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Inhibition effects of perfluoroalkyl acids on progesterone production in mLTC-1

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ABSTRACT

Perfluoroalkyl substances (PFASs) are a class of fluorine substituted carboxylic acid, sulfonic acid and alcohol, structurally similar to their corresponding parent compounds. Previous study demonstrated the potential endocrine disruption and reproductive toxicity of perfluorooctane sulfonic acid and perfluorooctanoic acid, two dominant PFASs in animals and humans. We explored the relationship between eleven perfluoroalkyl acids (PFAAs) with different carbon chain length and their ability to inhibit progesterone production in mouse Leydig tumor cells (mLTC-1). We found an obvious dose–response relationship between progesterone inhibition rate and PFAA exposure concentration in mLTC-1. The relative inhibition rate of progesterone by PFAAs was linearly related to the carbon chain length and molar refractivity of PFAAs. Mitochondrial membrane potential (MMP) decreased after PFAA exposure at the half-maximal inhibitory effect concentration (IC_{50}) of progesterone production in mLTC-1, while the reactive oxygen species (ROS) content increased significantly. These results imply that the inhibition effect of PFAAs on progesterone production might be due, in part, to ROS damage and the decrease in MMP in mLTC-1.

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Introduction

Perfluoroalkyl substances (PFASs) are a family of man-made, highly stable and hydrophobic organic compounds, widely used in fabrics and fast-food container material sand fire-resistant foams (Frisbee et al., 2009). This organic family shares common structural and physico-chemical characteristics and properties, but differs in backbone carbon chain length and functional group, the main features used to classify PFASs into perfluoroalkyl acids (PFAAs) and fluorotelomer alcohols (Buck et al., 2011). Fluorotelomer alcohols serve as precursors that can be transformed into PFAAs (Dinglasan et al., 2004). Perfluoroalkyl carboxylic acids (PFCAs) and perfluoroalkane sulfonic acids (PFSAs) are

the two main categories of PFAAs, and perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS) are the primary representatives of PFCAs and PFSAs, respectively. Their good properties have made them common industrial and consumptive products since the 1950s (Renner, 2001), leading to massive distribution of PFAAs not only in air, soil, and water, but also in biota globally (Giesy et al., 2001; Dai et al., 2006; Olsen et al., 2003, 2005).

Over the past few decades, PFAAs have drawn increasing attention from scientists due to their environmental persistency, bioaccumulation, and extensive distribution worldwide. Animal experiments and epidemiological studies have indicated that PFAAs exhibit considerable toxicities, including hepatotoxicity, developmental toxicity, immunotoxicity,

and glucose homeostasis disorder (Steenland et al., 2010; Lau et al., 2007; Rosen et al., 2013; Halldorsson et al., 2012; Maisonet et al., 2012; Corsini et al., 2014; Nelson et al., 2010; Taylor et al., 2013). The reproductive and developmental toxicities of PFAAs, which can result in broad and varied health consequences in offspring, are of particular concern. A nationwide cohort study in the US suggested an inverse association between maternal plasma PFOA levels and birth weight of newborns (Fei et al., 2007). Similarly, epidemiological studies on human cord blood from mothers found that PFOA was capable of traversing the human placental barrier and exposing the fetus *in utero*, implying a potential developmental toxicity of PFAAs (Apelberg et al., 2007). In addition, PFAAs may serve as endocrine disruptors and cause adverse effects (De Coster and van Larebeke, 2012; White et al., 2011). For example, sperm quality among 105 Danish men from the general population was reportedly diminished in association with more intense exposure to PFOA and PFOS (Joensen et al., 2009). Animal research has also demonstrated that *in utero* exposure to PFOA lowered sperm concentration and total sperm count, and increased

follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Vested et al., 2013).

Although extensive research on potential endocrine disruption and reproductive toxicology has been carried out with PFOA and PFOS, which are both dominant PFAAs in animals and humans, few reports have compared the endocrine disruption abilities among different PFAAs. Given that PFAAs of varying chain length (C4–C14) have been detected in humans, though at lower levels (Olsen et al., 2005), comparative study will be very helpful in clarifying the contribution of different backbone carbon chain lengths and functional groups to toxicity. Mouse Leydig tumor cells (mLTC-1) are commonly used cell line in steroidogenesis related research. To some degree, mLTC-1 can maintain the properties of the original Leydig cell and be responsive under certain stimuli, such as adenosine 3′,5′-cyclic monophosphate (cAMP), LH, and human chorionic gonadotropin (hCG), and are also capable of translating messages for the production of steroids, such as that done by normal Leydig cells (Rebois, 1982). In this study, we investigated the concentration–response
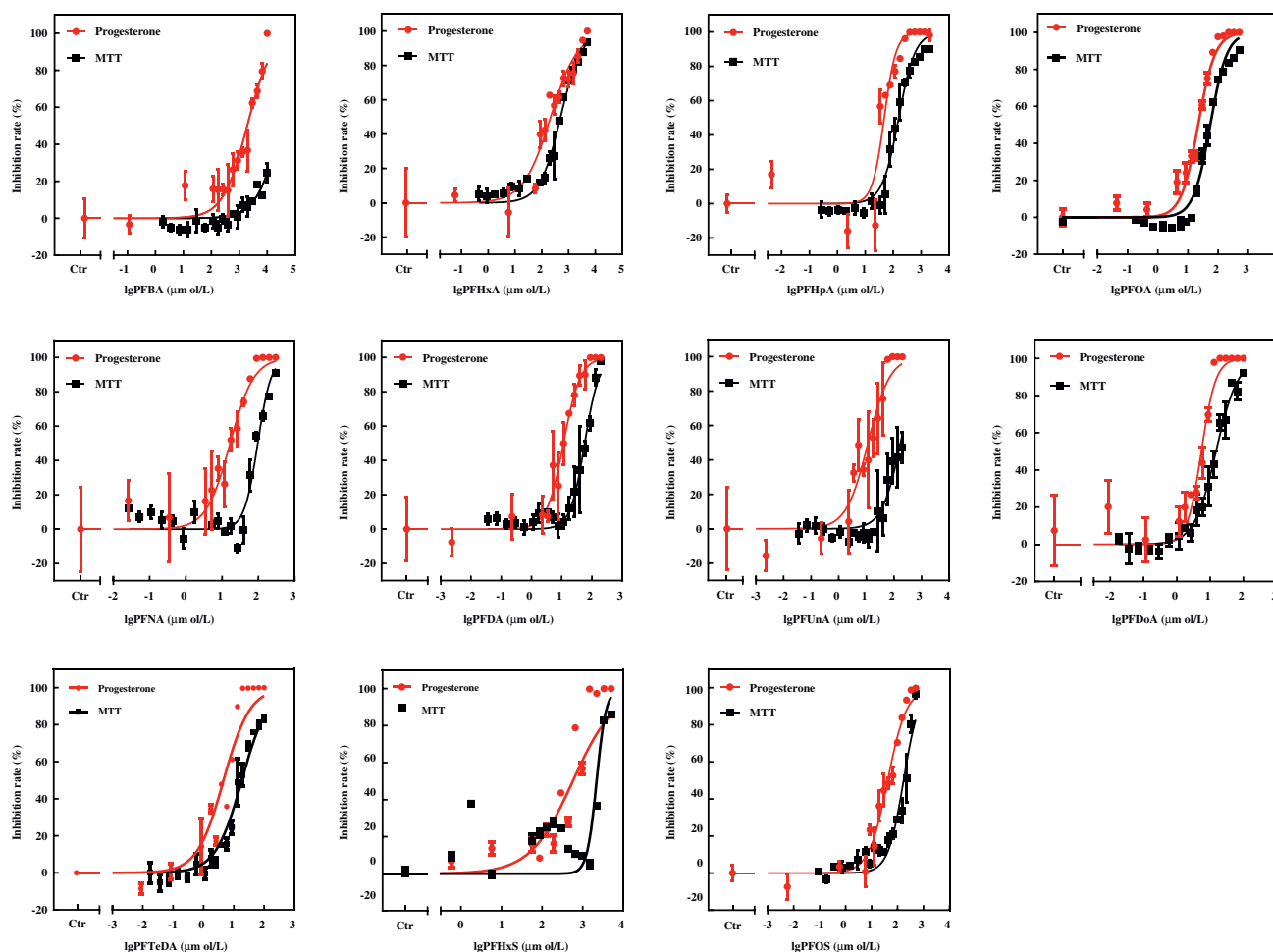

Fig. 1 – S-shape dose–response relationship between inhibition rate of progesterone production (red line and dots), and that of cell activity (black line and squares) and PFAA concentration after mLTC-1 were exposed to eleven individual PFAAs for 24 hr. Results are expressed as mean ± SEM (*n* = 3). Ctr: control; PFAA: perfluoroalkyl acid; mLTC-1: mouse Leydig tumor cells; SEM: standard error of the mean.

Table 1 – Goodness of fit dose–response curve and predicted IC$_{50}$ of PFAAs on progesterone production.

Chemicals	Goodness of fit (R^2)	Normality of residuals		Predicted IC$_{50}$ (μmol/L)
		Shapiro–Wilk	p Value	
PFBA	0.8171	0.976	0.4658	1930
PFHxA	0.9198	0.9761	0.4702	213.8
PFHpA	0.9018	0.9039	0.0013	47.75
PFOA	0.9661	0.9533	0.0678	21.73
PFNA	0.8823	0.9482	0.0435	16.61
PFDA	0.9314	0.9490	0.0465	11.52
PFUnA	0.8642	0.9663	0.2347	11.53
PFDoA	0.9288	0.9593	0.1222	5.47
PFTeDA	0.9283	0.9673	0.2312	5.141
PFHxS	0.9038	0.8935	0.0006	450.3
PFOS	0.9587	0.9661	0.2071	41.71

PFAAs: perfluoroalkyl acids; PFBA: perfluorobutyric acid; PFHxA: perfluorohexanoic acid; PFHpA: perfluoroheptanoic acid; PFOA: perfluorooctanoic acid; PFNA: perfluorononanoic acid; PFDA: perfluorodecanoic acid; PFUnA: perfluoroundecanoic acid; PFDoA: perfluorododecanoic acid; PFTeDA: perfluorotetradecanoic acid; PFHxS: potassium perfluorohexane sulfonate; PFOS: perfluorooctane sulfonic acid.

relationships between individual PFAAs and the progesterone production rate in mLTC-1, the quantitative structure–activity relationship (QSAR), and the reasons behind steroidogenesis change after PFAA exposure.

1. Materials and methods

1.1. Chemicals

Perfluorobutyric acid (PFBA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid (PFDoA), perfluorotetradecanoic acid (PFTeDA), PFOS, potassium perfluorohexane sulfonate (PFHxS), and cAMP were purchased from Sigma-Aldrich (St. Louis, MO, USA), and their purities were all greater than 95%. The PFAAs were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) and then diluted in Hyclone RPMI-1640 culture medium (UT, USA) as stock solution. The working solution for cell treatment was diluted with RPMI-1640 culture medium on the day of treatment, and the maximum concentration of DMSO in the working solution was not greater than 0.1%.

1.2. Cell culture

The mLTC-1 (Shanghai Institute of Cell Biology, Chinese Academy of Sciences, China) were grown in RPMI-1640 complete culture medium, supplemented with 10% fetal bovine serum (FBS) and 100 UI penicillin–streptomycin, in a humidified atmosphere of 5% CO_2 at 37°C.

1.3. Progesterone detection, cell activity assay and dose–response curve

For progesterone detection, mLTC-1 were plated in 48-well Corning plastic plates at a density of 5×10^4 cells per well and then treated by RPMI-1640 serially diluted PFAAs (Con$_n$/Con$_{n-1}$ =2/3) for 24 hr. After 24 hr incubation, the treatment solution was aspirated and then freshly prepared cAMP (1 mmol/L) was added to the mLTC-1 for another 3 hr treatment. The supernatant (total volume 200 μL) was collected for progesterone quantification using radioimmunoassay (RIA) by the Beijing North Institute of Biological Technology, China. The inter- and intra-assay coefficients of variation were not greater than 15%. For cell viability assess-
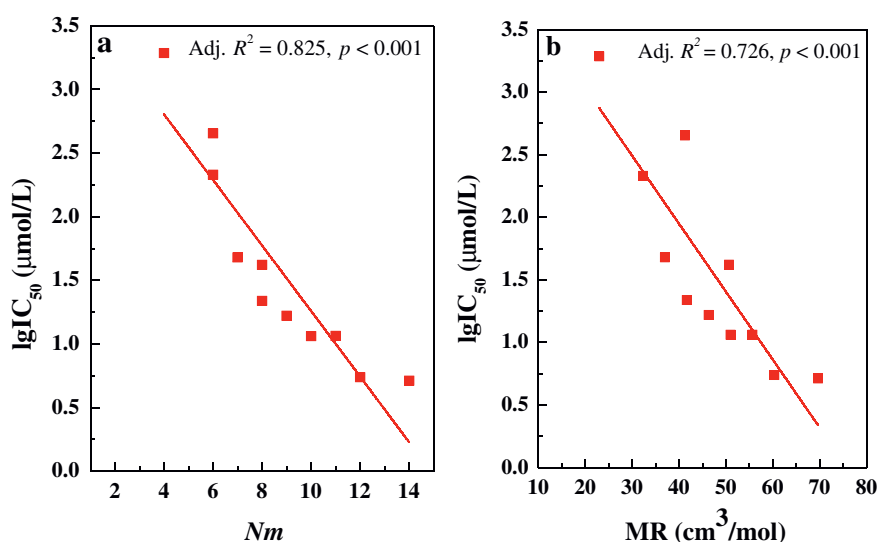

Fig. 2 – (a) Linear regression plot between lgIC$_{50}$ values of progesterone production and number of backbone carbon atoms (*Nm*) of the eleven PFAAs; (b) linear regression plot between lgIC$_{50}$ of progesterone production and molar refractivity (MR) of the eleven PFAAs.

ment, the mLTC-1 were plated into 96-well plates and incubated for 24 hr, then treated with PFAAs for an additional 24 hr. After treatment, cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA) assay. Briefly, 20 μL of MTT (5 mg/mL, dissolved in phosphate buffer saline (PBS)) was added to each well, followed by incubation at 37°C for 4 hr. The supernatants were then removed from the plates, and the produced formazan was dissolved in 150 μL of DMSO and corresponding optical density was measured at 550 nm using a spectrophotometer (BioTek, Synergy H1 Hybrid Microplate Reader, USA). The inhibition of cellular viability (I, %) was calculated as Eq. (1):

$$I = (1 - OD_{treated}/OD_{untreated}) \times 100\% \tag{1}$$

where, $OD_{treated}$ is the optical density in PFAAs treated group; $OD_{untreated}$ is the optical density in control group.

To calculate half-maximal inhibitory effect concentration (IC$_{50}$, μmol/L) on progesterone production, Prism 5.0 (GraphPad Software, San Diego, CA, USA) was used to fit a normalized dose–response curve. The S-shaped normalized dose–response curve was described by Eq. (2), as follows:

$$R_i = 100/\left(10^{(lgIC_{50}-x)} + 1\right) \tag{2}$$

where, R_i means inhibition rate, x means lg(PFAA concentration).

Relative progesterone level change and relative cell activity inhibition were calculated by Eq. (3):

$$R_i = 1 - (C_{treatment} - C_{blank}/C_{control} - C_{blank}) \tag{3}$$

where, $C_{treatment}$ (ng/mL) is the progesterone concentration in PFAAs and cAMP treated group; C_{blank} (ng/mL) is the blank value of progesterone concentration in substance-free sample; $C_{control}$ (ng/mL) is the progesterone concentration in control group that only treated by cAMP.

Regression analysis was performed using non-linear least-squares fit. The higher the coefficient of determination (R^2) and the lower the Chi-square, the better was the fit. As a quantitative measure of uncertainty, the observation-based 95% confidence interval was also determined.

1.4. Calculation of molecular descriptors and QSAR analysis

Molecular descriptors of PFAAs, which mainly included physicochemical parameters and topological structure indices, were computed with ChemBioOffice (PerkinElmer Inc., Waltham, MA, USA). Because these PFAAs shared a common skeleton structure, many of their molecular descriptors (or their log transformation values) were linearly related with each other. Therefore, only molar refractivity (MR) and some topological connectivity indices were adopted for QSAR analysis. The relationship between biological activities and physicochemical parameters was obtained by linear regression with a confidence interval of 95% (Zhu et al., 2009).

Table 2 – Molecular descriptors of PFAAs computed by ChemBioOffice.

Chemical	lgP* (by Crippen's fragmentation)	Total valence connectivity	Balaban index	Wiener index	Molecular refractory(MR) (cm³/mol)	Molecular topological index	Gibbs free energy (kcal/mol)	Dipole_Dipole	Henry's constant	Molecular weight	lgK$_{ow}$
PFBA	2.04	1.26E−05	66800	216	22.99	891	−1716.26	49.68	2.31	214.04	3.39
PFHxA	3.25	6.42E−08	402908	594	32.33	2364	−2472.98	81.67	0.87	314.06	4.90
PFHpA	3.85	4.58E−09	808226	882	36.99	3483	−2851.34	96.87	0.15	364.06	5.71
PFOA	4.46	3.27E−10	1485222	1248	41.66	4905	−3229.7	112.13	−0.57	414.06	6.44
PFNA	5.06	2.34E−11	2550579	1701	46.33	6666	−3608.06	127.57	−1.29	464.08	7.17
PFDA	5.66	1.67E−12	4150063	2250	51.00	8802	−3986.42	144.94	−2.01	514.08	7.90
PFUnA	6.27	1.19E−13	6461730	2904	55.67	11349	−4364.78	160.88	−2.73	864.09	8.63
PFDoA	6.87	8.52E−15	9699125	3672	60.33	14343	−4743.14	176.55	−3.45	614.10	9.36
PFTeDA	8.08	4.35E−17	20001927	5586	69.67	21,816	−5499.86	208.14	−4.89	714.11	10.82
PFHxS	3.93	4.58E−09	991921	988	41.30	4073	−2652.67	126.55	0.00	399.94	5.25
PFOS	5.14	2.34E−11	3002732	1864	50.64	7580	−3409.39	160.29	0.00	538.22	7.03

lgP: logarithm of partition coefficient.

1.5. Measurement of mitochondrial membrane potential (MMP)

The mLTC-1 were plated in black-wall 96-well plates and exposed to PFAA concentrations around their respective IC_{50} values of progesterone production for 24 hr after 24 hr incubation at 37°C and 5% CO_2. The mLTC-1 were then treated with 1 mmol/L cAMP for 3 hr. After treatment, cells were washed with PBS once and incubated with DMSO dissolved tetrachloro-tetraethyl benzimidazol carbocyanine iodide (JC-1) (5 μg/mL) (Beyotime, Shanghai, China) for 20 min at room temperature, then washed with PBS twice and analyzed using fluorescence plate reader (Synergy H1 Multi-Mode Reader, BioTek, Winooski, VT, USA). Settings were as follows: red ($Ex_{530\ nm}$; $Em_{590\ nm}$); green ($Ex_{480\ nm}$;

$Em_{530\ nm}$). The relative MMP ($\Delta\Psi$) was expressed as the ratio of red fluorescence intensity and green fluorescence intensity ($RFU_{590\ nm}/RFU_{530\ nm}$) (RFU, relative fluorescence unit). The fluorescent photos were collected under a Nikon fluorescence microscope (40×) (Nikon, Tokyo, Japan).

1.6. Reactive oxygen species (ROS) level in mLTC-1

Fluorescein 2′,7′-dichlorodihydro-fluorescein diacetate (DCFH-DA) can permeate into cells and is cleaved by non-specific esterase, and can be turned to high fluorescence upon oxidation by ROS. Using DCFH-DA (Beyotime Inc., Shanghai, China) as a probe, ROS changes in the cells after treatment were analyzed by a spectrophotometer (Synergy H1 Multi-Mode Reader, BioTek, Winooski, VT, USA).
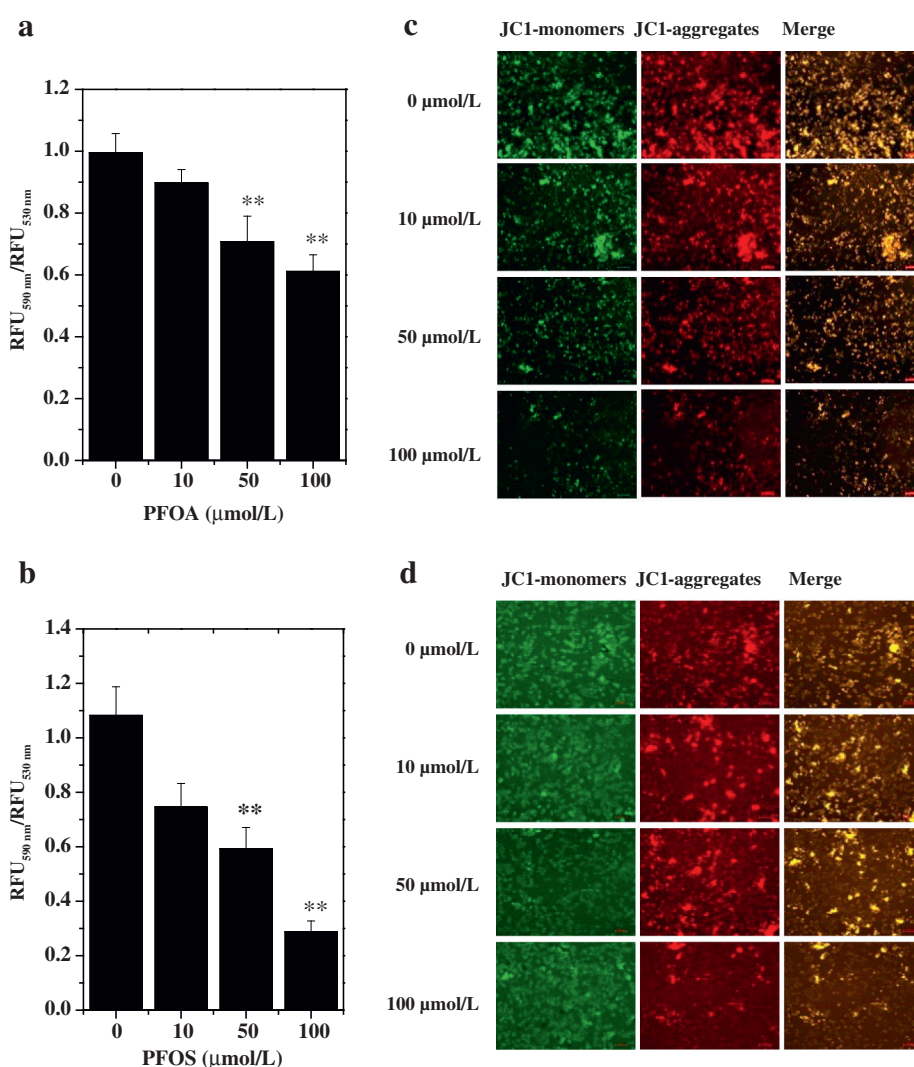

Fig. 3 – mLTC-1 mitochondrial membrane potential ($\Delta\Psi_m$) changes after exposure to PFOA and PFOS for 24 hr. The graphical representation (a, b) of the ratio of JC-1 aggregates to JC-1 monomers ($Em_{590\ nm}:Em_{530\ nm}$) revealed $\Delta\Psi_m$ dissipation after PFOA (a) and PFOS exposure (b). Results are expressed as the mean ± SEM of six measurements in one representative experiment. Significant difference between control and treatments is indicated by [**]$p < 0.01$. Microscope photos show the mitochondrial status dyed by JC-1 under fluorescence microscope after PFOA (c) and PFOS exposure (d). Red fluorescence represents the aggregate of JC-1; green fluorescence represents monomer of JC-1; the orange fluorescence, merged by red and green fluorescence. Scale bar: 100 μm. SEM: standard error of the mean.

1.7. Data processing and statistical analyses

All experimental results were presented as mean ± standard error of the mean (SEM). Statistical significance was determined using one-way analysis of variance (ANOVA) with Fisher's least significant difference (LSD) test. The equal variance of data was checked by the Levene homogeneity of variance test before ANOVA test. Differences were considered significant at $p < 0.05$ (*) and highly significant at $p < 0.01$ (**).

2. Results and discussion

2.1. PFAAs inhibited progesterone levels with an S-shaped dose–response curve

PFAAs may traverse the blood–testis barrier and are implicated in male reproductive dysfunction, including sperm count reduction and semen quality decrease, with Leydig cell steroidogenesis
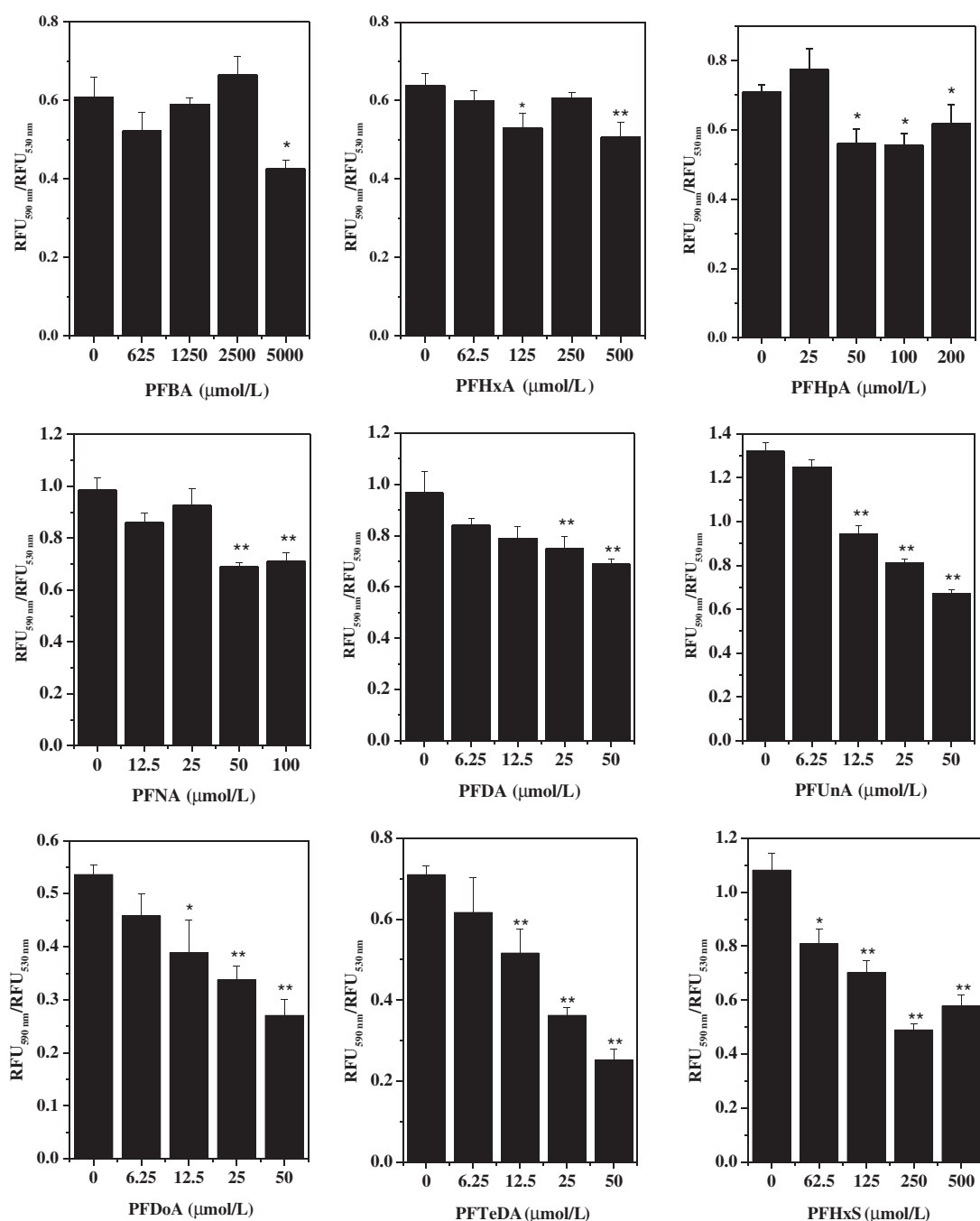

Fig. 4 – Mitochondrial membrane potential change after 24 hr exposure to different PFAAs under concentrations around their respective IC_{50} values of progesterone production. Results are expressed as the mean ± SEM of six measurements in one representative experiment. Significant difference between control and other concentrations is indicated by $^*p < 0.05$ or $^{**}p < 0.01$. SEM: standard error of the mean.

suggested as a primary target of these endocrine disrupting chemicals (Shi et al., 2010; Wan et al., 2011). Because normal Leydig cells located in situ only comprise a very low percentage of cell mass in the testis, which makes the enrichment of large quantities of viable Leydig cells a difficult task, mLTC-1 are commonly used in steroidogenesis related research (Rebois, 1982). Under stimulation (such as cAMP in the present study), mLTC-1 mainly produce progesterone rather than testosterone. The differences in tumor and normal Leydig cells may have arisen from alterations in mutations or gene regulation, involving the pathways responsible for the conversion of progesterone to testosterone (Ascoli and Puett, 1978). In the present study, we observed a dose–response relationship between progesterone content and PFAA exposure concentration in mLTC-1. As shown in Fig. 1 (red line), progesterone content in mLTC-1 decreased with the increase in PFAA treatment concentration. This relationship was illustrated by a standardized S-shape curve (dose–response curve) with lower asymptote zero and upper asymptote 100 and unstrained IC_{50} and Hill slope. The dose–response curve fitting results (Table 1) verified the goodness of fit. Different carbon length PFAAs presented similar effect curves on relative progesterone level, which coincided with the structural similarity of PFAAs.

PFAAs can affect cell activity and exert dose–response effects on cell viability (Hu et al., 2014). In the present study, cell viability accompanied by PFAA treatment was also detected by MTT assay. Similarly, standardized S-shape curves were fitted (Fig. 1, black line). The very similar Hill slopes of the dose–response curves for progesterone and cell activity following all PFAAs treatments indicated comparable change tendencies for progesterone content and cell viability with PFAAs exposure. For each individual PFAA, however, the decreasing degree of relative progesterone content was greater than that of cell activity, demonstrating that the adverse effects on cell viability loss only partially explained the relative progesterone content decrease after PFAAs

exposure. For example, progesterone content decreased by nearly 20% under PFAAs exposure concentrations at which cell viability was slightly or little affected, indicating that progesterone content was more sensitive than cell viability to PFAAs exposure.

2.2. Inhibitory effect on progesterone increased with the elongation of the carbon chain

Although different carbon length PFAAs presented similar S-shape effect curves for relative progesterone level in mLTC-1, as the carbon chain length increased, the equivalent effect concentration became smaller. For example, the IC_{50} was about 2 mmol/L for PFBA and only 5 μmol/L for PFTeDA (Fig. 1). To further explore the relationship of PFAA chain length with progesterone inhibitory effects, a linear regression analysis was carried out between $lgIC_{50}$ and the number of carbon atoms in a backbone (defined as Nm) for the eleven PFAAs. Results showed that with the elongation of the PFAA backbone, the progesterone inhibitory effect became stronger (Fig. 2a). Evidence suggests that the molecular property and toxicological effects of PFAAs are closely correlated with carbon chain length. For example, the actions of PFAAs with varying chain length on cultured rat hippocampal neurons showed that the increase in spontaneous miniature postsynaptic current (mPSC) frequency was in proportion to carbon chain length (Liao et al., 2009). The transthyretin binding affinity of PFAAs was also shown to be associated with their carbon chain (Weiss et al., 2009). Wolf and his colleagues evaluated the ability of numerous PFAAs to induce mouse and human peroxisome proliferator-activated receptor alpha (PPAR alpha) activity in a transiently transfected COS-1 cell assay, the result showed that the activation of PPAR alpha by PFAAs was positively correlated with carbon chain length, up to C9 (Wolf et al., 2008). In addition, the transcriptional activation of peroxisome proliferation genes in primary rat hepatocytes by PFAAs was also related to the length of each compound's carbon
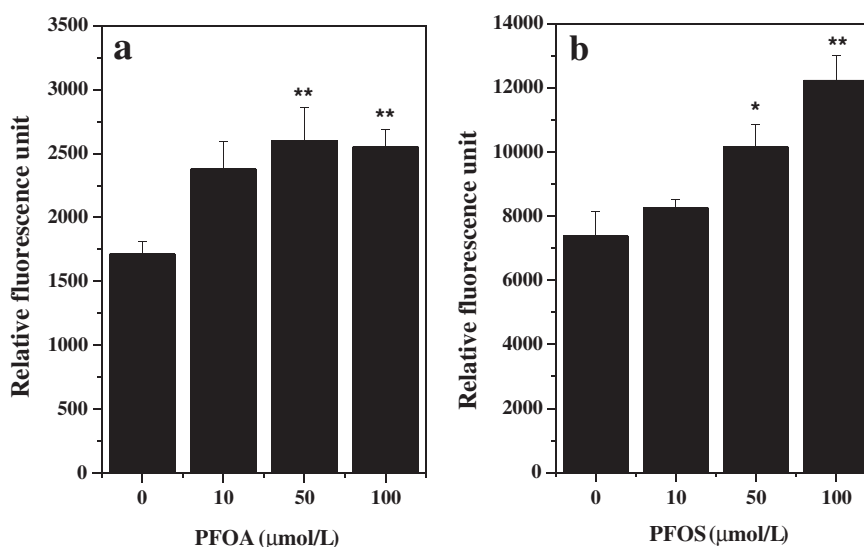

Fig. 5 – ROS content in mLTC-1 after 24 hr exposure to PFOA (a) and PFOS (b). Results are expressed as the mean ± SEM of six measurements in one representative experiment. Significant difference between control and treatments is indicated by [*]p < 0.05 or [**]p < 0.01. ROS: reactive oxygen species; SEM: standard error of the mean.

chain (Bjork and Wallace, 2009). For QSAR analysis, the molecular descriptors of the PFAAs, which mainly included physicochemical parameters and topological structure indices, were computed with ChemBioOffice (Table 2). The QSAR analysis indicated that the $lgIC_{50}$ values of the eleven PFAAs were linearly related with MR (Fig. 2b), a measure of molecular polarity that increases with the elongation of the PFAA backbone. The results indicated that the MR of PFAAs played an important role in their inhibitory effect on progesterone in mLTC-1.

2.3. MMP reduction and ROS increase contributed to the progesterone inhibitory effect of PFAAs in mLTC-1

Mitochondria are important organelles, providing the location, energy, and precursor needed for progesterone production. MMP ($\Delta\Psi_m$), maintained by the proton concentration gradient between the outer- and inner-membrane of mitochondria, is a primary indicator of mitochondrial health and is utilized for numerous processes, including the powering of mitochondrial adenosine triphosphate (ATP) synthesis and protein import (Wittig and Schagger, 2009). Studies have shown that mitochondria must be energized, polarized, and actively respiring to support steroidogenesis in Leydig cells, and agents that disrupt $\Delta\Psi_m$, such as protonophore carbonyl cyanide m-chlorophenylhydrazone, inhibit steroidogenesis (Allen et al., 2006; King and Stocco, 1996). As the fluorescence intensity ratio ($RFU_{590\,nm}/RFU_{530\,nm}$) results show (Fig. 3a and b), MMP decreased gradually with the dose increase of PFOA and PFOS. At concentrations close to IC_{50} (50 μmol/L) of progesterone production by PFOA and PFOS, MMP reduced to about 70% and 50% of the level in the control, respectively, and more dramatic decreases in MMP were observed after higher doses of chemical treatment (100 μmol/L). Similar conclusions could be drawn with the representative fluorescence microscope photos (Fig. 3c and d). At higher concentrations (50 and 100 μmol/L) of PFOA and PFOS, the red fluorescein in the cell population became dim. To explore MMP changes after different PFAA exposure, the exposure concentration around IC_{50} in the progesterone production assay of individual PFAAs was chosen. Results showed an evident decreasing tendency in MMP with dose increase for most PFAAs, except PFBA (Fig. 4). The significant reduction in MMP by PFAAs indicated the possible occurrence of mitochondrial permeability transition, which can lead to impairment of mitochondrial functionality, release of apoptogenic signal molecules, and loss of matrix components (Kowaltowski et al., 2001). Previous study showed that the decrease in $\Delta\Psi_m$ likely plays a significant role in the disturbance of basal and hCG-induced steroidogenesis caused by brominated flame retardant, hexabromocyclododecane, in rat Leydig cells (Fa et al., 2015).

ROS can be generated due to mitochondrial functionality impairment and loss of components in the electron transport chain (Kowaltowski et al., 2001). Furthermore, PFAAs can induce ROS formation (Panaretakis et al., 2001; Reistad et al., 2013), leading to deoxyribonucleic acid (DNA) strand breaks and oxidative DNA lesions *in vitro* (Yao and Zhong, 2005). We measured ROS alteration after PFOA and PFOS exposure in mLTC-1. Results indicated that PFOA and PFOS exposure induced ROS increase in mLTC-1 (Fig. 5). Exposure to ROS has been reported to mediate decline in steroid hormone production and senescence of Leydig cell function during aging (Chen et al., 2001;

Midzak et al., 2009; Zirkin and Chen, 2000), and to affect steroidogenic function in primary Leydig cells (Tsai et al., 2003) and MA-10 tumor Leydig cells (Chen et al., 2010; Stocco et al., 1993).

In summary, PFAAs decreased progesterone production in mLTC-1, which showed a dose–response pattern and was linearly related to the characteristic properties of PFAAs, such as carbon backbone length and MR. With the elongation of the carbon chain in PFAAs, their ability to inhibit progesterone strengthened. In addition, PFAAs led to a higher ROS level and lower MMP. The results implied that oxidative stress and MMP ($\Delta\Psi_m$) disturbance exerted by PFAAs at least partially contributed to their inhibition effect on progesterone production in mLTC-1.

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