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Perfluorononanoic acid induces apoptosis involving the Fas death receptor signaling pathway in rat testis

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ABSTRACT

Perfluorononanoic acid (PFNA, C9), a synthetic perfluorinated chemical containing nine carbons, accumulates and is biomagnified through food webs. This compound has been detected in the serum of humans and wildlife and has the potential for reproductive interference. Few studies, however, have reported the effects of PFNA exposure on male reproduction. To determine this, male rats were orally dosed for 1, 3 and 5 mg/kg day PFNA or with vehicle for 14 days. In the present study, serum testosterone levels were decreased, while estradiol levels were increased dramatically in rats receiving 5 mg PFNA/kg day. Spermatogenic cells from rats that received 5 mg PFNA/kg day exhibited apoptotic features including crescent chromatin condensation and chromatin margination. Flow cytometric analysis and TUNEL assays revealed a dose-dependent increase of apoptotic cell numbers. In addition, expression of Fas and Bax mRNA levels were upregulated significantly, and Bcl-2 mRNA levels were downregulated markedly in the 3 and 5 mg/kg day groups. A dose-dependent increase in levels of active caspase-8 and no significant changes of active caspase-9 were observed. Our results indicate that PFNA exposure can lead to cell apoptosis in rat testis, and this apoptosis was probably associated with the Fas death receptor-dependent apoptotic pathway.

1. Introduction

The perfluoroalkyl acids (PFAA) are a class of perfluorinated chemicals that consist of a carbon backbone of various lengths, which mainly include 4–14 carbons (Lau et al., 2007). Because of the strong carbon–fluorine bonds, these chemicals are hydrophobic and oleophobic and also exhibit resistance to hydrolysis, photolysis, and microbial degradation (Kleszczyński et al., 2007; Parsons et al., 2008). Perfluorochemicals have been used in numerous products including lubricants, surfactants, papers, paints, textile coating, polishes, cosmetics, and fire-retarding foams since the 1950s (Renner, 2003). PFAAs have been detected in environmental media, wildlife, and human serum from around the globe (Giesy and Kannan, 2001; Taniyasu et al., 2003; Shoeib et al., 2005; Houde et al., 2006; Young et al., 2007).

Perfluorononanoic acid (PFNA), a synthetic perfluorinated chemical containing nine carbons, has wide environmental distributions and is biomagnified through food webs (Lau et al., 2007). Occupational studies of employees from a PFNA-production plant, no adverse clinical effects were detected from occupational exposure to PFNA blend (Mundt et al., 2007). Monroy et al. (2008) also evaluated PFNA in 101 pregnant women in Canada and reported no effect on birth weight. A study of polyfluoroalkyl chemicals about 2094 participants from U.S. population reported the existence of PFNA in about 98% of the samples. The serum concentration of PFNA in these participants ranged from 0.1 to 11.5 μg/L (Calafat et al., 2007). However, no report on the effect of PFNA exposure on male reproduction has been described.

PFAAs have been reported to cause the changing of sex steroid hormone biosynthesis. Perfluorodecanoic acid (PFDA) exposure resulted in decreased plasma androgen and 5-dihydrotestosterone concentrations in male rats. Moreover, the weight and epithelial height of the accessory sex organs were also decreased (Bookstaff et al., 1990). Sprague–Dawley male rats exposed to perfluorododecanoic acid (PFDoA) for 14 days experienced a marked decrease in testosterone levels (Shi et al., 2007). Serum testosterone levels of male rats were also remarkably decreased after exposure to PFDoA for 110 days (Shi et al., 2009). Hormone alterations might be related to the induction of Leydig cell adenomas in rats exposed to PFOA (Biegel et al., 2001). In addition, PFAAs have been shown estrogenic activity. Wei et al. (2007) have described that PFOA could induce the increasing expression of vitellogenin in hepatic and testis-ova gonads in male rare minnows. High levels of PFAAs were also associated with fewer normal sperm (Joensen et al., 2009).

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Table 1
Sequences of primers used for real-time RT-PCR amplification.

Target gene	GenBank accession number	Product length (bp)	Primer sequences	Temperature (°C)
Fas	NM_139194	101	Sense: 5′-ATGGCTGTCCTGCCTCTGGT-3′ Antisense: 5′-CGAACGCTCCTCTTCAACTCC-3′	60
Fas-L	NM_012908	410	Sense: 5′-CCACCTCCATCACCACT-3′ Antisense: 5′-TACAACCCAGCCTCATT-3′	55
Bax	NM_017059	143	Sense: 5′-GGTTGCCCTCTTCTACTTT-3′ Antisense: 5′-AGCCACCCTGGTCTTG-3′	55
Bcl-2	NM_016993	217	Sense: 5′-ACTTTGCAGAGATGTCCAGT-3′ Antisense: 5′-CGGTTCAGGTACTCAGCAT-3′	55
β-actin	NM_031144	110	Sense: 5′-CGTTGACATCCGTAAAGAC-3′ Antisense: 5′-TAGGAGCCAGGGCAGTA-3′	55

Apoptosis is related to physiological and pathological events (Saikumar et al., 1999). During different stages of spermatogenesis, the apoptosis of germ cells in testis is responsible for the maintaining of normal quantity and quality of sperm (Print and Loveland, 2000). Excessive apoptosis of germ cells resulted in abnormal spermatogenesis (Tesarik et al., 1998). During the process of apoptosis, a family of cysteine proteases are activated (Yuan and Horvitz, 2004). Two main cascades have been recognized in mammals. The first pathway links caspase-8 to death receptors. The Fas receptor belongs to the family of tumor necrosis factor-related death receptors, and binding of this death receptor to its corresponding ligand FasL triggers caspase-8-mediated cleavage of procaspase-3, and then further induces the subsequent cell apoptosis (Gupta, 2003). In the second pathway, mitochondrial damage induced by extracellular stress causes the releasing of cytochrome *c* from mitochondria into the cytoplasm. In the cytosol, cytochrome *c* will recruit procaspase-9 and then activates "apoptosome". Both active caspase-9 and caspase-8 can cause the activation of downstream effectors, caspase-3 and caspase-7, and induce cell apoptosis (Danial and Korsmeyer, 2004). Spermatogenic cell apoptosis can be induced by many factors, including the changing of sex hormone biosynthesis. Our previous study has indicated that Leydig cells, Sertoli cells, and spermatogenic cells exhibited apoptotic features in the rats exposed to PFDoA for 14 days (Shi et al., 2007). The induction of apoptosis was also observed in the HepG2 cell line exposed to PFOA (Shabalina et al., 1999; Panaretakis et al., 2001). So we hypothesized that PFNA exposure might lead to the induction of apoptosis in male rats.

The aims of this study were to investigate the reproductive toxicity of PFNA, examine whether PFNA exposure would induce apoptosis in male testis, and determine the mechanism of PFNA-induced apoptosis. We examined both the extent of induction of apoptosis and explored the possible involvement of mitochondria-dependent and Fas death receptor-dependent signaling in male rats following 14 days exposure to three dosages of PFNA.

2. Materials and methods

2.1. Animals

Seven-week-old male Sprague–Dawley rats with body weights of 230–240 g were obtained from Weitong Lihua Experimentary Animal Central, Beijing, China. Six animals were assigned to each treatment and control group. Animals were housed one per cage (polycarbonate cages with wire lid and solid bottom) and maintained in a mass air displacement room with a 12-h light–dark cycle at 20–26 °C with a relative humidity of 30–70%. Animals had access to food and water *ad libitum*. All rats were acclimatized for 1 week before experiments begun.

2.2. Chemicals and treatments

PFNA (acid, CAS No. 375-95-1, 97% purity) was purchased from Sigma–Aldrich. All other chemicals and reagents were analytical grade. PFNA was prepared in 0.2% Tween-20 (Beijing Chemical Reagent Co., Beijing, China) and administered orally via gavage to rats in the treatment group for 2 weeks at doses of 1, 3, or 5 mg/kg body weight day. Control rats were treated similarly with the vehicle only. The chosen doses were based on a preliminary trial in which all rats died during a 14-day treatment with 20 mg PFNA/kg day. The PFNA and the control Tween-20 solution were administered in a volume of 6 ml/kg of body weight. The gavage dosing was selected based on the accuracy of delivery of this volume. At the end of the experiment, all rats were euthanized by decapitation. Trunk blood was collected and centrifuged at 2000 × *g* at 4 °C for 15 min. Serum was stored at −80 °C until analysis. Testes were immediately isolated and weighed. One part of the right testis was fixed in Bouin's and another part was fixed in 70% ethanol while the left testis was frozen immediately in liquid nitrogen and stored at −80 °C for RNA isolation and protein extraction.

2.3. Flow cytometric analysis

Testes fixed in 70% ethanol at 4 °C were minced in 1 ml phosphate-buffered saline (PBS, pH 7.4) and filtered through 120 and 300 μm nylon mesh to obtain a monocellular suspension. Supernatants were centrifuged at 1000 × *g* for 5 min, and then the cells were stained with 50 μg/ml propidine iodide (PI) in the presence of 100 μg/ml DNAse-free RNAse (Sigma–Aldrich, St. Louis, MO, USA). After 30 min at 4 °C in the dark, the cells were analyzed using an Epics-XLII flow cytometer (Beckman Coulter). Data were analyzed with the Muticycle AV software (Beckman Coulter, CA, USA).

2.4. Serum hormone levels

Concentrations of serum testosterone and estradiol were measured by ELISA using commercial rat ELISA kits (RapidBio Lab, Calabasas, CA, USA). Since the FSH and LH levels of some rats were lower than the detection limit of the ELISA kits, these two hormones were detected by radioimmunoassay (RIA) using commercial kits from Beijing North Institute of Biological Technology, China. The assay detection limit was 1.0 mIU/ml for FSH and LH. Both ELISA and RIA were performed according to the manufacturers' protocols.

2.5. Reverse transcription-polymerase chain reaction

Total RNA was isolated from the testis using the TRIZOL reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration of total RNA was measured by absorbance at 260 nm using a UV1240 spectrophotometer (Shimadzu, Japan). The purity was estimated by the 260/280 nm absorbance ratio. Reverse transcription was performed using oligo-$(dT)_{15}$ primer and M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. Reverse transcription was achieved by incubation for 60 min at 42 °C; followed by 5 min at 95 °C. Real-time PCR reactions were performed with the Stratagene Mx3000P qPCR system (Stratagene). SYBR Green PCR Master Mix reagent kits (Takala, Dalian, China) were used according to the manufacturer's instructions for quantification of gene expression. Rat-specific primers were designed for the genes of interest: Fas, Fas-L, Bax, and Bcl-2 (Table 1). The housekeeping gene β-actin was used as an internal control. Cycling conditions were as follows: 95 °C for 10 s followed by 40 cycles of 95 °C for 5 s, 55 °C (or 60 °C depending on the primer) for 15 s, and 72 °C for 10 s. After PCR, a melting curve analysis was performed to demonstrate the specificity of the PCR product, which was displayed as a single peak (data not shown). Every sample was analyzed in triplicate. The relative expression ratio (R) of a target gene was expressed for the sample versus the control in comparison to the β-actin gene. R is calculated based on the following equation (Livak and Schmittgen, 2001): $R = 2^{-\Delta\Delta C_t}$, where C_t represents the cycle number at which the fluorescence signal is first significantly different from background and $\Delta\Delta C_t$ is $(C_{t,target} - C_{t,actin})$ treatment $-$ $(C_{t,target} - C_{t,actin})$ control.

2.6. Histology

Testes were dissected, fixed in Bouin's fixative, embedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin as described (Matzuk et al., 1992).

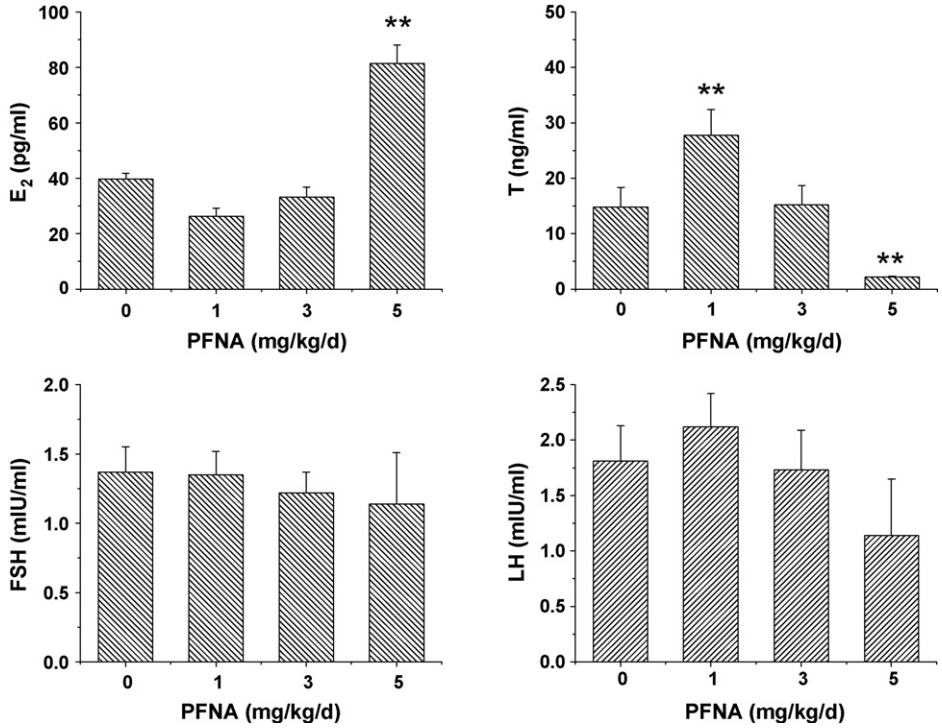

Fig. 1. Serum levels of estradiol (E_2), testosterone (T), FSH and LH from control- and PFNA-exposed male rats. Values represent the average ± SEM from six rats per group. Asterisks indicate a statistically significant difference: $^{**}p < 0.01$.

2.7. TUNEL assay

Deparaffinized and rehydrated sections (5 μm) were treated with 3% hydrogen peroxide for 10 min at room temperature to quench endogenous peroxidase before treatment with 20 μg/ml proteinase K for 10 min at 37 °C. Sections were then washed three times in PBS. Subsequent steps for TUNEL (terminal deoxynucleotide transferase mediated dUTP-biotin nick end labeling) staining were carried out using the *In Situ* Cell Death Detection, POD Kit (Roche, Mannheim, Germany) according to the supplier's instructions, except that the terminal transferase enzyme (TdT) was diluted 1:10 with sterile water. The sections were incubated with the diluted TUNEL reaction mixture for 60 min at 37 °C. The slides were then treated with 3% BSA blocking solution for 25 min at room temperature and incubated with the secondary antifluorescein-POD-conjugate for 30 min. After washing four times in PBST (0.01 M PBS, 0.1% Tween-20), diaminobenzidine (DAB) chromogenic reagent was added to the sections. The sections were then counterstained with hematoxylin, dehydrated in a graded series of ethanol, and cleared in xylene. The positive cells were stained brown.

2.8. Tissue extraction and protein contents

Testis tissue extracts were obtained by homogenizing sample in RIPA Buffer (50 mM Tris–HCl, pH 7.4; 150 mM NaCl; 1% NP-40; 0.1% SDS) supplemented with a protease inhibitor mixture (100 mg/ml) for 3 min. The homogenates were placed on ice for about 10 min and then centrifuged at 12,000 × *g* for 10 min at 4 °C. The supernatant was collected and stored at −20 °C for further experiments. The protein content of the testis extract was determined using the Bradford protein assay (Beijing Applygen Limited Company, Beijing, China). Changes in optical density were monitored at 595 nm.

2.9. Western blotting analysis

Tissue extract samples were prepared as described above. Each sample containing 200 μg protein was loaded onto sodium dodecyl sulfate (SDS)-polyacrylamide gel. Electrophoresed proteins were transferred to polyvinylidine difluoride membranes (PVDF, Millipore, Bedford, MA, USA). The membranes were first blocked with 7% milk in PBST (phosphate-buffered saline added 0.1% Tween-20) for 2 hr at RT and then incubated with primary antibody overnight at 4 °C. Rabbit anti-caspase-8 polyclonal IgG (Santa Cruz Biotechnology, CA, USA) and rabbit anti-caspase-9 polyclonal IgG (Cell Signal Technology, CA, USA) were used as the primary antibodies. The membranes were then washed and incubated with the secondary goat anti-rabbit IgG-HRP antibody (Beijing Zhongshan Goldenbridge Biotechnology, Beijing, China). Protein was visualized by enhanced chemiluminescence (Tiangen Biotechnology, Beijing, China) according to the manufacturer's instructions.

2.10. Statistical analysis

Data were analyzed using SPSS for Windows 13.0 Software (SPSS, Inc., Chicago, IL, USA) and presented as means with standard errors (mean ± SEM). Differences between the control and the treatment groups were determined using a one-way ANOVA followed by the LSD multiple range test. A *p*-value of <0.05 was considered statistically significant.

3. Results

3.1. Hormone levels

The effects of PFNA on hormone levels in male rats were measured (Fig. 1). The serum estradiol levels increased by 104% in rats exposed to 5 mg PFNA/kg day; however, no significant differences were observed in the 1 and 3 mg/kg day groups compared with the control group. Testosterone was increased significantly by 87.5% in the 1 mg PFNA/kg day group ($p < 0.01$). At doses of 5 mg/kg day, testosterone was decreased by 85.4% ($p < 0.01$). In the 3 mg PFNA/kg day group, no significant differences were observed. PFNA exposure did not significantly affect the serum concentrations of FSH and LH in any treated groups compared to the control group.

3.2. Light microscopy

Testicular histological examination was performed for the four experimental groups. The tubule showed organized histoarchitecture and an absence of germ cells detached in the control tubular lumen (Fig. 2A and C). In the 1 and 3 mg PFNA/kg day groups, no morphologic changes of the seminiferous epithelium were noted (figure not shown). In the 5 mg PFNA/kg day group, germ cells sloughed into the lumen of the seminiferous tubules (Fig. 2B). Crescent chromatin condensation and chromatin margination were observed in the germ cells of male testes (Fig. 2D).

The disorganization and atrophy of the seminiferous epithelium, suggested that PFNA exposure caused disruption of spermatogen-

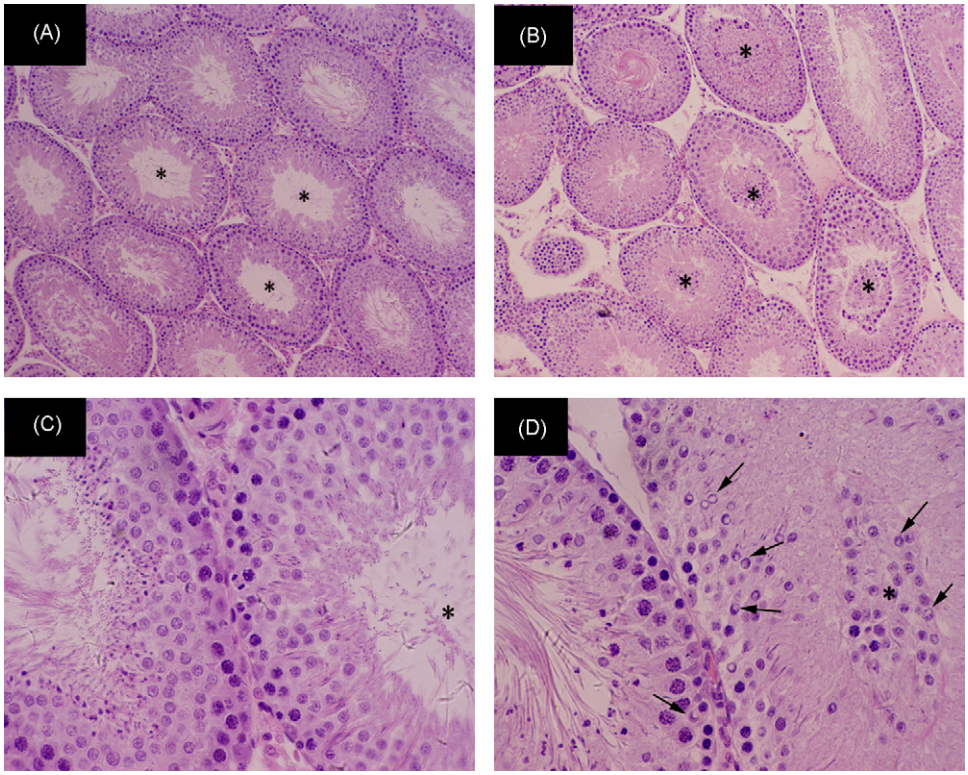

Fig. 2. Testis histopathology in male rats following PFNA exposure. Photomicrographs of seminiferous tubule sections (5 μm) stained with hematoxylin and eosin. Testes were obtained from rats treated with vehicle only (A and C) or 5 mg PFNA/kg day for 14 days (B and D). (A and C) The tubule shows organized histoarchitecture and an absence of germ cells detached in the tubular lumen (asterisk) (A, magnification 100×; C, magnification 400×). (B and D) Germ cells sloughed into the lumen of the seminiferous tubules (asterisk). Crescent chromatin condensation and chromatin margination (arrow) were observed in the germ cells of male testes (B, magnification 100×; D, magnification 400×).

esis. To evaluate the impact of PFNA on germ cell survival, testes sections were examined for DNA fragmentation indicative of cell death using the TUNEL reaction. Seminiferous tubules of control animals had very few TUNEL-positive cells, indicating very low level germ cell attrition in normal testes (Fig. 3A). In the 1 mg PFNA/kg day group, only a few TUNEL-positive cells were observed (Fig. 3B), but the damage was more severe and the TUNEL-positive cells were increased in testes of animals receiving 3 and 5 mg PFNA/kg day (Fig. 3C and D). The TUNEL-positive germ cells were mainly spermatocytes and spermatogonia, and these cells seemed to be initially more susceptible to PFNA toxicity.

3.3. Flow cytometric analysis

The effects of PFNA treatment on spermatogenic cell counts were determined by flow cytometric DNA analysis (Fig. 4). In the 3 and 5 mg PFNA/kg day groups, the percentage of apoptotic cells was increased dramatically ($p < 0.01$) compared with the control group. No significant differences were detected in the 1 mg/kg day group.

3.4. Gene expression

The effects of PFNA exposure on mRNA expression of genes involved in apoptosis in male rats were determined (Fig. 5). Bax and Bcl-2 are members of the Bcl-2 family, which mediates the mitochondria-dependent pathway. Expression levels of Bax were increased by 35.7% in the 5 mg PFNA/kg day group ($p < 0.05$), but no significant differences were observed in the 1 and 3 mg PFNA/kg day groups compared to the control group. In addition, Bcl-2 expression levels were downregulated significantly ($p < 0.05$) in the 3 and 5 mg PFNA/kg day groups. To investigate whether PFNA-induced apoptosis occurred *via* Fas and FasL interaction, which is known to mediate the caspase-8-dependent apoptotic pathway, we examined the changes in Fas and FasL mRNA expression levels in testis following PFNA exposure. Compared to the control group, expression levels of Fas in the 1 and 3 mg PFNA/kg day groups were higher, but no statistical differences were documented. In the 5 mg PFNA/kg day group, Fas expression was markedly upregulated about 90% ($p < 0.05$) compared with the control group. Moreover, expression of FasL was significantly downregulated in the 3 mg PFNA/kg day dosage group ($p < 0.05$); however, no significant differences were observed in the 1 and 5 mg PFNA/kg day groups.

3.5. Western blot analysis

Western blot analysis was employed to compare changes in the active caspase-8 and caspase-9 protein levels in total protein extracts from testes (Fig. 6). The levels of active caspase-8 were significantly increased in the 3 and 5 mg PFNA/kg day groups ($p < 0.05$). At doses of 1 mg PFNA/kg day, the levels of active caspase-8 were increased, but the difference was not statistically significant. PFNA treatment did not affect the levels of active caspase-9 in any of the exposed groups.

4. Discussion

In the 5 mg PFNA/kg day group, testosterone level was decreased significantly by 85.4%. PFDA has been shown to exert an anti-androgenic effect *in vivo* by acting directly on the interstitial Leydig cells of the testis (Boujrad et al., 2000). Inhibition of Leydig cell steroidogenesis may occur via inhibition of the peripheral-type benzodiazepine receptor (PBR) expression and cholesterol transport into the mitochondria, and thus, this inhibition could affect subsequent steroid formation. PFDA, however, did not affect

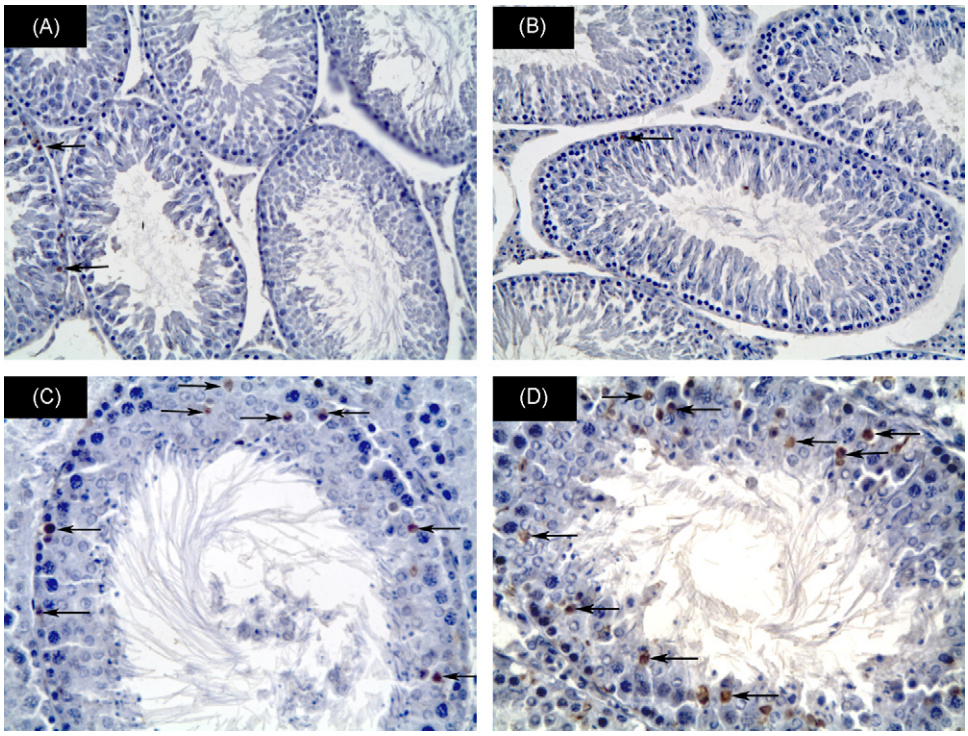

Fig. 3. Representative photomicrographs of TUNEL staining of apoptotic cells in testes exposed to vehicle (A), 1 mg PFNA/kg day (B), 3 mg PFNA/kg day (C) and 5 mg PFNA/kg day (D). Arrows indicate TUNEL-positive cells in the testis (A and B, magnification 200×; C and D, magnification 400×).

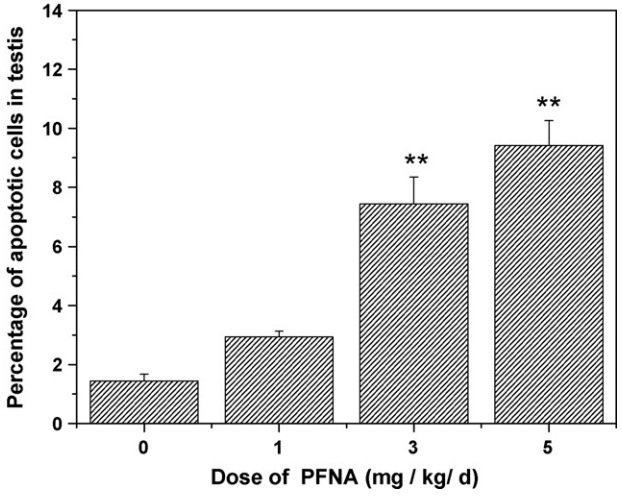

Fig. 4. Percentages of apoptotic cells in the testes from control and PFNA-exposed male rats. The data are presented as the mean ± SEM for six rats per group. $^{**}p < 0.01$.

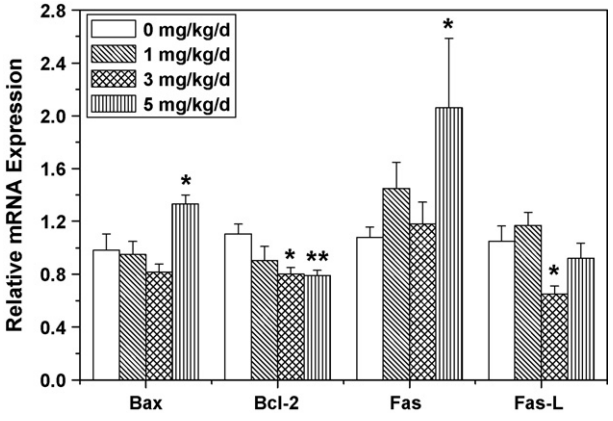

Fig. 5. Real-time quantitative RT-PCR analysis of testis mRNA expression levels of Bax, Bcl-2, Fas, and Fas-L from control and PFNA-exposed male rats. Gene expression levels represent the relative mRNA expression compared with the controls. Values are given as the mean ± SEM for six rats per group. $^{*}p < 0.05$; $^{**}p < 0.01$.

cholesterol side-chain cleavage enzyme ($P450_{SCC}$) enzyme activity or the steroidogenic acute regulatory protein (StAR) (Boujrad et al., 2000). This finding indicates that the steroid biosynthetic pathway was not affected by PFDA exposure. In another study, different mechanisms for the decreased testosterone biosynthesis were found. In male rats exposed to 5 or 10 mg PFDoA/kg day for 14 days, serum testosterone levels were significantly decreased with a concomitant reduction in expression of StAR, $P450_{SCC}$, 3-beta-hydroxysteroid dehydrogenase (3β-HSD), and 17-beta-hydroxysteroid dehydrogenase (17β-HSD), which are responsible for cholesterol transport and steroidogenesis (Shi et al., 2007). These results indicated that decreased testosterone biosynthesis was likely a result of decreased steroidogenesis gene expression. The PFDA-induced decreases in plasma androgen concentrations did not appear to result from a decrease in plasma luteinizing hormone (LH) concentrations, because plasma LH concentrations were not significantly altered by PFDA treatment (Bookstaff et al., 1990). Similar results were observed in our study since PFNA exposure did not significantly affect the serum concentrations of either FSH or LH in any of the treated groups. However, another study observed controversial results that LH levels were significantly decreased in rats exposed to 10 mg PFDoA/kg day (Shi et al., 2007). Whether testosterone production was affected by the pituitary is not entirely clear. Thus, additional work is needed to investigate the mechanisms of PFAA inhibition of steroid biosynthesis.

Examination of the testis structure in rats exposed to 5 mg PFNA/kg day revealed features of apoptosis in spermatogenic cells. These cells exhibited dense nuclei, crescent chromatin condensation, and chromatin margination. Furthermore, in some seminiferous tubules, the germ cells underwent a loss of adhesion

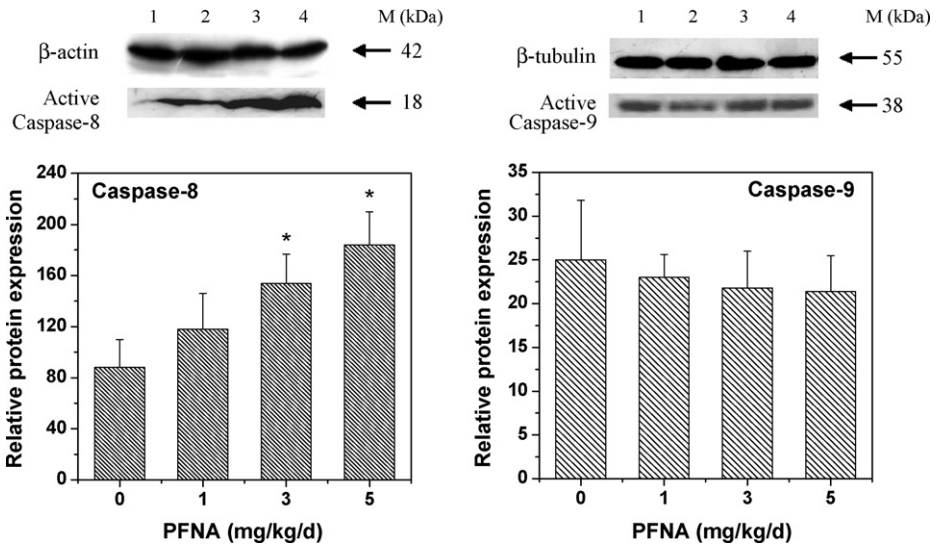

Fig. 6. Western blot analysis of active fragments of caspase-8 and caspase-9 in the testis of male rats following PFNA exposure for 14 days. Lane 1 is no treatment (control), and Lanes 2–4 contain protein from testes exposed to 1, 3, and 5 mg PFNA/kg day, respectively. The intensities of the caspase-8 and caspase-9 bands were normalized with respect to the intensities of β-actin and β-tubulin, respectively. Values are presented as the mean ± SEM for six rats per group. $^{*}p < 0.05$.

to the Sertoli cells and sloughed into the lumen of the seminiferous tubules. A similar phenomenon was also observed in male rats exposed to cimetidine (Sasso-Cerri and Cerri, 2008). Increases of TUNEL-positive cells were dose-dependent. These results were consistent with flow cytometric analysis that indicated that the apoptotic cells were sharply increased in the 3 and 5 mg PFNA/kg day groups, although no morphological changes of the seminiferous epithelium were noted in the 3 mg/kg day group. Together, these results provide evidence for PFNA induction of germ cell apoptosis. Degeneration of spermatogenic epithelium is associated with the decreasing testosterone concentrations (Tapanainen et al., 1993; Leung et al., 1997; Kim et al., 2001). Testosterone, which is considered a trophic factor for testes, played a key role in the induction of apoptosis in the testis (Young and Nelson, 2001).

The balance between estrogen and androgen action is important for spermatogenesis (Sharpe, 1998). The distortion of the normal androgen/estrogen balance results in men infertility (Luboshitzky et al., 2002). Exposure to diethylstilbestrol, an estrogen-like chemical, in male rats induced Fas death receptor-dependent spermatogenic cell death (Nair and Shaha, 2003). In addition, 17β-estradiol exposure induced upregulation of FasL in response to the apoptosis of spermatogenic cells (Mishra and Shaha, 2005). Apoptosis was also induced by increased Fas expression in human coronary artery endothelial cells in response to E_2 (Seli et al., 2006). Testosterone withdrawal from the testis has also been shown to increase germ cell apoptosis (Billig et al., 1995; Shetty et al., 1996). An increase in germ cell apoptosis related to the upregulation of Fas protein that is induced by reduced testosterone levels in rats was also observed (Nandi et al., 1999). The conclusion that Fas may play a role in germ cell apoptosis following testosterone withdrawal is consistent with the observation that Fas-mediated apoptosis occurs in the prostate and epididymis after testosterone withdrawal (Suzuki et al., 1996). In the current study, the serum levels of testosterone were decreased by 85.4%, and the estradiol levels were increased by 104% in rats in the highest dose group compared to the control group. Thus, the balance between testosterone and estradiol in the testes was clearly altered. Perhaps, this imbalance was the main cause of the spermatogenic cell apoptosis that occurred via the upregulation of the death receptor-dependent apoptotic pathway.

The current study suggested that exposure to PFNA triggered germ cell apoptosis. To further investigate the possible mechanisms, markers of both major apoptotic signaling cascades, the death receptor- and the mitochondrial-mediated pathways, were examined. Although PFNA exposure resulted in some alterations to the mitochondrial pathway, downregulation of Bcl-2 mRNA expression and upregulation of Bax mRNA expression in the testes occurred without accompanying changes in the activation of caspase-9. Bcl-2 is an antiapoptotic protein, whereas Bax is a proapoptotic Bcl-2 family protein. The balance between these two factors determines the fate of cells in many apoptosis systems (Danial and Korsmeyer, 2004). An increased ratio between pro- and antiapoptotic Bcl-2 family members leads to cytochrome *c* release from mitochondria, which triggers the final execution of cell death by the caspase cascade (Sjöström et al., 2002). Without caspase-9 activation, downstream signaling events such as cleavage of procaspase-3 to its active form and subsequent apoptosis do not occur (Danial and Korsmeyer, 2004). The Fas receptor belongs to the family of tumor necrosis factor-related death receptors, and binding of this death receptor to its corresponding ligand FasL triggers caspase-8-mediated cleavage of procaspase-3 (Timmer et al., 2002; Wallace, 2005). Marked upregulation of Fas mRNA expression observed in this study in the 5 mg PFNA/kg day group, although no changes in FasL expression was observed in the 5 mg/kg day group and downregulation of expression of FasL was observed in the 3 mg/kg day group. However, caspase-8 was activated dramatically in the 3 and 5 mg PFNA/kg day groups. These data suggest that the mitochondrial pathway was not activated in the testis by PFNA exposure and that, instead, the PFNA-induced testis toxicity in this animal model was mediated by the death receptor pathway. Similarly, testis apoptosis in response to nonylphenol has also been shown to be mediated by the Fas/FasL pathway (Han et al., 2004).

In summary, our results indicated that PFNA exposure leads to cell apoptosis in male rat testis, and these data implicate the death receptor pathway as the principal mediator of testis apoptosis in rats following PFNA exposure. Whether PFNA exposure directly induced the alterations in Fas and FasL expression and whether the imbalance between testosterone and estradiol that induced germ cell apoptosis involved the Fas/FasL pathway is still not clear and requires further investigation.

Conflict of interest

The author declares that there are no conflicts of interest.

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References

Biegel, L.B., Hurtt, M.E., Frame, S.R., O'Connor, J.C., Cook, J.C., 2001. Mechanisms of extrahepatic tumor induction by peroxisome proliferators in male CD rats. Toxicol. Sci. 60, 44–55.

Billig, H., Furuta, I., Rivier, C., Tapanainen, J., Parvinen, M., Hsueh, A.J., 1995. Apoptosis in testis germ cells: developmental changes in gonadotropin dependence and localization to selective tubule stages. Endocrinology 136, 5–12.

Bookstaff, R.C., Moore, R.W., Ingall, G.B., Peterson, R.E., 1990. Androgenic deficiency in male rats treated with perfluorodecanoic acid. Toxicol. Appl. Pharmacol. 104, 322–333.

Boujrad, N., Vidic, B., Gazouli, M., Culty, M., Papadopoulos, V., 2000. The peroxisome proliferator perfluorodecanoic acid inhibits the peripheral-type benzodiazepine receptor (PBR) expression and hormone-stimulated mitochondrial cholesterol transport and steroid formation in Leydig cells. Endocrinology 141, 3137–3148.

Calafat, A.M., Wong, L.Y., Kuklenyik, Z., Reidy, J.A., Needham, L.L., 2007. Polyfluoroalkyl chemicals in the US population: data from the National Health and Nutrition Examination Survey (NHANES) 2003–2004 and comparisons with NHANES 1999–2000. Environ. Health Perspect. 115, 1596–1602.

Danial, N.N., Korsmeyer, S.J., 2004. Cell death: critical control points. Cell 116, 205–219.

Giesy, J.P., Kannan, K., 2001. Global distribution of perfluorooctane sulfonate in wildlife. Environ. Sci. Technol. 35, 1339–1342.

Gupta, S., 2003. Molecular signaling in death receptor and mitochondrial pathways of apoptosis. Int. J. Oncol. 22, 15–20.

Han, X., Tu, Z., Wang, X., Shen, S., Hou, Y., 2004. Nonylphenol induced apoptosis in rat testis through the Fas/FasL pathway. Bull. Environ. Contam. Toxicol. 73, 620–627.

Houde, M., Martin, J.W., Letcher, R.J., Solomon, K.R., Muir, D.C., 2006. Biological monitoring of polyfluoroalkyl substances: a review. Environ. Sci. Technol. 40, 3463–3473.

Joensen, U.N., Bossi, R., Leffers, H., Jensen, A.A., Skakkebaek, N.E., Jørgensen, N., 2009. Do perfluoroalkyl compounds impair human semen quality? Environ. Health Perspect. 117, 923–927.

Kim, J.M., Ghosh, S.R., Weil, A.C., Zirkin, B.R., 2001. Caspase-3 and caspase activated deoxyribonuclease are associated with testicular germ cell apoptosis resulting from reduced intratesticular testosterone. Endocrinology 142, 3809–3816.

Kleszczyński, K., Gardzielewski, P., Mulkiewicz, E., Stepnowski, P., Składanowski, A.C., 2007. Analysis of structure–cytotoxicity in vitro relationship (SAR) for perfluorinated carboxylic acids. Toxicol. In Vitro 21, 1206–1211.

Lau, C., Anitole, K., Hodes, C., Lai, D., Pfahles-Hutchens, A., Seed, J., 2007. Perfluoroalkyl acids: a review of monitoring and toxicological findings. Toxicol. Sci. 99, 366–394.

Leung, A., Wang, C., Swerdloff, R.S., 1997. Significance of apoptosis in the temporal and stage-specific loss of germ cells in the adult rat after gonadotropin deprivation. Biol. Reprod. 57, 1193–1201.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_t}$ method. Methods 25, 402–408.

Luboshitzky, R., Kaplan-Zverling, M., Shen-Orr, Z., Nave, R., Herer, P., 2002. Seminal plasma androgen/oestrogen balance in infertile men. Int. J. Androl. 25, 345–351.

Matzuk, M.M., Finegold, M.J., Su, J.G., Hsueh, A.J., Bradley, A., 1992. Alpha-inhibin is a tumour-suppressor gene with gonadal specificity in mice. Nature 26, 313–319.

Mishra, D.P., Shaha, C., 2005. Estrogen-induced spermatogenic cell apoptosis occurs via the mitochondrial pathway: role of superoxide and nitric oxide. J. Biol. Chem. 280, 6181–6196.

Monroy, R., Morrison, K., Teo, K., Atkinson, S., Kubwabo, C., Stewart, B., Foster, W.G., 2008. Serum levels of perfluoroalkyl compounds in human maternal and umbilical cord blood samples. Environ. Res. 108, 56–62.

Mundt, D.J., Mundt, K.A., Luippold, R.S., Schmidt, M.D., Farr, C.H., 2007. Clinical epidemiological study of employees exposed to surfactant blend containing perfluorononanoic acid. Occup. Environ. Med. 64, 589–594.

Nair, R., Shaha, C., 2003. Diethylstilbestrol induces rat spermatogenic cell apoptosis in vivo through increased expression of spermatogenic cell Fas/FasL system. J. Biol. Chem. 278, 6470–6481.

Nandi, S., Banerjee, P.P., Zirkin, B.R., 1999. Germ cell apoptosis in the testes of Sprague–Dawley rats following testosterone withdrawal by ethane 1,2-dimethanesulfonate administration: relationship to Fas? Biol. Reprod. 61, 70–75.

Panaretakis, T., Shabalina, I.G., Grandér, D., Shoshan, M.C., DePierre, J.W., 2001. Reactive oxygen species and mitochondria mediate the induction of apoptosis in human hepatoma HepG2 cells by the rodent peroxisome proliferator and hepatocarcinogen, perfluorooctanoic acid. Toxicol. Appl. Pharmacol. 173, 56–64.

Parsons, J.R., Saez, M., Dolfing, J., de, V.P., 2008. Biodegradation of perfluorinated compounds. Rev. Environ. Contam. Toxicol. 196, 53–71.

Print, C.G., Loveland, K.L., 2000. Germ cell suicide: new insights into apoptosis during spermatogenesis. Bioessays 22, 423–430.

Renner, R., 2003. Concerns over common perfluorinated surfactant. Environ. Sci. Technol. 37, 201A–202A.

Saikumar, P., Dong, Z., Mikhailov, V., Denton, M., Weinberg, J.M., Venkatachalam, M.A., 1999. Apoptosis: definition, mechanisms, and relevance to disease. Am. J. Med. 107, 489–506.

Sasso-Cerri, E., Cerri, P.S., 2008. Morphological evidences indicate that the interference of cimetidine on the peritubular components is responsible for detachment and apoptosis of Sertoli cells. Reprod. Biol. Endocrinol. 6, 18.

Seli, E., Guzeloglu-Kayisli, O., Cakmak, H., Kayisli, U.A., Selam, B., Arici, A., 2006. Estradiol increases apoptosis in human coronary artery endothelial cells by up-regulating Fas and Fas ligand expression. J. Clin. Endocrinol. Metab. 91, 4995–5001.

Shabalina, I.G., Panaretakis, T., Bergstrand, A., DePierre, J.W., 1999. Effects of the rodent peroxisome proliferators and hepatocarcinogen, perfluorooctanoic acid, on apoptosis in human hepatoma HepG2 cells. Carcinogenesis 20, 2237–2246.

Sharpe, R.M., 1998. The roles of oestrogen in the male. Trends Endocrinol. Metab. 9, 371–377.

Shetty, J., Marathe, G.K., Dighe, R.R., 1996. Specific immunoneutralization of FSH leads to apoptotic cell death of the pachytene spermatocytes and spermatogonial cells in the rat. Endocrinology 137, 2179–2182.

Shi, Z., Ding, L., Zhang, H., Feng, Y., Xu, M., Dai, J., 2009. Chronic exposure to perfluorododecanoic acid disrupts testicular steroidogenesis and the expression of related genes in male rats. Toxicol. Lett. 188, 192–200.

Shi, Z., Zhang, H., Liu, Y., Xu, M., Dai, J., 2007. Alterations in gene expression and testosterone synthesis in the testes of male rats exposed to perfluorododecanoic acid. Toxicol. Sci. 98, 206–215.

Shoeib, M., Harner, T., Wilford, B.H., Jones, K.C., Zhu, J., 2005. Perfluorinated sulfonamides in indoor and outdoor air and indoor dust: occurrence, partitioning, and human exposure. Environ. Sci. Technol. 39, 6599–6606.

Sjöström, J., Blomqvist, C., von Boguslawski, K., Bengtsson, N.O., Mjaaland, I., Malmström, P., Ostenstadt, B., Wist, E., Valvere, V., Takayama, S., Reed, J.C., Saksela, E., 2002. The predictive value of bcl-2, bax, bcl-xL, bag-1, fas, and fasL for chemotherapy response in advanced breast cancer. Clin. Cancer. Res. 8, 811–816.

Suzuki, A., Matsuzawa, A., Iguchi, T., 1996. Down regulation of Bcl-2 is the first step on Fas-mediated apoptosis of male reproductive tract. Oncogene 13, 31–37.

Taniyasu, S., Kannan, K., Horii, Y., Hanari, N., Yamashita, N., 2003. A survey of perfluorooctane sulfonate and related perfluorinated organic compounds in water, fish, birds, and humans from Japan. Environ. Sci. Technol. 37, 2634–2639.

Tapanainen, J.S., Tilly, J.L., Vihko, K.K., Hsueh, A.J., 1993. Hormonal control of apoptotic cell death in the testis: gonadotropins and androgens as testicular cell survival factors. Mol. Endocrinol. 7, 643–650.

Tesarik, J., Greco, E., Cohen-Bacrie, P., Mendoza, C., 1998. Germ cell apoptosis in men with complete and incomplete spermiogenesis failure. Mol. Hum. Reprod. 4, 757–762.

Timmer, T., de Vries, E.G., de, J.S., 2002. Fas receptor-mediated apoptosis: a clinical application? J. Pathol. 196, 125–134.

Wallace, D.C., 2005. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. Annu. Rev. Genet. 39, 359–407.

Wei, Y., Dai, J., Liu, M., Wang, J., Xu, M., Zha, J., Wang, Z., 2007. Estrogen-like properties of perfluorooctanoic acid as revealed by expressing hepatic estrogen-responsive genes in rare minnows (*Gobiocypris rarus*). Environ. Toxicol. Chem. 26, 2440–2447.

Young, C.J., Furdui, V.I., Franklin, J., Koerner, R.M., Muir, D.C., Mabury, S.A., 2007. Perfluorinated acids in Arctic snow: new evidence for atmospheric formation. Environ. Sci. Technol. 41, 3455–3461.

Young, K.A., Nelson, R.J., 2001. Mediation of seasonal testicular regression by apoptosis. Reproduction 122, 677–685.

Yuan, J., Horvitz, H.R., 2004. A first insight into the molecular mechanisms of apoptosis. Cell 23 (116), S53–S56, 1 p following S59.