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Original Article

Anti-androgenic and hormetic effects of perfluorooctyl sulfonate on male rats

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Abstract: Objective: To determine the anti-androgenic effect of perfluorooctane sulfonate (PFOS) on male rat pubertal development. Method: According to a male rat pubertal assay, rats were divided into four groups (15 males per dose group), exposed to 0, 20, 60 and 180 ppm PFOS respectively per group, by gavage from postnatal day 23 to 53. Hematology, biochemistry, preputial separation (PPS), sperm quantity and sperm deformities, organs and accessory sex organs weight, pathology were analyzed. Results: Compared with control rats, PFOS caused reduction of body weight gain, hemoglobin, monocyte, basophil percent, basophil number, testosterone level, sperm quantity, weight of accessory sex organs, kidney and pituitary, lipid metabolic abnormalities, sperm deformities, liver and renal functional and pathological injury, and delayed PPS time, at 180 ppm; conversely, PFOS advanced PPS time and improved accessory sex organs weight, in 20 ppm. Conclusions: PFOS caused anti-androgenic and hormetic effects, systemic toxicity, liver, kidney, and immune toxicity, in pubertal male rats.

Keywords: PFOS, anti-androgenic effect, hormesis, pubertal male assay, systemic toxicity, pituitary

Introduction

Perfluorooctyl substances (PFASs) are a class of hydrocarbons that contain thousands of compounds. PFASs belong to persistent organic pollutants (POPs), which are widely distributed in the world, and can be detected in humans, wild animals, and the natural environment, including drinking water, food, air, and soil [1]. Perfluorooctyl sulfonate (PFOS) is a commonly used one of typical PFASs, which is the final decomposition product of some PFASs. Some developed countries, including the United States, have stopped producing and using compounds like PFOS since 2000 [1]. However, some countries continue to produce PFOS which continues the pollution of the environment. Pollution of PFASs in the environment is continuing, for some countries are still producing and using PFOS. Consumers commonly ingest PFASs through food, drinking water and air which is hazy. Therefore, PFASs are generally harmful to all of the population's health [2]. In particular, reproductive toxicity caused by PFASs is mainly manifested as retarding growth and development of sexual organs and accessory sexual organs, delaying puberty, and impairment of sexual function and sperm activity [3, 4]. In an *in vivo* study, PFOA inhibited Leydig cell development of rats [5, 6]. Meanwhile in an *in vitro* study, it was found that PFASs bound androgen receptors (AR), by competing with androgens and were demonstrated to bind AR, showing a dose-response relationship. In addition, some studies have also shown PFASs inhibit androgen-related cell signaling pathways [7]. PFASs show an inhibitory effect on the hypothalamus and pituitary of adult rats [8]. PFOS inhibits the expression of LH receptor (LHR) and AR gene in the hypothalamus of adult rats, and inhibits the expression of AR genes in the pituitary of adult rats [9]. The above *in vitro* studies suggest that PFOS may have anti-androgen effects, but PFOS anti-androgenic effects have not been determined *in vivo*. Steroidogenesis has been shown in H295R cells [10].

In this study, a Male Pubertal Assay in rats was carried out to determine the anti-androgen effect of PFOS *in vivo*.

Materials and methods

Chemicals

Potassium Perfluorooctyl sulfonate (purity 99.8%, purchased from Beijing Jinghua Yaobang Pharmaceutical Technology Co., Ltd) was used as the representative PFOS.

Animals and treatment

All studies were conducted according to the laboratory animal management regulations of Beijing, regarding animal experimental welfare and ethical inspection and approved by the China National Center for Food Safety Risk Assessment Standing Committee on Ethics in Animal Experimentation (2014016). In a Male Pubertal Assay (OCSPP 890.1500) [11], sixty 19-day-old Sprague Dawley (SD) male rats (grade: SPF) weighing 31.5-56.2 g were purchased from the Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China), and fed at Beijing Stomatological Hospital (Beijing, China). Rats were held 3 days for adaptation to the laboratory environment and then randomly divided into 4 groups, respectively, control, low, middle and high-dose, with 15 animals per group. The four groups were treated daily via food with PFOS in the diet at doses of 0, 20, 60, 180 ppm from the post-natal day (PND) 23 to 53. Animals were examined for preputial separation (PPS) daily beginning on PND 30 and weight at day of attainment was recorded. Following euthanization sacrifice on PND 53, organ weights were recorded and microscopic examination of the testes, epididymides, prostate, liver, and kidneys were performed. On the last day of exposure, rats were weighed after 24 hours with fasting treatment, and anesthetized with 2% sodium pentobarbital. Blood samples were collected from the abdominal aorta of all animals, analyzed for hematology and serum chemistry parameters. Hematology parameters from whole blood were measured using an automatic blood cell counter (Japan photoelectric MEK-6813K). For serum chemistry analysis, the serum was separated from blood and examined using an automatic biochemical analyzer (AU680, Beckman Coulter Co., Ltd.). Total serum testosterone level was analyzed using a competitive ELISA assay (Huaxing Co., Ltd., China). After collecting the blood, we took the testes, epididymis, ventral prostate, dorsal prostate-seminal vesicle-urethral gland (DPS), levatorani- bulbocavernosus muscles (LABC), brain, liver, kidney, adrenal glands, spleen, and pituitary gland. After weighing, the organs were placed in 10% neutral formalin buffer.

Sperm quantity

The epididymis was cut into pieces to let the sperm spread freely into Hanks buffer to make sperm suspension, then we dropped the sperm suspension into the blood cell count plate, and the concentration of sperm in each epididymal sperm hanks buffer was counted according to the blood cell count method.

Sperm malformation rate: smear sperm suspension was smeared on glass slide, after air drying, and the slide was dehydrated by 70%, 95% and 100% ethanol successively, and 50% silver nitrate (containing 0.3% formaldehyde) was added. Cover glass was added for a 65°C temperature bath for 3~5 h. After silver nitrate staining was complete, the cover glasses were removed, slides were rinsed with distilled water and stained with 1% Giemsa solution for 10-20 seconds. One thousand sperm were observed in each rat, and amorphous, big-head, banana-shaped, hookness, double head and double tail, and the percentage of total sperm deformities were recorded respectively.

Statistical analysis

The data are expressed as the mean ± SD. Homogeneity of variance was examined by the Levene's test. If the Levene's test indicated no significant deviations from homogeneity in the variance, the data were analyzed by one-way analysis of variance followed by the least significant difference method with multiple comparisons to determine whether the difference was significant. In the case of significant deviations from variance with the Levene's test, the significant difference between groups was identified by the Dunnett's *t*-test. The data were analyzed using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA), and $P<0.05$ was considered statistically significant.

Table 1. Effect of PFOS on food intake (mean ± SD, n=15)

Project		0 ppm	20 ppm	60 ppm	180 ppm
Food intake (g)	PND30	277.9±37.5	262.9±53.6	305.5±32.8	261.6±16.6
	PND37	327.5±26.6	331.2±26.6	354.8±27.5	288.9±16.6*
	PND44	365.4±46.6	375.1±47.2	384.7±14.4	271.4±80.2*
	PND51	523.0±58.7	539.5±64.6	536.5±22.7	352.4±74.3*
	PND53	176.0±18.0	181.4±19.8	184.3±16.7	120.1±40.8*

Note. *Compared with the control group, $P<0.05$.

Table 2. Effect of PFOS on Preputial Separation (mean ± SD, n=15)

Group	PPS (PND)	Weight of PPS (g)
0 ppm	43.15±3.37	195.22±32.95
20 ppm	39.13±2.11*	193.12±31.23
60 ppm	42.53±2.78	190.23±34.12
180 ppm	47.13±2.48*	171.46±35.26*

Note. *Compared with the control group, P<0.05.

Results

Observations

During the experiment, the animals presented significant clinical adverse clinical signs, including emaciation, ruffled fur, and reduced activity, at the 180 ppm group. **Table 1** shows that from PND 37 to 53, the body weight gain and food intake of rats were significantly decreased in the 180 ppm group compared to that in the the control ($P<0.05$).

Preputial Separation (PPS)

Table 2 shows that, compared with the controls, the PPS time advanced in the 20 ppm group ($P<0.05$), and was delayed in the 180 ppm group ($P<0.05$). The terminal body weight of the 180 ppm was lower than in controls ($P<0.05$).

Organ weights

As shown in **Table 3**, compared with the control group, the liver absolute and relative weight increased ($P<0.05$), with a dose-response relationship; the absolute and relative weight of the pituitary and kidney decreased ($P<0.05$); the accessory sex organs, including ventral prostate, DPS, LABC absolute and relative weight decreased in the 180 ppm group ($P<0.05$); the absolute and relative weight of ventral prostate, DPS, LABC increased slightly at 20 ppm, compared with controls without significant difference; however, there was significant difference compared with the 180 ppm group ($P<0.05$).

Sperm quantity and abnormality

As shown in **Table 4**, compared with the control group, sperm quantity decreased ($P<0.05$), with a dose-response relationship; sperm abnormality including amorphous, big-head, banana-shaped, hookness, 2 heads, 2 tails and abnormality percentage increased ($P<0.05$), with a dose-response relationship.

Correlation between pituitary and accessory sex organs

As shown in **Table 5**, pituitary absolute weight was correlated with ventral prostate, DPS and LABC.

Clinical hematology

Compared with the control group, hematocrit (HCT), basophil percent (BASOP), basophil number (BASO-N), reticulocyte number (RETN) and reticulocyte percent (RETP) levels significantly decreased in the 60 and 180 ppm groups ($P<0.05$), mononuclear number (MONON) decreased in the treated groups, hemoglobin (HGB), Mean corpuscular volume (MCV) and mean corpuscular- hemoglobin concentration (MCH) of the high dosage group decreased ($P<0.05$); all parameters above decreased with a dose-response relationship (**Table 6**).

Clinical biochemistry and testosterone

Compared with the controls, alanine aminotransferase (ALT) of the 60 and 180 ppm groups was changed, while aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (TBIL), total protein (TP) and blood urea nitrogen (BUN) and high-density lipopro-

Table 3. Effect of PFOS on Organ Weights at Necropsy (mean ± SD, n=15)

Organ	Project	Control	20 ppm	60 ppm	180 ppm
Liver	A (g)	9.11±0.82	10.23±0.94*	11.72±1.2*	14.85±0.90*
	R (%)	21.25±3.17	24.28±4.29	28.06±4.74*	34.04±4.45*
Kidney	A (g)	2.27±0.19	2.37±0.18	2.16±0.20	1.89±0.22*
	R (%)	5.30±0.75	5.59±0.72	5.20±0.97	4.30±0.50*
Pituitary	A (mg)	8.4±1.3	8.4±1.0	7.7±1.3	6.1±0.8*
	R (10^{-6})	1.82±0.60	1.84±0.58	1.85±0.42	1.38±0.17*
Adrenal	U (g)	42.4±7.6	43.2±7.4	42.3±5.9	42.5±5.4
	R (10^{-5})	9.27±3.45	10.22±2.05	10.05±1.55	9.71±1.56
DPS	A (mg)	643.3±170.6	685.7±92.2	628.5±185.7	359.7±124.7*
	R (10^{-3})	1.47±0.29	1.61±0.22	1.47±0.32	0.82±0.25*
Ventral prostate	A (mg)	237.0±42.2	254.9±56.3	241.2±56.9	136.3±39.1*
	R (10^{-4})	5.55±1.27	5.91±0.89	5.66±1.03	3.08±0.77*
LABC	A (mg)	417.8±088.2	436.2±086.1	361.9±80.9	248.5±74.1*
	R (10^{-4})	9.72±2.21	9.62±3.17	8.56±1.68	5.64±1.48*
Epididymis	A (mg)	230.5±31.8	249.3±35.4	222.8±38.1	206.7±45.6
	R (10^{-3})	1.07±0.19	1.18±0.20	1.05±0.14	0.94±0.17
Testis	A (g)	2.34±0.26	2.49±0.23	2.42±0.29	2.46±0.22
	R (%)	5.43±0.69	5.87±0.76	5.78±0.97	5.63±0.63

Note. *Compared with the control group, $P<0.05$. A = Absolute weight. R = Relative weight to body weight on PND 23.

Table 4. Effects on sperm quantity and sperm abnormality (mean ± SD, n=10)

Parameters	Control	20 ppm	60 ppm	180 ppm
sperm count (10^6/ml)	111.4±18.9	135.2±20.7	72.2±11.9*	41.3±8.1*
Amorphous	15.3±1.3	18.3±2.1*	46.8±4.3*	60.5±4.1*
Big-head	2.4±0.5	11.1±1.4*	31.0±3.2*	46.8±3.5*
Banana-shaped	4.6±0.7	5.7±1.3	7.8±1.5*	11.2±2.7*
Hookness	4.1±0.7	5.6±1.0*	6.5±1.9*	7.7±1.7*
2 heads	1.7±0.5	2.3±0.5*	3.4±1.2*	4.2±1.3*
2 tails	0.4±0.5	1.3±0.5*	4.6±0.5*	5.2±1.8*
Abnormality percentage	28.5±2.3	44.3±2.6*	100.1±6.5*	135.6±4.5*

Note. *Compared with the control group, $P<0.05$.

Table 5. Correlation between pituitary, androgen dependent organs

Organ	ventral prostate	DPS	LABC
Pearson correlation	0.616	0.582	0.572
P value	0.000	0.000	0.000

tein cholesterol (HDL-C) of the 180 ppm group increased ($P<0.05$). Low-density lipoprotein cholesterol (LDL-C) and triglyceride (TG) albumin (ALB) of the 180 ppm group decreased ($P<0.05$) and the cholesterol (CHO) of the 60 and 180 ppm groups decreased ($P<0.05$), with a dose-response relationship. The direct bilirubin (DBIL) decreased ($P<0.05$) in the 20 ppm and 60 ppm groups but DBIL significantly increased ($P<0.05$) at a dose of 180 ppm (**Table 7**).

Histopathology

As shown in **Table 8**; **Figures 1** and **2**, hepatocyte edema and liver inflammation occurred in the liver; the tubules dilated and glomeruli atrophy occurred in the kidneys; revealing that PFOS caused liver and kidney toxicity with dose-response relationship.

Discussion

The present study discovered the antiandrogen and hormetic effects of PFOS for the first time *in vivo*, and in addition, confirmed the reproduc-

Table 6. Effect of PFOS on Hematology (mean ± SD, n=15)

Parameters	0 ppm	20 ppm	60 ppm	180 ppm
HGB (g/L)	136.14±7.77	133.43±8.92	129.63±11.98	124.36±6.09*
HCT (%)	42.01±2.19	41.74±2.17	40.00±3.51*	37.81±1.88*
MCV (fl)	63.99±2.01	63.81±3.73	63.64±3.03	60.45±2.68*
MCH (pg)	20.71±0.47	20.36±0.71	20.61±0.67	19.89±0.70*
MONON (10^9/L)	0.31±0.11	0.21±0.07*	0.19±0.12*	0.18±0.10*
BASON (10^7/L)	1.1±0.8	0.8±0.7	0.4±0.6*	0.4±0.7*
BASOP (%)	0.16±0.12	0.12±0.11	0.05±0.08*	0.05±0.09*
RETN (10^8/L)	5.5±0.8	5.2±0.8	4.4±0.9*	3.2±0.4*
RETP (%)	8.42±1.29	7.92±1.20	6.59±1.32*	5.19±0.59*

Note. *Compared with the control group, $P<0.05$.

Table 7. Effect of PFOS on Blood biochemical of Male Rats (mean ± SD, n=15)

Parameters (unit)	0 ppm	20 ppm	60 ppm	180 ppm
ALT (U/L)	44.23±9.79	50.95±7.87	56.83±12.24*	92.66±16.75*
AST (U/L)	190.98±23.69	189.00±32.16	214.76±37.67	231.31±46.23*
ALP (U/L)	293.40±89.75	258.93±49.76	311.93±64.38	418.10±53.29*
ALB (g/L)	28.63±1.45	27.87±1.34	29.72±1.06*	34.11±1.69*
TBIL (μmol/L)	2.14±0.96	2.09±0.27	1.87±0.39	2.91±0.69*
DBIL (μmol/L)	1.03±0.57	0.48±0.18*	0.48±0.20*	1.76±0.39*
HDL-C (mmol/L)	13.58±0.12	13.56±0.18	13.69±0.14	14.15±0.13*
LDL-C (mmol/L)	3.67±0.12	3.62±0.10	3.62±0.08	3.51±0.07*
CHO (mmol/L)	1.45±0.27	1.46±0.36	1.20±0.27*	0.49±0.17*
TG (mmol/L)	0.36±0.12	0.40±0.14	0.27±0.13	0.15±0.03*
TP (g/L)	49.45±2.19	48.99±2.35	51.01±2.71	53.85±2.04*
BUN (mmol/L)	5.37±1.57	5.32±1.20	5.64±1.44	8.14±1.14*
Testosterone (ng/ml)	3.97±0.40	3.98±0.40	3.44±0.30	2.50±0.28*

Note. *Compared with the control group, $P<0.05$.

Table 8. Incidence of histopathological lesions in male rats (n=15)

Parameters	0 ppm	20 ppm	60 ppm	180 ppm
liver	1	3*	7*	10*
kidney	2	3	2	9*

Note. *Compared with controls, number of animals with pathological lesions increased.

tive toxicity and systemic toxicity caused by PFOS.

Animal and epidemiological studies have confirmed that PFOS causes weight loss [11, 12]. This is consistent with the conclusion of the present study, which PFOS caused weight loss in animals with a dose-response relationship. It is unclear whether body weight loss is due to endocrine disruption effects or systemic toxicity. An epidemiological study also showed that PFASs caused body weight loss in both boys and girls [13]. In general, endocrine disruptors induce liver and renal toxicity [14, 15]. Salihovic and colleagues (2018) [14] reported that PFAS concentration was in positive correlation with ALT, ALP, and AST, while PFAS concentration was in negative correlation with bilirubin. In the present study, PFOS increased levels of ALT, ALP, and AST and presented a dose-response relationship. These results are consistent with population epidemiological studies. Low dose PFOS was negatively correlated with direct bilirubin, however, total bilirubin and direct bilirubin increased in the high-dose group, and PFOS caused liver edema, and showed an obvious dose-response relationship. Elevated BUN levels were associated with decreased absolute renal weight, as well as glomerular atrophy and renal tubule swelling, suggesting that high doses of PFOS induce significant renal toxicity.

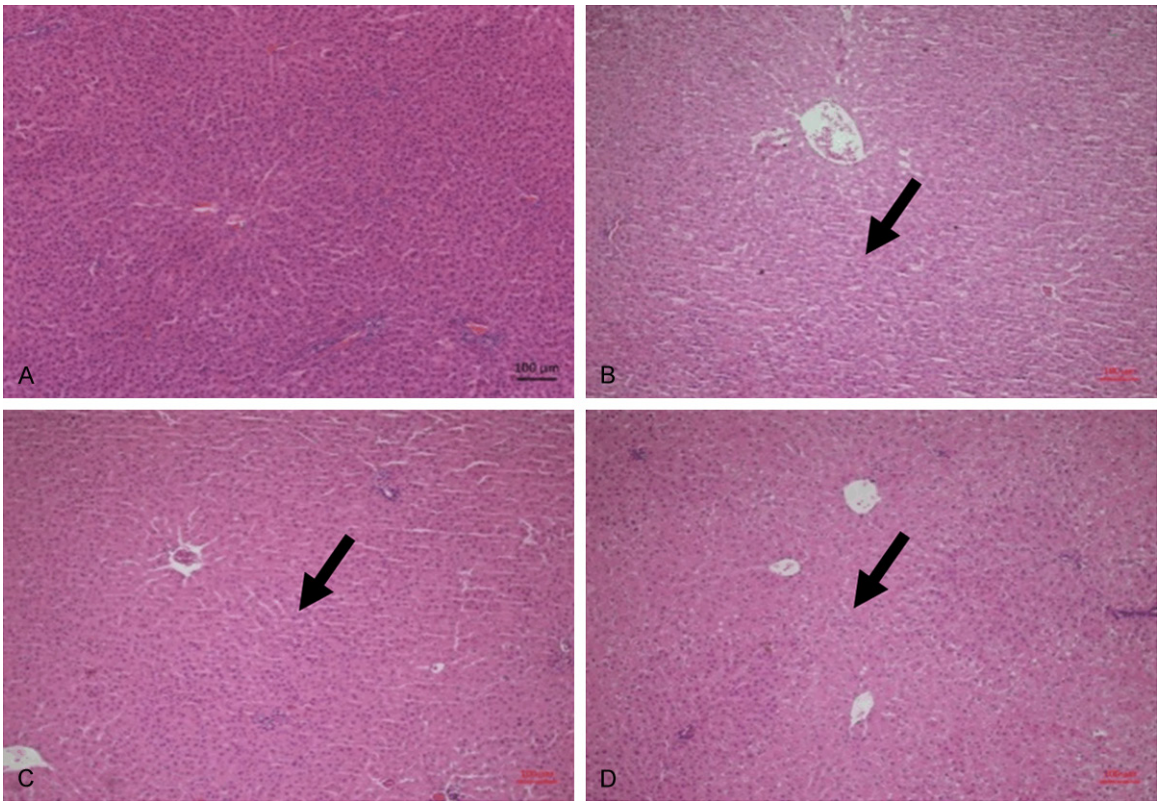

Figure 1. Liver histopathology in male rats. All dosage caused liver histopathological damage. Note. A: Control group haematoxylin and eosin (H&E) 100×, B: Edema in the 20 ppm dose group H&E 100×, C: Edema in the 60 ppm group H&E 100×, D: Edema in the 180 ppm group H&E 100×.

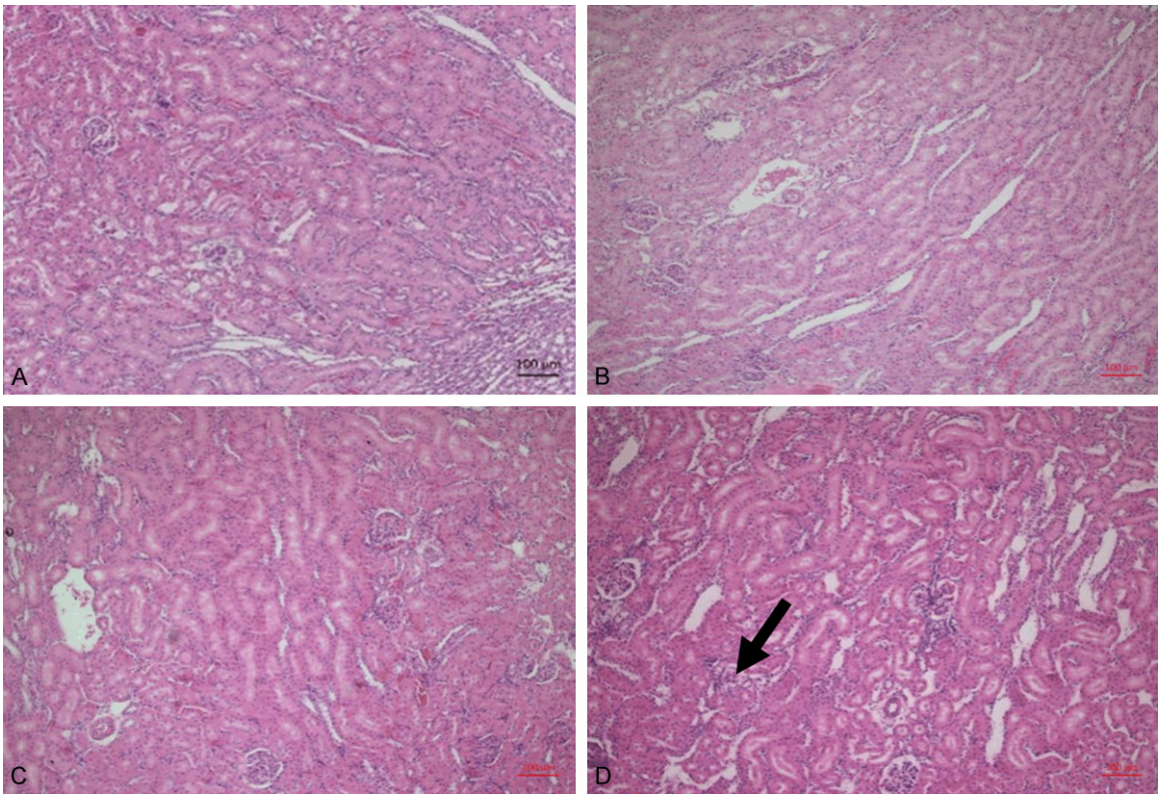

Figure 2. Kidney histopathology in male rats. Dosage of 180 ppm caused histopathological kidney damage. Note. A: Control group H&E 100×, B: 20 ppm dose group H&E 100×, C: 60 ppm group H&E 100×, D: Glomerular atrophy in 180 ppm group H&E 100×.

In addition, PFOSs have obvious immune-toxicity, which shows leukocyte's abnormal proportions and lymphocyte subgroup reductions [16]. The immune system is sensitive to PFOS, and the reference dose level based on long-term exposure to immune system suppression in the population is only 1.8×10^{-6} mg/kg/day [9]. In this study, PFOS caused obvious anemia, reduction of MONON, BASOP and BASON. The studies above suggest that the immune system is sensitive to PFOS.

Animal experiments showed that PFASs cause changes in sexual behavior, decreased fertility, degenerative changes in sexual organs, and decreased sperm quantity and quality in male rats. Epidemiological studies showed that PFASs delay puberty in boys [12]. A cross-sectional study found that PFASs reduced peripheral testosterone (T), sperm quantity, testicular volume, penis length and AGD [4]. The high dose in this study resulted in significant delay of PPS' time, which was consistent with the results of the above study. Also, low dose PFOS promoted weight increase of the accessory sex organs, including ventral prostate, DPS, LABC muscles and epididymis, although there was no statistically significant difference because of the large standard deviation. The results indicated that low dose PFOS induced hormetic effects.

Weight loss in two or more accessory sex organs can be considered as an anti-androgen effect [8]. In the present study, PFOS caused the weight of DPS, Ventral prostate, LABC to decrease. Although there was no statistically significant difference in the weight reduction of the epididymis in the medium and high dose group, there was an obvious dose-response relationship, so PFOS could be considered to have adverse effects on the epididymis. The weight loss of accessory sex organs might be directly attributed to the decrease of androgens. In present study, PFOS reduced testosterone. *In vitro* studies have shown that PFOS inhibits anabolic steroids in H295R cells [6]. When the concentration of PFOS was greater than 50 nM, the synthesis of testosterone in testicular mesenchymal cells was inhibited, and the expression of inhibitory genes decreased and promoting genes increased [17]. In the present study, the stunted development of the accessory sex organs might be due to a sex hormone level decrease.

PFOS reduced androgen levels, which may be an important mechanism of androgen effects. PFOS inhibited serum testosterone levels in juvenile male rats (35 days old), the expression of interstitial chorionic gonadotropin receptor genes and cytochrome oxidase (P450) 11A1 and 17A1 genes were inhibited also in the process of catalytic steroid hormone production [9]. Intrauterine PFASs exposure can reduce testosterone in mouse offspring [18]. Epidemiological studies have shown that levels of PFASS in the mother were negatively correlated with androgens of the offspring in adulthood [19]. Epidemiological studies revealed a negative correlation between PFASs levels in serum and male testosterone levels [20]. The above studies suggest that exposure to PFASs in early life (embryonic), adolescence, or adulthood has adverse effects on the testosterone level. Cholesterol is an important precursor to the synthesis of sex hormones. In the present study, total cholesterol triglycerides and low-density lipoprotein decreased with a dose-response relationship; total testosterone reduction was consistent with the trend of cholesterol.

The examination of sperm activity and deformity is a common indicator for the epidemiological evaluation of the effects of compounds on male germ cells. *In vitro* studies have shown that PFASs inhibited sperm stem cells to differentiate into primary spermatozoa and sperm cells [21]. Intrauterine PFOS exposure in female mice reduced sperm quantity when male offspring mature and grow up [22]. Adult rats exposed to PFASs, had decreased sperm quantity as well [23]. Epidemiological studies have shown that PFASs in the blood of pregnant mothers leads to decreased sperm concentration and sperm activity in the offspring during puberty [24]. In addition, PFASs also have adverse effects on sperm morphology, resulting in decreased head area and cir-

cumference of sperm, sperm head deformity, and percentage of immature sperm and ratio of curly sperm tail increase [8]. In the present study, PFOS was also found to cause sperm abnormality and sperm quantity reduction in adolescent rats, which was consistent with previous animal experiments and epidemiological studies. Sperm abnormality might be due to PFOS' reproductive toxicity, and sperm quantity reduction might be due to testosterone reduction caused by PFOS.

The hypothalamic pituitary testicular (HPG) axis regulates testosterone synthesis and the pituitary secrets luteinizing hormone (LH), which regulates testosterone (T) synthesis. Therefore, the hypothalamus, pituitary gland and testis may be the target organs of anti-androgen action. Animal experiments have shown that PFASs had inhibitory effects on the hypothalamus and pituitary gland of adult rats, and PFOS inhibited the expression of AR genes in the pituitary gland of adult rats [8]. An epidemiological study shows that plasma PFOS is negatively correlated with LH in 269 adult males [19]. In the present study, the correlation of the pituitary gland and accessory sex organs indicated that the pituitary gland might be an important anti-androgenic target organ of PFOS.

PFOS induced anti-androgenic and hermetic effects in pubertal male rats, accompanied by effects of PFOS which included reduction in pituitary weight, testosterone and cholesterol levels, and also systemic toxicity. It is suggested that the anti-androgen effect of PFOS may have multiple targets, including: the HPG axis, testis, sperm quantity, teratospermia, accessory sex organs, lipid metabolism, etc., and PFOS' specific anti-androgenic mechanisms needs to be further explored.

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Disclosure of conflict of interest

None.

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