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

Meiping Tian[a,b], Qingyu Huang[a], Heng Wang[a], Francis L Martin[c], Liangpo Liu[a], Jie Zhang[a], Heqing Shen[a*]

[a]*Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China;*

[b]*University of Chinese Academy of Science, Beijing 100049, China;*

[c]*School of Pharmacy and Biomedical Sciences, University of Central Lancashire, Preston PR1 2HE, UK*

[1]**To whom correspondence may be addressed:** Prof. Heqing Shen, Institute of Urban Environment, Chinese Academy of Sciences, 1799 Jimei Road, Xiamen 361021, China; Telephone/Fax: (86)-592-6190771; E-mail: hqshen@iue.ac.cn

20   **ABSTRACT**

21   Perfluorooctanoic acid (PFOA) is a persistent organic pollutant, which may possess

22   endocrine disrupting properties. Herein, we investigated the possible mechanism(s) of

23   toxicity and steroidogenesis in mouse Leydig cells. MLTC-1 (mouse Leydig tumour cells)

24   cells were exposed to 0, 50, 100 or 200 μM PFOA for 48 h to ascertain their effects on the

25   nuclear (membrane) receptor responses, steroidogenesis pathway and related regulated gene

26   expression and steroid hormone secretion profiles. Our results reveal that nuclear receptor

27   *PXR*, *SR-B1* and *LHR* are sensitive to PFOA exposure. PFOA can accumulate in

28   mitochondria and alter cholesterol precursor (fatty acid) mitochondrial transport

29   process-related gene expression and thus inhibit steroid hormone precursor (cholesterol)

30   production. In particular, PFOA exhibits biphasic effects on testosterone and progesterone

31   production at differing levels of exposure. These findings indicate the potential

32   endocrine-related effects of PFOA on steroid hormone secretion in Leydig cells and point to a

33   novel disruption model.

34

35   ***Key Words***: Perfluorooctanoic acid; endocrine disruption; biphasic effects; MLTC-1 cells;

36   steroidogenesis; steroid hormone

37

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

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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

82 disruption *via* molecular initiating events (receptor response) and endpoints (steroid hormone

83 secretion) related to reproductive toxicity, by using mouse *in vitro* Leydig MLTC-1 cells.

84

85 **2. Materials and methods**

86 2.1. *Chemicals* and reagents

87   PFOA ($C_8F_{15}O_2H$; Chemical Abstract Service, no. 335-67-1; purity >96%) and isotope

88 PFOA $^{13}C_8$ was purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

89 Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich Chemical Co. (St. Louis,

90 Mo. USA). Human chorionic gonadotrophin (hCG) was obtained from PROSPECT

91 (Ness-Ziona, Israel). The steroid hormone standards of testosterone and 17-OH progesterone

92 were purchased from Dr. Ehrenstorfer GmbH (Germany) and the isotope D3-testosterone was

93 purchased from Cerilliant (Promochem, Wesel, Germany). All other chemicals of appropriate

94 grades were commercially available.

95 2.2. *Cell culture and cell viability assay.*

96   The MLTC-1 cell line was obtained from the Cell Institute of Shanghai (Shanghai,

97 China) and cultured in RPMI-1640 medium (Gibco BRL, Grand Island, NY) supplemented

98 with 100 unit/mL penicillin, 100 unit/mL streptomycin and 10% (v/v) foetal bovine serum

99 (Hyclone, USA). The cells were grown at 37℃ with 5% CO_2 in a humidified incubator

100 (SANYO, Japan).

101   Cell viability was evaluated by the MTT proliferation assay. Cells were plated at a

102 density of $1.5×10^4$ per well in 96-well plates. After 48-h incubation at different

103 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$^\text{o}$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: $95^{\circ}C$ for 10 min followed by 40 cycles at $95^{\circ}\!C$

131   for 15 s, and $60^{\circ}\!C$ 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°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°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

170 MTBE was collected each time. All three extracts were combined, and evaporated to dryness

171 under a gentle stream of nitrogen at 45℃. Finally the dried residue was re-suspended in 0.5

172 mL of methanol/water (50:50, v:v) before analysis. Calibration standards and QC samples

173 were analysed concurrently with unknown samples using the same sample preparation

174 procedure.

175 *2.7. Statistical analysis*

176   Measurement data of cholesterol, progesterone, testosterone levels and gene

177 expression by real-time RT PCR analysis were analysed using SPSS for Windows 11.5

178 Software (SPSS, Inc., Chicago, IL) and were presented as mean with standard errors (mean ±

179 SE). The experiments were repeated four times, each in duplicate. Data were analysed by

180 one-way ANOVA with Tukey's multiple comparisons test. Asterisks above columns indicate

181 a significant difference in comparison to the control, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

182

183 **3. Results**

184 3.1. *Cell viability assay*

185   To analyse the effects of PFOA on MLTC-1 cell viability, the cells were treated with

186 different doses (from 0 to 300 μM) of PFOA for 48 h. Results of cytotoxicity studies are

187 shown in Figure 1. Taking into account effects on cell viability, the PFOA concentrations

188 were kept below the levels (*i.e.*, 300 μM) at which significant lethal effects occurred.

189 Exposure concentrations in subsequent experiments were as follows: 0, 50, 100 or 200 μM.

190 *3.2. Nuclear (membrane) receptor responses to PFOA exposure*

191   As nuclear (membrane) receptor is involved in environmental exposure and

192 toxicological effects, transcript profiles of nuclear (membrane) receptor were investigated in

193 MLTC-1 cells exposed to varying levels of PFOA. The mRNA expression of *PPARα*

194 (PPAR-alpha; controls the peroxisomal *β*-oxidation pathway of fatty acids) was unaltered

195 following PFOA exposure compared to control (Fig. 2). *AR* (androgen receptor; is activated

196 by binding androgenic hormones and then regulates male sexual phenotype gene expression)

197 gene exhibits significantly reduced expression following 50 or 100 μM PFOA, while no

198 significant alteration is observed at the higher dose (*i.e.*, 200 μM) PFOA treatment. *PXR*

199 (Pregnane X receptor; regulates a broad range of genes involved in the transport, metabolism

200 and elimination of foreign toxic substances) gene was significantly up-regulated in a

201 dose-related fashion after PFOA exposure (p <0.05) (Fig. 2). There was no significant

202 alteration found at lower levels of PFOA (*i.e.*, 50 μM) on *LHR* (luteinizing hormone receptor;

203 allows Leydig cells to respond to luteinizing hormone that triggers these cells to produce

204 androgens) gene expression. However, 100 μM PFOA significantly induced (p <0.05) *LHR*

205 expression (Fig. 2). However, significant down-regulation (p <0.01) was observed following

206 200 μM PFOA exposure. Considering Figure 2, it can be identified that *SR-B1* (scavenger

207 receptor B1; regulates cholesterol uptake) mRNA expression was significantly

208 down-regulated (p <0.01) in all PFOA exposure groups, noticeably following 200 μM PFOA

209 which declined to 0.16±0.04 folds compared to controls. *SREBP2* (sterol regulatory

210 element-binding protein 2; controls cholesterol homeostasis) result is consistent with the

211 *SR-B1* observation; *SREBP2* expression significantly declined (0.74- to 0.51-fold compared

212 to controls, p <0.01; Figure 2) with various concentrations of PFOA exposure in MLTC-1

213 cells.

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 and C). Our results confirm that PFOA can transport into subcellular mitochondria *via* PFOA accumulation. Figure 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, *CPTii* (carnitine-palmitoyltransferase ii) 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, 3C). CACT (carnitine-acylcarnitine translocase) is a 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, 3C). However, *CPTii* (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

236 (Fig. 3B). Mitochondrial matrix enzyme *CRAT* (carnitine acetyltransferase), that catalyses the

237 inter-conversion of acetyl-CoA and acetylcarnitine, was also significantly (p <0.01) induced

238 (1.71-fold) following 200 μM PFOA exposure (Fig. 3B, 3C).

239 *3.4. PFOA disturbs cholesterol synthesis transcriptional profile and secretion*

240 Cholesterol and steroid hormone biosynthesis is regulated by steroidogenic genes. The

241 effects of PFOA exposure on expression of genes involved in cholesterol biosynthesis or

242 steroidogenesis in MLTC-1 were determined (Fig. 4 and Table 2). Quantitative real-time

243 RT-PCR assays results show that cholesterol biosynthesis pathway-related genes expression

244 were down-regulated (0.29-0.87-fold) significantly (p <0.01) in the 200 μM PFOA-treated

245 group compared to vehicle control. Interestingly, 50 μM PFOA exposure significantly (p

246 <0.05) induced *MVK* (1.52-fold), *PMVK* (1.52-fold), *MVD* (1.23-fold), *FOPS* (1.35-fold) and

247 *CYP5* (1.74-fold) gene expression (Fig. 4, Table 2). Especially, HMGCR, an enzyme

248 involved in mevalonate synthesis and is rate-limiting in the cholesterol synthesis pathway,

249 was significantly down-regulated in a dose-related manner after PFOA exposure: 0.85-, 0.82-,

250 and 0.36-fold for 50, 100 and 200 μM PFOA-treatment groups, respectively (Fig. 4, Table 2).

251 Moreover, the effects of PFOA on cholesterol levels in MLTC-1 cells are shown as Figure 4C.

252 Cholesterol was markedly decreased (p <0.05) to 0.83-, 0.87- and 0.85-fold of control

253 following exposures of 50, 100 and 200 μM PFOA, respectively.

254 *3.5. Effects of PFOA on steroidogenesis pathway gene expression and steroid hormone*

255 *secretion*

256 The expression of genes involved in steroidogenesis were significantly (p <0.01)

257 decreased (0.37-0.71-fold) in the 200 μM PFOA-treatment groups compared to the vehicle

258 controls (Fig. 5A, 5B). The *StAR* gene, which is responsible for cholesterol transport to the

259 inner mitochondrial membrane, was also significantly reduced ($p < 0.01$) to 0.80-, 0.65- and

260 0.37-fold of the controls in the 50, 100 and 200 μM PFOA-treated groups, respectively (Fig.

261 5A, 5B). *3β-HSD* gene, responsible for converting pregnenolone to progesterone, was

262 markedly reduced ($p < 0.01$) to 0.73-, 0.80- and 071-fold of the control in 50, 100 and 200 μM

263 PFOA-treated groups, respectively (Fig. 5A, 5B). Similarly, *CYP17α*, which plays a

264 significant role in steroid hormone synthesis, was also markedly reduced ($p < 0.01$) to

265 0.63-fold of the controls in the 200 μM PFOA-treated group (Fig. 5A, 5B). Interestingly, no

266 significant differences between the PFOA treatment groups and controls are observed for

267 *P450SCC* (catalyses cholesterol side-chain cleavage to pregnenolone) and *17β-HSD*

268 (catalyses androstenedione to testosterone) mRNA expression (Fig. 5A, 5B).

269 　　The levels of 17-OH progesterone following 50 μM PFOA were above control levels,

270 but no statistical difference was found. For the 100 μM PFOA groups, progesterone levels

271 were significantly increased ($p < 0.01$) to 1.31-fold of the control, whereas at 200 μM,

272 progesterone were significantly reduced ($p < 0.01$) to 0.53-fold of control (Fig. 5C). Similar to

273 17-OH progesterone, PFOA exposure induced biphasic effects on testosterone production in

274 MLTC-1 cells. PFOA effects at medium dose (100 μM) have significantly stimulatory effects

275 ($p < 0.01$) on testosterone production, *i.e.*, 1.84-fold compared to controls, while significantly

276 inhibitory ($p < 0.01$) effects at higher exposures are noted, *i.e.*, 0.50-fold at 200 μM compared

277 to controls (Fig. 5D).

278

279

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

324 (Fig. 3). According to our observation, it is shown that PFOA can inhibit mitochondrial outer

325 membrane fatty acid import-related gene (*CPTii*), while inducing mitochondrial matrix fatty

326 acid retransformation and oxidation metabolism products export-related gene (*CPTii*, *CRAT*)

327 expression. Our results are consistent with a previous study that showed that PFOA induced

328 *CPTii* and *CRAT* expression in human, rat and mouse liver cells [31-33]. Because of the

329 structural resemblance of PFOA and endogenous fatty acids, PFOA can be taken up in the

330 *in-vitro* cell model [34]. Kudo and co-authors imply that both PFOA and fatty acids can

331 transfer into mitochondria, but PFOA is unable to metabolize *via β*-oxidation [35]. In

332 agreement, we find that PFOA is transported into MLTC-1 cell cytoplasm and mitochondria.

333 We speculate that MLTC-1 cell reduces PFOA uptake by lowering *CPTii* expression, while

334 facilitating PFOA elimination by increase *CPTii* and *CRAT* expression, which leads to

335 disturbed fatty acids transport; further research is necessary to demonstrate this.

336 Cholesterol is a main substrate for testosterone biosynthesis. Leydig cells can synthesise

337 cholesterol in endoplasmic reticulum and use several potential sources of cholesterol for

338 steroidogenesis in mitochondria [36]. Previous studies suggest that PFOA can disrupt

339 cholesterol content by altering cholesterol transport and biosynthesis routes [9, 31, 32]. Our

340 results show that PFOA significantly weakens the cholesterol content in MLTC-1 cells. The

341 PXR and SR-B1 nuclear receptor as well as SREBP transcription factors responses

342 associated with cholesterol uptake are been proposed in the MTC-1 cells with PFOA

343 exposure. In order to further investigate the effects of PFOA on the cholesterol biosynthesis

344 pathway in MLTC-1 cells, the expression of a series of important genes in this pathway

345 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* 3β-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

368 alteration of MLTC-1 cell viability. This can be explained by the fact that many

369 environmental endocrine disruptor chemicals are reported to exhibit the ability to induce U

370 and/or invert U dose-response trends, which results into low-dose stimulation responses [39].

371 Similar studies have shown that exposures to low or high levels of phthalates or prolactin

372 have biphasic effects on testosterone production in MLTC-1 cells [40-42]. Alteration of

373 cholesterol transport and steroidogenic enzymes in MLTC-1 cells may be involved in the

374 biphasic effects of PFOA on androgen production. Our findings agree with the hypothesis

375 that low-dose stimulation corresponds to a negative feedback compensation mechanism that

376 counterbalances the endocrine disrupting chemicals-induced inhibition of gene expression of

377 the steroidogenic enzymes [43-44]. In conclusion, our results suggest that PFOA disrupts

378 cholesterol precursor fatty acid transport into mitochondria and then alters cholesterol

379 synthesis. Meanwhile, PFOA-regulated nuclear (membrane) receptor response and

380 steroidogenesis result in disruption of sex hormones secretion. In particular, PFOA has a

381 biphasic effect on testosterone and progesterone production.

382 **Conflict of interest statement**

383 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.

Figure 4. PFOA affect 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. 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 5. PFOA affect steroid hormone biosynthetic pathways in MLTC-1 cells, steroidogenesis gene expression (A), steroidogenesis gene pathway (B), 17-OH progesterone secretion (C), and testosterone secretion (D).

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, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

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

	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

Data are represented as mean ± SEM 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, * $p \leq 0.05$, ** $p \leq 0.01$
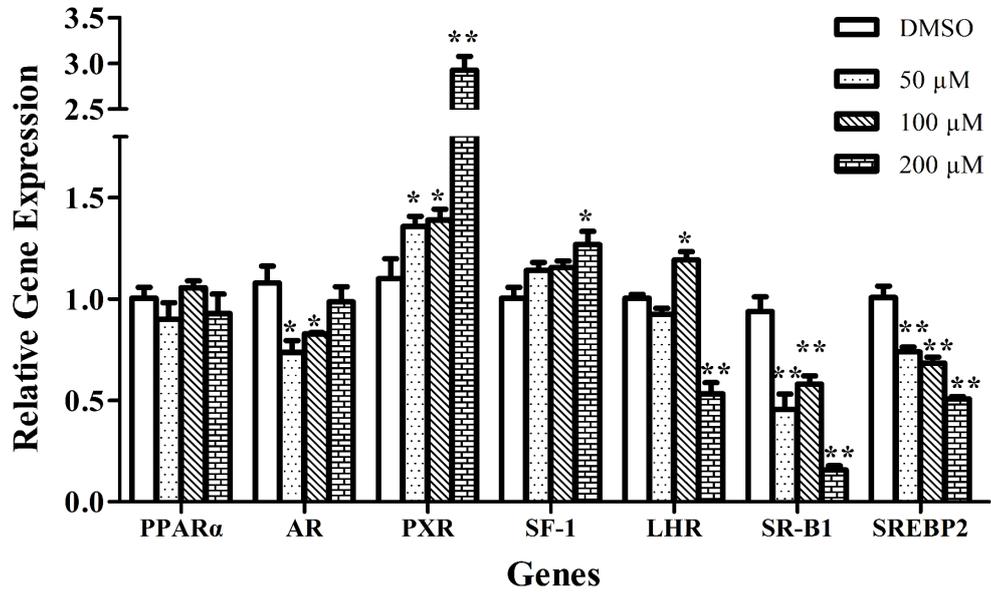
582 **Figure 1**

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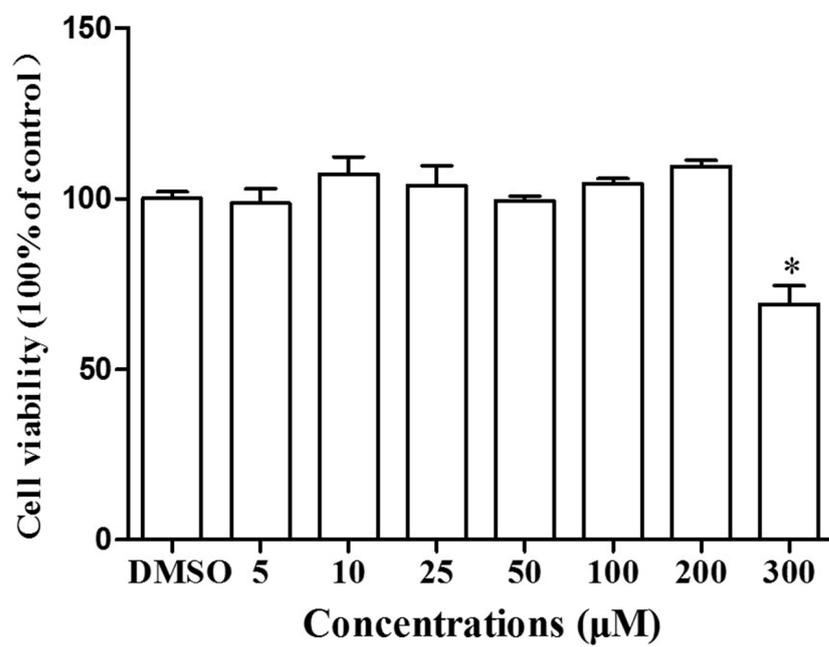
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586   **Figure 2**
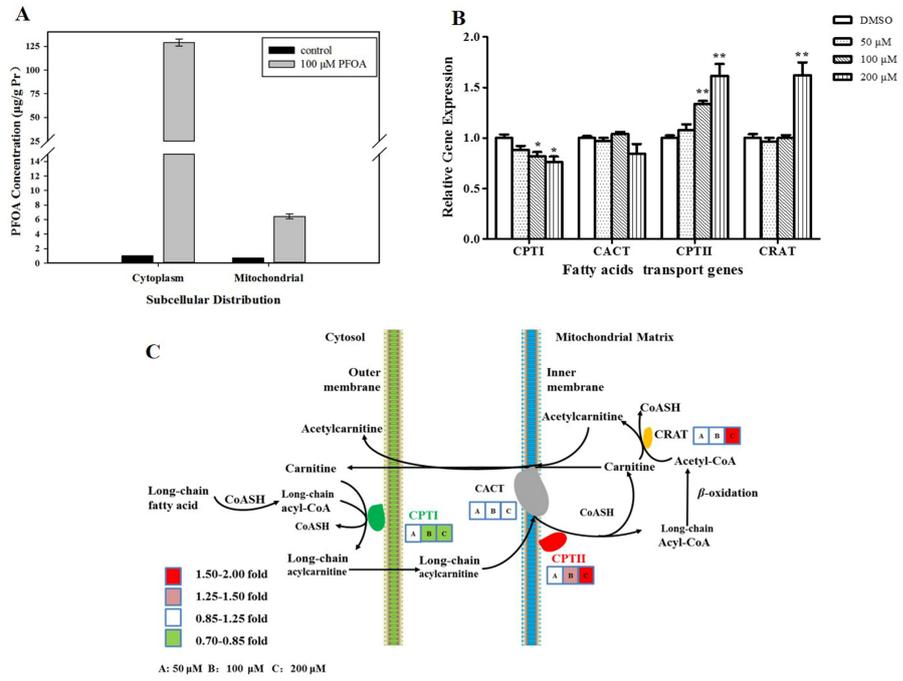
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Figure 3
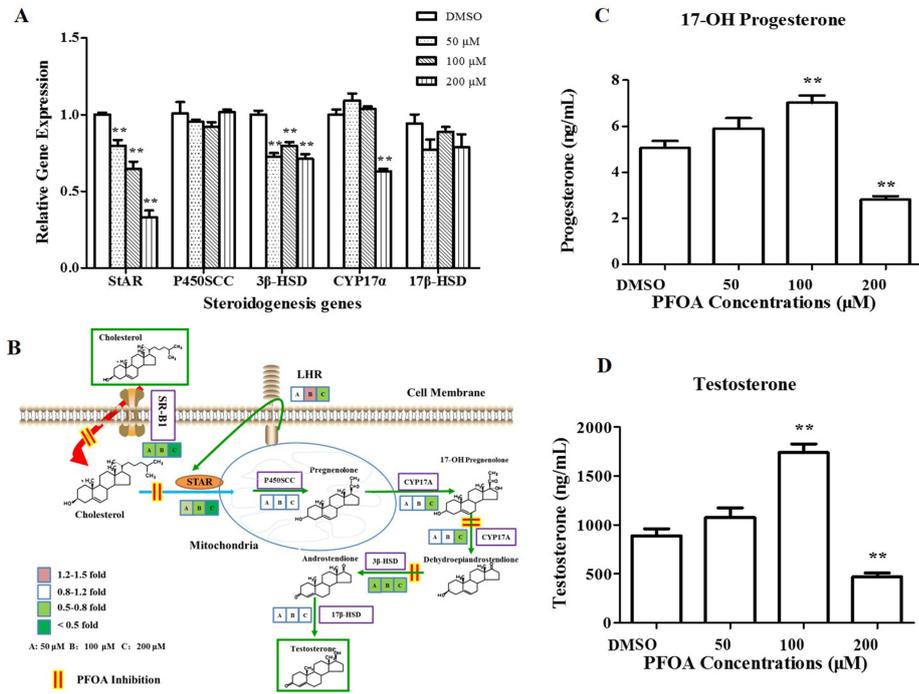
594   **Figure 4**

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Figure 5