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Subchronic Effects of Perfluorooctane Sulphonate on the Testicular Morphology and Spermatogenesis in Mice

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ABSTRACT

To determine the subchronic male reproductive toxicity of PFOS, male mice were administrated with a serial dosage of PFOS for 90 days for testicular observation and spermatogenesis evaluation. PFOS ≥ 11 mg/kg (accumulative dosage) resulted in visible histopathological changing in testis, including disorder and vacuolization of spermatogenic cells. Changed testicular organ coefficient (for dosage ≥ 55 mg/kg), decreased sperm concentration (for 110 mg/kg dosage), decreased motility (for 110 mg/kg dosage) and increased sperm malformation (for all of the treated groups) were also confirmed ($p<0.05$). Sperm malformation showed high sensitivity to PFOS exposure. A sperm malformation percentage, varying from 11.00% to 51.20%, was confirmed for the treated groups. As to various sperm malformation types, curved body type constituted the most proportion. The treated groups showed a curved body malformation percentage varied from 8.00% to 43.5%, due to different PFOS dosage. The observations indicate that subchronic exposure of PFOS can interfere with spermatogenesis process and affect sperm quality in mammals.

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Authors' Contribution
DYZ and XLX designed the study, performed experimental work and analyzed the data. QR and XYS helped in sperm checking. DYZ and YL wrote the article.

Key words
PFOS, Mice, Reproductive toxicity, Testis, Spermatogenesis

INTRODUCTION

Perfluorooctane sulphonate (PFOS) has been used widely as a surface protector, or surfactant, for more than half a century and has entered environmental water, soil, the atmosphere, and various organisms including humans (Lindstrom *et al.*, 2011). Because of its special chemical structure, PFOS is resistant to most environmental degradation means and is easily to accumulate in organisms. The environmental half-life of PFOS has been estimated to over 41 years (Olsen *et al.*, 2007). PFOS shows high accumulation potential in mammals. The elimination half-life of PFOS is 100 days in rats, 200 days in monkeys, and years in humans(Organisation for Economic Co-operation and Development, 2002). PFOS binds mainly to proteins, and prefers to accumulate in such organs as liver and plasma, causing severe harm (Lindstrom *et al.*, 2011).

Environmental pllutants pose a potential threat to human reproductive health. According to statistics, human semen quality has decreased sharply in recent years and the global mean sperm density has decreased by half since the 1960s (Sengupta *et al.*, 2017). Epidemiological investigations have indicated that these changes may be related to massive exposure to hazardous environmental pollutants (Dohle *et al.*, 2004). On the other hand, exposure to PFOS has been found to cause damages to male reproductive organs, disturb related hormone secretion in many animals. For example, PFOS was confirmed to affect development of the thyroid and gonads in *Xenopus laevis*, causing hyperplasia and vacuolization of thyroid follicular epithelial cells and deformity of the gonads (Liu *et al.*, 2008; Lou *et al.*, 2013). For example, chronic PFOS exposure alted the sex ratio (causing female dominance) and damaged the male gonad in zebrafish (Wang *et al.*, 2011; Chen *et al.*, 2013; Chen *et al.*, 2016). PFOS exposure on male quail and mallard resulted in slightly greater incidence of small testes in length (Newsted *et al.*, 2007). As to mammals, PFOS exposure in rats was confirmed to cause damage to spermatogenesis and sperm maturation process including decreased testis weight, lowered sperm counts, poor sperm activity, poor lactate dehydrogenase (LDH) and succinate dehydrogenase (SDH) activity, increased sperm deformity rate and malondialdehyde (MDA) levels (Fan *et al.*, 2005; Li *et al.*, 2012; Liu *et al.*, 2010;*et al.* Qu *et al.*, 2016).

It is of great importance to conduct more research in

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more species to explore PFOS's reproductive toxic effects and corresponding mechanism. In previous studies on mammals, PFOS was administrated usually for short-time exposure (less than 2 months), which is different from the real exposure model. In this study, we examined the testis development and sperm quality, as a measure of male reproductive toxicity, of male mice after subchronic exposure to a serial dosage of PFOS for 90 days.

MATERIALS AND METHODS

Chemicals and animals

PFOS in potassium salt form was bought from J&K (China) and was dissolved using 2% tween-80 for animal experiments. ICR male mice with average body weight of 13.06 (±0.08) g were provided by provided by Zhejiang Provincial Center of Experimental Animals, China [license SCXK (Zhe) 2008-0033]. The animals were housed in an environmentally controlled facility (temperature 20 to 24°C; humidity 55% ± 5%; 12h light/ dark cycle) with access to food and water ad libitum in the SPF animal laboratory. All experiments were performed in accordance with the China Guidelines for Ethical Review of Laboratory Animal Welfare (GB/T 35892-2018)

Subchronic exposure experiment

ICR male mice were divided randomly to 5 groups with 20 mice in each group. The mice in different groups were exposed to 0, 0.5, 11, 55 and 110 mg/kg PFOS (accumulative dose) for 90 days respectively by freely drinking method. The general behavior of the animals was recorded during the experiment. At the end of the treatment period, the animals were weighted and then killed for the histopathological observation.

Evaluation of relative organ weight

After subchronic exposure to PFOS, the testes, epididymides, liver and heart of the mice were isolated and weighed. The relative organ weight (organ coefficient) was defined as the ratio of the organ weight (mg) to the body weight (g), respectively.

Evaluation of the concentration, motility and malformation of sperm

Epididymis was isolated, sheared and filtered for suspension preparation using physiological saline. Sperm counting and sperm motility evaluation were performed at 25 °C using phase contrast microscope (OLYMPUS CX41, Japan) with chamber of 25*16 grids (Kutluyer, 2018). For motility evaluation, 200 sperms were observed for each group. The motility of sperm was divided into 4 levels according to its swimming activity (WHO, 1999). Sperm activity = (I+II+III)/ (I+II+III+IV) ×100%.

For sperm malformation analysis, three semen smears per animal were prepared for microscopy, with 400 sperms being observed per smear. Sperm malformation was assessed according to a previous report. Malformations may occur to 4 locations in sperms respectively: head (fat- head, banana-shape head, irregular-shape head, hook-missing head, double-head or small- head), neck (curved-neck), body (curved-body or multiple body), tail (folded-tail) or acrosome (broken-acrosome or acrosome-missing). In addition, lesion occurring to more than one location in a sperm was defined as multiple malformations (CNSMC, 2003).

Histopathological observation of the testis

After being washed with saline, the mouse testis was fixed with 10% formaldehyde and subsequently embedded in paraffin and sliced. After staining with hematoxylin and eosin (H&E), the samples were observed using microscope.

Statistical analysis

To determine the statistical significance between different groups, one-way ANOVA analysis with Duncan's multiple comparisons test was employed using SPSS (V13.0) software.

RESULTS

Relative organ weights of the testis and epididymis

As shown in Table I, the total body weight of the exposed animals showed no significant difference compared with that of negative control. The heart coefficient showed no difference either. Difference with statistical significance was only observed for testis coefficient, epididymis coefficient and liver coefficient in those animals treated with high dosage of PFOS. PFOS of 55 mg/kg dosage only resulted in increased testis coefficient ($p<0.05$). PFOS of 110 mg/kg dosage resulted in increased testis coefficient ($p<0.05$), epididymis coefficient ($p<0.05$), as well as liver coefficient ($p<0.01$).

Histopathological observation of the testis

The testicular histopathological results were shown in Figure 1. There are 5-8 layers of regular spermatogenic cells in the seminiferous tubules of the mice in the negative control group. The spermatogenic cells are undergoing normal development from spermatogonia to mature spermatozoa. Also, there are a large number of spermatozoa could be observed in the lumen (Fig. 1A). Almost no significant difference between 0.5 mg/kg group and control group was observed except for vacuolation

in the body of some spermatogenic cell (Fig. 1B). As to 11 mg/kg dosage group, the spermatogenic cells were decreased to be only 4-6 layers in seminiferous tubules, which were less than those in the negative control group (Fig. 1C). As to 55mg/kg dosage group, loosened cell arrangement, more disordered structure and more vacuolar cells and more serious vacuolization degree were observed (Fig. 1D). As to 110 mg/kg dosage group, it indicated that the wall of seminiferous tubule of testis was deformed, the whole seminiferous tubule had disordered spermatogenic cell layers, the cell layers in the tubules were significantly reduced, the cell vacuolation was serious, and the number of sperm in the lumen was reduced (Fig. 1E, 1F).

Sperm concentration

As shown in Table II, the mean value of sperm quantity in most treated groups was lower than in the negative control group. However, this was only significantly lower in the 110 mg/kg dosage group ($p<0.01$), in which the mean sperm concentration decreased by 42.39%.

Sperm motility

The mean motility percentage of the sperms in all treated groups decreased compared with the negative control group, as shown in Table II. Again, this was only significant for the 110 mg/kg dosage group (p<0.01). The motility percentage in the 110 mg/kg group was decreased by 43.67% compared to the negative control group.

Sperm malformation

As shown in Table II and Table III, the total sperm malformation percentage increased in all of the treated groups ($p<0.05$). The sperm malformation percentage in the treated groups varied from 11.00% to 51.20%,

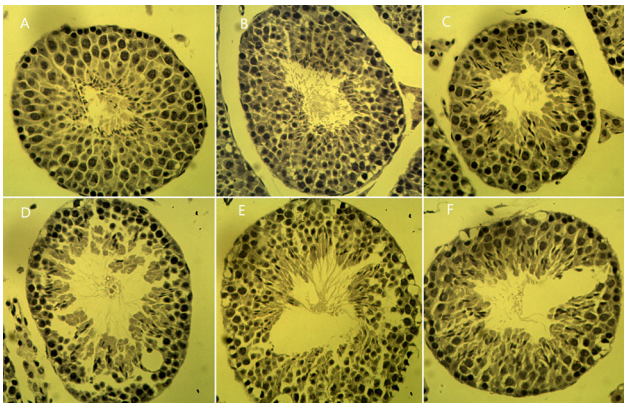

Fig. 1. Histopathological observation of the testis of mice (400×). A, 0 mg/kg PFOS group; B, 0.5 mg/kg PFOS group; C,11 mg/kg PFOS group; D,55 mg/kg PFOS group; E and F, 110 mg/1kg PFOS group.

Table I.- Effects of PFOS on organ coefficient ($\bar{x}$±s, n=20).

PFOS Dose (mg/kg)	d0 body weight (g)	d90 body weight (g)	Testis coefficient (%)	Epididymis coefficient (%)	Liver coefficient (%)	Heart coefficient (%)
0	25.89±1.22	47.09±2.78	0.74±0.11	0.36±0.05	5.47±0.50	0.53±0.09
0.5	24.37±2.14	44.66±3.30	0.80±0.16	0.38±0.06	5.58±0.46	0.52±0.10
11	24.40±1.47	45.17±2.80	0.78±0.10	0.38±0.04	5.58±0.58	0.55±0.10
55	24.44±2.21	44.73±4.24	0.92±0.09*	0.37±0.05	5.50±1.93	0.55±0.10
110	25.64±1.15	44.89±3.60	0.94±0.12*	0.52±0.49*	7.15±0.20**	0.57±0.07

*$p<0.05$; *$p<0.01$

Table II.- Effects of PFOS on the sperm quantity and quality ($\bar{x}$±s,n=20).

PFOS Dose (mg/kg)	Sperm concentration (×10⁶/mL)	Sperm motility percentage (%)	Sperm malformation percentage (%)
0	12.88 ±4.43	58.62 ±8.70	7.77 ±1.31
0.5	12.27 ±2.50	57.98 ±7.69	11.00±1.55*
11	10.78 ±1.84	54.47 ±9.18	18.30 ±2.83*
55	11.13 ±2.39	49.55 ±4.71	30.33 ±3.68**
110	7.42 ±3.08**	33.02 ±4.64**	51.20 ±4.90**

*$P<0.05$; **$P<0.01$

Table III.- The proportion of various sperm malformation.

PFOS Dose (mg/kg)	Malformation ratio (%)												
	Fat head	Banana-shape head	Irregular head	Hook-missing head	Double head	Small head	Curved neck	Multiple body	Curved body	Folded tail	Acrosome-missing	Broken acro-some	Multiple malformation
0	0.11	0.33	0.00	0.33	0.11	0.00	0.22	0.00	5.22	0.67	0.78	0.00	0.00
0.5	0.40	0.10	0.00	0.70	0.00	0.00	0.20	0.00	8.00	0.50	0.80	0.20	0.10
11	0.80	0.00	0.00	0.70	0.00	0.10	0.50	0.00	13.10	1.10	1.60	0.30	0.10
55	0.67	0.22	0.00	1.11	0.00	0.11	1.33	0.00	19.11	5.11	2.00	0.56	0.11
110	0.80	0.10	0.00	1.60	0.10	0.20	0.70	0.00	43.50	1.40	2.10	0.70	0.00 0.08

which was obviously higher than the 7.77% in the negative control group. Malformation occurred to the head, neck, body, tail or multiple parts (compound type) in the sperms respectively. Table III summarizes the detailed proportion of various malformation types in the treated groups. Due to different dosage, PFOS exposure resulted in 8.00% to 43.50% of curved-body malformation percentage, 0.50% to 5.11% of folded head malformation proportion, and 0.70% to 1.60% of hook-missing head malformation proportion. Curved-body showed to be the largest proportion of sperm malformation for all of the treated groups. PFOS exposure also induced increased fat-head malformation and broken-acrosome malformation. On the other hand, banana-shape head, irregular head, double head and multiple body showed nearly no difference between the treated groups and the negative control group.

DISCUSSION

PFOS has been detected in the environment and organisms globally. Contamination of PFOS in various organisms, from invertebrates to mammals, including human, has been confirmed. Most investigations were performed in fish, birds and marine mammals, showing high level of PFOS contamination in almost all of the sampled organisms (Blake *et al*, 2007; Bossi *et al.*, 2005; Van de Vijver *et al.*, 2007). PFOS in animals was found to prefer to accumulate in the livers and sera. Generally, the PFOS concentrations in animals are hundreds to thousands of ng/g in their livers and dozens of ng/mL in their sera. Extensive PFOS contaminations were also found even in non-occupational exposure humans globally. For example, a detection of 473 blood samples sampled from 9 contries showed >30 $ng \cdot mL^{-1}$ PFOS concentrations for Americans, 3-29 $ng \cdot mL^{-1}$ for Koreans, Belgians, Malaysians, Brazilians, Italians and Colombians, and <3$ng \cdot mL^{-1}$ for Indians (Kannan *et al.*, 2004). Another checking of human sera between 1980s and 2000s showed an increasing trend of PFOS concentration too (Harada *et al.*, 2010). In addition to serum or plasma, PFOS was also frequently detected in breast and liver (Olsen *et al.*, 2003; Völkel *et al.*, 2008). Therefore, it is of great importance to evaluate how subchronic exposure of PFOS affect the mammals.

There were some previous studies indicated PFOS's male reproductive toxicity in mammals. For example, male rats received PFOS for 65 days showed decreased testis weight, sperm count, LDHx and SDH activity in testis, and increased sperm deformity ($p<0.05$) for 1.5 and 4.5 mg/kg per day dosages (Fan *et al.*, 2005). Adolescent SD rats on postnatal day 21 were exposed to PFOS by gavage for 7 days for reproductive evaluation on postnatal day 56. Lowered sperm counts were observed for 10 mg/kg and 20 mg/kg dosage (<0.05) and lowered serum testosterone concentration for 20 mg/kg dosage (Li *et al.*, 2012). Adult mice were given PFOS by gavage to for 5 weeks showed decreased serum testosterone level and sperm counts for 10mg/kg per day group. Also, increased apoptosis of germ cells were observed and were thought to be related to mitochondrial pathway (Qu *et al.*, 2016). In this study, subchronic exposure of PFOS was confirmed to be able to change the organ coefficient of testis and epididymis in mice, which support the main findings above that PFOS affect testis and sperms in mammals Histopathological observations indicated that PFOS exposure destroyed the normal histological structure of testis, resulting in a poor spermatogenesis ability. Evaluation of sperm furtherly supported the histopathological observations, as decreased sperm concentrations and motility and an increased sperm malformation percentage were confirmed.. Sperm malformation in mice seemed to be the most significant

changing after being exposed to PFOS. Furtherly, curved-body, fat-head and broken-acrosome were found to be the most frequently occurred sperm malformation caused by PFOS exposure.

Spermogenesis is a special process during which germ cells undergo a series of meiosis, mitosis and cell differentiation. As a highly ordered process, spermogenesis is always under precise control and regulation. The external regulation factor is mainly hormone, and the internal regulation is mainly the regulation of specific gene expression in germ cells. Sex hormones play an important role in spermatogenesis and maturation. For example, testosterone (T), follicle stimulating hormone (FSH) and luteinizing hormone (LH) are the main hormones regulating and maintaining testicular activity. Therefore, changing of their level can affect spermatogonial differentiation obviously (Shetty *et al.*, 1996). The mechanism how PFOS affect the function of testis and spermatogenesis is still poorly known. By alkaline comet assay *in vitro*, it was confirmed that PFOS can not cause significant levels of cytotoxicity in human sperms directly (Emerce and Cetin, 2018). Therefore, indirect toxicity mechanisms should be taken into account. For example, the mechanism might involve secretion and function of sex hormone. As to an investigation in 247 healthy men, PFOS level showed to be negatively associated with the level of testosterone (T), calculated free testosterone (FT), free androgen index (FAI), as well as the ratios of T/LH, FAI/LH and FT/LH (Joensen *et al.*, 2013). Also, a previous study on mouse leydig cell cultured in vitro indicated that PFOS, at dozens of μg/mL level, can destroy the proliferation and the physiological state of mouse leydig cell which is responsible for the synthesis and secretion of sex hormones. There are also some other studies support the above points that PFOS in mammals can affect their spermatogenesis accompanied by disturbed sex hormone activity. For example, PFOS of 1.5 mg/kg and 4.5mg/kg dose for 65 days in rats were confirmed to cause significant decreasing of sperm count, mean activities of LDHx and SDH, and increasing of sperm malformation percentage (Fan *et al.*, 2005). Administration with ammonium perfluorooctanoate on rats also caused decreased serum and testicular interstitial fluid testosterone levels and increased serum estradiol levels (Biegel *et al.*, 1995). A recent study on adult male rats indicated that PFOS can modify the relative gene and protein expressions of such receptors as gonadotropin-releasing hormone receptor (GnRHr), luteinizing hormone receptor (LHr), follicle-stimulating hormone receptor (FSHr) and the androgen receptor (Ar), in several tissues of the reproductive axis (López-Doval *et al.*, 2016) . Study in female animals support the observations too. PFOS exposure for 24h caused apoptosis of human placental syncytiotrophoblasts and decreased secretion of steroid and human chorionic gonadotropin by placental syncytiotrophoblasts (Zhang *et al.*, 2015). In a recent study, PFOS was also found to be able to induce Sertoli cell injury *in vitro*. The injury was related to perturbd actin cytoskeleton, changed spatial expression of actin regulatory proteins, mis-localization of Arp3 and paladin, disorganization of F-actin, truncation of actin microfilament, and so on. It supports a previous viewpoint that Sertoli cell is a potential target for PFOS-induced reproductive dysfunction in male mice (Gao *et al.*, 2017; Qiu *et al.*, 2013)

CONCLUSIONS

This studyconfirmed that subchronic exposure of relatively low dosage of PFOS for 90 days damages the morphology and physiological function of testis. Sperm malformation is very sensitive to PFOS exposure, and the detailed proportion of various sperm malformation was furtherly summarized. The findings are helpful to understand the detailed adverse effects by subchronic exposure of PFOS in male mammals. It provided a reference for exploring the mechanism of PFOS's reproductive toxicity in future.

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Statement of conflict of interest

We declare no conflict of interest in this study.

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