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The effect of perfluorododecanonic acid on endocrine status, sex hormones and expression of steroidogenic genes in pubertal female rats

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ABSTRACT

Perfluorododecanoic acid (PFDoA), one of a number of commercially important perfluoroalkyl acids, has been detected in sera from humans and other animals; however, the effects of PFDoA on female reproduction remain unclear. To assess the impact of PFDoA on puberty and endocrine status, we exposed weaned pre-pubertal female rats to PFDoA, administered orally at doses of 0, 0.5, 1.5 and 3 mg/kg-d for 28 days, and measured body weight, reproductive organ weight and morphology, pubertal indicators, endocrine hormones, total serum cholesterol levels and steroidogenic enzyme gene expression. At 3 mg/kg-d, PFDoA significantly decreased body weight and serum estradiol levels, increased cholesterol levels ($p < 0.05$), and altered ovarian expression of genes responsible for cholesterol transport and steroidogenesis, including steroidogenic acute regulatory protein, cholesterol side-chain cleavage enzyme and 17-beta-hydroxysteroid dehydrogenase ($p < 0.05$). PFDoA at the highest dose also reduced estrogen receptor α and β expression levels in the ovary ($p < 0.05$), whereas a lower concentration of PFDoA (0.5 mg/kg-d) decreased estrogen receptor β mRNA levels in the uterus ($p < 0.05$). PFDoA treatment did not affect serum follicle-stimulating hormone or luteinizing hormone (LH) levels at any concentration, although PFDoA at 3 mg/kg-d reduced LH receptor mRNA levels. There were no marked changes in sexual organ weight, age and weight at vaginal opening or first estrous cycle, or ovarian/uterine histology at any PFDoA concentration. These data show that PFDoA does not affect the endocrine status of pubertal rats, but at higher doses it does impact estradiol production and the expression of some key genes responsible for estrogen synthesis.

1. Introduction

The perfluoroalkyl acids (PFAAs) are commercially important perfluorinated chemicals that are used in a variety of products, such as lubricants, paints, cosmetics and fire-fighting foams. These specialty chemicals consist of a carbon backbone of different chain lengths, and include the eight-carbon perfluorooctanoic acid (PFOA), the eight-carbon perfluorooctane sulfate (PFOS), the ten-carbon perfluorodecanoic acid (PFDA) and the twelve-carbon perfluorododecanonic acid (PFDoA) [1]. These chemicals are highly resistant to degradation and are ubiquitous in the environment; their presence has been demonstrated in wildlife and in humans [2–4]. For example, in remote regions of the high arctic ice caps, PFOA, and several other PFAA intermediates have been detected in concentrations ranging from the low to mid picogram-per-liter range [5]. A Swedish study calculated that the total amount of PFAAs transferred to breast-feeding infants was approximately 200 ng/day [6]. Although PFOA and PFOS are the two main PFAAs detected in worldwide samples, the existence of longer forms, such as PFDoA, is also consistently reported in the recent scientific literature [7,8]. The toxic effects of PFAAs in experimental animals has generated public concern about the possible health risks for humans and wildlife posed by environmental PFAAs.

Several studies have demonstrated that PFAAs, such as PFOA and PFOS, induce hepatic peroxisome proliferation and perturb hepatic lipid metabolism in rats [9,10], and cause hepatocellular adenomas in male rats [11]. In our previous study, PFDoA exposure also caused prominent hepatotoxicity in male rats [12]. In addition to hepatotoxicity, PFAAs with eight to twelve carbon chains also exhibited adverse effects on the immune system, and altered hormone levels, and reproduction and development in a rodent model [13,14]. Both inhibition of testosterone synthesis and testicular dysfunction have been observed in male rats following exposure to PFAAs, such as PFOA, PFDA and PFDoA [15–17]. To date, toxicological studies have focused mainly on the effects of PFAAs on the male reproductive system; thus, the effects of PFAAs on female reproductive function are much less well understood. There have been few such toxicological studies of PFOS in females, and those that have been conducted have reached controversial conclusions. For example, PFOS significantly altered estrous cycles and endocrine status in adult rats

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treated via intraperitoneal injections at doses of 1 and 10 mg/kg-d for 14 days [18]. However, the same dose of PFOS (1 and 10 mg/kg-d) administered to adult female rats over the course of 28 days via oral gavage did not affect estrous cycles [19]. One potential shortcoming of these studies on the effects of PFOS on reproductive function is the age of the rats used (i.e., adults), since studies have shown that the reproductive system of young (neonatal/pubertal) rats may be more susceptible to exposure to environmental pollutants [20,21].

Reports have also shown that PFAAs with shorter length carbon chains are more rapidly eliminated and thus exhibit less bioaccumulation than those with longer chains, suggesting that PFDoA might possess toxicologic properties that are different from those of PFOA, PFOS or PFDA [22,23]. To address the specific toxicity of PFDoA in an appropriate animal model, we evaluated the effect of PFDoA on reproductive function in pubertal female rats. Weaned female Sprague–Dawley rats were treated with PFDoA, administered orally, for 28 days, and the animals were investigated for endpoints related to sexual organ development and endocrine characteristics. The parameters measured included reproductive tissue weights, reproductive organ morphology, estrous cyclicity and sex hormone levels. Additionally, the expression of genes responsible for steroid biosynthesis was measured in the ovary of females exposed to PFDoA.

2. Materials and methods

2.1. Animals

Weaned 21-day-old female Sprague–Dawley rats (35–40 g), obtained from Weitong Lihua Experimental Animal Central, Beijing, China, were maintained in a mass air-displacement room with a 12-h/12-h light/dark cycle at 20–26 °C and a relative humidity of 40–60%. Animals were housed separately in stainless steel cages and allowed to acclimatize for 3 days before the start of experiments at postnatal day (PND) 24 (day of birth defined as PND 0). Food and water were provided *ad libitum* for the duration of the study.

2.2. Treatment

PFDoA (CAS No. 307-55-1, 95% purity, Sigma–Aldrich) was used as acid and dissolved in 0.5% Tween-20 in distilled water. Rats were randomly divided into four groups of eight rats each. Rats in the three exposure groups were orally administrated PFDoA at doses of 0.5, 1.5 or 3 mg/kg-d for 28 days in a total volume of 8 ml/kg body weight. The control animals were treated similarly using 0.5% Tween-20 (vehicle).

2.3. Observation of vaginal opening and cyclicity

Female rats were monitored on a daily basis from the start of the study for vaginal opening (VO). The age at the start of VO as well as the body weight on that day were recorded for each female. Daily vaginal smears were collected beginning the day following VO for each animal to determine the age and body weight at first estrous for each rat. Smear-collection continued until all animals had been sacrificed. Vaginal smears were read wet on a low-power light microscope for the presence of leukocytes (diestrous), nucleated epithelium (proestrous) or cornified epithelial cell (estrous) to determine cyclicity patterns. Rats with irregular cyclicity were defined as those not presenting at least two consecutive normal cycles during the experiment.

2.4. Necropsy

At the conclusion of the 28-day treatment, females at the diestrous stage of their estrous cycle in each group were weighed and euthanized by decapitation. Samples for evaluating alterations in serum hormone concentrations were collected during the diestrous stage (PND 52–55), a time that has previously been shown to be optimal for detecting changes in hormone levels in female rats [24]. Trunk blood was collected and centrifuged at 2000 × *g* at 4 °C for 15 min. Serum was stored at −20 °C until analysis. The right and left ovaries, and uterus (wet) were removed and weighed. The left ovary and a part of the uterus were fixed in 10% buffered formalin for histological observation. The right ovary and remaining uterus segment were frozen immediately in liquid nitrogen and stored at −80 °C until analyzed.

2.5. Histological analysis of follicle numbers

The ovaries and uteri from three rats in each group were embedded in paraffin after fixed (48 h), and 5-μm sections from each tissue were serially cut and mounted on glass slides. Slides were routinely stained with hematoxylin and eosin (H&E). Histological analyses were performed using an AX80 microscope (Olympus, Tokyo, Japan). A necropsy evaluation showed that ovaries from control and treated animals did not differ in size. In every 20th section of ovary (eight sections/ovary), primordial, primary, preantral and antral follicles were identified and counted as previously described [25–27]. Only follicles with a visible nucleus were counted to avoid double counting.

2.6. Serum hormone and cholesterol levels

Estradiol, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels were detected using commercial kits from Beijing North Institute of Biological Technology, China. Estradiol was analyzed by chemiluminescence, and FSH and LH levels were determined by radioimmunoassay (RIA) according to the manufacturer's instructions. Total cholesterol (TCHO) in serum was measured using a commercial TCHO kit (Biosino Bio-technology and Science Inc., Beijing, China). TCHO was quantitated colorimetrically by measuring absorbance at 500 nm using a UV1240 spectrophotometer (Shimadzu, Japan). The assay detection limit was 8 pg/ml for estradiol and 1.0 mIU/ml for FSH and LH. For both FSH and LH, inter- and intra-assay coefficients of variation were <10% and <15%, respectively; for estradiol, inter- and intra-assay coefficients of variation were <8% and <10%, respectively; and for TCHO, the corresponding values were <3% and <5%.

2.7. Reverse transcription and real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from individual ovaries and uteri using TRIZOL reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. The concentration and integrity of total RNA was calculated by measuring absorbance at 260 nm using a UV1240 spectrophotometer (Shimadzu, Japan) and agarose gel electrophoresis. RNA purity was estimated from the 260/280 nm absorbance ratio. An aliquot of total RNA was reverse transcribed to generate cDNA using oligo-$(dT)_{15}$ primers (Promega, USA) and M-MuLV reverse transcriptase (Promega, USA) as described by the manufacturers. Gene expression was quantified by real-time PCR using the Stratagene Mx3000P qPCR system (Stratagene, USA) and SYBR Green PCR Master Mix reagent kits (Takara, Dalian, China) as described by the manufacturers. Rat-specific primers were designed for the following genes of interest: luteinizing hormone receptor (LHR), scavenger receptor class B type 1 (SR-B1), steroidogenic acute regulatory protein (StAR), cholesterol side-chain cleavage enzyme (P_{450}SCC), 3-beta-hydroxysteroid dehydrogenase (3β-HSD), cytochrome P_{450}C17 subfamily a (CYP17a), 17-beta-hydroxysteroid dehydrogenase (17β-HSD), aromatase, estrogen receptors α and β (ER-α and ER-β) and follicle-stimulating hormone receptor (FSHR) (Table 1). Amplification products were all within the 100–200-base-pair range. The housekeeping gene, β-actin, was used as an internal control. Cycling conditions were as follows: 95 °C for 10 min followed by 40 cycles of 94 °C for 5 s, 53–57 °C for 15 s, and 72 °C for 10 s. After PCR, all products exhibited a single peak in melting curves and were identified as single bands of the appropriate size on ethidium bromide gels. A C_t value, representing the first cycle at which the fluorescence signal is significantly above background, was obtained for each amplification reaction. Each sample was analyzed in duplicate. The relative expression ration (R) of a target gene is calculated based on the description of Barlow et al. [28].

2.8. Statistical analysis

All data were analyzed using SPSS for Windows 13.0 Software (SPSS, Inc., Chicago, IL). All values are expressed as mean ± SEM. The ratio of ovary or uterus to body weight was calculated to yield relative organ weights. The normality of the data was analyzed by means of a Shapiro-Wilk test. Significant differences were analyzed using a one-way ANOVA followed by a Dunnett's post hoc two-sided *t*-test between treatment and control groups. Differences in body weight at VO and first estrous cycle were analyzed by a general linear model, adjusted for age, as previously described [29]. In all experiments, the number of follicles in each stage was obtained by individually totaling the number of primordial, primary, preantral and antral follicles in each section. A probability (p) of less than 0.05 was chosen as the limit for statistical significance.

3. Results

3.1. Weight and general toxicity

None of the PFDoA-exposed animals showed visible signs of toxicity in the present study. As shown in Table 2, no significant changes in mean body weight were observed for females exposed to 0.5 mg or 1.5 mg PFDoA/kg-d compared with controls. However, for females exposed to 3 mg PFDoA/kg-d, the mean body weight was markedly decreased following 28 days exposure ($p < 0.05$). There were no significant differences in the absolute or relative weights of ovary or uterus at any PFDoA concentration. There were also no

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

Target gene	GenBank accession no.	Product length (bp)	Primer sequences	T_m (°C)
SR-B1	AY451993	156	Sense: 5′-ACAGGTCCCAGGGCTCAG-3′ Anti-sense: 5′-CGTGCGGTTCATAAAGG-3′	57.0
LHR	NM_012978	130	Sense: 5′-CATTCAATGGGACGACTCTA-3′ Anti-sense: 5′-GCCTGCAATTTGGTGGA-3′	53.0
StAR	NM_031558	111	Sense: 5′-GGGCATACTCAACAACCAG-3′ Anti-sense: 5′-ACCTCCAGTCGGAACACC-3′	57.0
P_{450}SCC	J05156	115	Sense: 5′-CTTTGGTGCAGGTGGCTAG-3′ Anti-sense: 5′-CGGAAGTGCGTGGTGTTT-3′	57.0
3β-HSD	M38178	145	Sense: 5′-TGTGCCAGCCTTCATCTAC-3′ Anti-sense: 5′-CTTCTCGGCCATCCTTTT-3′	53.0
Cyp17a	NM_012753	114	Sense: 5′-CTCTGGGCACTGCATCAC-3′ Anti-sense: ′-CAAGTAACTCTGCGTGGGT-3′	53.0
17β-HSD1	NM_012851	115	Sense: 5′-GGTTATGAGCAAGCCCTGAG-3′ Anti-sense: 5′-GGAAGCGGTTTGTGGAGAA-3′	57.0
Aromatase	NM_017085	142	Sense: 5′-GCCTGTCGTGGACTTGGT-3′ Anti-sense: 5′-GGTAAATTCATTGGGCTTGG-3′	57.0
ER-α	NM_012689	146	Sense: 5′-GACTCGCTACTGTGCTGTG-3′ Anti-sense: 5′-CGATGGTGCATTGGTTT-3′	53.0
ER-β	NM_012754	200	Sense: 5′-TGTGCCAGCCCTGTTACTA-3′ Anti-sense: 5′-TTACGCCGGTTCTTGTCTA-3′	53.0
FSHR	NM_199237	109	Sense: 5′-TGACAGCCATCACCCTAG-3′ Anti-sense: 5′-AAAGTCCAGCCCAATACC-3′	54.0
β-actin	NM_031144	134	Sense: 5′-TCGTGCGTGACATTAAAGAG-3′ Anti-sense: 5′-ATTGCCGATAGTGATGACCT-3′	53.0–57.0

statistically significant differences in daily food intake between any treatment group and controls (data not shown).

3.2. Pubertal development and endocrine status

As shown in Table 3, there were no significant differences in the age or body weight at VO and first estrous cycle between control and PFDoA-exposed females. During the experiment, no rats exhibited irregular estrous cycles and the total number of each estrous cycle stage from the first cycle to necropsy did not differ markedly between the control and treatment groups.

3.3. Histological analysis and evaluation of follicle populations

No abnormal ovarian or uterine structures were evident in PFDoA-exposed rats (data not shown), and the ovaries of female

Table 2
Body weight and organ weight of female rats treated with PFDoA for 28 days.

Parameter	PFDoA (mg/kg-d)			
	0	0.5	1.5	3
BW (g)	182.5 ± 2.4	184.6 ± 2.9	185.9 ± 1.7	171.0 ± 4.1[a]
UW (mg)	255.88 ± 11.43	265.00 ± 13.89	312.25 ± 20.44	247.25 ± 10.13
RUW (mg/g)	1.40 ± 0.06	1.43 ± 0.07	1.68 ± 0.10	1.45 ± 0.07
OW (mg)	112.25 ± 5.77	115.38 ± 4.85	109.00 ± 5.94	101.13 ± 5.81
ROW (mg/g)	0.62 ± 0.04	0.630 ± 0.03	0.58 ± 0.03	0.59 ± 0.04

Note: *Abbreviations*: BW, Body weight; UW, uterus weight; RUW, relative uterus weight; OW, ovary weight; ROW, relative ovary weight. Data are presented as mean ± SEM (n = 8 rats/group). Significance was analyzed using a one-way ANOVA followed by a Dunnett's post hoc two-sided t-test between treatment and control groups.
[a] Indicates the significant difference between exposure group and control ($p < 0.05$).

Table 3
Pubertal development of female rats exposed to PFDoA for 28 days.

Parameter	PFDoA (mg/kg-d)			
	0	0.5	1.5	3
Age at vaginal opening (day)	30.5 ± 0.5	31.0 ± 0.5	29.8 ± 0.5	30.3 ± 0.7
BW at vaginal opening[a] (g)	112.4 ± 4.3	109.0 ± 3.0	103.8 ± 3.8	109.6 ± 1.8
Age at first estrous (day)	31.6 ± 0.3	32.1 ± 0.4	30.8 ± 0.31	31.4 ± 0.4
BW at first estrous[a] (g)	125.6 ± 4.2	120.0 ± 3.0	116.8 ± 3.8	122.6 ± 1.8
No. of proestrous	4.5 ± 0.2	4.5 ± 0.2	4.9 ± 0.1	4.8 ± 0.2
No. of estrous	4.5 ± 0.2	5.1 ± 0.1	4.9 ± 0.1	4.8 ± 0.2
No. of diestrous	4.9 ± 0.1	5.2 ± 0.1	5.5 ± 0.2	5.4 ± 0.2

Note: Data are presented as mean ± SEM (n = 8 rats/group).
[a] BW, Body weight. Significance of body weight differences was analyzed using a general linear model, adjusted for age; differences among other parameters were evaluated using a one-way ANOVA followed by a Dunnett's post hoc two-sided t-test between treatment and control groups.

Table 4
Follicular number at different stages in female rats exposed to PFDoA for 28 days.

Follicle	PFDoA (mg/kg-d)			
	0	0.5	1.5	3
No. of primordial follicles	55.3 ± 4.3	57.3 ± 5.9	50.0 ± 6.4	54.0 ± 10.0
No. of primary follicles	61.0 ± 6.0	57.7 ± 3.4	54.3 ± 4.2	51.0 ± 5.3
No. of preantral follicles	39.3 ± 4.3	33.3 ± 3.0	35.3 ± 3.2	36.7 ± 0.9
No. of antral follicles	50.0 ± 5.5	49.3 ± 5.4	54.0 ± 5.5	52.7 ± 4.6

Note: Data are presented mean ± SEM ($n = 3$ rats/group). Significance was analyzed using a one-way ANOVA followed by a Dunnett's post hoc two-sided *t*-test.

rats in all groups had a normal complement of growing follicles and corpus luteum. After exposing to PFDoA for 28 days, rats in control and treatment groups exhibited no significant differences in the number of primordial, primary, preantral or antral follicles in the ovary (Table 4).

3.4. Serum cholesterol and hormone levels

The effects of PFDoA exposure on serum cholesterol and hormone levels are shown in Fig. 1. At a dose of 3 mg/kg-d, PFDoA treatment induced a significant increase in serum cholesterol levels in females compared with untreated rats ($p < 0.05$), whereas PFDoA at 0.5 or 1.5 mg/kg-d had no effect. The levels of the sex hormones, FSH and LH, in serum were also unchanged in rats exposed to PFDoA for 28 days compared with controls. However, there was a significant decline in serum estradiol levels in rats exposed to 3 mg PFDoA/kg-d ($p < 0.05$), in which estradiol levels were reduced to approximately 60% those of controls. At doses of 0.5 and 1.5 mg PFDoA/kg-d, there were no marked differences in circulating estradiol between control and treatment groups.

3.5. Analysis of gene expression

The effects of PFDoA exposure on gene expression in rat ovaries are shown in Fig. 2. Following exposure of pubertal female rats to PFDoA for 28 days, there were no significant alterations in the levels of SR-B1, 3β-HSD, aromatase or FSHR mRNA in the ovaries of treated rats compared to control rats. PFDoA significantly decreased the levels of LHR (66% of control), StAR (72% of control) and P_{450}SCC (62% of control) mRNA at a concentration of 3 mg/kg-d ($p < 0.05$), but had no effect at 0.5 or 1.5 mg/kg-d. The expression of 17β-HSD was markedly increased in the ovaries of PFDoA-treated rats over that in controls at doses of 0.5, 1.5 and 3 mg/kg-d ($p < 0.05$). PFDoA exposure also significantly decreased ER-α mRNA levels at doses of 1.5 and 3 mg/kg-d, whereas ER-β expression was only decreased at 3 mg PFDoA/kg-d ($p < 0.05$). Expression of ER-α in the uterus was relatively constant across all groups, and uterine expression of ER-β exhibited a significant decline only at dose of 0.5 mg PFDoA/kg-d ($p < 0.05$). Neither ovarian nor uterine FSHR expression was affected by PFDoA at any dosage (Fig. 3).

4. Discussion

In the current study, we showed that PFDoA exposure for 28 days did not affect the histological morphology of ovaries or uteri, or alter the onset of puberty (e.g., age at first estrous cycle) or endocrine status in female rats. However, at the highest dose (3 mg/kg-d), PFDoA led to a decrease in serum estrogen levels accompanied by changes in serum total cholesterol levels and ovarian expression of genes responsible for cholesterol transport and steroidogenesis. The expression of ER-α and ER-β in the ovary was also affected by PFDoA treatment in females. These observations suggest that high-dose PFDoA may disrupt estrogen biosynthesis and estrogen signal transduction at the molecular level in the ovary. Our data are the first to demonstrate that PFDoA exposure may affect the expression

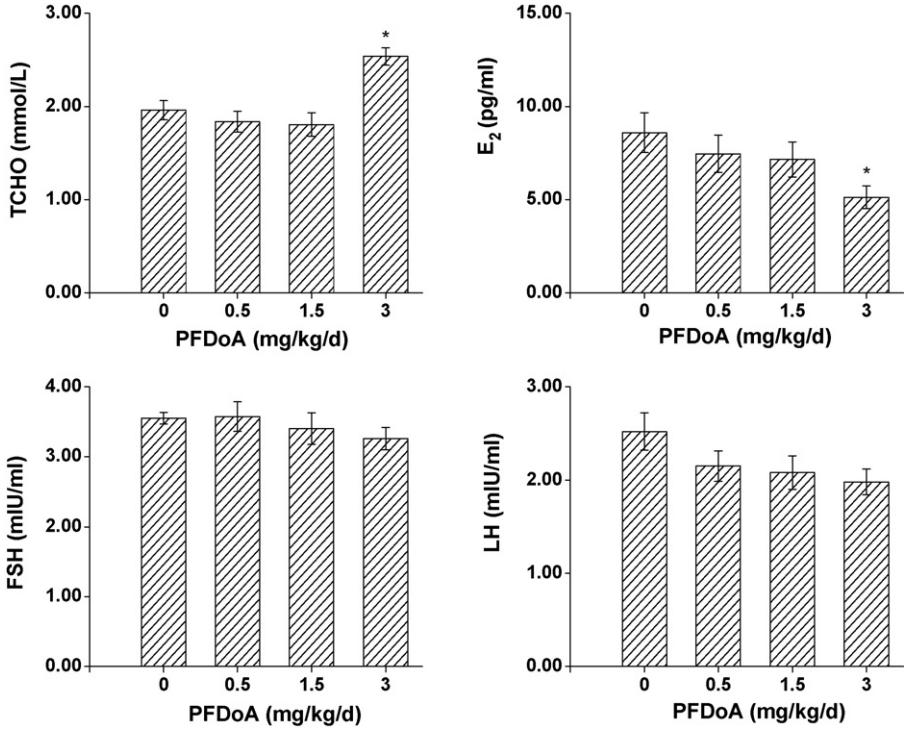

Fig. 1. Serum levels of total cholesterol (TCHO), estradiol (E_2), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in control and PFDoA-exposed female rats. Values represent the mean ± SEM ($n = 8$ rats/group). Significance was analyzed using a one-way ANOVA followed by a Dunnett's post hoc two-sided *t*-test between treatment and control groups. Asterisk (*) indicates a significant difference between exposure group and control ($p < 0.05$).

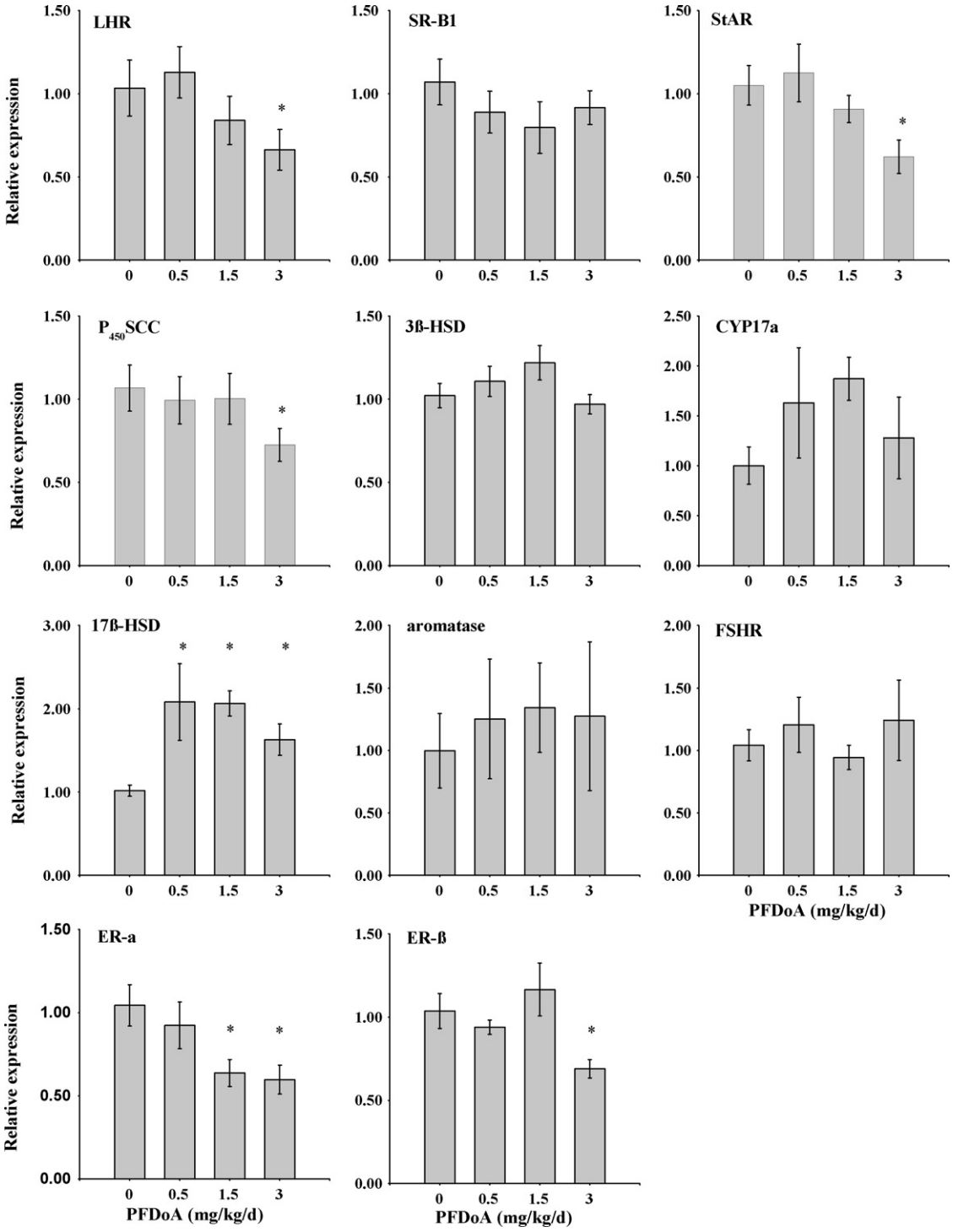

Fig. 2. Real-time quantitative RT-PCR analyses of ovarian mRNA for luteinizing hormone receptor (LHR), scavenger receptor class B type 1 (SR-B1), steroidogenic acute regulatory protein (StAR), cholesterol side-chain cleavage enzyme (P_{450}SCC), 3-beta-hydroxysteroid dehydrogenase (3β-HSD), cytochrome P450c17 subfamily a (CYP17a), 17-beta-hydroxysteroid dehydrogenase (17β-HSD), aromatase, estrogen receptor α or β (ER-α or ER-β) and follicle-stimulating hormone receptor (FSHR) in control and PFDoA-exposed female rats. Values represent the mean ± SEM ($n = 8$ rats/group). Gene expression levels correspond to β-actin-normalized mRNA expression relative to controls. Significance was analyzed using a one-way ANOVA followed by a Dunnett's post hoc two-sided *t*-test between treatment and control groups. Asterisk (*) indicates a significant difference between exposure group and control ($p < 0.05$).

of genes related to estrogen production and estrogen signaling in pubertal female rats.

PFDoA exposure at a dose of 3 mg/kg-d decreased the body weight of female rats but did not influence food intake, demonstrating that the reduction in body weight was not related to food consumption. We speculate that this adverse effect might be caused by the direct action of PFDoA in this experiment. These results contrast with other studies that reported that body weight was reduced in conjunction with a reduction in food intake in PFOA or PFOS-treated rats [1,18]. These apparent discrepancies in the relationship between food consumption and body weight may reflect differences in the effects of PFDoA on body metabolism compared to those of PFOA or PFOS. In addition, PFDoA did not affect the absolute or relative weight of ovaries or uteri at any dose tested, suggesting

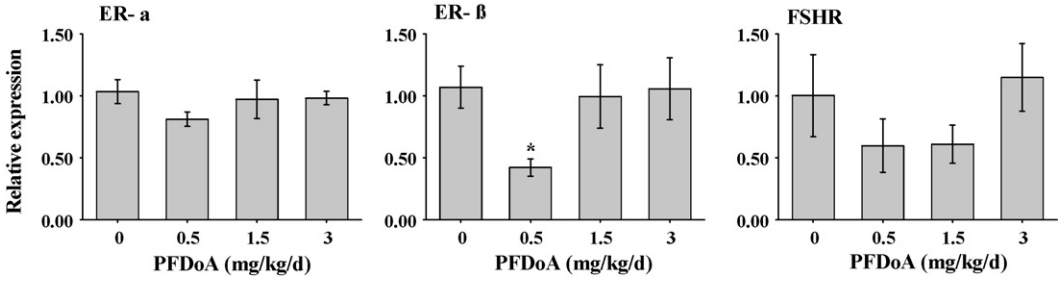

Fig. 3. Real-time quantitative RT-PCR analyses of uterine mRNA for estrogen receptor α or β (ER-α or ER-β) and follicle-stimulating hormone receptor (FSHR) in control and PFDoA-exposed female rats. Gene expression levels correspond to β-actin-normalized mRNA expression relative to controls. Values represent the mean ± SEM ($n = 8$ rats/group). Significance was analyzed using a one-way ANOVA followed by a Dunnett's post hoc two-sided t-test between treatment and control groups. Asterisk (*) indicates a significant difference between exposure group and control ($p < 0.05$).

these endpoints may not be sensitive to PFDoA in pubertal female rats.

VO is a reliable index of puberty development in female rats and usually occurs on the day after the first preovulatory surge of gonadotropins [30]. We thus used VO and the first estrous cycle as measures of the end of puberty onset. Estrous cycles may reflect endocrine status in female rats and are highly correlated with the endocrine hormones, FSH and LH. In this study, the absence of any significant change in the ages at VO or first estrous observed across all groups suggests that exposure of pre-pubertal female rats to PFDoA does not affect puberty onset. Moreover, PFDoA did not lead to irregular estrous cycles or changes in the total number of any estrous cycle stages during the experiment, indicating that PFDoA does not have general adverse effects on endocrine status. These findings may be explained by the maintenance of relatively stable serum LH and FSH levels across all groups, since these two hormones are necessary for maintaining normal estrous cycles in females. FSH is a crucial hormone in the stimulation of both follicle growth and differentiation, and thus plays a pivotal role in luteo-follicular transition [31]. The LH surge is essential for initiating ovulation in the mature follicle [32]. Although LHR mRNA levels were decreased by exposure to 3 mg PFDoA/kg-d, this decrease apparently did not affect LH activity or cause irregular cycling in females. The absence of an effect of PFDoA on the estrous cycles is consistent with the report of Luebker et al. [19], which showed that PFOS administered via oral gavage for 28 days produced no significant estrous cycle changes in adult female rats. However, our results contrast with those of Austin et al. [18], who reported that intraperitoneal administration of 1 and 10 mg/kg-d PFOS to adult female rats for 2 weeks decreased the percentage of rats exhibiting regular estrous cycles to approximately 66% and 42% of controls, respectively, and increased the percentage of rats with persistent diestrous. The discrepancy between these two studies may be attributable to differences in chemical properties of PFOS and PFDoA, dosage, and/or animal ages. Differences in the route of administration may have also contributed to different physiological responses, with intraperitoneal PFOS and oral PFDoA producing different chemical burdens in the body that differentially affected estrous cycling.

The size and number of follicles in the ovary is related to changes in serum FSH concentration, reflecting the function of FSH in promoting the growth of preovulatory follicles [33,34]. Our finding of no obvious changes in the number of follicles at different stages in PFDoA-treated rats also implies that PFDoA exposure might not affect follicle development and transition in the ovary. Estrogen also plays an important role in follicular growth. However, our finding that the decrease in serum estradiol induced by 3 mg PFDoA/kg-d was not accompanied by a change in the number of primordial, primary, preantral or antral follicles in the ovary indicates that the extent of the estradiol decrease was not sufficient to affect follicular growth and development. Because estrogen is principally produced and secreted by follicles, the estrogen level in the serum of females is highly correlated with follicle number. Thus, the absence of a change in the number of follicles following PFDoA exposure also rules out the possibility that the decrease in serum estradiol is due to a decrease in follicle number. Taken together, these data suggest that exposure to 3 mg PFDoA/kg-d decreases circulating estradiol levels by reducing the ability of individual follicles to produce estrogen. In addition, the hypothalamus and pituitary are responsible for regulating the synthesis of both LH and FSH, which may, in turn, regulate ovarian function, including estrogen biosynthesis [31]. The fact that serum LH and FSH levels remained unchanged in all groups demonstrates that 3 mg/kg-d PFDoA did not disrupt estradiol production in pubertal female rats indirectly through effects on the activity of the pituitary and hypothalamus. The fact that FSHR mRNA levels in the ovary and uterus were not altered by PFDoA treatment, as noted above, provides further evidence that FSH signaling was not involved in the observed effects on estrogen production.

To explore other possible mechanisms to explain the inhibition of estrogen production at the highest PFDoA dosage, we measured the levels of serum cholesterol, the precursor in steroid biosynthesis, and analyzed mRNA expression levels for key genes involved in cholesterol transport and steriodogenesis in the ovary. The concentration of serum cholesterol remained unchanged after exposure to 0.5 or 1.5 mg/kg-d PFDoA compared to controls, but was significantly increased at 3 mg/kg-d PFDoA. Since the liver has been shown to be a target organ of PFAAs' action [1], it is possible that increased cholesterol levels at the highest dose of PFDoA could be related to the disruption of cholesterol metabolism in the liver. In a previous study on adult male rats, we also observed that cholesterol levels were elevated after exposure to PFDoA for 14 days, and further showed that this increase was associated with abnormal fatty acid metabolism in the liver [12]. However, our results are contrast to serum cholesterol reduction in rats exposed to PFOA or PFOS [1]. This discrepancy may be associated with the difference in molecular structure characteristics among different PFAAs, such as carbon chain length. Collectively, our results are inconsistent with PFDoA inhibiting estradiol biosynthesis through effects on cholesterol production; if anything, the PFDoA effects on cholesterol production shown here would be predicted to increase estradiol synthesis.

Cholesterol transport processes include transfer of cholesteryl esters in serum into ovarian cells by the cholesterol transporter, SR-B1 [35], and then intracellular transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane by StAR. This latter step is the rate-limiting regulated step in steroidogenesis [36], and decreased StAR expression often leads to the disruption of cholesterol transportation within ovarian cells and inhibition of estrogen biosynthesis. In the current study, SR-B1 mRNA levels in the ovary were not altered by any

dosage of PFDoA tested, suggesting that PFDoA may not inhibit the transportation of cholesterol from serum into ovarian cells. However, ovarian StAR expression was significantly decreased by PFDoA at 3 mg/kg-d, suggesting that PFDoA may reduce estradiol synthesis in ovarian cells by inhibiting StAR-mediated transport of cholesterol across the mitochondrial membrane. In male rats, we observed a similar decrease in StAR expression after exposure to PFDoA (5 or 10 mg/kg-d) for 14 days [17], consistent with the idea that StAR may be a sensitive target for PFDoA action. LH signaling through its receptor, LHR, plays an important role in stimulating StAR expression [37]. In the current study, we found that LHR mRNA expression, but not the level of serum LH, was decreased by PFDoA at 3 mg/kg-d, suggesting that a reduction in LHR expression may contribute to StAR mRNA suppression. Although additional studies will be required to clarify the exact mechanism by which PFDoA inhibits ovarian StAR expression in female rats, this reduction in LHR and StAR expression may decrease cholesterol transport capacity and disrupt StAR-mediated delivery of cholesterol in ovarian cells.

P_{450}SCC is a rate limiting enzyme responsible for the conversion of cholesterol to pregnenolone in estrogen biosynthesis. In this study, P_{450}SCC mRNA levels were significantly decreased by PFDoA at a dose of 3 mg/kg-d, suggesting that the reduction in P_{450}SCC expression may contribute, along with decreased expression of LHR and StAR, to the inhibition of estradiol production. Decreased expression of P_{450}SCC may be partially associated with the reduced expression of LHR and StAR as the expression of these two genes responsible for conversion of cholesterol to the next intermediate may have been down-regulated.

Other genes, including 3β-HSD, CYP17a, 17β-HSD1 and aromatase, also play essential roles in estrogen biosynthesis in females [38]. In the ovary, 3β-HSD is responsible for catalyzing the conversion of pregnenolone to progesterone, which is subsequently converted to hydroxyprogesterone and androstenedione by CYP17a. Aromatase catalyzes the aromatization of androstenedione to estrone, which is then converted to estradiol, a step catalyzed by 17β-HSD1. In this study, 3β-HSD and CYP17a expression was relatively constant across all groups, implying that the products of these two genes did not likely contribute to the decrease in estradiol levels induced by exposure to 3 mg/kg-d PFDoA. Elevated 17β-HSD1 expression and activity may stimulate estradiol production [38,39]. Thus, the finding that 17β-HSD1 expression was significantly enhanced in females following exposure to 0.5, 1.5 or 3 mg/kg-d PFDoA was unexpected. However, estradiol has been shown to exhibit an inhibitory effect on 17β-HSD1 expression in aromatase knockout (ArKO) mice [40], leading us to speculate that decreased circulating estradiol in the 3 mg/kg-d PFDoA group may feedback to stimulate 17β-HSD1 mRNA expression, at least at this higher PFDoA dosage. But 17β-HSD1 expression was also increased at lower PFDoA dosages that had no effect on serum estradiol levels, raising the possibility that increased 17β-HSD1 mRNA levels in all PFDoA-treated groups might result from a combination of various factors. Among the factors that have been shown to regulate 17β-HSD1 expression are FSH, human chorionic gonadotrophin, retinoic acids and activin-A [41–43]. Clearly, additional studies employing multiple experimental strategies will be needed to elucidate the mechanisms by which PFDoA induces 17β-HSD1 expression. Consistent with the absence of an effect of PFDoA on serum FSH levels and FSHR mRNA expression, mRNA for aromatase, an FSH-dependent cytochrome P-450 enzyme involved in estradiol synthesis, was unchanged in pubertal female rats. In male rats, no significant changes in aromatase mRNA levels were observed after a 14-day exposure to PFDoA dosages of 1, 5 or 10 mg/kg-d [17]. Taken together, these findings suggest that aromatase is not a target of PFDoA action; thus, this enzyme is not likely to be responsible for decreased estradiol in the 3 mg/kg-d group.

Estradiol is known to act mainly through binding to ER-α and ER-β in the rodent ovary and uterus [44]. The observation that PFDoA significantly reduced both ER-α and ER-β expression in the ovaries of female rats suggests that PFDoA may alter ovarian estrogen signal transduction at the molecular and cellular levels. Moreover, the effects of PFDoA on ER-α mediated signaling may be greater than those of ER-β in the ovary, as evidenced by the fact that ER-α mRNA expression was decreased following exposure to 1.5 or 3 mg/kg-d PFDoA, but ER-β expression was only decreased at 3 mg/kg-d. In addition, estrogen may regulate its receptors expressions by negative feedback manner and sustain the balance between estrogen and its receptors, in turn, this balance may maintain estrogen homeostasis [45,46]. The changes in mRNA levels for both ovarian ER forms imply that the balance between estrogen and its receptors has been disrupted by PFDoA. Thus, the imbalance may contribute to the decreased estradiol levels at 3 mg/kg-d PFDoA. However, in the uterus, only ER-β expression was significantly decreased by PFDoA (0.5 mg/kg-d). The difference in the ER-α and ER-β expression pattern between ovary and uterus implies that PFDoA may act through different mechanisms to modulate ER expression in these two tissues.

In conclusion, the present data extend our understanding of the effects of PFDoA exposure on endocrine status and gene expression to a consideration of estradiol production in pubertal female rats. Although the serum level of estradiol in female rats was significantly decreased by PFDoA at 3 mg/kg-d, biological meaningful changes in estrous cycles, follicle number at different stages or ovarian or uterine histology were not observed. Since PFDoA inhibited the expression of ovarian LHR, StAR and P_{450}SCC, the changes in the level of mRNA for these three genes may have contributed to the decrease in estradiol production observed in the highest dose group. In addition, the imbalance between estrogen and its ovarian receptors may also play a role in estradiol inhibition at the highest concentration of PFDoA. The reduction in ovarian ER-α and ER-β mRNA levels at the highest dose implies that PFDoA may disrupt estrogen signal transduction in the ovaries of pubertal rats.

Conflict of interest

The authors declare that there are no conflicts of interest.

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