Biphasic effects of perfluorooctanoic acid on steroidogenesis in mouse Leydig tumour cells

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ABSTRACT

Perfluorooctanoic acid (PFOA) is a persistent organic pollutant, which may possess endocrine disrupting properties. Herein, we investigated the possible mechanism(s) of toxicity and steroidogenesis in mouse Leydig cells. MLTC-1 (mouse Leydig tumour cells) cells were exposed to 0, 50, 100 or 200 μM PFOA for 48 h to ascertain their effects on the nuclear (membrane) receptor responses, steroidogenesis pathway and related regulated gene expression and steroid hormone secretion profiles. Our results reveal that nuclear receptors PXR, SR-B1 and LHR are sensitive to PFOA exposure. PFOA can accumulate in mitochondria and alter cholesterol precursor (fatty acid) mitochondrial transport process-related gene expression and thus inhibit steroid hormone precursor (cholesterol) production. In particular, PFOA exhibits biphasic effects on testosterone and progesterone production at differing levels of exposure. These findings indicate the potential endocrine-related effects of PFOA on steroid hormone secretion in Leydig cells and point to a novel disruption model.

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1. Introduction

Perfluorinated compounds (PFCs) are a group of synthetic chemical substances consisting of carbon-fluorine bonds, and well known for their uses in a wide range of industrial applications due to their unique properties of stability, lipophobicity and hydrophobicity. In recent years, widespread distribution of PFCs into different environmental matrices has become an important concern due to their bioaccumulation in different tissues of humans and wildlife [1]. Given its longer half-life, perfluorooctanoic acid (PFOA) is one of the most widely reported PFCs in exposed biological species [1]. The general population has both PFOA and perfluorooctanesulfonic acid (PFOS) typically present at blood concentrations ranging from approximately 10–100 nM [2,3]. However, levels of PFOA in serum of occupationally-exposed workers can be 10 μM or higher [2,4].

Recently, it has been suggested that PFOA might cause several health effects in animals and humans, including reproductive impairments, neurological disorders, liver toxicity and development abnormalities [5–7]. An in vivo animal toxicity study has shown that PFOA has the ability to cause several types of tumours, including in Leydig cells [8]. In particular, PFOA has been considered a potential endocrine disrupting chemical, causing male reproductive system-related abnormalities. Taking this into consideration, many studies have reported that PFOA may interrupt sex hormone functions either by decreasing serum testosterone (T) levels and/or increasing serum oestradiol (E2) levels in rodents [9,10] and increasing serum testosterone and oestrone levels in fish species [11], disruption of gonad development in male fish [12], and altered human and rat steroidogenic enzyme activities [9,10,13]. That said, human epidemiology studies into the relationship between PFOA concentrations and hormone levels in humans have been inconsistent. Some studies suggest a negative association of PFOA levels with serum total testosterone and free testosterone, and a positive association with oestradiol [14–16]. In contrast, a positive relationship between total testosterone with concentrations of PFOA have also been reported [17], while no such associations were found in human epidemiology or in vitro toxicology studies [18,19]. Similarly, a similarly ambiguous association of PFOA levels and semen quality has also been documented. For example, lower sperm concentrations and total sperm count per ejaculate were associated with in utero PFOA exposure levels [20]. High levels of PFOA were also associated with reduced numbers
of normal human sperm [21]. Contrary to this, some studies suggest that there is no correlation between PFOA and human semen quality, including sperm concentration, count, volume, motility and morphology [21,22].

Studies investigating the impact of PFOA on male reproductive health are controversial. Further studies will need to be undertaken to clarify the biological mechanisms underlying PFOA endocrine disruption. Testicular Leydig cells are the primary source of steroid hormone in the male. Steroid hormone production starts with cholesterol, which is converted into an intermediate prior to generation of the end product sex hormone, testosterone [23]. Mouse Leydig tumour cells (MLTC-1) are a useful model to study effects on steroidogenesis because of their steroidogenesis potency. In vivo and in vitro experiments also suggest that mouse Leydig cells appear to be more similar to human Leydig cells in their responses to environmental exposure than are those of the rat [24]. Consequently, mouse Leydig MLTC-1 cells were selected for the in vitro model towards assessment of PFOA endocrine disruption.

The aim of this study is to improve our understanding of PFOA-induced endocrine disruption via molecular initiating events (receptor response and endpoints (steroid hormone secretion) related to reproductive toxicity, by using mouse in vitro Leydig MLTC-1 cells.

2. Materials and methods

2.1. Chemicals and reagents

PFOA (C8F15O2H; Chemical Abstract Service, no. 335-67-1; purity > 96%) and isotope PFOA13C8 was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). The cells were grown at 37 °C USA). The cells were grown at 37 °C, 5% CO2 in humidified incubator (SANYO, Japan).

Cell viability was evaluated by the MTT proliferation assay. Cells were plated at a density of 1.5 × 104 per well in 96-well plates. After 48-h incubation at different concentrations of PFOA (0–300 μM), 50 μM MTT (5 mg/ml) was added to each well and the cells were incubated for 4 h at 37°C. Untreated cells were used as a negative control. The medium was removed and 150 μL DMSO was added to each well and gentle shaking was then performed for 10 min. Absorbance was determined at 490 nm. Four replicates for each PFOA exposure were performed. Results were presented as percentage of the values measured in untreated control cells. To ensure absence of cytotoxicity, the concentrations 50 μM, 100 μM, and 200 μM were selected for the following PFOA exposure experiments.

2.3. PFOA treatment

MLTC-1 cells were seeded in 6-cm petri dishes and cultured for 24 h prior to treatment. PFOA was dissolved in DMSO. Cells were exposed to 50 μM, 100 μM or 200 μM PFOA for 48 h, with DMSO (0.1%) alone employed as a vehicle control. Four replicates for each dose of PFOA exposure were performed. Then, the cells were washed with PBS and serum-free medium. Subsequently, cells were stimulated for 4 h with hCG in serum-free medium supplemented with 0.1% BSA. The medium was collected for progesterone and testosterone determination, and the cells for cholesterol measurements and other biochemical assays.

2.4. RNA extraction and quantitative real-time RT PCR analysis

To determine mRNA expression levels, a quantitative real-time RT PCR assay was performed. Total RNA was extracted from the cells using an RNA extraction kit (Promega, USA), following the manufacturer’s protocol. Extracted RNA samples were stored at −80 °C, for subsequent use.
analyses. The NanoDrop spectrophotometer (NanoDrop Technologies Inc., USA) was used to measure RNA concentration and purity.

Reverse transcription of cDNA synthesis was performed with 1 µg total RNA using PrimeScript® RT reagent Kit with gDNA Eraser cDNA synthesis Kits (Takara, Japan) employing oligo dT primer. Real-time RT PCR was carried out in a 20 µL final volume and performed in triplicate using SYBR Green Master Mix reagents (Roche, USA) in a LC 480 system (Roche Applied Science, Germany), according to the manufacturer’s protocol. Primer sets and product sizes used for amplification PCR analysis are shown as Table 1. The conditions for quantitative PCR were as follows: 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s, and 60 °C for 30 s. Gene expression levels were normalized to GAPDH expression levels. Three replicates of quantitative PCR were performed for each sample. Four replicates for each dose of PFOA exposure were performed. The fold changes of the tested genes were determined by the 2^−ΔΔCt algorithm approach.

2.5. Cholesterol, progesterone and testosterone determination

Total cholesterol (TCHO) content in MLTC-1 cells in the control and PFOA groups were measured using commercial kits according to the manufacturer’s instructions (Beihua Kangtai Clinical Reagent, China). Total cholesterol concentration was normalized to Leydig cell protein concentration. Steroid hormones testosterone and progesterone levels in cell culture medium were detected by LC-ESI-MS/MS. In brief, each sample of 1 mL medium was diluted with 3 mL of ammonium acetate buffer (1 mol/L), and 20 µL of 100 ng/mL D3-testosterone internal standard was added (progesterone was semi-quantified). Then the diluted samples were extracted by adding 3 mL ethyl acetate and vortexed vigorously for 15 s in a glass tube. The liquid-liquid extraction was repeated three times. The following phase separation was completed by centrifugation at 1500 rpm for 10 min. The ether phase was transferred to another glass tube with a Pasteur pipette. The three times extract was combined and washed with 5 mL water, then the combined extracts were evaporated under a gentle stream of nitrogen gas at 40 °C. The residue was re-suspended in 0.5 mL of methanol/water (50:50, v:v) by vortexing vigorously for 15 s and transferred into a HPLC vial. The sample residue was re-suspended in 0.5 mL of methanol/water (50:50, v:v) before analysis. Calibration standards and QC samples were analysed concurrently with unknown samples using the same sample preparation procedure.

2.7. Statistical analysis

Measurement data of cholesterol, progesterone, testosterone levels and gene expression by real-time RT PCR analysis were analysed using SPSS for Windows 11.5 Software (SPSS, Inc., Chicago, IL) and were presented as mean with standard errors (mean ± SE). In each experiment there were 3 reps per sample and 4 samples per group and all experiments were performed in duplicate. Data were analysed by one-way ANOVA with Tukey’s multiple comparisons test. Asterisks above columns indicate a significant difference in comparison to the control, **p ≤ 0.05, ***p ≤ 0.01.

3. Results

3.1. Cell viability assay

To analyse the effects of PFOA on MLTC-1 cell viability, the cells were treated with different doses (from 0 to 300 µM) of PFOA for 48 h. Results of cytotoxicity studies are shown in Fig. 1. Taking into account effects on cell viability, the PFOA concentrations were kept below the levels (i.e., 300 µM) at which significant lethal effects occurred. Exposure concentrations in subsequent experiments were as follows: 0, 50, 100 or 200 µM.

3.2. Nuclear (membrane) receptor responses to PFOA exposure

As nuclear (membrane) receptor is involved in environmental exposure and toxicological effects, transcript profiles of nuclear (membrane) receptor were investigated in MLTC-1 cells exposed to varying levels of PFOA. The mRNA expression of PPARα (PPAR-alpha; controls the peroxisomal β-oxidation pathway of fatty acids) was unaltered following PFOA exposure compared to control (Fig. 2). AR (androgen receptor; is activated by binding androgenic hormones and then regulates male sexual phenotype gene expression) gene exhibits significantly reduced expression following 50 or 100 µM PFOA, while no significant alteration is observed at the higher dose (i.e., 200 µM) PFOA treatment. PXR (Pregnane X receptor; regulates a broad range of genes involved in the transport, metabolism and elimination of foreign toxic substances) gene was significantly up-regulated in a dose-related fashion after PFOA exposure (p < 0.05) (Fig. 2). There was no

![Fig. 1. The viability of MLTC-1 cells exposed to various concentrations of PFOA (0–300 µM) for 48 h. The values are expressed as the means (± SE) of survival (% of control cells). Data were analysed by one-way ANOVA with Tukey’s multiple comparisons test. Asterisks above columns indicate a significant difference in comparison to the control, **p ≤ 0.05, ***p ≤ 0.01.](image-url)
significant alteration found at lower levels of PFOA (i.e., 50 μM) on LHR (luteinizing hormone receptor; allows Leydig cells to respond to luteinizing hormone that triggers these cells to produce androgens) gene expression. However, 100 μM PFOA significantly induced (p < 0.05) LHR expression (Fig. 2). However, significant down-regulation (p < 0.01) was observed following 200 μM PFOA exposure. Considering Fig. 2, it can be identified that SR-B1 (scavenger receptor B1; regulates cholesterol uptake) mRNA expression was significantly down-regulated (p < 0.01) in all PFOA exposure groups, noticeably following 200 μM PFOA which declined to 0.16 ± 0.04 folds compared to controls. SREBP2 (sterol regulatory element-binding protein 2; controls cholesterol homeostasis) expression was significantly decreased (0.74- to 0.51-fold compared to controls, p < 0.01; Fig. 2) in MLTC-1 cells with various concentrations of PFOA exposure, which is consistent with the
3.3. PFOA alters fatty acids transport into mitochondria

Considering PFOA’s structural similarity to endogenous fatty acids, the potential interactive-relationship of PFOA and fatty acids in mitochondria were investigated by investigating PFOA subcellular mitochondrial distribution (Fig. 3A) and then determining candidate gene expression coding for enzymes involved in fatty acids mitochondrial transport (Fig. 3B, C). Our results confirm that PFOA can transport into subcellular mitochondria via PFOA accumulation. Fig. 3A shows the subcellular accumulation of PFOA in cytoplasm and mitochondria. Following PFOA exposure, it was detected in both cytoplasmic and mitochondrial fractions. Following 100 μM PFOA exposure, the medium concentration of PFOA was 41.3 ± 1.1 ppm, the PFOA content was 129.0 ± 3.8 μg/g Pr (μg PFOA/g protein) in the cytoplasm and 6.4 ± 0.3 μg/g Pr in mitochondria, respectively. Meanwhile, the control group PFOA content was 0.09 ± 0.01 ppb, 1.0 ± 0.1 μg/g Pr, and 0.7 ± 0.1 μg/g Pr in medium, cytoplasm and mitochondria, respectively.

Meanwhile, CPT I (carnitine-palmitoyltransferase I) responsible for connecting carnitine to long-chain fatty acids, which facilitates them crossing the outer mitochondrial membrane, was significantly down-regulated (p < 0.05) (Fig. 3B, C). CACT (carnitine-acylcarnitine translocase), coding carnitine carrier protein, a component of the mitochondrial inner membrane and transfers fatty acylcarnitines into the mitochondria, was not significantly altered following different PFOA exposure levels (Fig. 3B, C). However, CPT II (carnitine-palmitoyltransferase II), coding for the inner mitochondrial membrane protein that converts acylcarnitine to acyl-CoA for further fatty acid metabolism, was significantly (p < 0.01) induced following 100 μM (1.34-fold) or 200 μM (1.69-fold) PFOA (Fig. 3B). Mitochondrial matrix enzyme CRAT (carnitine acetyltransferase), that catalyses the inter-conversion of acetyl-CoA and acetylcarnitine, was also significantly (p < 0.01) induced (1.71-fold) following 200 μM PFOA exposure (Fig. 3B, C).

3.4. PFOA disturbs cholesterol synthesis transcriptional profile and secretion

Cholesterol and steroid hormone biosynthesis is regulated by

SR-B1 alteration.

Fig. 4. PFOA affects cholesterol biosynthetic pathways gene expression and content in MLTC-1 cells. Heat map displays fold-changes of PFOA exposure on cholesterol biosynthetic pathways gene expression profiles (A), effects of PFOA on the cholesterol biosynthesis pathway gene expression in MLTC-1 cells in 200 μM treatment (B), and effects of PFOA exposure on cholesterol level in MLTC-1 cells (C). Colour scales range from bright red to bright green corresponding to up- or down-regulation of gene expression, respectively. In each experiment there were 3 reps per sample and 4 samples per group and all experiments were performed in duplicate. Data were analysed by one-way ANOVA with Tukey’s multiple comparisons test. Asterisks above columns indicate a significant difference in comparison to the control, *p ≤ 0.05, **p ≤ 0.01.
steroidogenic genes. The effects of PFOA exposure on expression of genes involved in cholesterol biosynthesis or steroidogenesis in MLTC-1 were determined (Fig. 4 and Table 2). Quantitative real-time RT-PCR assays results show that cholesterol biosynthesis pathway-related genes expression were down-regulated (0.29-0.87-fold) significantly ($p < 0.01$) in the 200 μM PFOA-treated group compared to vehicle control. Interestingly, 50 μM PFOA exposure significantly ($p < 0.05$) induced MVK (1.52-fold), PMVK (1.52-fold), MVD (1.23-fold), FOPS (1.35-fold) and CYP5 (1.74-fold) gene expression (Fig. 4, Table 2). Especially, HMGCR, coding the enzyme involved in mevalonate synthesis and is rate-limiting in the cholesterol synthesis pathway, was significantly down-regulated in a dose-related manner after PFOA exposure: 0.85-, 0.82-, and 0.36-fold for 50, 100 and 200 μM PFOA-treatment groups, respectively (Fig. 4, Table 2). Moreover, the effects of PFOA on cholesterol levels in MLTC-1 cells are shown as Fig. 4C. Cholesterol was markedly decreased ($p < 0.05$) to 0.83-, 0.87- and 0.85-fold of control following exposures of 50, 100 and 200 μM PFOA, respectively.

3.5. Effects of PFOA on steroidogenesis pathway gene expression and steroid hormone secretion

The expression of genes involved in steroidogenesis were significantly ($p < 0.01$) decreased (0.37–0.71-fold) in the 200 μM PFOA-treatment groups compared to the vehicle controls (Fig. 5A, B). The STAR gene, which is responsible for cholesterol transport to the inner mitochondrial membrane, was also significantly reduced (p < 0.01) to 0.80-, 0.65- and 0.37-fold of the controls in 50, 100 and 200 μM PFOA-treated groups, respectively (Fig. 5A, B). 3β-HSD gene, responsible for converting pregnenolone to progesterone, was markedly reduced (p < 0.01) to 0.73-, 0.80- and 0.71-fold of the control in 50, 100 and 200 μM PFOA-treated groups, respectively (Fig. 5A, B). Similarly, CYP17α, which plays a significant role in steroid hormone synthesis, was also markedly reduced (p < 0.01) to 0.63-fold of the controls in the 200 μM PFOA-treated group (Fig. 5A, B). Interestingly, no significant differences between the PFOA treatment groups and controls are observed for P450Scc (catalyses cholesterol side-chain cleavage to pregnenolone) and 17β-HSD (catalyses androstenedione to testosterone) mRNA expression (Fig. 5A, B).

The contents of 17-OH progesterone following 50 μM PFOA were above control levels, but no statistical difference was found. For the 100 μM PFOA groups, progesterone levels were significantly increased ($p < 0.01$) to 1.31-fold of the control, whereas at 200 μM, progesterone were significantly reduced ($p < 0.01$) to 0.53-fold of control (Fig. 5C). Similar to 17-OH progesterone, PFOA exposure induced biphasic effects on testosterone production in MLTC-1 cells. PFOA effects at medium dose (100 μM) have significantly stimulatory effects ($p < 0.01$) on testosterone production, i.e., 1.84-fold compared to controls, while significantly inhibitory ($p < 0.01$) effects at higher exposures are noted, i.e., 0.50-fold at 200 μM compared to controls (Fig. 5D).

4. Discussion

In the present study, we evaluated the mode of toxicity and steroidogenesis in mouse Leydig MLTC-1 cells following PFOA exposure to understand toxicological effects in the mouse testis and effects on steroid production. We demonstrate that PFOA has the ability to disrupt fatty acids transport, maybe due to the structural similarity of PFOA and endogenous fatty acids, inhibition of exogenous cholesterol uptake and endogenous cholesterol de novo production via a decrease in transport and synthesis metabolism pathway genes expression respectively, interruption of sex hormones secretion by altering cholesterol mitochondrial transport and impacting steroidogenic enzyme activity in MLTC-1 cells. PFOA has a non-monotonic effect on testosterone and 17-OH progesterone production with different levels of exposure.

Multiple receptors are involved in the metabolic response to PFOA exposure in rodent liver cells. PPARα activation, involved in the regulation fatty acid β-oxidation, and Acox1 (acyl CoA oxidase) is the down-stream target genes [27]. PPARα activation has been demonstrated in rat and mice liver treated with PFOA [27,28]. In the present MLTC-1 cells study, PPARα receptor and Acox1 (data not shown) gene expression are not altered following any exposure of PFOA in Leydig cells. The results suggest that PPARα receptor in MLTC-1 Leydig cells is less sensitive to PFOA exposure compared to previous liver cells. PXR is the molecular target for a wide range of endogenous and xenobiotic compounds. It is responsible for regulation of lipid metabolism and cholesterol homeostasis by mediating genes for cholesterol uptake (SR-B1) and efflux (ABCA1) [27,29]. Our results reveal distinct patterns for the SR-B1 receptor and PXR gene expression after PFOA treatment in MLTC-1 cells. PXR and SR-B1 were sensitive to PFOA, which is noticeable, in the 200 μM PFOA treatment group and the changes were 2.78- and 0.16-fold for both PXR and SR-B1 compared to controls, respectively. We infer that PFOA disrupts cytoplasmic cholesterol transport via inhibiting SR-B1 uptake function. LH via binding to its receptor (LHR) then controls steroidogenesis. It is noteworthy that the present findings provide novel evidence that PFOA plays a dual role in regulating LHR function in MLTC-1 cells, exhibiting induction at lower exposures (100 μM) PFOA and inhibition at higher levels (200 μM). Our results are also in agreement with previously reported findings, which also show that acute triiodothyronine exposure stimulates LHR expression, whereas chronic exposure attenuates LHR expression [30]. A possible mechanism may be related to the fact that high LHR levels sensitize testicular cells to LH and facilitate steroidogenesis, whereas, lower LHR levels differ in their effect, and maintenance of normal testosterone secretion requires additional LH secretion [30]. Our testis-steroid and progesterone results agree with this, and correlate with LHR expression. Hence, it cannot be ignored that PFOA has possible effects on sex hormones biosynthesis via LHR regulation, although the mechanism requires further exploration.

PFCs have structural similarity with endogenous fatty acids, which can alter lipid profiles in liver via induction of hepatic fatty acids metabolism. The gene expression profile in PFOA-exposed rat liver also shows those largest categories of induced genes, which are involved in transport and metabolism of lipids, particularly fatty acids [31]. Our previous work also revealed that PFOA can alter the transport of long-chain fatty acids from the cytosol to mitochondrial matrix via carnitine shuttle [32]. Genes coding for enzymes responsible for unsaturated

### Table 2

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<th>Gene</th>
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<th>200 μM PFOA</th>
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<tr>
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Data are represented as mean ± SE of four independent experiments that were performed in duplicate. The one-way analysis of variance (ANOVA) with Tukey’s multiple comparisons test was applied to estimate for statistical significance between controls versus treatment groups. Asterisks indicate a significant difference relative to control.

* $p < 0.05$
** $p < 0.01$
fatty acids transport have been altered by PFOA in MLTC-1 cells (Fig. 3). According to our observation, it is shown that PFOA can inhibit mitochondrial outer membrane fatty acid import-related gene (CPT I), while inducing mitochondrial matrix fatty acid retransformation and oxidation metabolism products export-related gene (CPT II, CRAT) expression. Our results are consistent with a previous study that showed that PFOA induced CPT II and CRAT expression in human, rat and mouse liver cells [31–33]. Because of the structural resemblance of PFOA and endogenous fatty acids, PFOA can be taken up in the in-vitro cell model [34]. Kudo and co-authors imply that both PFOA and fatty acids can transfer into mitochondria, but PFOA is unable to metabolize via β-oxidation [35]. In agreement, we find that PFOA is transported into MLTC-1 cell cytoplasm and mitochondria. We speculate that MLTC-1 cell reduces PFOA uptake by lowering CPT I expression, while facilitating PFOA elimination by increase CPT II and CRAT expression, which leads to disturbed fatty acids transport; further research is necessary to demonstrate this.

Cholesterol is a main substrate for testosterone biosynthesis. Leydig cells can synthesise cholesterol in endoplasmic reticulum and use several potential sources of cholesterol for steroidogenesis in mitochondria [36]. Previous studies suggest that PFOA can disrupt cholesterol content by altering cholesterol transport and biosynthesis routes [9,31,32]. Our results show that PFOA significantly weakens the cholesterol content in MLTC-1 cells. The PXR and SR-B1 nuclear receptor as well as SREBP transcription factors responses associated with cholesterol uptake are proposed in the MTC-1 cells with PFOA exposure. In order to further investigate the effects of PFOA on the cholesterol biosynthesis pathway in MLTC-1 cells, the expression of a series of important genes in this pathway were determined by quantitative real-time RT PCR. The general down-regulation gene expression profiles are in agreement with the cells’ cholesterol metabolism content. Specifically, HMGCR is an enzyme involved in mevalonate synthesis and is a rate limiting enzyme in cholesterol synthesis pathway. It was down-regulated by PFOA in rat liver with resulting decreased cholesterol content, while up-regulated in human liver cells resulting in increased cholesterol content [31,32]. These inconsistent effects may result from different species and experimental models, which have unique metabolic mechanisms. Meanwhile, acyl-CoA is an important raw material for cholesterol biosynthesis, which is altered via fatty acids transport [32]. This study is consistent with previous MLTC-1 cells results showing that PFOA can inhibit cholesterol biosynthesis in vitro [9]. Herein, PFOA may disrupt cholesterol by both exogenous cholesterol uptake and endogenous cholesterol biosynthesis.

Regarding male reproductive function, the level of testosterone in MLTC-1 cells is significantly stimulated to 1.84-fold of the control at lower PFOA concentrations (100 μM) and inhibited to 0.50-fold of the control at higher treatment concentrations (200 μM). We observed significant decreases in mRNA levels of three genes (StAR, 3β-HSD,
CYP17a1) that play pivotal roles in testosterone production in MLTC-1 cells exposed by PFOA. STAR is responsible for carrying cholesterol into the inner mitochondrial membrane from the outer mitochondrial membrane, which subsequently converts into pregnenolone by P450SCC in the inner mitochondrial membrane and finally into progesterone via 3β-HSD catalysis (Fig. 5B). Previous studies have shown that PFCs inhibit the expression of several key enzymes, including STAR, 3β-HSD and CYP17a1 [39,37,38]. However, in the present study, low exposure concentrations of PFOA stimulated testosterone production and high concentrations of PFOA inhibited testosterone production, though the process occurred without any alteration of MLTC-1 cell viability. This can be explained by the fact that many environmental endocrine disruptor chemicals are reported to exhibit the ability to induce U and/or invert U dose-response trends, which results into low-dose stimulation responses [39]. Similar studies have shown that exposures to low or high levels of phthalates or prolactin have biphasic effects on testosterone production in MLTC-1 cells [40–42]. Alteration of cholesterol transport and steroidogenic enzymes in MLTC-1 cells may be involved in the biphasic effects of PFOA on androgen production. Our findings agree with the hypothesis that low-dose stimulation corresponds to a negative feedback compensation mechanism that counterbalances the endocrine disrupting chemicals-inhibited induction of gene expression of the steroidogenic enzymes [43,44].

In conclusion, our results suggest that PFOA disrupts cholesterol precursor fatty acid transport into mitochondria and then alters cholesterol synthesis. Meanwhile, PFOA-regulated nuclear (membrane) receptor response and steroidogenesis result in disruption of sex hormones secretion. In particular, PFOA has a biphasic effect on testosterone and progesterone production.

Conflict of interest statement
The authors declare that there are no conflicts of interest.

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