2% vs. 0.0±0.0%; P<0.05; Fig. 3A and C; cross‑sectional area, 412.6±47.4 µm² vs. 212.4±12.0 µm²; P<0.05; Fig. 3B and D). These results were matched by the aforementioned changes in the percentage of heart weight to body weight.

PFOS is associated with myocardial apoptosis in rats. The cardiotoxicity effect of PFOS on apoptosis was explored using TUNEL staining. As presented in Fig. 4A and B, rats subjected to 1 mg/kg PFOS exhibited no difference in myocardial apoptosis compared with the control group (P>0.05). In addition, in the 10 mg/kg PFOS group, the percentage of TUNEL‑positive nuclei were significantly increased compared with the control group (P<0.05). Furthermore, the expression levels of p53 and Bax were significantly upregulated in the 10 mg/kg PFOS group compared with the control group (P<0.05; Fig. 4C and D), and p53 protein was also significantly upregulated in the 1 mg/kg PFOS group (P<0.05; Fig. 4C and D).

PFOS is associated with inflammatory infiltration in rat heart tissues. The influence of PFOS on the cardiac inflammation in rats was investigated. Immunohistochemical staining for pro‑inflammatory cytokine IL‑1β indicated that the expression profile of IL‑1β was significantly increased in the 10 mg/kg PFOS group compared with the control group (6.60±0.65 vs. 3.84±0.76 pg/mg protein; P<0.05; Fig. 5A and B). Consistent with the apparent IL‑1β accumulation, there was also a significant upregulation of TNF‑α expression in the 10 mg/kg PFOS group compared with the control group (1.41±0.09 vs. 1.00±0.10; P<0.05; Fig. 5A and C). In addition, there was little expression of IL‑1β and TNF‑α in heart tissues exposed to 1 mg/kg PFOS (P>0.05).

Discussion

The current study revealed several main findings. Firstly, exposure of PFOS at the dosage of 10 mg/kg caused myocardial damage in rats. Secondly, 10 mg/kg PFOS significantly increased cardiac fibrosis and myocardiac hypertrophy in rats.
Finally, it was demonstrated that 10 mg/kg PFOS treatment upregulated myocardial apoptosis and expression levels of IL-1β and TNF-α in heart tissue of rats.

PFOS, a type of fluorine-saturated eight-carbon compound, is a persistent, bioaccumulative and organic pollutant as a result of its ubiquitous distribution and extreme stability (26). For humans, dietary intake is the main source of exposure to PFOS. Pollution data by the National Health and Nutrition Examination Survey has demonstrated that serum concentrations of PFOS range from 0.8-0.9 µM, while an increased concentration (0.3-6.9 µM) has been detected in Minnesota Mining and Manufacturing company employees (3,27). Serum PFOS concentrations are generally far lower compared with 1 mg/kg in this study. However, based on the average weight of adults in China (60 kg) and their average blood content (~6,400 ml), the serum PFOS concentration of fluorination plant workers is 1,386 ng/ml (28); therefore, the serum PFOS concentration of fluorination plant workers is 1,386 ng/ml (28); therefore, the average concentration of PFOS has been calculated to reach 0.1 mg/kg body weight. Studies have demonstrated that concentrations of PFOS that are lower than the oral PFOS LD₅₀ of 250 µg/g (29) were associated with significant injury in rats or in mice (4,25). In the current study, the dosage of 10 mg/kg was a little higher compared with the accumulated doses in human blood.

The cardiovascular toxicity of PFOS has been rudimentarily studied in vitro and in vivo. Harada et al (30) reported that the action potential duration and peak potential were markedly reduced when exposed to PFOS in guinea-pig ventricular myocytes (30). The sinus venosus-bulbus arteriosus distance was also demonstrated to be increased in a marine medaka when exposed to PFOS (9). Moreover, PFOS can enlarge the right atrium of mice and rats (31). PFOS not only affects heart malformation, but also heart function. PFOS exposure also changes heart rates in zebrafish embryos (32). Our previous study demonstrated that PFOS induced toxicity of ESCs through mitochondrial structure injury and abnormal Ca²⁺ shuttle (33). However, there is only a small amount of research performed to assess the cardiovascular toxicity of PFOS in rats. To investigate what effects PFOS exposure exerted on cardiac toxicity, the present study detected biochemical indices and pathological changes in rats. It was demonstrated that PFOS treatment augmented the percentage of heart to body weight in rats. Moreover, 10 mg/kg PFOS induced significant cardiac fibrosis and myocardiac hypertrophy that was matched by increased biochemical indices associated with myocardial damage in rats. The results of the present study suggested that PFOS at a dosage of 10 mg/kg could exert a pronounced cardiotoxicity in rats; however the mechanism underlying PFOS toxicity in the cardiovascular system remains unclear.

Figure 3. PFOS is associated with cardiac fibrosis and myocardiac hypertrophy in rats. (A) Masson staining and (B) wheat germ agglutinin staining of the rat hearts exposed to different doses of PFOS. Statistical graph of the (C) fibrosis area and the (D) cardiomyocyte cross-sectional area. White arrows point out Masson-positive regions (A magnification, x400; scale bars, 50 µm; B magnification, x200, scale bars, 100 µm). n=5. *P<0.05. PFOS, perfluorooctane sulfonate.
Apoptosis is an important process in various human diseases and has been implicated in PFOS toxicity (34). A small number of basal studies have been carried out to examine the toxicity of PFOS associated with apoptosis (35-37). PFOS treatment can induce cell apoptosis in murine N9 cells (35) and in hepatoma Hep G2 cells (36). It can also upregulate the number of apoptotic cells in liver of adult rats (37). However, PFOS exposure has been reported to influence the protein and mRNA expression levels of Bax and Bcl-2 in adults (38). In addition, the genes associated with apoptosis, such as p53 and Bax, were significantly upregulated in zebrafish embryos that were raised in an environment with PFOS, the mechanism of which was associated with ROS generation and MAPK activity (21). A previous
study revealed that PFOS exposure results in the apoptosis of rat cardiocytes via TUNEL analysis (18). In line with this study, the current study revealed that 10 mg/kg PFOS induced increases of TUNEL-positive cells, p53 and Bax protein expression, suggesting an increase in apoptosis in the heart tissues of adult rats.

Increasing evidence has demonstrated that inflammation serves a notable role in Polyfluoroalkyl chemical-induced toxicity (22,39). Perfluorononanoic acid is hypothesized to increase liver weight and upregulate large quantities of IL-1β and TNF-α (39). In addition, it is reported that PFOS exposure leads to hepatocyte proliferation accompanied by an increase of serum IL-6 and TNF-α (22). On the contrary, clinical research has revealed that lower gut inflammation is closely associated with higher perfluoroalkyl substances exposure, which can be regarded as a risk factor for inflammatory bowel disease (40). However, whether pro-inflammatory cytokines are involved in PFOS-stimulated cardiac toxicity remains unclear. Notably, the results of the current study revealed that proinflammatory cytokines IL-1β and TNF-α were significantly elevated in rat heart tissues after exposure to 10 mg/kg PFOS.

Previous studies have reported that the exposure of female rats to perfluorooctane sulfonate increased the estrogen receptor α (ERα) expression, suggesting that PFOS acts as estrogenic compounds to activate ERα (41,42). In adult male and female B6C3F1 mice, daily PFOS exposure could induce immunotoxicity, with certain differences between male mice and female mice being identified in regards to immune parameters (43). Further study should be conducted to provide evidence towards the difference of male and female rats in cardiac toxicity. In addition, as an organic compound, the concentration of PFOS in heart tissue needs to be detected with high performance liquid chromatography in future experiments.

In summary, the current study demonstrated that PFOS exposure caused pathological changes, reflected by cardiac fibrosis and myocardial hypertrophy in the hearts of adult rats, which was possibly associated with an increase in apoptosis and proinflammatory cytokines, such as IL-1β and TNF-α. The present study provided preliminary data for further study of cardiovascular system subjected to a PFOS challenge.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.
Authors' contributions

JY and MG designed the current study and wrote the manuscript. DX, LL and LT performed experiments and analyzed data. DX and JY confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Procedures of animal experiments were approved by The Ethics Committee of Laboratory Animal Care and Welfare, Zhejiang Academy of Medical Sciences (Zhejiang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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