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Perfluorooctane sulfonate impairs rat Leydig cell development during puberty

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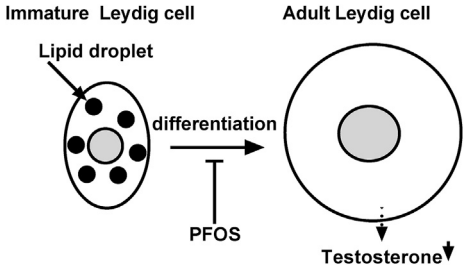
HIGHLIGHTS

- Perfluorooctane sulfonate delays Leydig cell development during puberty.
- Perfluorooctane sulfonate disrupts Leydig cell specific gene expression.
- Perfluorooctane sulfonate promotes immature Leydig cell apoptosis.

GRAPHICAL ABSTRACT



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ABSTRACT

Perfluorooctane sulfonate (PFOS) possibly delays male sexual development. However, its effects on pubertal Leydig cell development are unclear. The objective of the present study was to investigate the effects of *in vivo* PFOS exposure on rat Leydig cell development during puberty. Immature male Sprague Dawley rats were gavaged 5 or 10 mg/kg PFOS on postnatal day 35 for 21 days. Compared to the control (0 mg/kg), PFOS lowered serum testosterone levels without altering luteinizing hormone and follicle-stimulating hormone levels on postnatal day 56. PFOS *in vivo* downregulated mRNA or protein levels of Leydig cells (*Lhcgr*, *Cyp11a1*, and *Cyp17a1*). PFOS *in vitro* inhibited androgen secretion in immature Leydig cells at $\geq$ 50 nM, most possibly via downregulating *Hsd17b3* mRNA level. At $\geq$ 500 nM, PFOS downregulated *Lhcgr*, inhibited BCL-2 and increased BAX levels to cause Leydig cell apoptosis. In conclusion, PFOS at a lower dose directly inhibited pubertal development of Leydig cells.

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1. Introduction

Perfluorooctane sulfonate (PFOS) is one of widely used chemicals with many fluoro groups. Because it has a unique characteristics of surface activity, PFOS is used as a material for the coating of textiles, umbrella, paper, and upholstery, and as a reaction additive (Johnson et al., 1984; Abdellatif et al., 1990; Jensen and Leffers, 2008). PFOS is classified as a persistent organic pollutant, since it has very long elimination half-life (Giesy and Kannan, 2002) with the geometric mean elimination half-life of 4.8 years in humans (Olsen et al., 2007). A study showed that the mean PFOS level of the US general population was 14.7 ng/ml in 2006 (Olsen et al., 2008) and that of Chinese people was 39.6 ng/mL in 2006 (Yeung et al., 2006).

It has been reported that PFOS had developmental toxicity (Saikat et al., 2013). Recently, several studies claimed PFOS-mediated reproductive toxicities. PFOS induced the structural abnormalities and disturbed blood-testis barrier of rat testis (Cheng et al., 2011) and caused the reproductive toxicity of male animals (Qiu et al., 2013). Indeed, PFOS inhibited some rat and human androgen-biosynthetic enzymes in the testis (Zhao et al., 2010) and blocked fetal Leydig cell development in the rat (Zhao et al., 2014).

PFOS may also be an endocrine disrupter to delay pubertal sexual development. A cross-sectional study to determine the association of PFOS with sexual maturation indicators in 3076 boys showed that it increased the odds to delay pubertal sexual development (Lopez-Espinosa et al., 2011). However, whether PFOS affected the pubertal sexual development and the underlying mechanism is unclear.

The pubertal sexual development depends on the Leydig cell differentiation during puberty. In rats, Leydig cell differentiation during puberty progressed through postnatal day 35 when immature Leydig cells were formed and completed on postnatal day 56 when adult Leydig cells were well developed and produced testosterone with full capacity (Chen et al., 2017). Immature Leydig cells were differentiated by expressing the following proteins: 1) steroidogenesis-regulatory signaling unit [luteinizing hormone receptor (LHCGR, gene *Lhcgr*)], 2) cholesterol transportation unit [high-density lipoprotein receptor (SCARB1, gene *Scarb1*) and steroidogenic acute regulatory protein (STAR, gene *Star*)], and 3) androgen biosynthetic unit [cholesterol side chain cleavage (CYP11A1, gene *Cyp11a1*), 3β-hydroxysteroid dehydrogenase 1 (HSD3B1, gene *Hsd3b1*), 17α-hydroxylase/20-lyase (CYP17A1, gene *Cyp17a1*), and 17β-hydroxysteroid dehydrogenase 3 (HSD17B3, gene *Hsd17b3*)]. Immature Leydig cells can respond to luteinizing hormone (LH) to increase testosterone and 5α-androstane-3α,17β-diol (DIOL) production (Ge and Hardy, 1998). Interestingly, a glucocorticoid-metabolizing enzyme, 11β-hydroxysteroid dehydrogenase 1 (HSD11B1, gene *Hsd11b1*), appears in rat immature Leydig cells starting on postnatal day 28 (Phillips et al., 1989b), thus being a biomarker of Leydig cells at the advanced stage. Immature Leydig cells also had a proliferative capacity to increase the Leydig cell number (Ge and Hardy, 1997). In the present study, we investigated the effects of PFOS on pubertal development after male immature rats were exposed to PFOS *in vivo* and dissected the action site of PFOS using *in vitro* treatment of immature Leydig cells.

2. Materials and methods

2.1. Materials and animal

[^{3}H] Pregnenolone, [^{3}H] progesterone, and [^{3}H] androstenedione were purchased from DuPont-New England Nuclear (Boston, MA). Unlabeled pregnenolone, progesterone, androstenedione, and testosterone were obtained from Steraloids (Newport, RI). PFOS were purchased from Sigma-Aldrich (St. Louis, MO). Sixty-nine 28-day-old male Sprague-Dawley rats were purchased from Shanghai Laboratory Animal Center (Shanghai, China). All studies were approved by the Wenzhou Medical University's Animal Care and Use Committee and were performed in accordance with the Guide for the Care and Use of Laboratory Animals.

2.2. Animal experiment

Fifteen 28-day-old male Sprague-Dawley rats were raised in a 12 h dark/light cycle temperature at 23 ± 2 °C and relative humidity of 45%–55%. Water and food were accessed *ad libitum*. Rats were acclimated for a week before they were randomly assigned into 3 groups (5 animals per group). PFOS was dissolved in 2% Tween 20. Rats in group 1 were gavaged 2% Tween 20 serving as the control, while rats in group 2 and 3 were gavaged 5 or 10 mg/kg PFOS, respectively. The doses were selected based on the previous references (Wan et al., 2011; Zhao et al., 2014). In an adult mouse study with 1, 5, and 10 mg/kg PFOS were gavaged for 21 days, only 10 mg/kg caused lower testosterone levels and 5 and 10 mg/kg PFOS also downregulated *Lhcgr* (Wan et al., 2011). An *in utero* exposure to 5 and 20 mg/kg PFOS also caused lower testosterone levels in fetal rat Leydig cells (Zhao et al., 2014). Thus, 5 and 10 mg/kg doses of PFOS were selected in the present study. Twenty-one days after gavage, the rats were sacrificed on postnatal day 56 by asphyxiation with CO_2. Trunk blood was collected, placed in a gel glass tube, and centrifuged at 2000 × g for 10 min to collect serum. Serum samples were labeled and stored at −80 °C until being analyzed for testosterone, LH and follicle-stimulating hormone (FSH). Besides, each pair of testes was separated and weighted. One testis per animal was punched three holes using a G27 syringe needle and then fixed in Bouin's solution for one day for the histochemical analysis. The contralateral testis was frozen in the liquid nitrogen for subsequent analysis of gene and protein levels.

2.3. Sperm quantification

The distal cauda epididymides were immediately cut out and transferred to 35-mm organ culture dishes containing 1 ml mixture of Dulbecco's modified Eagle medium and 3% bovine serum albumin. Then, they were cut finely and incubated at 37 °C for 5 min in CO_2 incubator to allow the sperm to release into the medium. Sperm concentration analysis was conducted by computer assisted semen analyzer with the HTM-IVOS system (Hamilton-Thorne Research, Beverly, MA, USA).

2.4. Immature Leydig cell isolation

Immature Leydig cells had all testosterone biosynthetic enzymes (Ge and Hardy, 1998). Eighteen 35-day-old Sprague-Dawley rats were sacrificed by asphyxiation with CO_2. Immature Leydig cells were purified as described previously (Ge and Hardy, 1998). In brief, the removed testis was perfused with collagenase via a testicular artery, and digested with collagenase and DNase (Sigma-Aldrich, St. Louis, MO, USA) for 15 min. Then, the cell suspension was filtered through 100 μm nylon mesh to remove the tissue debris and the cells were separated under Percoll gradient (Sigma-Aldrich, St. Louis, MO). The cells with a density of 1.07–1.088 g/ml were collected and washed. Purified cells were evaluated by the histochemical staining for HSD3B1 activity, with 0.4 mM etiocholanolone as the steroid substrate and NAD^+ as a cofactor (Payne et al., 1980). More than 95% Leydig cells were intensely stained, indicating that the purity of immature Leydig cells is high. Total