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 receptor *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.

***Key Words*:** Perfluorooctanoic acid; endocrine disruption; biphasic effects; MLTC-1 cells; steroidogenesis; steroid hormone

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 (E_2) 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 ($C_8F_{15}O_2H$; Chemical Abstract Service, no. 335-67-1; purity >96%) and isotope PFOA $^{13}C_8$ was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, Mo. USA). Human chorionic gonadotrophin (hCG) was obtained from PROSPECT (Ness-Ziona, Israel). The steroid hormone standards of testosterone and 17-OH progesterone were purchased from Dr. Ehrenstorfer GmbH (Germany) and the isotope D3-testosterone was purchased from Cerilliant (Promochem, Wesel, Germany). All other chemicals of appropriate grades were commercially available.

2.2. *Cell culture and cell viability assay.*

The MLTC-1 cell line was obtained from the Cell Institute of Shanghai (Shanghai, China) and cultured in RPMI-1640 medium (Gibco BRL, Grand Island, NY) supplemented with 100 unit/mL penicillin, 100 unit/mL streptomycin and 10% (v/v) foetal bovine serum (Hyclone, USA). The cells were grown at 37℃ with 5% CO_2 in a humidified incubator (SANYO, Japan).

Cell viability was evaluated by the MTT proliferation assay. Cells were plated at a density of 1.5×10^4 per well in 96-well plates. After 48-h incubation at different concentrations of PFOA (0-300 μM), 50 μL MTT (5 mg/mL) was added to each well and the

104 cells were incubated for 4 h at 37℃. Untreated cells were used as a negative control. The
105 medium was removed and 150 μL DMSO was added to each well and gentle shaking was
106 then performed for 10 min. Absorbance was determined at 490 nm. Four replicates for each
107 PFOA exposure were performed. Results were presented as percentage of the values
108 measured in untreated control cells. To ensure absence of cytotoxicity, the concentrations 50
109 μM, 100 μM, and 200 μM were selected for the following PFOA exposure experiments.
110 2.3. *PFOA treatment*
111 MLTC-1 cells were seeded in 6-cm petri dishes and cultured for 24 h prior to treatment.
112 PFOA was dissolved in DMSO. Cells were exposed to 50 μM, 100 μM or 200 μM PFOA for
113 48 h, with DMSO (0.1%) alone employed as a vehicle control. Four replicates for each dose
114 of PFOA exposure were performed. Then, the cells were washed with PBS and serum-free
115 medium. Subsequently, cells were stimulated for 4 h with hCG in serum-free medium
116 supplemented by 0.1% BSA. The medium was collected for progesterone and testosterone
117 determination, and the cells for cholesterol measurements and other biochemical assays.
118 2.4. *RNA extraction and quantitative real-time RT PCR analysis*
119 To determine mRNA expression levels, a *quantitative* real-time RT PCR assay was
120 performed. Total RNA was extracted from the cells using an RNA extraction kit (Promega,
121 USA), following the manufacturer's protocol. Extracted RNA samples were stored at -80^{o}C,
122 for subsequent analyses. The NanoDrop spectrophotometer (NanoDrop Technologies Inc.,
123 USA) was used to measure RNA and/or DNA concentration and purity.
124 Reverse transcription of cDNA synthesis was performed with 1 μg total RNA using
125 PrimeScript® RT reagent Kit with gDNA Eraser cDNA synthesis Kits (Takara, Japan)

126 employing oligo dT primer. Real-time PCR was carried out in a 20 μL final volume and
127 performed in triplicate using SYBR Green Master Mix reagents (Roche, USA) in a *LC 480*
128 system (Roche Applied Science, Germany), according to the manufacturer's protocol. Primer
129 sets and product sizes used for amplification PCR analysis are shown as Table 1. The
130 conditions for quantitative PCR were as follows: 95°C for 10 min followed by 40 cycles at 95℃
131 for 15 s, and 60℃ for 30 s. Gene expression levels were normalized to *GAPDH* expression
132 levels. Three replicates of quantitative PCR were performed for each sample. Four replicates
133 for each dose of PFOA exposure were performed. The fold changes of the tested genes were
134 determined by the $2^{-\Delta\Delta Ct}$ algorithm approach.
135 2.5. *Cholesterol, progesterone and testosterone determination*
136 Total cholesterol (TCHO) content in MLTC-1 cells in the control and PFOA groups
137 were measured using commercial kits according to the manufacturer's instructions (Beihua
138 Kangtai Clinical Reagent, China). Total cholesterol concentration was normalized to Leydig
139 cell protein concentration. Steroid hormones testosterone and progesterone levels in cell
140 culture medium were detected by LC-ESI-MS/MS. In brief, each sample of 1 mL medium
141 was diluted with 3 mL of ammonium acetate buffer (1 mol/L), and 20 μL of 100 ng/mL
142 D3-testosterone internal standard was added (progesterone was semi-quantified). Then the
143 diluted samples were extracted by adding 3 mL ethyl acetate and vortexed vigorously for 15
144 seconds in a glass tube. The liquid-liquid extraction was repeated three times. The following
145 phase separation was completed by centrifugation at 1500 rpm for 10 min. The ether phase
146 was transferred to another glass tube with a Pasteur pipette. The three times extract was
147 combined and washed with 5 mL water, then the combined extracts were evaporated under a

148 gentle stream of nitrogen gas at 40^{o}C. The residue was reconstituted with 200 µL
149 methanol/water (50:50, v:v) by vortexing vigorously for 15 sec and transfer into a HPLC vial.
150 The sample was stored at -20^{o}C until LC-MS-MS analysis. Two quality control samples and
151 two sets of standards were analysed together with unknown samples in each analytical batch.
152 2.6. Quantification of PFOA in cytoplasmic and mitochondrial fractions
153 Mitochondrial isolation was performed using the Cell Mitochondria Isolation Kit
154 (Beyotime Institute of Biotechnology, China) according to the manufacturer's instructions.
155 Briefly, MLTC-1 cells were pelleted, washed, and re-suspended in ice-cold mitochondria
156 isolation buffer. The cells were homogenized and centrifuged at 600 g for 10 min at 4℃. The
157 supernatant was centrifuged at 11,000 g for 10 min at 4℃ to obtain mitochondrial pellets.
158 Mitochondrial-free cytoplasm was obtained from the supernatant. Mitochondrial pellets were
159 lysed in a lysis buffer.
160 Mitochondrial lysate and cytoplasm were extracted by ion-pair extraction and
161 solid-phase extraction (SPE) with subsequent HPLC-MS/MS quantification method, as
162 previously outlined [25, 26]. In brief, 0.03 ml of mitochondrial lysate was made up to 1 mL
163 with distilled water in a 15 mL PP tube (containing 10 ng C_{13}PFOA internal standard). Before
164 extraction, the spiked samples were allowed to equilibrate overnight at room temperature
165 (26℃). Then, 1 mL tetra-*n*-butylammoniumhydrogen sulfate and 2 mL sodium carbonate
166 (0.25 M, pH 10) were added. After mixing, 5 mL MTBE was added, and the mixture was
167 shaken for 15 min at 250 rpm. The organic and the aqueous layers were separated by
168 centrifugation at 3000 rpm for 15 min. Then, 4 mL MTBE supernatant was removed and
169 transferred to another 15 mL PP tube. This procedure was twice repeated, except that 5 mL

MTBE was collected each time. All three extracts were combined, and evaporated to dryness under a gentle stream of nitrogen at 45℃. Finally the dried 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). The experiments were repeated four times, each 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, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

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 Figure 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 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 Figure 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) result is consistent with the *SR-B1* observation; *SREBP2* expression significantly declined (0.74- to 0.51-fold compared to controls, p <0.01; Figure 2) with various concentrations of PFOA exposure in MLTC-1 cells.

214 *3.3. PFOA alters fatty acids transport into mitochondria*

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

227 Meanwhile, *CPTii* (carnitine-palmitoyltransferase ii) responsible for connecting
228 carnitine to long-chain fatty acids, which facilitates them crossing the outer mitochondrial
229 membrane, was significantly down-regulated (p <0.05) (Fig. 3B, 3C). CACT
230 (carnitine-acylcarnitine translocase) is a carnitine carrier protein, a component of the
231 mitochondrial inner membrane and transfers fatty acylcarnitines into the mitochondria, was
232 not significantly altered following different PFOA exposure levels (Fig. 3B, 3C). However,
233 *CPTii* (carnitine-palmitoyltransferase ii), coding for the inner mitochondrial membrane
234 protein that converts acylcarnitine to acyl-CoA for further fatty acid metabolism, was
235 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, 3C).

3.4. PFOA disturbs cholesterol synthesis transcriptional profile and secretion

Cholesterol and steroid hormone biosynthesis is regulated by 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, an 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 Figure 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, 5B). 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 the 50, 100 and 200 μM PFOA-treated groups, respectively (Fig. 5A, 5B). *3β-HSD* gene, responsible for converting pregnenolone to progesterone, was markedly reduced (p <0.01) to 0.73-, 0.80- and 071-fold of the control in 50, 100 and 200 μM PFOA-treated groups, respectively (Fig. 5A, 5B). 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, 5B). 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, 5B).

The levels 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* reduced 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 testosterone 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 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 (*CPTii*), while inducing mitochondrial matrix fatty acid retransformation and oxidation metabolism products export-related gene (*CPTii*, *CRAT*) expression. Our results are consistent with a previous study that showed that PFOA induced *CPTii* 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 *CPTii* expression, while facilitating PFOA elimination by increase *CPTii* 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 been 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

346 expression profiles are in agreement with the cells' cholesterol metabolism content.
347 Specifically, HMGCR is an enzyme involved in mevalonate synthesis and is a rate limiting
348 enzyme in cholesterol synthesis pathway. It was down-regulated by PFOA in rat liver with
349 resulting decreased cholesterol content, while up-regulated in human liver cells resulting in
350 increased cholesterol content [31, 32]. These inconsistent effects may result from different
351 species and experimental models, which have unique metabolic mechanisms. Meanwhile,
352 acyl-CoA is an important raw material for cholesterol biosynthesis, which is altered *via* fatty
353 acids transport [32]. This study is consistent with previous MLTC-1 cells results showing
354 that PFOA can inhibit cholesterol biosynthesis *in vitro* [9]. Herein, PFOA may disrupt
355 cholesterol by both exogenous cholesterol uptake and endogenous cholesterol biosynthesis.

356 Regarding male reproductive function, the level of testosterone in MLTC-1 cells is
357 significantly stimulated to 1.84-fold of the control at lower PFOA concentrations (100 μM)
358 and inhibited to 0.50-fold of the control at higher treatment concentrations (200 μM). We
359 observed significant decreases in mRNA levels of three genes (*StAR*, *3β-HSD*, *CYP17α*) that
360 play pivotal roles in testosterone production in MLTC-1 cells exposed by PFOA. StAR is
361 responsible for carrying cholesterol into the inner mitochondrial membrane from the outer
362 mitochondrial membrane, which subsequently converts into pregnenolone by P450SCC in the
363 inner mitochondrial membrane and finally into progesterone *via* 3*β*-HSD catalysis (Fig. 5B).
364 Previous studies have shown that PFCs inhibit the expression of several key enzymes,
365 including StAR, 3β-HSD and CYP17α [9, 37, 38]. However, in the present study, low
366 exposure concentrations of PFOA stimulated testosterone production and high concentrations
367 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-induced inhibition 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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Figure Legends

Figure 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 (±SEM) 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, * $p \leq 0.05$, ** $p \leq 0.01$.

Figure 2. Effects of PFOA on the mRNA expression of receptor genes. MLTC-1 cells were exposed to different doses of PFOA (0, 50, 100 or 200 μM) for 48 h. The relative mRNA expression of *PPARα*, *AR*, *PXR*, *LHR* and *SR-B1* gene were measured by quantitative real-time RT PCR. The experiments were repeated four times 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, * $p \leq 0.05$, ** $p \leq 0.01$.

Figure 3. Effects of PFOA on the subcellular accumulation (A), fatty acids mitochondria transport genes expression (B) in MLTC-1 cells and schematic diagram of fatty acids mitochondrial transport (C).

Mean ± SEM is derived from four independent experiments. Fraction isolated: cytoplasm and mitochondria.

553 **Figure 4.** PFOA affect cholesterol biosynthetic pathways gene expression and content in
554 MLTC-1 cells. Heat map displays fold-changes of PFOA exposure on cholesterol
555 biosynthetic pathways gene expression profiles (A), effects of PFOA on the cholesterol
556 biosynthesis pathway gene expression in MLTC-1 cells in 200 μM treatment (B), and effects
557 of PFOA exposure on cholesterol level in MLTC-1 cells (C).
558 Colour scales range from bright red to bright green corresponding to up- or down-regulation
559 of gene expression, respectively. The experiments were repeated four times in duplicate. Data
560 were analysed by one-way ANOVA with Tukey's multiple comparisons test. Asterisks above
561 columns indicate a significant difference in comparison to the control, * $p \leq 0.05$, ** $p \leq 0.01$.
562
563 **Figure 5.** PFOA affect steroid hormone biosynthetic pathways in MLTC-1 cells,
564 steroidogenesis gene expression (A), steroidogenesis gene pathway (B), 17-OH progesterone
565 secretion (C), and testosterone secretion (D).
566 The experiments were repeated four times in duplicate. Data were analysed by one-way
567 ANOVA with Tukey's multiple comparisons test. Asterisks above columns indicate a
568 significant difference in comparison to the control, * $p \leq 0.05$, ** $p \leq 0.01$.
569

570 **Table 1.** Sequence of primers used for quantitative real-time PCR.

Gene	Accession number	Primer sequence (5'-3')	Product size (bp)
GAPDH	NM_001289746.1	F：GTATTGGGCGCCTGGTCACC R：CGCTCCTGGAAGATGGTGATGG	202
CPTii	XM_006531658.3	F: AACAACCGTAGGCTCCACCGT R: ATTCAAAAGACTTCGGGGGAC	99
CACT	NM_020520.4	F: CAGATTCAGGCTTCTTCAGGG R: ACTGGCAGGAACATCTCGCAT	135
CPTii	NM_009949.2	F: TGGCTTTCCTGCGACAGTATG R: GGCGAATAGTCTCTGTGCGGC	93
CAT	XM_006497646.3	F: AAGTCAAAGAGACCACCCACG R: GGAGGTTAGGATGCCAACAGG	177
PPARα	XM_006520624.3	F: GGAGAACAAGAGACGAGGGTG R: CAGGGACTGAGGAAAAGGGAC	157
AR	NM_013476.4	F: CTTTCAAGGGAGGTTACGCCA R: ACAGAGACAGAGAGGACGGGA	111
PXR	XM_006521848.3	F: TGAAAGACAGGGTTCCAATGA R: GTGTGGCAGAAGAGGGATGAT	119
LHR	XM_006523723.2	F：GAGAAGCGAATAACGAGACG R：AGCCAAATCAACACCCTAAG	178
SR-B1	XM_017320764.1	F：TTGTTCTACCTCCTCTCTCGC R：CTGACCCCCCACCTCTACCTT	179
StAR	NM_011485.5	F：TGGAAAAGACACGGTCATCA R：CTCCGGCATCTCCCCAAAAT	154
P450SCC	NM_001346787.1	F：CGTGACCAGAAAAGACAACA R：AGGATGAAGGAGAGGAGAGC	152
3β-HSD	NM_001304800.1	F：AGTGATGGAAAAAGGGCAGGT R：GCAAGTTTGTGAGTGGGTTAG	167
CYP17α	NM_007809.3	F：TGGGCACTGCATCACGATAA R：GCTCCGAAGGGCAAATAACT	122
17β-HSD	NM_008291.3	F：AACGCAACATCAGCAACAGA R：CAGCCCCACCTCACCCTACC	88
HMGCS1	NM_001291439.1	F：GCTGTCATCAGTAACGGGGAG R：CCAAGACATCCATTCCTCCAA	99
HMGCR	XM_006517531.1	F：ACCAAACCCCGTAACCCAAAG R：GCCAAAAGGAAGGCTAAACTC	255
MVK	XM_006530185.3	F：GAGCAATGGGAAAGTGAGCGT R：GGAGGTCCCCCATCTTCTTTA	161
PMVK	NM_026784.3	F：AGGCTCTTTCCCTTCCAGTTT R：GTCCTTCCCGGATTTTCTCTT	255
MVD	NM_138656.2	F：ACAAGAAGCAGACGGGCAGTA R：AGGTAGGAGATCGGTGGGAAG	217
IDI1	XM_006498513.3	F：ATCCACCTTCCTCTGACTCCC R：AGCCCTACTCCTTCCCACTTC	161
FDPS	NM_001253751.1	F：ACAGTGGGCTGGTGTGTAGAA R：CAGAAGCAGAGCGTCGTTGAT	147
FDFT1	XM_006518547.3	F：GAACTCATAACCAACACCCTA R：CCTTCCGAATCTTCACTACTC	175
SQLE	NM_009270.3	F：ACAGCCACATTCGCACCCCTC R：CATTTAAAGCCTGCCTACCCC	107

LSS	XM_006513284.3	F：CTCCAGAATGAGTTGGGTCGG R：GCTGTTTGCGCTTTTGGTAAG	143
CYP51A1	NM_020010.2	F：TTTCCGAGAAGCGGTGTGCGA R：ACGGCGAGACGGAACAGGTAG	207
SC4MOL	NM_025436.2	F：TTTGGCAAGGTGTTTGGGCTG R：CAAGGGATGTGCGTATTCTGC	157
NSDHL	NM_010941.3	F：CTGAAGACCTCCCTTACGCCA R：TTCTTAGGGTCGTTGGCATCC	97
SC5DL	XM_006510253.2	F：GCTTTTCACCCTGTGGACGGC R：CTGGGGAACCCGAAAATCACC	153
DHCR7	XM_006508479.3	F：TTGAAGAAGGGAGGCTTTTTT R：AGGTGGATGAGCTGCTAGGTG	191

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572

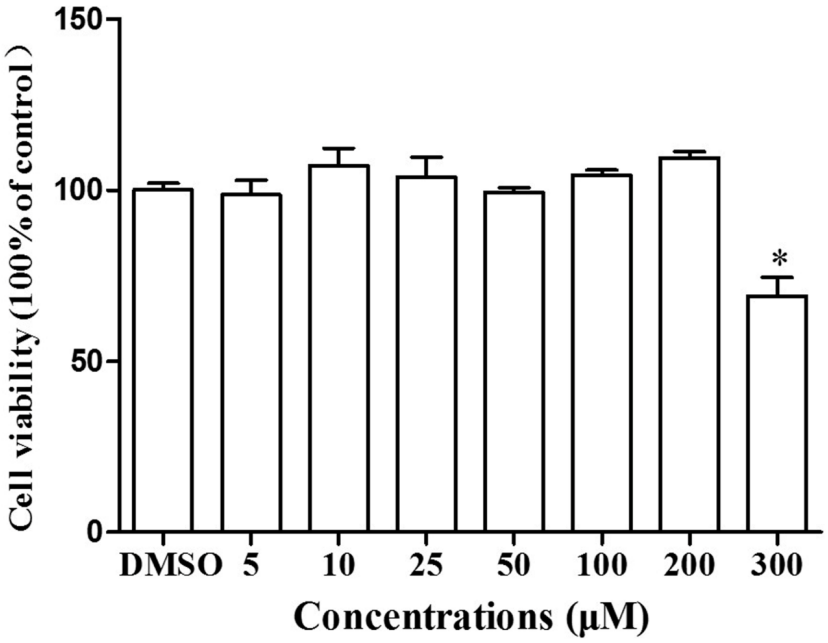
573 **Table 2** Effect of PFOA on mRNA expression of cholesterol biosynthetic pathways genes in
574 MLTC-1 cells
575

	DMSO	50 μM PFOA	100 μM PFOA	200 μM PFOA
HMGCS	1.00 ± 0.02	0.92 ± 0.04	0.82 ± 0.05**	0.49 ± 0.07**
HMGCR	1.00 ± 0.05	0.85 ± 0.11*	0.82 ± 0.05**	0.36 ± 0.05**
MVK	1.00 ± 0.04	1.52 ± 0.14**	1.27 ± 0.12*	0.87 ± 0.07
PMVK	1.00 ± 0.03	1.52 ± 0.15**	1.26 ± 0.09*	0.73 ± 0.03*
MVD	1.00 ± 0.08	1.23 ± 0.14*	1.17 ± 0.11	0.58 ± 0.07**
IDI1	1.00 ± 0.08	0.90 ± 0.08	0.75 ± 0.04**	0.37 ± 0.05**
FDPS	1.00 ± 0.10	1.35 ± 0.09**	1.09 ± 0.05	0.43 ± 0.08**
FDFT1	1.00 ± 0.06	0.89 ± 0.04*	0.84 ± 0.03**	0.58 ± 0.03**
SQLE	1.00 ± 0.04	0.87 ± 0.07*	0.80 ± 0.05**	0.36 ± 0.04**
LSS	1.00 ± 0.05	1.13 ± 0.08	1.22 ± 0.11	0.43 ± 0.08**
CYP5	1.00 ± 0.06	1.74 ± 0.08**	1.35 ± 0.17*	0.29 ± 0.04**
SC4MOL	1.00 ± 0.03	1.07 ± 0.07	0.98 ± 0.06	0.44 ± 0.08**
NSDHLC	1.00 ± 0.09	0.97 ± 0.02	1.09 ± 0.09	0.53 ± 0.05**
SC5DL	1.00 ± 0.07	0.92 ± 0.08	0.87 ± 0.02*	0.48 ± 0.07**
DHCR7	1.00 ± 0.08	1.05 ± 0.02	1.01 ± 0.06	1.10 ± 0.02

576
577 Data are represented as mean ± SEM of four independent experiments that were performed in
578 duplicate. The one-way analysis of variance (ANOVA) with Tukey's multiple comparisons
579 test was applied to estimate for statistical significance between controls *versus* treatment
580 groups. Asterisks indicate a significant difference relative to control, * $p \leq 0.05$, ** $p \leq 0.01$
581

582 **Figure 1**

583



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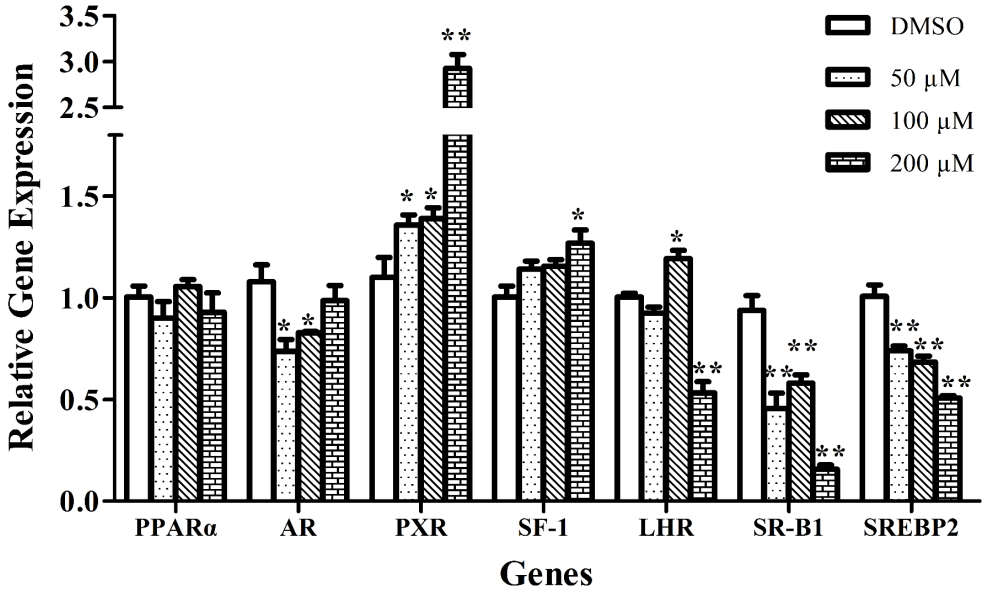
586 **Figure 2**

587



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590 **Figure 3**

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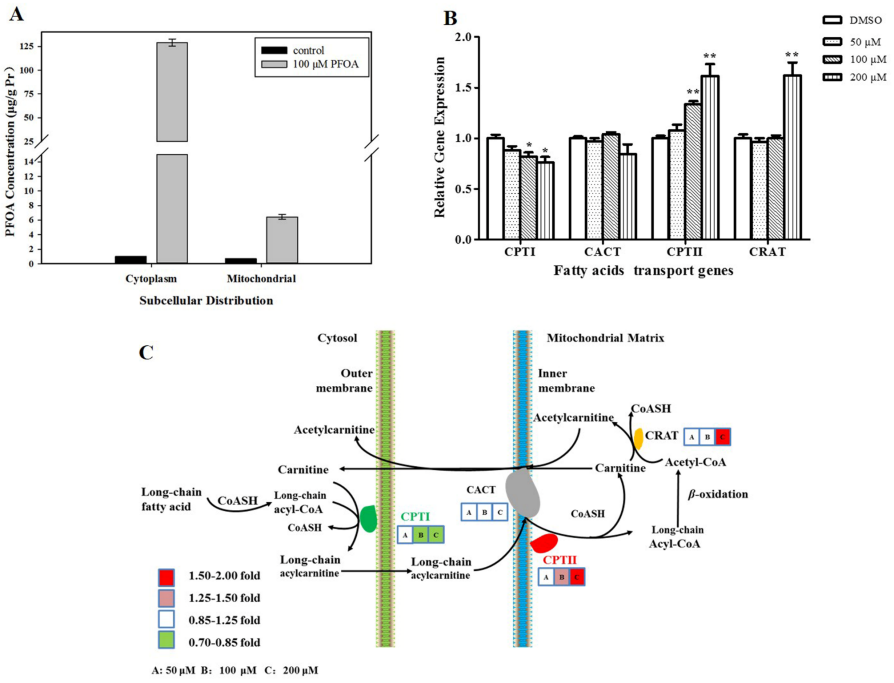

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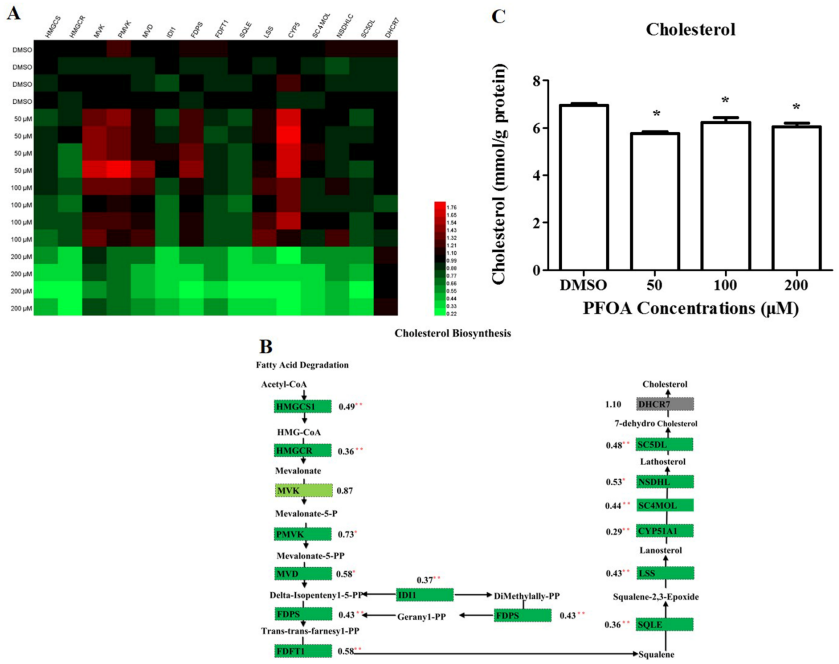
Figure 4


A
HMGCS
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MVD
IDI
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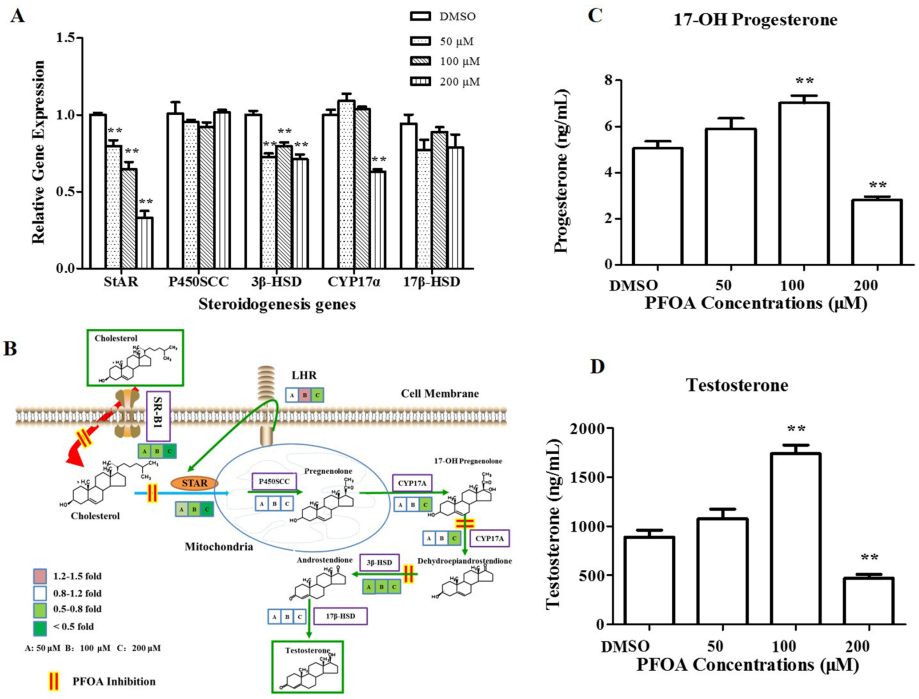
NSDHL/C
SC5DL
DHCR7
DMSO
50 μM
100 μM
200 μM
C
Cholesterol
Cholesterol (mmol/g protein)
DMSO
50
100
200
PFOA Concentrations (μM)
B
Cholesterol Biosynthesis
Fatty Acid Degradation
Acetyl-CoA
HMGCS1
HMG-CoA
HMGCR
Mevalonate
MVK
Mevalonate-5-P
PMVK
Mevalonate-5-PP
MVD
Delta-Isopentenyl-5-PP
IDI1
DiMethylally-PP
FDPS
Geranyl-PP
Trans-trans-farnesyl-PP
FDFT1
Squalene
SQLE
Squalene-2,3-Epoxide
LSS
Lanosterol
CYP51A1
SC4MOL
NSDHL
Lathosterol
SC5DL
7-dehydro Cholesterol
DHCR7
Cholesterol

598 **Figure 5**

599



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