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Subchronic reproductive effects of 6:2 chlorinated polyfluorinated ether sulfonate (6:2 Cl-PFAES), an alternative to PFOS, on adult male mice

Xiujuan Zhou[a], Jianshe Wang[a,*], Nan Sheng[a], Ruina Cui[a], Yiqun Deng[b], Jiayin Dai[a]

[a] Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, PR China
[b] College of Life Sciences, South China Agricultural University, Guangzhou, 510642, PR China

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ABSTRACT

With a similar structure to perfluorooctane sulfonate (PFOS), 6:2 chlorinated polyfluorinated ether sulfonate (6:2 Cl-PFAES) has been widely used as a mist suppressant in the chromium plating industry in China since the 1970s. After being disregarded for the past 30 years, 6:2 Cl-PFAES has now been detected in environmental matrices and human sera, suggesting potential health concerns. We carried out a subchronic exposure study to investigate the reproductive toxicity of 6:2 Cl-PFAES exposure (0, 0.04, 0.2, and 1.0 mg/kg/d body weight, 56 d) in adult male BALB/c mice. Results showed that relative epididymis and testis weights decreased in the 1.0 mg/kg/d group compared with the control. However, no changes were observed in the serum levels of testosterone, estradiol, follicle-stimulating hormone (FSH), or luteinizing hormone (LH), nor in the histopathological structure of the epididymis and testis and sperm count. In addition, 56 d of consecutive gavage of 1.0 mg/kg/d of 6:2 Cl-PFAES did not affect male mouse fertility. RNA sequencing showed that no genes were significantly altered in the testes after 6:2 Cl-PFAES exposure. Several testicular genes, which are sensitive to PFOS exposure, were also detected using Western blotting, and included steroidogenic proteins, STAR, CYP11A1, CYP17A1, and 3β-HSD and cell junction proteins, occludin, β-catenin, and connexin 43; however, none were changed after 6:2 Cl-PFAES exposure. Except for a decrease in the relative epididymis and testis weights in the 1.0 mg/kg/d group, 6:2 Cl-PFAES exposure for 56 d exerted no significant effect on the serum levels of reproductive hormones or the testicular mRNA profilesin adult male mice, implying a relative weak reproductive injury potential compared with that of PFOS.

1. Introduction

Per- and polyfluoroalkyl substances (PFASs) are a class of man-made compounds with wide applications in industrial and consumer products [1]. The carbon-fluoride bonds that characterize PFASs make them highly stable and environmentally persistent, resulting in their global detection in wildlife, environmental matrices, and diverse human populations [2–5]. The persistence, bioaccumulation, and toxicity of PFASs, especially long-chain substances (e.g., ≥ C6 PFSAs and ≥ C8 PFCAs) observed in laboratory animal studies are of considerable concern [6], and have led to the phase-out of several long-chain PFSAs, e.g., eight-carbon-chain perfluorooctane sulfonate (PFOS) and its precursor, in Europe and North America [7].

The restriction of these chemicals has motivated the commercial replacement by short carbon-chain or semi-fluorinated analogues with shifted formulations, although some analogues have been used long-term [8–10]. With a similar chemical structure to PFOS, 6:2 chlorinated polyfluorinated ether sulfonate (6:2 Cl-PFAES) (CAS # 756426-58-1) has been used as a substitute (Fig. 1). Potassium 6:2 Cl-PFAES ($C_8ClF_{16}O_4SK$) is the major component in the commercial product F-53B (trade name). F-53B has been used as a mist suppressant in the electrolytic process of metal plating in China since the 1970s, even before the introduction of PFOS [9,10]. Despite this, 6:2 Cl-PFAES was relatively overlooked until its detection in influent and effluent wastewater at comparable levels to PFOS [9]. It has similar physicochemical properties and environmental fates as PFOS, including partition coefficient (Kow, Kaw, and Koa), acid-dissociation constant (pKa), bioconcentration factor (BCF), and bioaccumulation factor values (BAF) [11]. Recently, 6:2 Cl-PFAES has been widely detected in China in municipal sewage sludge [12], surface water [13,14], atmospheric [15], and biological samples [16,17], as well as in the liver of marine mammals in Greenland, indicating possible long-range transport [18]. In addition, 6:2 Cl-PFAES has been detected in serum and umbilical cord samples from paired pregnant women in Wuhan, China, with levels only lower than that of PFOS and perfluorooctanoic acid (PFOA) [19]. Furthermore, 6:2 Cl-PFAES can be transported efficiently across

* Corresponding author.
E-mail address: jianshewang@ioz.ac.cn (J. Wang).

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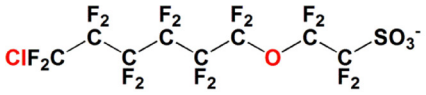
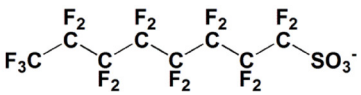


6:2 Cl-PFAES



PFOS

Fig. 1. 6:2 Cl-PFAES and PFOS structures. Differences between the two chemicals are highlighted in red (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

the placenta [20]. Human serum and urine analyses suggest that Cl-PFAES is the most biopersistent PFAS in humans reported to date [21].

Growing evidence suggests that PFASs act as endocrine disruptors to the reproductive system [22]. PFOS can reduce testosterone production and epididymal sperm counts in adult male rodents [22,23]. Several cross-sectional studies have also shown negative associations between blood PFOS and the proportion of morphologically normal sperm cells and testosterone levels in human adults [24–26]. Although many studies on laboratory animals support that exposure to PFOS can lead to toxicological hazards, includingon the reproductive system [27,28], relatively limited toxicological data are available for the alternative 6:2 Cl-PFAES [9,29,30]. Given that 6:2 Cl-PFAES is widely distributed in the environment and human serum at a similar concentration as PFOS, we carried out a subchronic exposure study to exploreitspotential reproductive hazards on male mice.

2. Materials and methods

2.1. Animal treatment

Male BALB/c mice (aged 6–8 weeks) were purchased from the Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Animals were housed in a mass air displacement room, with food and water provided *ad libitum* throughout the study. After one week of adaptation, the animals were randomly grouped (n = 15 per group) and dosed by oral gavage with 0, 0.04, 0.2, or 1.0 mg/kg/d body weight of potassium 6:2 Cl-PFAES (CAS # 73606-19-6, purity > 96%) for 56 d. The chemical was synthesized at the Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences (CAS). The 6:2 Cl-PFAES (50 mg) was dissolved in 5 mL of Tween 20 as stock solution, with the working solution then obtained by serial dilution with Milli-Q water. At the end of the exposure experiment, some animals from the control and 1.0 mg/kg/d group (n = 6 per group) were used for the mating experiment. All other animals were fasted overnight, then weighed and sacrificed by cervical dislocation. Their testes and epididymides were collected and weighed. Blood was collected and then coagulated at room temperature. Serum was collected and stored at −80 °C until analysis. Six mice from each group were randomly used for sperm analysis. The right testes and epididymides from the remaining animals were either fixed in 4% paraformaldehyde or stored at −80 °C after being immediately frozen in liquid nitrogen. All procedures were approved by the Ethics Committee of the Institute of Zoology, CAS.

2.2. Instrument analysis and quality assurance and control

Testes and epididymides from four animals per group were selected randomly to detect 6:2 Cl-PFAES content via an Acquity ultra-performance liquid chromatograph (UPLC) coupled to a Xevo TQ-S triple quadrupole mass spectrometer (Waters, Milford, MA, USA). In brief, testes and epididymides were spiked with $^{13}C_4$-PFOS as an internal standard, and extracted with 4 mL of methyl tert-butyl ether (MTBE). After shaking for 20 min, the mixture was then centrifuged at 4000 rpm for 15 min to collect the supernatant. After concentration by nitrogen gas, the supernatant was dissolved in 200 µL of methanol. Chromatography was performed with an Acquity UPLC BEHC18 column (1.7 µm, 2.1 mm × 100 mm). Electrospray ionization (ESI) was in negative mode, and the transitions monitored for 6:2 Cl-PFAES and $^{13}C_4$-PFOS were *m/z* 427→407 and 429→409, respectively. Quality assurance and control protocols included matrix spikes and continuing calibration verification.

2.3. Histopathological examination, hormone levels, and sperm quality analyses

Testes and epididymides from six mice from each group were fixed in 10% Bouin's fixative, embedded in paraffin wax, sectioned (4–5 µm), and stained with hematoxylin and eosin for pathological observation. Luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone, and estradiol levels in serum were commercially measured by radioimmunoassay. Testicular testosterone concentration was also measured and normalized to total protein concentration in the testis homogenate. For sperm quality analyses, fresh cauda epididymides (n = 6 per group) were immersed in 1.5 mL of T6 solution at 37 °C. Pricks were made with a syringe to release sperm into the medium for 15 min. The sperm suspension was diluted using phosphate buffered saline (PBS), and sperm were counted with a hemocytometer under an optical microscope.

2.4. Litter size, birth weight, sex ratio, and survival rate of pups

After treatment, six male mice from the control and from the 1.0 mg/kg/d 6:2 Cl-PFAES group were randomly selected for mating with normal females. Each male mouse was cohabitated overnight with three females in a cage. Vaginal plugs were checked in the morning, with a plug considered as successful breeding. The mating index was calculated as number of females mating/cohabited females. After delivery, the total number of pups delivered (litter size), gender ratio (female/male) per litter, and birth weight of the pups were recorded, as was the weaning weight on lactation day 21 (LD 21).

2.5. RNA isolation, library, and sequencing

Testis tissues from two individual animals in each group were pooled into one sample, with three samples (six animals) in each group used for RNA sequencing. Total RNA was isolated using TRIzol (Life Technologies-Invitrogen, Carlsbad, CA, USA), and RNA-sequencing was performed commercially by Annoroad Genomics (Beijing, China). In brief, poly(A) mRNA was isolated, fragmented, and transcribed into cDNA. Using T4 quick DNA ligase, the double-stranded cDNA was then end-repaired and ligated with an adaptor. Adaptor ligated fragments were selected by size, and the desired fragment range was then amplified by polymerase chain reaction (PCR). Finally, the cDNA library was sequenced using an Illumina HiSeq 2500 platform.

The raw reads were cleaned by filtering adaptors and low-quality reads (> 5% unknown nucleotides or > 50% of low-quality bases (Q ≤ 19)). Using the TopHat program, the reads were mapped to whole genome reference sequences [31]. Transcript expressions were calculated using FPKM (fragments per kb per million mapped fragments) [32], and differential transcripts between the control and treatment groups were tested using the DEGseq program [33]. The *p*-value was corrected using a false discovery rate (FDR) [34]. Fold change ≥ 2 or ≤ 0.5 and FDR < 0.05 were the thresholds for differential expression. Based on Pearson correlation efficiency, hierarchical cluster analysis of gene expression was also carried out.

2.6. Western blotting

Several genes, which are reported to change in the testes after PFOS exposure [23,35], were selected for Western blotting analysis. Testes were homogenized in RIPA buffer, run on a SDS-PAGE, and then transferred onto a PVDF membrane (GE Healthcare Bio-Sciences AB, Sweden). The membrane was incubated with primary antibodies for cytochrome P450 17α-hydroxylase/20-lyase (CYP17A1) (Abcam, USA, ab125022), cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11A1) (Millipore, USA, ab1794), 3 beta-hydroxysteroid dehydrogenase (3β-HSD) (Abcam, USA, ab65156), steroidogenic acute regulatory protein (STAR) (Abcam, USA, ab58013), occludin (Abcam, USA, ab167161), β-catenin (Santa Cruz, USA, sc-7963), and connexin 43 (Abcam, USA, ab11370), and then with appropriate secondary IgG-HRP antibodies. Protein bands were analyzed with Quantity One software (v4.6.3, Bio-Rad, USA) and β-tubulinwas used as an internal control.

2.7. Statistical analysis

All data were analyzed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA) and were presented as means with standard errors (means ± SE). Differences between groups in body and organ weights, hormone levels, and Western blotting analyses were detected using one-way analysis of variance (ANOVA), followed by Duncan's multiple range tests. Differences in the mating experiment between the control and 1.0 mg/kg/d 6:2 Cl-PFAES group were determined using students *t*-tests. A *p*-value of ≤ 0.05 was considered statistically significant.

3. Results

3.1. Effect of 6:2 Cl-PFAES on body and organ weights

There was a moderate increase in body weight in the 6:2 Cl-PFAES-exposed groups compared with the control (Fig. 2A, B). No change in absolute testis weight was observed in the three 6:2 Cl-PFAES-dosed groups (Fig. 2C); however, the relative testis weights showed a significant decrease of 8% in the 1.0 mg/kg/d 6:2 Cl-PFAES group compared with the control (Fig. 2D). In addition, the absolute and relative epididymis weights also diminished markedly by 14% and 16%, respectively, in the 1.0 mg/kg/d 6:2 Cl-PFAES group (Fig. 2E, F).

3.2. 6:2 Cl-PFAES concentration in sera, testes, and epididymides

After exposure with 0, 0.04, 0.2 or 1 mg/kg/d of 6:2 Cl-PFAES for 56 d, the sera level of 6:2 Cl-PFAES in the animals was 0.07, 5.89, 18.89, and 58.33 μg/mL, respectively (Fig. 3A). The 6:2 Cl-PFAES levels in the testes increased in a dose-dependent manner after treatment. The concentration of 6:2 Cl-PFAES in the testes was 0.095 ng/mL in the control group, and 5.6, 14.4, and 49.93 μg/g in the 0.04, 0.2, and 1.0 mg/kg/d 6:2 Cl-PFAES groups, showing a 5.9×10^4, 1.5×10^5, and 5.3×10^5-fold increase, respectively, compared with the control (Fig. 3B). The 6:2 Cl-PFAES level in the epididymides was 0.065 μg/g in the control, and 1.69, 2.68, and 9.64 μg/g in the 0.04, 0.2, and 1.0 mg/kg/d 6:2 Cl-PFAES groups, showing a 26.1, 41.4, and 149-fold increase, respectively, compared with the control (Fig. 3C).

3.3. Hormone levels in sera and testes

The testosterone levels in sera and testes are shown in Fig. 4A and B. No changes were observed in the three 6:2 Cl-PFAES treatment groups compared with the control group. The levels of other hormones, including estradiol, LH, and FSH, showed no differences among the four groups (Fig. 4C–E).

3.4. Histopathology, sperm quality, and mating experiment

Mice exhibited normal histological features in the testes after exposure to 6:2 Cl-PFAES, with successive stages of spermatogenesis in seminiferous tubules (Fig. 5). We also observed the histopathological structures in cauda and caput epididymides, which exhibited no significant morphometric differences after 6:2 Cl-PFAES treatment (Fig. 6). In addition, there were no obvious malformation differences in sperm images between the control and 6:2 Cl-PFAES treatment groups (Fig. 7A–D). Sperm quality also showed no significant changes, though a decrease tendency was observed in the middle and high dose treatment groups compared with that in the control (Fig. 7E). After treatment, no mating differences were observed between the control and 1.0 mg/kg/d 6:2 Cl-PFAES group (Supplementary Table S1). There were no differences in litter number, gender ratio per litter, or birth weight of the pups, and no weight changes in pups at weaning on LD 21 between the two groups.

3.5. RNA-seq data, DEGs analyses, and Western blotting results

A total of 557,245,354 paired-end reads were obtained from all 12 samples, with a range of 40.0 to 54.3 million from each sample. A total of 483,434,298 clean reads (86.7%) were generated after removal of adaptor, ambiguous, and low-quality sequences. More detailed information is shown in Supplementary Table S2. Using the thresholds of FDR < 0.05 and fold change ≥ 2 or ≤ 0.5, no transcripts were identified as significantly changed after treatment. The hierarchical cluster of expressed transcripts from the 12 samples is shown in Supplementary Fig. 1.Clustering analysis did not delineate treated samples from different treatment groups and control. The gene expression profiles after 6:2 Cl-PFAES exposure were similar to those of control while biological variations segregated treated samples into different clusters. The result indicated that 6:2 Cl-PFAES exposure at the current doses did not induce strong and specific expression pattern changes in the mouse testes. The level of some transcripts in RNA-seq involved in hormone steroidogenesis and cell junctions is listed in Supplementary Table S3. The effect of 6:2 Cl-PFAES on the levels of proteins involved in hormone steroidogenesis, including CYP11A1, CYP17A1, 3β-HSD, and STAR, and proteins involved in cell junctions, including occludin, β-catenin,and connexin 43, were further evaluated in the testes with Western blotting. Results showed that their levels were unchanged in the 6:2 Cl-PFAES treatment groups compared with the control (Fig.8).

4. Discussion

Due to its lower production costs compared to PFOS, 6:2 Cl-PFAES has been widely used in the metal plating industry in China [36–38], with the phase-out of PFOS increasing demand for 6:2 Cl-PFAES as an alternative. Average concentrations of PFOS in the blood of general populations are reported to range from 10 to 80 ng/mL, whereas the level in occupational workers reached 12.83 mg/L [27,39–42]. In China, 6:2 Cl-PFAES has been detected at a similar level as that of PFOS in general population serum [19]. Toxicological studies suggest that PFOS exerts a wide range of adverse effects, including reproductive and developmental impairments [27,43,44]. Epidemiological reports also indicate that elevated PFOS burdens may contribute to decreased testosterone, increased abnormal sperm cells, and infertility in male humans [26,45,46]. Although relatively limited toxicological data are available on 6:2 Cl-PFAES, studies indicate that it has a strong bioaccumulation propensity in fish [16] and moderate toxicity to zebrafish [9]. Median BAF values for 6:2 Cl-PFAES are higher than those of PFOS [16]. Furthermore, acute intracerebroventricular administration of Cl-PFAES is comparable to that of PFOS in disturbing long-term potentiation in the central nervous system of rats [29]. Embryotoxicity and disruption of cardiac development following 6:2 Cl-PFAES

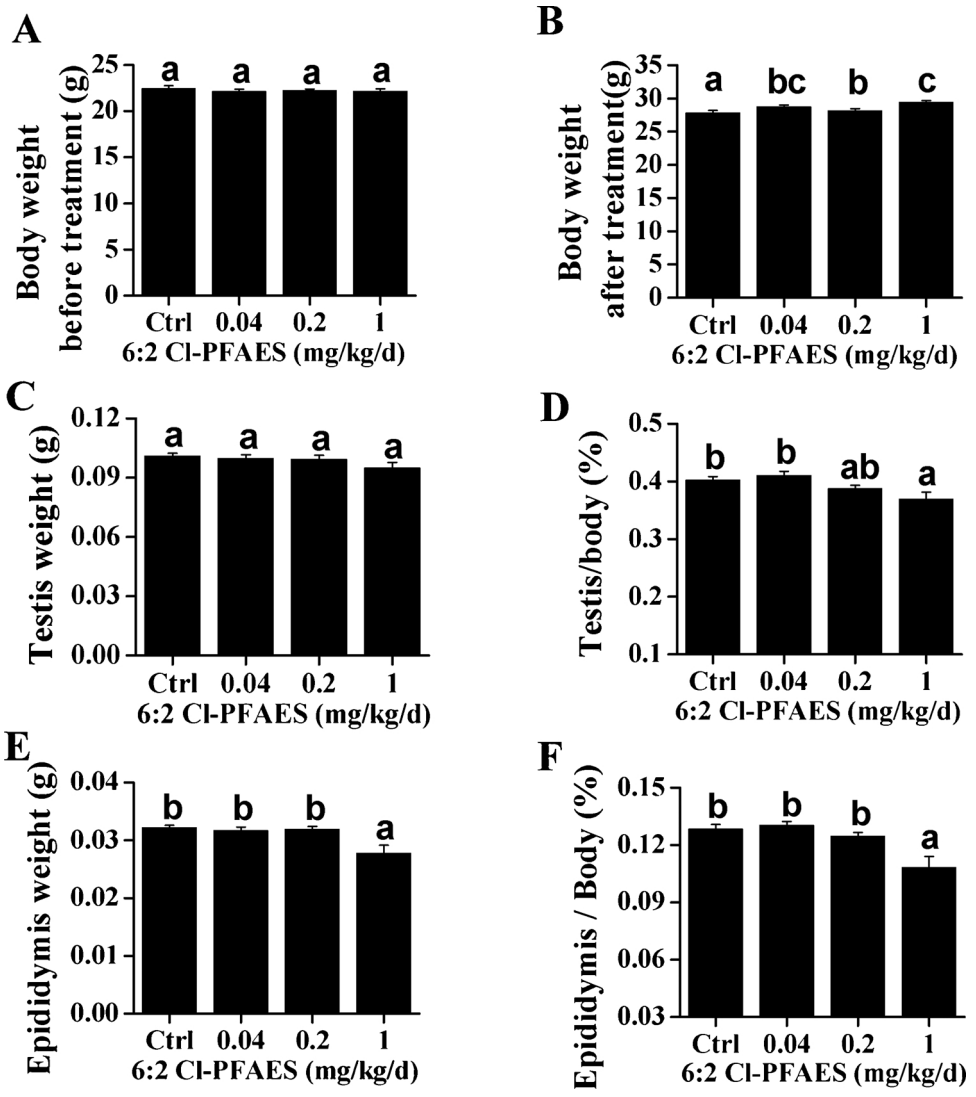


Fig. 2. Body and organ weights. Body weights before and after 6:2 Cl − PFAES treatment are shown in (A) and (B), respectively. Testis weight (C), epididymis weight (D), testis/body (E), and epididymis/body (F) after 6:2 Cl − PFAES treatment. Data are means ± SE (n = 9). Values not sharing a common superscript differ significantly at $p < 0.05$ by ANOVA and Duncan's multiple range tests.

exposure has also been reported in zebrafish embryos [30].

Given the evidence of the deleterious effects of PFOS on the male reproductive system, as well as the structural similarity between PFOS and 6:2 Cl-PFAES, we investigated the effects of 6:2 Cl-PFAES on adult male mice. After exposure, the relative testis weights in the 1.0 mg/kg/d 6:2 Cl-PFAES group exhibited a significant decrease compared with the control, and the absolute and relative epididymis weights also diminishedin the 1.0 mg/kg/d 6:2 Cl-PFAES group. PFOS is reported to induce histopathological alterations in the testes of adult male mice, such as increased vacuolization in Sertoli cells, after exposure to 2.5 mg/kg/d body weight and higher PFOS doses for four weeks [43]. Therefore, we explored the pathological alteration in 6:2 Cl-PFAES-

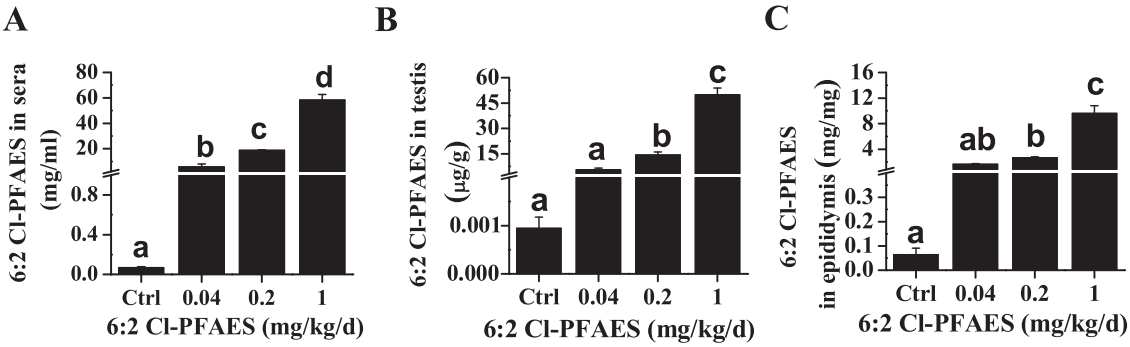


Fig. 3. Content of 6:2 Cl − PFAES in sera, testes and epididymides after exposure. Data are means ± SE (n = 4). Values not sharing a common superscript differ significantly at $p < 0.05$ by ANOVA and Duncan's multiple range tests.

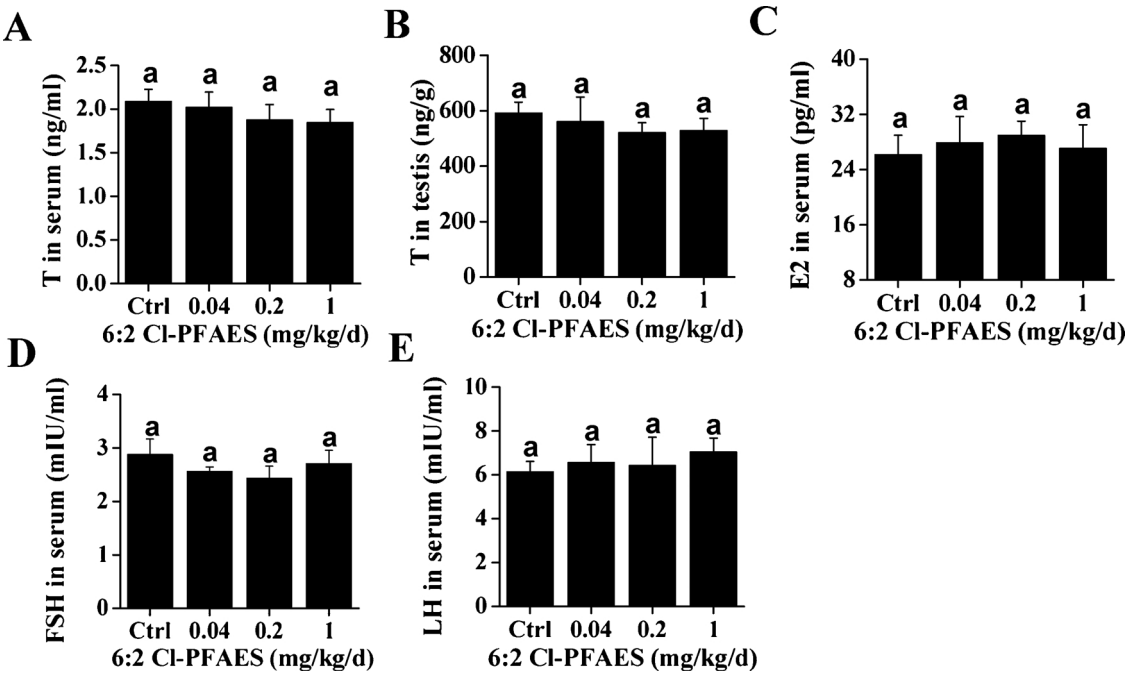


Fig. 4. Hormone levels in sera and testes after 6:2 Cl-PFAES treatment. Levels of testosterone (T) in sera (A) and testes (B), and levels of estrogen (E2) (C), follicle-stimulating hormone (FSH) (D), and luteinizing hormone (LH) (E) in sera after 6:2 Cl-PFAES treatment. Data are means ± SE (n = 6). Values not sharing a common superscript differ significantly at $p < 0.05$ by ANOVA and Duncan's multiple range tests.

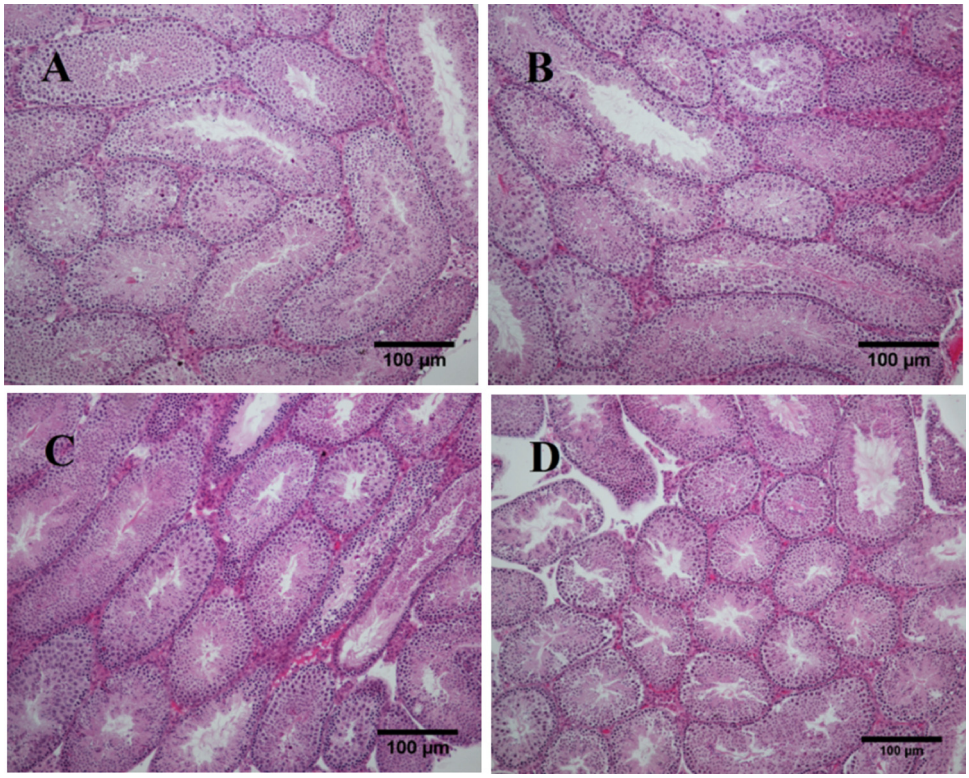


Fig. 5. Histopathological sections of testes with hematoxylin and eosin staining after 0 (A), 0.04 (B), 0.2 (C), and 1.0 mg/kg/d (D) 6:2 Cl-PFAES treatment. n = 6, only representative photos are shown.

exposed male mice. The mice exhibited no histopathological lesions in the testis or epididymis, and no significant malformation or quality differences in sperm compared with that of the control after treatment with various doses of 6:2 Cl-PFAES (maximum of 1.0 mg/kg/d body weight) for eight weeks. Notably, a decrease in epididymal sperm counts has been reported after PFOS exposure, but with relatively higher exposure doses (at least 2.5 mg/kg/d) and shorter exposure times (3–5 weeks) [23,35,43,47].

Previous studies have shown that PFOS exposure can lead to abnormal sex hormone levels in the blood, as well as impaired sperm quality and fertility, in adult male rodents and monkeys [23,35,48]. High doses of PFOS exposure (10 mg/kg/d) can also decrease serum

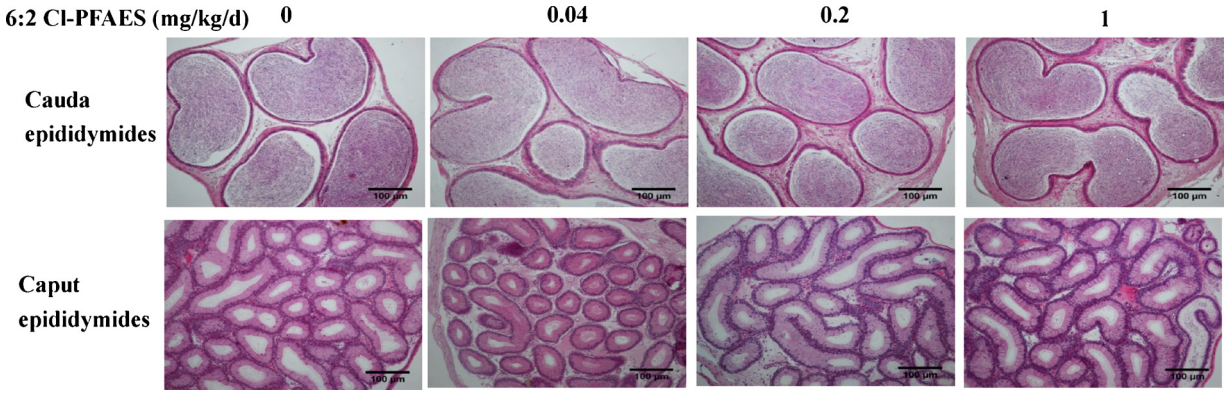


Fig. 6. Histopathological sections of epididymides with hematoxylin and eosin staining after 0 (A), 0.04 (B), 0.2 (C), and 1.0 mg/kg/d (D) 6:2 Cl-PFAES treatment. n = 6, only representative photos are shown.

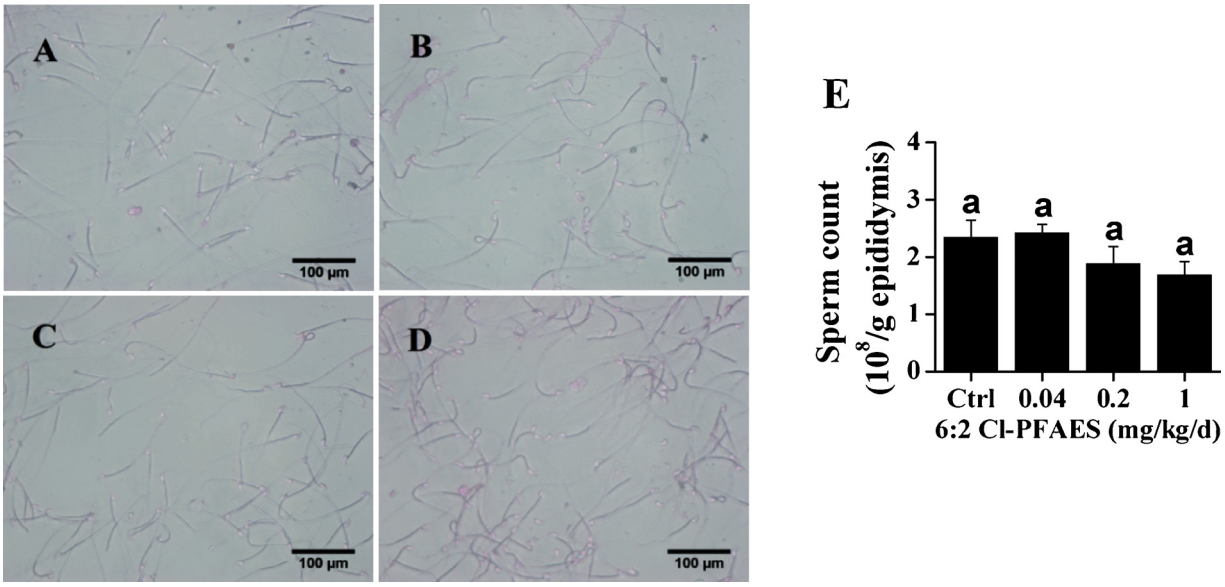


Fig. 7. Histopathological images and counts of sperm. Histopathological images of sperm in 0 (A), 0.04 (B), 0.2 (C), and 1.0 mg/kg/d (D) 6:2 Cl − PFAES treatment groups. Only representative photos are shown. Sperm were counted after exposure (E). Sperm counts are presented as means ± SE (n = 6). Values not sharing a common superscript differ significantly at $p < 0.05$ by ANOVA and Duncan's multiple range tests.

testosterone levels [23,47]. Similar results have been reported with relatively low doses of PFOS. For example, oral exposure to PFOS (0.5, 1.0, 3.0, and 6.0 mg/kg/d) in adult male rats disrupted the reproductive axis at different levels, including in the pituitary gland by stimulating FSH release and inhibiting LH secretion, and in the testes by inhibiting testosterone secretion, which may be related to the pathological lesions in the reproductive axis [22]. In our study, however, hormone levels, including testosterone in sera and testes, and testosterone, estradiol, LH, and FSH in sera were not changed in the three 6:2 Cl-PFAES treatment groups compared to the control.

Spermatogenesis is driven by LH, which stimulates steroidogenesis in Leydig cells, and by FSH, which acts on Sertoli cells. Many proteins involved in steroidogenesis, including StAR, CYP11A1, 3β-HSD, CYP17A1, and 17 beta-hydroxysteroid dehydrogenases (17β-HSD), transport and catalyze a series of reactions in the conversion of cholesterol to testosterone [49]. Studies have shown that the mRNA levels of steroidogenic enzymes are notably reduced after 5 and 10 mg/kg/d PFOS treatment for 21 d, implying that their inhibition may contribute to the effects of PFOS on androgen secretion in the testes [23]. However, no expression changes in these steroidogenic-related genes were observed in the RNA-sequencing and Western blottingresults after 6:2 Cl-PFAES treatment.

Some environmental pollutants can interfere with the function of estrogen receptors (ERs) [50]. *in vitro* studies have indicated that PFOS might interact with ER and change ER-dependent transcriptional activation of spermatogenesis-related genes to induce reproductive impairment [47,51,52]. In adult male rats orally treated with 1.0, 3.0, or 6.0 mg/kg/d of PFOS for 28 d, not only were the mRNA levels of ER modified, but so were several other reproductive hormone receptors in the testes, including the inhibition of FSH and androgen receptors and stimulation of LH receptors [53]. In another study, the expression levels of testicular receptors for FSH and LH were reduced after 21 d of exposure to 5 or 10 mg/kg of PFOS in adult mice, accompanied with a reduction in epididymal sperm counts and testosterone content in the sera of the 10 mg/kg PFOS group [23]. In our RNA-sequencing results, the transcripts of these receptors were not changed in any of the 6:2 Cl-PFAES treatment groups.

Sertoli cells play a key role in maintaining the spermatogenesis microenvironment [54]. The blood-testis barrier (BTB) between Sertoli cells protects germ cells from various endogenous antigens and exogenous hazardous substances [54,55]. Impairment of the BTB is considered a toxic mechanism exerted by reproductive toxicants, and junction proteins in Sertoli cells might be early targets of these substances [56]. PFOS (5–10 μg/mL) is reported to disrupt tight junction permeability without detectable cytotoxicity [57]. Studies have shown that male mice orally administered with PFOS (5 and 10 mg/kg groups)

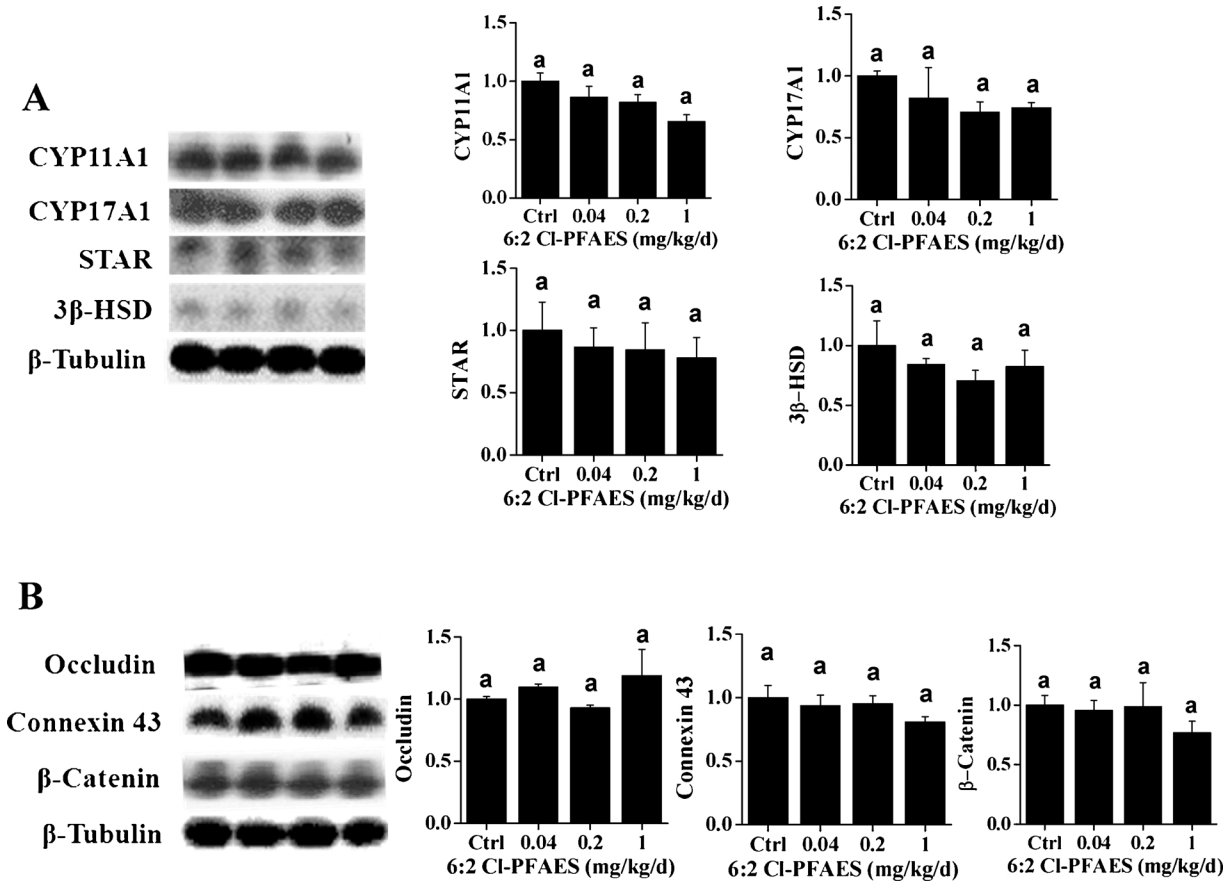


Fig. 8. Western blottingresults of selected genes in the testes after 6:2 Cl−PFAES treatment. (A) Steroidogenic proteins. (B) Cell junction proteins. Data are means ± SE (n = 6). Values not sharing a common superscript differ significantly at $p < 0.05$ by ANOVA and Duncan's multiple range tests.

for four weeks exhibit decreased levels of cell junction proteins, such as occludin and connexin 43 [35]. The notable alteration in junction proteins implies that they might be sensitive to PFOS exposure in the testes. To explore the effect of 6:2 Cl-PFAES, cell junction proteins, including occludin, β-catenin, and connexin 43, were selected. However, Western blotting showed no significant alteration in the testes after exposure to 1.0 mg/kg/d of 6:2 Cl-PFAES. These junction proteins play critical roles in the homeostasis maintenance of the BTB [58,59]. There are several reasons why 6:2 Cl-PFAES treatment did not alter these junction proteins includes: (1) compared with PFOS studies, the exposure dose of 6:2 Cl-PFAES was relatively low, though with a longer exposure duration, which may result in 6:2 Cl-PFAES producing no effect on these proteins; (2) although 6:2 Cl-PFAES and PFOS have a similar structure and physicochemical properties [11], our steroid hormone, testis pathology, and transcriptional profile results implied that 6:2 Cl-PFAES exerted a weaker reproductive effect on male mice, and even under the highest exposure dose (1.0 mg/kg/d) did not decrease the junction protein levels.

Although some *in vivo* studies have been undertaken on the reproductive effect of PFOS, few have explored internal substance levels in the testes after exposure [43]. The concentration of 6:2 Cl-PFAES in the testes and epididymides increased in a dose-dependent manner after 56 d of 6:2 Cl-PFAES treatment. The 6:2 Cl-PFAES concentration in the testes was 14.4 μg/g in the 0.2 mg/kg/d group and 49.93 μg/g in the 1.0 mg/kg/d group after 56 d of treatment, which was half and equal to the testicular PFOS concentration after 28 d of exposure to 25 and 50 mg/kg/d PFOS, respectively [43]. Compared with the concentration used in experimental animals, humans currently show a relatively lower burden of 6:2 Cl-PFAES [19]. In our study, although no obvious pathological or transcriptional changes were observed in the testes after eight weeks of exposure in adult male mice, 6:2 Cl-PFAES decreased the relative weights of testes and epididymides in the high dose group. In addition, it needs to be very cautious to interpret the data and draw conclusions with our negative findings, due to the relative small sample size in a single study. Furthermore, given that 6:2 Cl-PFAES is considered the most biopersistent PFAS in humans reported to date, with an estimated median half-life for renal clearance of 280 years and median total elimination time of 15.3 years [21], high dose 6:2 Cl-PFAES may accumulate over time, and the potential reproductive hazard of long-term exposure cannot be excluded.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2018.07.004.

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