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Effects of PFNA exposure on expression of junction-associated molecules and secretory function in rat Sertoli cells

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ABSTRACT

Perfluorononanoic acid (PFNA, C9), a synthetic perfluorinated chemical containing nine carbons, has been identified in humans and wildlife worldwide. Sertoli cell plays a key role in spermatogenesis; however, the toxic effects of PFNA on Sertoli cells have not been studied. The aim of this study was to investigate the effects of PFNA exposure on cell junction molecules and factors specifically secreted by Sertoli cells. Primary Sertoli cells from 20- to 21-day-old rats were exposed to increasing PFNA concentrations (0, 1, 10, 25, 50, or 75 μM) for 24 h. No significant changes in the expression of cytoskeleton-associated molecules were noted, although the mRNA levels of vimentin were upregulated dramatically in cells exposed to 50 and 75 μM PFNA. Meanwhile, the mRNA levels of Sertoli cell-specific secretions, such as Mullerian inhibiting substance (MIS), androgen binding protein (ABP), inhibin B, transferrin, and follicle-stimulating hormone receptor (FSH-R) changed significantly in experimental groups. Wilms' tumor gene (WT1), a transcription factor, was upregulated significantly in cells exposed to 1–75 μM PFNA. In additional studies, male rats were exposed to 0, 1, 3, or 5 mg/kg-d PFNA for 14 days. Vacuoles in the cytoplasm of Sertoli cells were observed in the ultrastructure of testis. Furthermore, the changes in the concentrations of MIS and inhibin B in serum and the protein levels of WT1 and transferrin in testis were similar to the mRNA expression levels of those observed after *in vitro* treatment. In conclusion, these findings demonstrated that PFNA treatment led to the damage of specific secretory functions of Sertoli cells and that these effects might be an underlying cause of the male-specific reproductive toxicity of PFNA.

1. Introduction

The perfluorinated alkyl acids (PFAAs), which have been manufactured for over 50 years, are widely used in industry as surfactants, carpet cleaners, fire retardants, polymers, and paper and textile coatings [1,2]. In recent years, human and wildlife monitoring studies have detected PFAAs worldwide. Due to the stability of these chemicals, non-biodegradable nature and their consequent persistence in the environment [3,4] as well as to their global distribution [5], increasing attention has been focused on understanding of the toxicology of this class of compounds.

Perfluorononanoic acid (PFNA), a compound with nine carbons, is widely distributed in the environment and is biomagnified through food webs [5]. A study of polyfluoroalkyl chemicals reported the presence of PFNA in about 98% of 2094 participants from the U.S. population. In addition, the serum concentrations decreased for perfluorooctansulfonate (PFOS) and perfluorooctanoic acid (PFOA) and increased for PFNA during the period between 1999/2000 and 2003/2004 [6]. PFNA has also been detected in human seminal plasma [7]. Furthermore, the concentration of PFNA increased 10-fold from 2004 to 2007 in northern sea otters [8]. Biodegradation of some precursors may be responsible for the increasing concentrations of PFNA in the environment, since recent studies indicated that PFNA and PFOA were generated together from the same precursors during wastewater treatment [9]. Lange also suggested that biodegradation of telomere alcohols yielded mainly PFOA but also PFNA [10]. The metabolism of 8:2 FTOH also produce PFNA [11]. In addition, ammonium perfluorononanoate (APFN) manufactured about 70–200 tons from 1975 to 2004 worldwide [2], is yet another source of PFNA, which rendered this compound more available to wildlife and human beings.

The Sertoli cell, which is the only somatic cell in the seminiferous tubule of the testicle, plays an important role in spermatogenesis [12]. Morphometric analysis has shown that each Sertoli cell in the adult rat testis provides structural and nutritional support to about 30–50 developing germ cells [13,14]. Moreover, some specific secretions synthesized by Sertoli cells regulate or respond to pituitary hormone release and further influence spermatogenesis [15]. In addition, the blood-testis barrier (BTB) created by the tight junctions between adjacent Sertoli cells provides a specialized and relative stable environment for germ cell development [16]. Due to the importance of Sertoli cells in the testis, any agent

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that impairs the viability and/or function of these cells may have profound effects on spermatogenesis [17].

Previous studies have demonstrated that PFAAs are potential endocrine disruptors [18], and the effects on the male reproductive system by PFAAs mainly include the alteration of sex hormone release [19,20], disorganization of seminiferous epithelium, increased germ cell apoptosis [21,22], changes in expression of genes related to steroidogenesis [23,24] and decreased weight and epithelial height of the accessory sex organs [20]. The balance of testosterone and estradiol is critical in male reproductive toxicology [25], but the involvement of the break balance of androgen and estrogen may not be sufficient to elucidate all toxic phenomenon. Sertoli cells are a target for various toxicants and as such, serve as a well-established model for toxicity experiments in male reproductive systems *in vitro* [26]; however, until now, the effects of PFNA exposure on the morphology and function of Sertoli cells have not been reported.

Therefore, the goal of this study was to determine whether PFNA exposure has adverse effects as evidenced by changes in Sertoli cell structure, BTB-associated elements including claudin-11, occludin, vinculin, N-cadherin and β-catenin, and specific secretions including Wilms' tumor gene (WT1), Mullerian inhibiting substance (MIS), androgen binding protein (ABP), inhibin B, transferrin, follicle-stimulating hormone receptor (FSH-R), and Cacna1a. In addition, we determined whether Sertoli cells were involved in the spermatogenic failure induced by PFNA, to provide information regarding the molecular mechanisms of reproductive toxicity which results from PFAAs exposure.

2. Materials and methods

2.1. Antibody and reagents

PFNA (CAS No. 375-95-1, 97% purity) was purchased from Sigma Aldrich (St. Louis, MO). Fetal bovine serum (FBS) and collagenase IV were obtained from GIBCO BRL (Paisley, Scotland). DMEM/F12 cell culture medium was obtained from Hyclone (Waltham, MA). Dimethyl sulfoxide and hyaluronidase were purchased from Sigma. The polyclonal antibodies against N-cadherin (SC-7939), β-catenin (SC-7963), WT1 (SC-192), and trasferrin (SC-22597) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). All other chemicals and reagents were analytical grade.

2.2. Animal treatment and sample collection

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 and maintained in a mass air displacement room with a 12-h light–dark cycle at 20–26 °C with a relative humidity of 50–70%. Animals had access to food and water *ad libitum*. All rats were acclimatized for 1 week before experiments began. PFNA was prepared in 0.2% Tween-20 and administered orally via gavage to rats in the treatment groups for 2 weeks at doses of 1, 3, or 5 mg/kg body weight-day. Control rats were treated similarly but received the vehicle only. 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, frozen in liquid nitrogen, and stored at −80 °C for protein extraction.

2.3. Isolation and culture of rat primary Sertoli cells

Sertoli cells were isolated from the testes of 20–21-day-old male Sprague–Dawley rats. Briefly, testes were removed, decapsulated, and washed twice in cold PBS. Then, the testes were digested with 2 mg/ml collagenase IV at 37 °C for 30 min and centrifugated at 600 rpm for 5 min. The pellets were washed twice in PBS to remove blood and Leydig cells. To separate Sertoli cells and germ cells, the tubular pieces were incubated in a mixture of enzymes (2 mg/ml collagenase IV, 1 mg/ml hyaluronidase, and 0.25% trypsin) for about 10 min at 37 °C. FBS was used to stop the enzyme digestion, and the cells were then centrifugated at 800 rpm for 5 min. The sedimented cells were washed three times in PBS. The Sertoli cells were resuspended in the culture medium (DMEM plus F12 with 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin) and filtered through a 200-mesh filter. After counting, Sertoli cells were plated in 6-well plates (2.5×10^6 cells/well) or 24-well plates (2×10^5 cells/well) and cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The culture medium was changed after 24 h incubation. After an additional 24 h culture, contaminating spermatogenic cells were lysed with a hypotonic solution of 20 mM Tris–HCl (pH 7.4) for 1.5–2 min as described previously [27]. The medium was replaced with serum-free medium for 24 h. Then, the cells were treated with the toxicology agents.

2.4. Cell treatment

Sertoli cells were isolated as described above and cultured for 3 days. Then, the cells were incubated with PFNA at increasing concentrations (1, 10, 25, 50, and 75 μM). Control cells were treated with culture medium including 0.01% DMSO. The stock solutions of PFNA (10 mM) were made in 1% DMSO to increase their solubility. Solvents at the highest concentration (0.01%) in working solutions were not cytotoxic [28]. After 24 h of incubation with these chemicals, cells were prepared for cytotoxicity studies, and the protein and mRNA levels were determined.

2.5. Confocal immunohistochemistry

To determine the purity of the Sertoli cells, the Sertoli cells were evaluated by confocal immunohistochemistry of WT1 [29]. The Sertoli cells that had been treated with a hypotonic solution on the coverslips were cultured for an additional 24 h. After three washes in PBS, the cells were fixed in a freshly prepared mixture of methanol and acetone (1:1) for 10 min, followed by washes in PBS. The cells were then incubated in 3% BSA for 1 h at room temperature. Then the primary antibody against WT-1 (1:100) was added at 4 °C and incubated overnight. Fluorescein isothiocyanate-conjugated anti-rabbit IgG (1:200) was added at 37 °C for 1 h. After three washes in PBS, the coverslips were incubated in propidium iodide (PI) for 10 min to stain the nuclei. Finally, the slides were analyzed with a confocal laser scanning microscope (Carl Zeiss Inc., Thornwood, NY).

2.6. Cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was based on the protocol described by Carmichael [30]. MTT (5 mg/ml) was dissolved in PBS, sterilized by filtration through a 0.22-μM Millipore filter, and stored at −20 °C. After treatment as above, cells were washed twice in PBS, and then 100 μl of 0.5 mg/ml MTT in serum-free medium (final dilution 1:10) was added to each well. The cells were then incubated for 4 h at 37 °C to allow MTT metabolism. The produced formazan was dissolved in 100 μl DMSO and then measured at 570 nm. Results were presented as percentage of the control values obtained with untreated cells.

2.7. Transmission electron microscopy

The testes were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C for 24 h. Then the samples were washed with phosphate buffer (0.1 M, pH 7.4) for 12 h and post-fixed for 20 min in 1% OsO_4 in 0.1 M phosphate buffer (pH 7.4). The samples were then washed with phosphate buffer, dehydrated in a series of increasing (20–100%) ethanol solutions, and embedded in an Epon:alcohol mixture 1:1 for 2 h followed by 100% Epon for an additional 3 h. The samples were then incubated overnight in the oven. Thin sections (70 nm) were placed on copper grids and stained in a 2% uranyl acetate solution in a 1% solution of lead citrate for 30 min. A JEM 100CX transmission electron microscope operated at 50–60 kV was used to visualize the ultrastructure of the testes samples.

2.8. Real-time RT-PCR

Total RNA was isolated from the testis using the TRIZOL reagent (Invitrogen Corp., Carlsbad, CA) 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 of the RNA was estimated by the 260/280 nm absorbance ratio. Reverse transcription was performed using an oligo-$(dT)_{15}$ primer and M-MLV reverse transcriptase (Promega, WI) 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, USA). SYBR Green PCR Master Mix reagent kits (Takala, Dalian, China) were used according to the manufacturer's instructions for the quantification of gene expression. PCR primers (Table 1) were designed using Primer 5.0 software. The housekeeping gene β-actin was used as an internal control. The cycling conditions were as follows: 95 °C for 10 s followed by 40 cycles of 95 °C for 5 s, 55 °C (or 58 °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 PCR product specificity, 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: $R = 2^{-\Delta\Delta C_t}$, 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.

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

Target gene	GenBank accession number	Product length (bp)	Primer sequences	Temperature (°C)
Occludin	NM_031329	194	Sense: 5′-GGACAGAGCCTATGGAACG-3′ Antisense: 5′-CCAAGGAAGCGATGAAGC-3′	58
Vinculin	XM_223781	257	Sense: 5′-CGAGCCAGAGGACAAGG-3′ Antisense: 5′-TCATCACATAACTCAGCAATC-3′	58
Vimentin	NM_031140	453	Sense: 5′-CTCGTCCTCCTACCGC-3′ Antisense: 5′-CGCAACTCCCTCATCTC-3′	58
Laminin-α5	XM_215963	574	Sense: 5′-CGCTGTAAACCTGGGTATTG-3′ Antisense: 5′-TGGCAAGTAGTGGTCCTTCG-3′	58
Claudin-11	NM_053457	82	Sense: 5′-GCTTCGTGGGTTGGAT-3′ Antisense: 5′-CAGGTGGGGATGGTGTA-3′	55
FSH-R	NM_199237	325	Sense: 5′-CACAAGCCAATACAAACTAACT-3′ Antisense: 5′-AAAAGTCCAGCCCAATACC-3′	58
AR	NM_012502	98	Sense: 5′-AGGTTACGCCAAAGGGTT-3′ Antisense: 5′-GAGACAGTGAGGACGGGAT-3′	55
Sertolin	NM_022271	171	Sense: 5′-CTGACAGGGATGTTATAGTGCC-3′ Antisense: 5′-GGTTGAAATGTTCCTTCTTGC-3′	58
Testin	NM_173132	331	Sense: 5′-ACTTGGCTATGTGACTCCTGTG-3′ Antisense: 5′-CTCGCTTCCTGGGATTTG-3′	58
MIS	NM_012902	245	Sense: 5′-GGGAGCAAGCCCTGTTAG-3′ Antisense: 5′-GCGGGAATCAGAGCCAAA-3′	58
WT1	NM_031534	367	Sense: 5′-CCTCCATCCGCAACCAA-3′ Antisense: 5′-TGCCCTTCTGTCCATTTCA-3′	58
Transferrin	NM_001013110	136	Sense: 5′-ACATCCACAGACCCACTACT-3′ Antisense: 5′-TAAGCCAATGGGGATAAT-3′	55
Inhibin B	XM_344130	186	Sense: 5′-ACCTGAAACTGCTCCCCTAT-3′ Antisense: 5′-TCGCCTCGCTCAAACAA-3′	55
ABP	M38759	118	Sense: 5′-TCGGTTTCCGCTCGTTC-3′ Antisense: 5′-CAGGTCCACATCACAGTTCC-3′	55
Cacna1a	NM_012918	163	Sense: 5′-TTGGCAACATCGGCATT-3′ Antisense: 5′-CCGCTGAGGCAGGACA-3′	55
β-Actin	NM_031144	110	Sense: 5′-CGTTGACATCCGTAAAGAC-3′ Antisense: 5′-TAGGAGCCAGGGCAGTA-3′	55 58

2.9. Western blotting analysis

Protein was extracted from testis frozen at −80 °C by homogenizing the sample in RIPA Buffer (50 mM Tris–HCl, pH 7.4; 150 mM NaCl; 1% NP-40; 0.1% SDS) supplemented with protease inhibitor (PMSF, 10 μg/ml) for 5 min and lysates were clarified by centrifugation at 12,000 × *g* for 20 min. Protein was isolated from Sertoli cells following lysis in lysis buffer for 10 min and then also sedimented at 12,000 × *g* for 20 min. The protein content was determined using the Bradford protein assay (Beijing Applygen Limited Company, Beijing, China). Changes in optical density were monitored at 595 nm. Each sample, which contained 100 μg protein, was run on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and then transferred to polyvinylidine difluoride membranes (PVDF, Millipore, Bedford, MA). The membranes were first blocked with 7% milk in PBST for 2 h at RT and then incubated with the primary antibodies at a dilution of 1:1000 each for anti-N-cadherin, anti-β-catenin, anti-WT1, and anti-transferrin at 4 °C overnight. The membranes were then washed and incubated with the secondary 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. Serum MIS and inhibin B analysis

Concentrations of MIS and inhibin B in serum samples were detected by ELISA in accordance with the manufacturer's directions (R&D Systems, Minneapolis, MN). Both absorbance were measured with an ELISA plate reader at wavelength of 490 nm.

2.11. 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 value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Purity of the primary rat Sertoli cell

The purity of the isolated Sertoli cells was evaluated by confocal immunohistochemistry of WT1. WT1 is specifically expressed in the nuclei of Sertoli cells in testis (Fig. 1). This protein, which is expressed in early fetal life and then maintained throughout the life of the organism [31], is considered a marker of Sertoli cells. The purity of the Sertoli cell preparations was 94.9 ± 0.8% as calculated from three different experiments using three different Sertoli cell preparations.

3.2. Analysis of cell viability and mitochondrial integrity

Viability of Sertoli cells exposed to 0, 1, 10, 25, 50, 75, or 100 μM PFNA for 24 h was assessed by the MTT assay. Exposure to PFNA at a concentration of 100 μM resulted in significantly reduced cell viability (80.9 ± 2.5%). No significant differences were observed following exposure to the lesser concentrations of PFNA (Fig. 2).

3.3. Gene expression in primary cultured Sertoli cells

The effects of PFNA exposure on the mRNA expression of genes involved in the creation of the BTB were determined by real-time RT-PCR (Fig. 3A). The mRNA levels of claudin-11 and occludin, which are involved in the constitution of tight junctions (TJ), and vinculin are involved in the formation of adherens junctions (AJ), were not changed significantly in any exposure groups compared to the control group. In contrast, the intermediate filament protein vimentin was upregulated markedly in the 50 and 75 μM PFNA groups compared to the controls ($p < 0.01$). We also examined the components of the seminiferous tubule basement membrane including laminin α5 and found no statistical differences between treatment groups and the control group. Similarly, no significant differences in mRNA expression levels of sertolin and testin were detected in any exposure groups compared to the control.

One of the major functions of Sertoli cells is to secret important molecules, which play key roles in the process of germ cell development. Thus, we determined the expression levels of several Sertoli cell-specific genes in primary cultured Sertoli cells (Fig. 3B). MIS, a

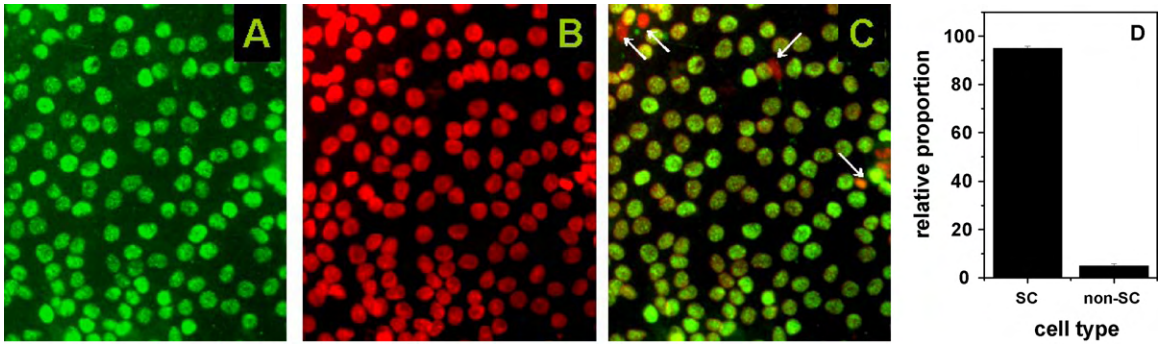

Fig. 1. Purity of cultured primary rat Sertoli cells. Cell purity was evaluated by confocal immunohistochemistry of WT1 in the nuclei of Sertoli cells. Sertoli cells were isolated from 20- to 21-day-old rat testes and cultured at 37 °C in a CO_2 incubator (5% CO_2/95% air). Forty-eight hours later, the cells were hypotonically treated with 20 mM Tris (pH 7.4, 22 °C) to remove the residual germ cells. (A) Positive staining of WT1. (B) Nuclei of the cells were stained with PI. (C) Overlap of images in panels A and B. (D) The relative proportion of Sertoli cells in the total cell population. The arrowhead points to non-Sertoli cells. The relative proportion of Sertoli cells was 94.9 ± 0.80% as calculated from three different experiments using three different Sertoli cell preparations.

testicular hormone, was increased significantly in cells exposed to 10, 25, 50, or 75 μM PFNA ($p < 0.05$). Consistent with the induction of MIS, WT1 was dramatically increased cells exposed to 1–75 μM PFNA ($p < 0.01$). In addition, levels of ABP were also significantly increased in the 50 and 75 μM PFNA groups ($p < 0.05$). On the other hand, mRNA levels of transferrin, which is secreted specifically by Sertoli cells in the testis, were significantly reduced in cells exposed to 25, 50, and 75 μM PFNA ($p < 0.05$) compared with the control group. Similar to transferrin, inhibin B levels were significantly downregulated in the cells exposed to 50 and 75 μM PFNA ($p < 0.01$), and FSH-R mRNA levels were also reduced in the 25 and 50 μM PFNA groups ($p < 0.05$). No significant differences were observed, however, in the mRNA expression levels of AR and Cacna1a between the treatment groups and the control group.

3.4. Ultrastructural analysis of testes

To investigate the effects of PFNA exposure on Sertoli cell structure, we examined the ultrastructural changes in rat seminiferous tubules after *in vivo* treatment of testes with 0, 3, and 5 mg/kg-d PFNA for 14 days. As shown by electron microscopy (Fig. 4), cell junctions in the normal testis were intact (Fig. 4A). In the 3 mg/kg-d PFNA group, vacuoles between Sertoli cells were observed in some sections (Fig. 4B). In the 5 mg/kg-d PFNA group, large vacuoles were apparent between the Sertoli cells and spermatogonia (Fig. 4C). Furthermore, more vacuoles in the cytoplasm of the Sertoli cells and increasing germ cell degeneration were observed (Fig. 4D).

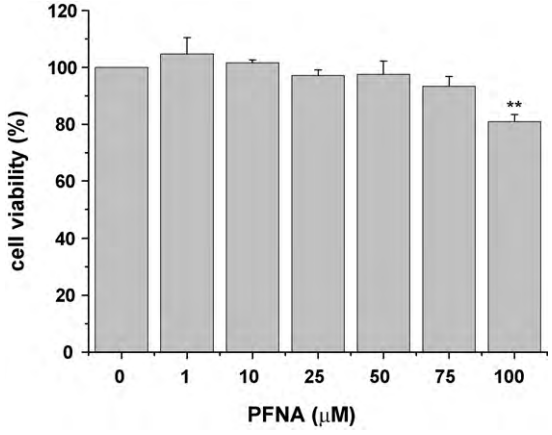

Fig. 2. Effects of mitochondrial integrity and cell viability of primary cultured Sertoli cells exposed to PFNA at the indicated concentrations for 24 h. At the end of the incubation, cells were washed and levels of mitochondrial integrity/cell viability were measured using the MTT assay. Values represent the means ± S.E.M. ($n = 3$). Asterisks indicate a statistically significant difference: $^{**}p < 0.01$.

3.5. Western blot analysis in primary cultured Sertoli cells and rat Sertoli cells

To further investigate the effects of PFNA exposure on the cytoskeleton-associated proteins in Sertoli cells, we measured the protein levels of N-cadherin and β-catenin (Fig. 5), which are involved in the formation of AJs. Following 24 h of exposure to PFNA, no significant effects on the protein levels of N-cadherin and β-catenin were observed in any treatment groups (1, 10, 25, 50, and 75 μM) compared to the control group.

To confirm the *in vitro* results for gene expression levels of WT1 and transferrin, we also investigated the effects of PFNA exposure on the protein expression levels of WT1 and transferrin in testis exposed to 1, 3, or 5 mg/kg-d PFNA for 14 days *in vivo*. Increased WT1 protein levels were observed in the rat cells exposed to 1, 3, or 5 mg PFNA/kg-d ($p < 0.05$, Fig. 6). In contrast, the transferrin protein levels were reduced significantly in all treated groups compared with the control group ($p < 0.05$).

3.6. Concentrations of MIS and inhibin B in serum

Alterations of the MIS and inhibin B levels in the serum of the experimental rats were evaluated by ELISA (Fig. 7). Rats dosed with 5 mg PFNA/kg-d exhibited increased serum WT1 concentrations ($p < 0.05$), although the increased concentrations of WT1 in rats receiving 1 and 3 mg/kg-d PFNA were not statistically significantly different ($p > 0.05$). On the contrary, the concentrations of inhibin B were significantly decreased in the serum of rats exposed to 1, 3, and 5 mg PFNA/kg-d compared with the concentrations in the serum of the control group ($p < 0.01$).

4. Discussion

One of the important functions of the Sertoli cell is to generate the BTB, whose functions were to modulate the passage of different molecules into and out of the adluminal compartment of the seminiferous epithelia and to serve as an immunological barrier to constitute a specialized and relative stable environment for the movement, development, and differentiation of germ cells [32]. The BTB is composed of coexisting AJs and TJs [33,34]. Disruption of these junctions leads to impairment of spermatogenesis [35] and may ultimately lead to sterility in the male [36]. In glycerol-treated Sertoli cells, the disruption of TJ-associated proteins and the impairment of spermatogenesis were observed [37]. Exposure of Sertoli

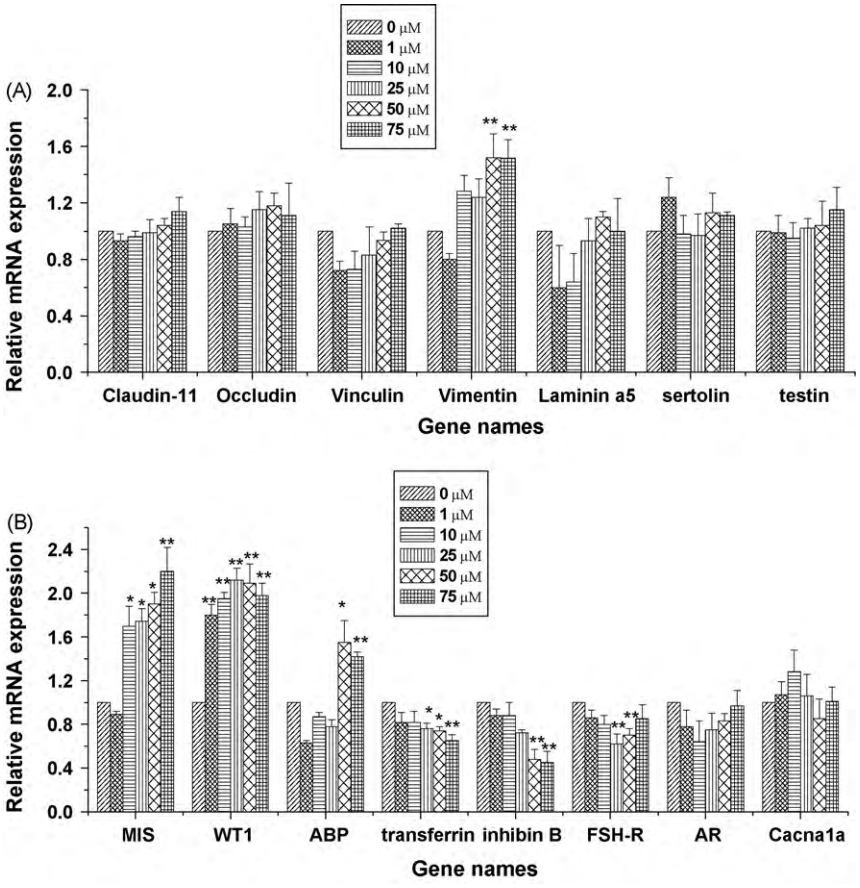

Fig. 3. Real-time quantitative RT-PCR analyses of gene expression in control and PFNA-exposed primary cultured Sertoli cells. (A) The mRNA levels of claudin-11, occludin, vinculin, vimentin, laminin-a5, Sertolin, and testin. (B) The mRNA levels of MIS, WT1, ABP, transferrin, inhibin B, FSH-R, AR, and Cacna1a. Gene expression levels represent the mRNA expression levels relative to control levels. Values represent the means ± S.E.M. ($n = 3$). Asterisks indicate a statistically significant difference: $^{*}p < 0.05$; $^{**}p < 0.01$.

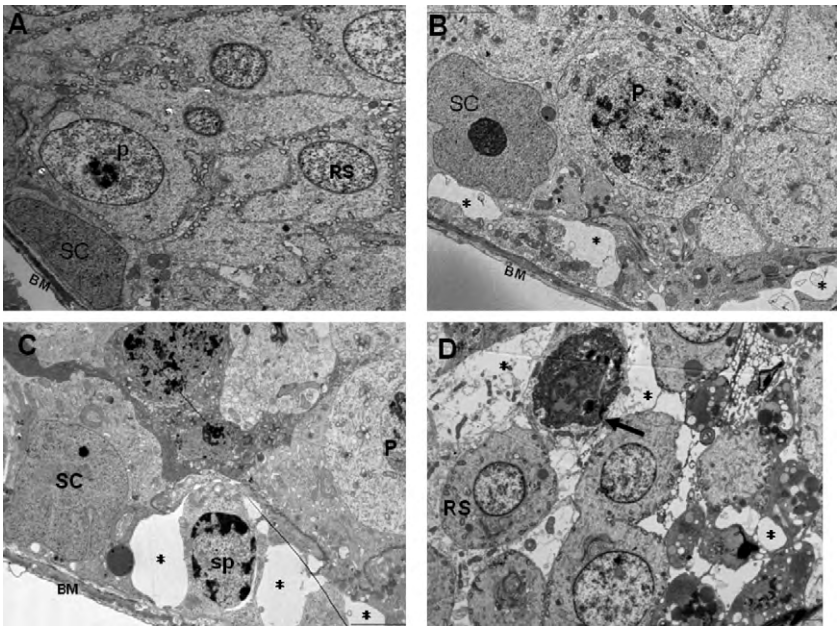

Fig. 4. Electron micrograph of ultrastructural changes in seminiferous epithelium of adult rats exposed to vehicle (A), 3 mg PFNA/kg-d (B), or 5 mg PFNA/kg-d (C and D). Magnification, 1500× (A and D) and 2000× (B and C). (A) Cross-section of the normal seminiferous tubule. No vacuoles were noted. (B) Vacuoles were observed between the Sertoli cells. (C) Vacuoles (*) were observed between Sertoli cells and spermatogonia. (D) More vacuoles (*) were observed in the Sertoli cell cytoplasm, and increasing germ cell degeneration (arrow) was observed. SC, Sertoli cell; P, spermatocyte; RS, round spermatid; SP, spermatogonia; BM, basal membrane.

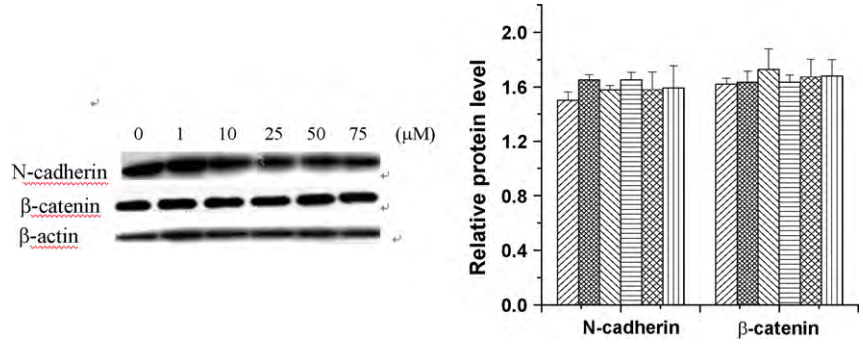

Fig. 5. Western blot analysis of N-cadherin and β-catenin in control and PFNA-exposed Sertoli cells. Lanes from left to right represent protein from primary cultured Sertoli cells exposed to 0, 1, 10, 25, 50, or 75 μM PFNA for 24 h, respectively. The intensities of the N-cadherin and β-catenin bands were normalized with respect to the intensities of the β-actin bands. Values are presented as the means ± S.E.M. ($n = 3$).

cells to $CdCl_2$ perturbed the inter-Sertoli TJ assembly [38]. Furthermore, lonidamine perturbed the AJs between Sertoli cells and germ cells and induced germ cell loss from the epithelium [39].

Due to the important roles of the BTB in spermatogenesis and the relative sensitivity of this barrier to toxic assault, we designed *in vitro* experiments to investigate the possible effects of PFNA exposure on junction-associated molecules in primary cultured Sertoli cells. Our study demonstrated that the mRNA expression levels of TJ-associated components, such as claudin-11 and occludin, as well as of AJ-associated proteins, such as vinculin, N-cadherin, and β-catenin, were not affected as no statistically significant differences between exposure groups and the control group were observed. Similarly, the mRNA levels of sertolin and testin were not changed markedly in any treatment group. These proteins have been reported to be markers for cell–cell interactions in rat testes [40,41]. However, in Hu and Upham's studies, they got the conclusion that PFAA could inhibit gap junction intercellular communication (GJIC) dependent on the chain lengths [42,43]. These

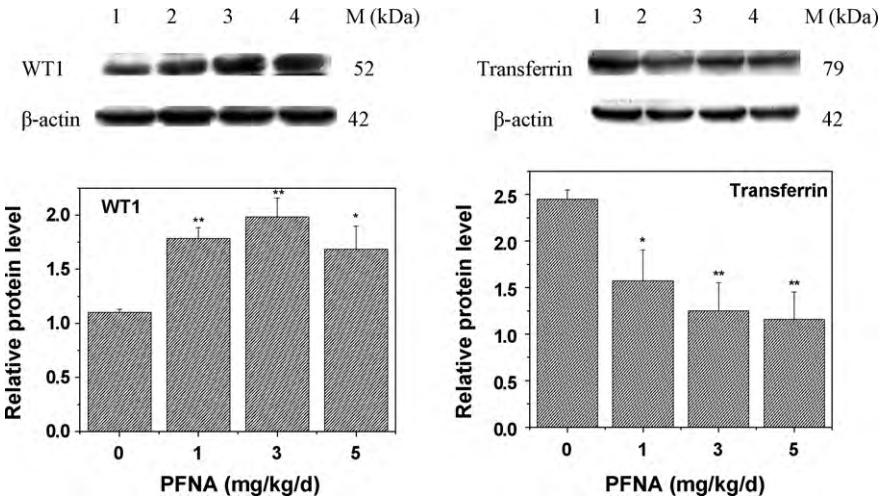

Fig. 6. Western blot analysis of WT1 and transferrin in the testis of male rats following PFNA exposure for 14 days. Lane 1 contains the control sample, and Lanes 2–4 contain protein from testes exposed to 1, 3, or 5 mg PFNA/kg-d for 14 days, respectively. The intensities of the WT1 and transferrin bands were normalized with respect to the intensities of β-actin bands. Values are presented as the means ± S.E.M. for six rats per group. Asterisks indicate a statistically significant difference: $^{*}p < 0.05$; $^{**}p < 0.01$.

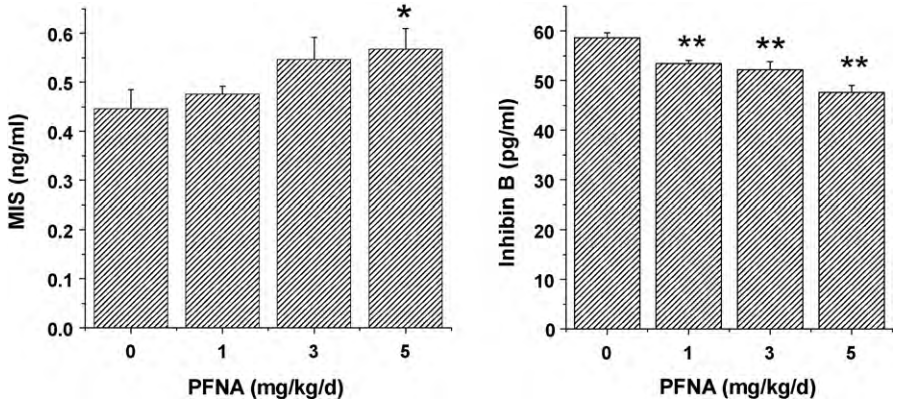

Fig. 7. Serum levels of MIS and inhibin B in control rats and rats exposed to 1, 3, and 5 mg/kg-d for 14 days. Values represent the mean ± S.E.M. from six rats per group. Asterisks indicate a statistically significant difference: $^{*}p < 0.05$; $^{**}p < 0.01$.

researches may indicate that the GJIC are more sensitive than the AJ and TJ at the exposure of PFAAs.

Interestingly, the mRNA levels of the intermediate filament protein, vimentin, were upregulated markedly in the Sertoli cells exposed to 50 and 75 μM PFNA. The role of vimentin in Sertoli cells is still not known, but studies have suggested that this protein may play a key role in mediating cell junction contact between Sertoli cells and Sertoli cells/germ cells [44]. The increased vimentin expression was accompanied with decreased cell adhesion [45,46], and the collapse of vimentin correlated with the loss of structural integrity of the seminiferous epithelium as well as with germ cell apoptosis [47,48]. Thus, our current data may partly explain the disrupted junction and the increasing intercellular space between Sertoli cells and Sertoli cells/germ cells detected through the transmission electron microscopy. In addition, these data may also help explain the observed disorganization of the seminiferous epithelium, sloughing of germ cells, and increased germ cells apoptosis, which were observed in rat testis following exposure to 3 and 5 mg/kg-d PFNA for 14 days [22]. Taken together, these results demonstrated that vimentin may be sensitive to PFNA treatment and may be a targeted substrate of PFNA exposure. The association of the altered vimentin expression with the reproductive toxicity due to PFNA exposure is still not clear; thus, further works need to be done to clarify the mechanism of action of this chemical that results in impaired spermatogenesis.

MIS was secreted by Sertoli cells specifically and the elevation of MIS was a marker of the decreasing of intratesticular androgen concentration [49]. Inhibition of testosterone production by MIS in cultured Leydig cells was observed in a dose-dependent manner [50]. In the current study, gene expression levels of MIS in primary cultured Sertoli cells were increased significantly following exposure to 10–75 μM PFNA. Consistent with this *in vitro* experiment, the serum concentration of MIS was also increased in rats exposed to 5 mg/kg-d PFNA. Considering the evidence that overproduction of MIS induces decreased expression of steroidogenic acute regulatory protein (StAR) in adult transgenic males [51], we speculate that increased expression of MIS may be indirectly involved in the inhibition of testosterone production in rats exposed to 5 mg/kg-d PFNA for 14 days [22] through influence of StAR expression. In fact, the regulation of testosterone biosynthesis were complex, evidence indicated that many nuclear receptors were activated by PFAAs exposure in mouse liver [52], such as androstane receptor (CAR) and pregnane X receptor (PXR). The activation of these nuclear receptors in liver may have relationships with the altered sex hormone by PFAA exposure [53]. However, in the 1 mg/kg-d PFNA group, the serum concentration of MIS was unchanged, the testosterone concentration was increased in serum. Research demonstrated that the production of MIS is not only androgen-dependent and also regulated by the germ cell and FSH [49]. Moreover, the present study of serum testosterone levels did not necessarily reflect real intratesticular androgen levels.

WT1 has a bifunctional role in embryonic mammalian gonadogenesis involving both sexual differentiation [54] and spermatogenesis [55]. Germ cell apoptosis and a loss of the AJ complexes were observed between Sertoli cells and germ cells in *siRNA-WT1* mice [55]. MIS has been proposed to be a transcriptional target of WT1, whereby the interaction of WT1 and steroidogenic factor (SF-1) activates the transcription of the MIS gene, which is a critical mediator of male sexual differentiation [56,57]. Furthermore, the development of Leydig cells requires WT1 function [58]. In this study, we found that gene expression levels of WT1 were significantly upregulated following treatment with 10, 25, 50, or 75 μM PFNA, and the protein levels of WT1 were also increased in the testis from rats exposed to 1, 3, or 5 mg/kg-d PFNA in *in vivo* experiments. These results suggest that increasing expression of WT1 further induced the upregulation of MIS in response to PFNA exposure. Additionally, the increased WT1 expression indicated a dramatic change of Sertoli cell function; however, the mechanism of this change appears to be complex and is not clear at this time.

ABP is a secretory product of Sertoli cells and is considered to be a biological marker of Sertoli cell function [59]. ABP binds, transports, and protects androgen from degradation [60,61] and also functions to control their bioavailability in the testis, which facilitates the development and maturation of spermatogenic cells [62]. Excess ABP upregulates the expression of aromatase in the germ cells and resulted in meiotic arrest from androgen deficiency [63]. In the current study, the expression levels of ABP mRNA increased in cells exposed to 50 and 75 μM PFNA for 24 h, suggesting that a change in Sertoli cell function as a result of PFNA treatment. Furthermore, although the levels of ABP expression in the rats exposed to PFNA was not determined in the present study, we hypothesized that increased ABP would further lead to the reduction of free testosterone by sequestering the hormone in testis and, thus, result in increased apoptosis of germ cells in the testes [22]. Similarly, in transgenic mice, overexpression of ABP in Sertoli cells resulted in androgen deficiency by possibly sequestering the free testosterone [64]. The consequence of increased ABP expression in the process of spermatogenesis is still not clear and remains to be elucidated.

Inhibin B, which is produced mainly by Sertoli cells, is a glycoprotein that modulates FSH secretion via a negative feedback loop [65]. This protein is considered as a biomarker of testicular toxicity in rodent toxicity studies [66]. The concentration of inhibin B correlates with testicular histology [67], sperm concentration [68], and sperm count [69]. Marked decreased inhibin B levels were associated with severe damage of testes [70,71]. In addition, inhibin B levels are lower in infertile individuals and in men with hypogonadotrophic hypogonadism [72]. In the present study, the mRNA levels of inhibin B were decreased dramatically in primary cultured Sertoli cells exposed to 50 and 75 μM PFNA. Similarly, serum concentrations of inhibin B were also decreased significantly in rats exposed to 1, 3, and 5 mg/kg-d PFNA *in vivo*. As a marker of spermatogenesis and testis toxicity, the decreased inhibin B levels may predict impaired secretory function of Sertoli cells and damaged testicular spermatogenesis that resulted from the PFNA exposure. These results also highlight the toxicity of PFNA to the male reproductive system.

Transferrin, which is also secreted by Sertoli cells, is thought to play a critical role in the delivery of iron from the somatic compartment to the germ cells sequestered by the BTB [73]. Iron is necessary for cell proliferation, differentiation, and metabolism. A mutant mouse that is unable to synthesize normal levels of transferrin exhibited a reduced ability to produce functional spermatozoa [74]. Our data showed that transferrin mRNA expression levels were significantly reduced with increasing doses of PFNA both *in vitro* and *in vivo*. Moreover, we observed a dramatic dose-dependent relationship between transferrin mRNA levels and PFNA dose. These data revealed that PFNA exposure impairs the synthesis and secretion of transferrin. This exposure may further affect the development of germ cells in testes and may underlie the reproductive toxicity caused by PFNA.

FSH is important for the maturation of Sertoli cells, production of specific proteins in Sertoli cells, and normal spermatogenesis [75]. FSH-R is expressed only on Sertoli cells in the testis, and this receptor regulates many functions of Sertoli cells through interaction with FSH [76]. In a study by Skinner et al. [77], FSH was shown to influence the mRNA expression levels of ABP and transferrin. In the present assay, the mRNA levels of FSH-R were reduced significantly in cells exposed to 25 and 50 μM PFNA compared to cells in the control group, indicating that the decreased FSH-R may be related to the changing expression levels of ABP and transferrin and the alteration of the secretory function of Sertoli cells.

AR plays an important role in normal spermatogenesis as well as in Sertoli cell secretory processes [78]. No significant changes in AR mRNA levels were observed in PFNA-treated primary cultured Sertoli cells, suggesting that AR is not involved in the altered secretory functions of Sertoli cells following PFNA exposure. The Cacna1a gene, which is responsible for the regulation of Ca^{2+} influx in Sertoli cells and plays a role in Sertoli cell junction dynamics [79], exhibited stable expression levels in all of the PFNA exposure groups *in vitro*. Thus, Cacna1a is not a sensitive or targeted gene of PFNA exposure.

In summary, we demonstrated that PFNA exposure affects multiple functions of Sertoli cells. Higher doses of PFNA influenced vimentin expression, which may be related to the disrupted junctions in seminiferous epithelium. The increasing mRNA levels of MIS, WT1, and ABP following PFNA exposure may indirectly reduce the concentration of free testosterone in testes and further affect the development and maturation of germ cells. In addition, transferrin and FSH-R are involved in multiple biological functions in the process of spermatogenesis, and changes in these proteins may, in part, be responsible for impairment of spermatogenesis. Moreover, reduced expression of inhibin B, a biomarker of testicular toxicity, predicted toxicity of PFNA on the male reproductive system. Thus, these results provide preliminary, but important, information for elucidating the molecular mechanisms of reproductive toxicity that result from PFNA exposure, although the detailed mechanism of action of this chemical remains to be elucidated.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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All experimental manipulations were approved by the Institute of Zoology, Chinese Academy of Sciences Institutional Animal Care Committee and were performed in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals.

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