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The Mechanism of Mitochondrial Damage in PFOS-Induced $HepG_2$ Cell Apoptosis

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ABSTRACT

To investigate the mechanism underlying the induction of apoptosis in $HepG_2$ cells following perfluorooctanesulfonate (PFOS) treatment. The inhibitory function of PFOS on $HepG_2$ cell proliferation was detected using a Cell Counting Kit-8 (CCK-8) reagent kit. Five PFOS dose groups (100, 150, 200, 250 and 300mg/L) were established. After a 48-h PFOS treatment, the cell apoptosis rates were analyzed with flow cytometry (FCM). The mitochondrial membrane potential (MMP) was measured with JC-1reagent kit. The morphological changes of apoptotic nuclei were observed using the Hoechst 33258 staining method. After treatment with PFOS, the mRNA expression levels of apoptosis-related proteins, including cytochrome C, caspase-3, apoptosis-inducing factor (AIF) and Bax, were detected using real-time fluorescence quantitative PCR. The results show that PFOS had obviously inhibitory effects on $HepG_2$ cell proliferation in a dose- and time-dependent manner. And with increasing of PFOS level, the cell apoptosis rate gradually increased. PFOS treatment reduced the MMP and increased the mRNA expression levels of apoptosis-related proteins. PFOS could inhibit proliferation and induce apoptosis in $HepG_2$ cells. The mechanism for this induction might be associated with the compound's influences on the mRNA expression levels of apoptosis-related factors, including cytochrome C, caspase-3, AIF and Bax.
KEY WORDS: PFOS; $HepG_2$; Apoptosis; Mitochondrion

INTRODUCTION

Perfluorooctanesulfonate (PFOS) is a representative compound of perfluorinated compounds (PFCs) and is a major metabolic product of other PFCs [1]. Because of its stable chemical properties, high surface activity and hydrophobic and hydrophobic characteristics, it has been applied extensively in textile, paper, food packaging, carpet, leather, shampoo and fire extinguisher industries and the civil industry since the mid-20th century [2, 3]. However, many studies in recent years have shown that, because of its recalcitrance and accumulation features, PFOS has caused water and air pollution worldwide; it can even be found in the Arctic region [4, 5]. Human can be exposed to PFOS through the digestive tract. After PFOS enters the body, it mainly accumulates in the blood, liver, kidney and spleen [6, 7]. Because the compound has low clearance rates in the kidney, it has a long half-life in the body, with a half-life in blood that can reach 5.4 years [8]; therefore, this compound receives a great deal of attention from scientists.

The liver is one of the major target organs of PFOS toxicity. *In vivo* and *in vitro* studies have all shown that PFOS is hepatotoxic and can cause hepatomegaly, hepatic steatosis and aggravation of hepatitis [9-11]. However, the mechanism underlying the induction of hepatocyte apoptosis by PFOS is not clear. Therefore, this study observed the mechanism underlying the induction of apoptosis in human hepatoma $HepG_2$ cells by PFOS to preliminarily investigate the compound's possible apoptosis mechanism to provide theoretical and experimental bases for PFOS toxicity studies in the future.

1. MATERIALS AND METHODS

1.1 Materials

The human hepatoma $HepG_2$ cell line was from the cell bank of the Chinese Academy of Sciences. Culture reagents, including 1640 culture medium, fetal bovine serum (FBS) and trypsin, were purchased from Gibco (USA). PFOS was purchased from Sigma (USA). A Cell Counting Kit-8 (CCK-8) reagent kit, a MMP detection reagent kit and Hoechst 33258 staining solution were purchased from Beyotime (China). A fluorescein isothiocyanate (FITC)-Annexin V apoptosis detection reagent kit was purchased from BD (USA). A Prime Script^{TM} RT reagent kit and a SYBR Premix Ex Taq^{TM} reagent kit were purchased from Takara (Japan). Primers

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were synthesized by Shanghai Generay Biotech (China). Dimethyl sulfoxide (DMSO) was purchased from Sigma (China). TRIzol was purchased from Life Technologies (USA).

1.2 Cell culture

$HepG_2$ cells were inoculated in 1640 culture medium containing 10% FBS and were cultured at 37°C with 95% humidity and 5% CO_2. Fresh culture medium was added to the cells every other day. When the cell confluence reached more than 80%, experiments were performed or cells were passaged.

1.3 Effects of PFOS on $HepG_2$cell proliferation

$HepG_2$cells in the logarithmic growth phase were prepared in a single-cell suspension at 5×10^4 cells/ml and inoculated onto a 96-well plate at 100μL/well. Cells were cultured at 37°C with 95% humidity and 5% CO_2 for 24h. Cells were treated with different concentrations of PFOS (3.125, 6.25, 12.5, 25, 50, 100, 200 and 400mg/L). In addition, a blank control group was established. After the cells were treated with PFOS for 24, 48.and 72h, the 96-well plate was washed with 1×PBS once, cultured medium was replaced with 100μL of serum-free culture medium, and 10μL of CCK-8 reagent was added into each well. Cells were then incubated at 37°Cfor 1h. The absorbance at 450nm wavelength was measured using an automated Enzyme-Linked Immunosorbent Assay (ELISA) reader (Thermo, USA), and cell survival rates were calculated.

1.4 Detection of cell apoptosis using flow cytometry (FCM)

Cells in the logarithmic growth phase were prepared in a single-cell suspension at 1×10^5 cells/ml and were inoculated onto 6-cm culture plates at 6ml/plate. After the cells cultured at 37°Cand 5% CO_2 for 24h, the cells were treated with different concentrations of PFOS (0, 100, 150, 200, 250 and 300mg/L) for 48h. Cells were washed with cold PBS twice and resuspended in 1×Binding Buffer. Cells were then stained according to the instruction of the Annexin V-FITC staining reagent kit. Finally, the conditions of cell apoptosis were detected using FCM.

1.5 Detection of mitochondrial membrane potential (MMP) and observation of morphological changes in cell apoptosis

Cells in the logarithmic growth phase were prepared in a single-cell suspension at 1×10^5 cells/ml and inoculated onto a 6-well plate at 2ml/well. After the cells cultured at 37°C and 5% CO_2 for 24h, the cells were treated with different concentrations of PFOS (0, 100, 200 and 400mg/L) for 48h. Cells were processed according to the instructions of the MMP detection reagent and Hoechst staining kits. Finally, the MMPs of cells and the cell morphologies were observed under an inverted fluorescent microscope.

1.6 Detection of the expression levels of related genes using real-time fluorescence quantitative PCR

Cells in the logarithmic growth phase were prepared in a single-cell suspension at 1×10^5 cells/ml and inoculated onto 6-cm culture plates at 6ml/plate. After the cells were cultured at 37°Cwith5% CO_2 for 24h, the cells were treated with different concentrations of PFOS (0, 100, 150, 200, 250 and 300mg/L) for 48h. Cells in all dose groups were collected, and the total RNA was extracted using TRIzol. RNA was reverse-transcribed into cDNA based on the manufacturer's instructions included with the reagent kit. The gene expression levels of cytochrome C, apoptosis-inducing factor (AIF), caspase-3 and Bax were detected using real-time fluorescence quantitative PCR according to the instructions of the SYBR Premix Ex Taq™ reagent kit. The primer sequences are shown in Table 1.

Table 1 The primer sequences

Name of Gene	Primer Sequences	Product Length（bp）
Cytochrome C	Forward：CGTTGAAAAGGGAGGCAAGC	124
	Reverse：TCCCCAGATGATGCCTTTGT	
Caspase-3	Forward：CTCTGGTTTTCGGTGGGTGT	138
	Reverse：CGCTTCCATGTATGATCTTTGGTT	
AIF	Forward：CCTGCTGCTCCTTTACTTCTCT	200
	Reverse：TAACCCCCTCTCGTCTGACT	
Bax	Forward：GCCCTTTTGCTTCAGGGTTTC	168
	Reverse：CATCCTCTGCAGCTCCATGT	
β-actin	Forward：CAGTTGCTGCCAGGTCTGAT	174
	Reverse：GCAGACACCTATGTGGTCCC	

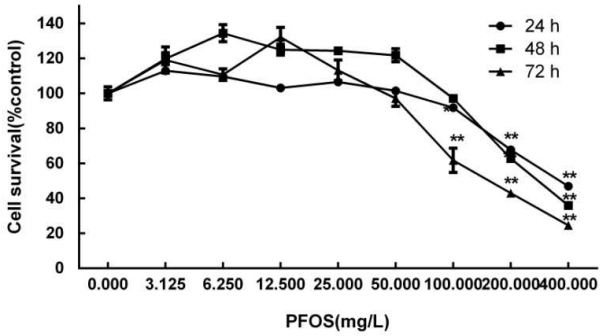
1.7 Statistical analysis

Statistical analysis was performed using Graph Pad Prism 5.0 software. Mean values and standard deviations were used to describe the indicators that conformed to normal distributions. The comparison of data among different groups was performed using one-way analysis of variance (ANOVA). Comparisons between all dose groups and the blank control group were performed using the Dunnett-t test.

2. RESULTS

2.1 PFOS-mediated inhibition of $HepG_2$ cell proliferation

Compared to the control cells, cells were treated with different concentrations of PFOS (3.125-400mg/L). When the PFOS concentration surpassed 100mg/L, the survival rates of cells gradually decreased with the increase of dosage and the extension of treatment time. These results indicated that PFOS and cell survival rates had dose- and time-effect relationships (Fig 1).



Note: compared to the blank control group, *P<0.05 and **P<0.01.

Fig 1 Effects of PFOS on the survival rate of $HepG_2$ cells

2.2 Effects of PFOS on the $HepG_2$ cell apoptosis rate

The FCM results are shown in Fig 2. After cells were treated with PFOS (100, 150, 200, 250 and 300mg/L) for 48h, the total cell apoptosis rates were 15.637%, 10.584%, 6.32%, 8.492% and 33.63%, respectively, indicating that PFOS could induce $HepG_2$ cell apoptosis. When the dose was within the 200-300mg/L range, with the increasing of PFOS concentrations, the cell apoptosis rate also gradually increased.

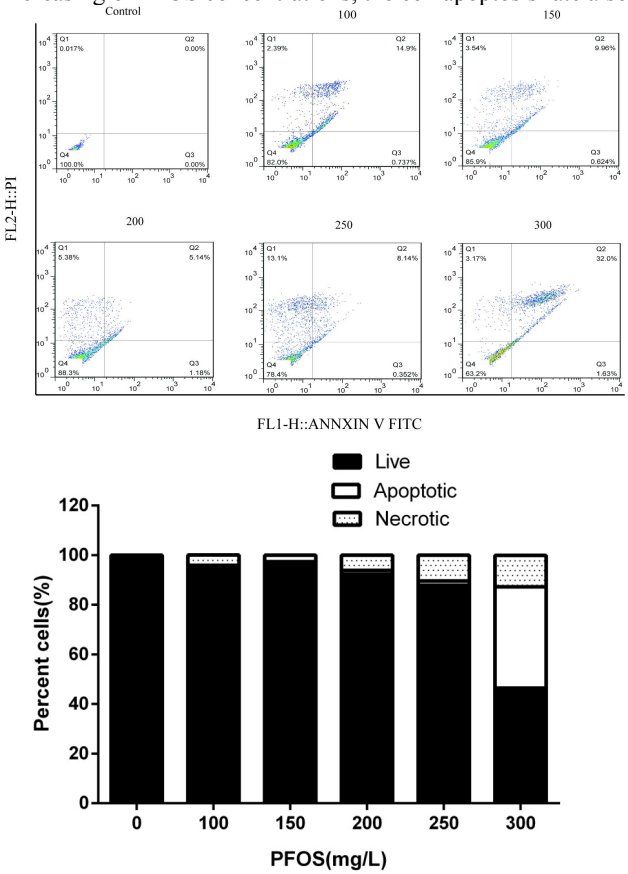


Fig 2 Effects of PFOS on $HepG_2$ apoptosis

2.3 Effects of PFOS on MMP

The changes in MMP after cells were treated with different concentrations of PFOS for 48hare shown in Fig 3. Exposure to 200mg/L PFOS reduced the fluorescence intensity in cells in a dose-dependent manner.

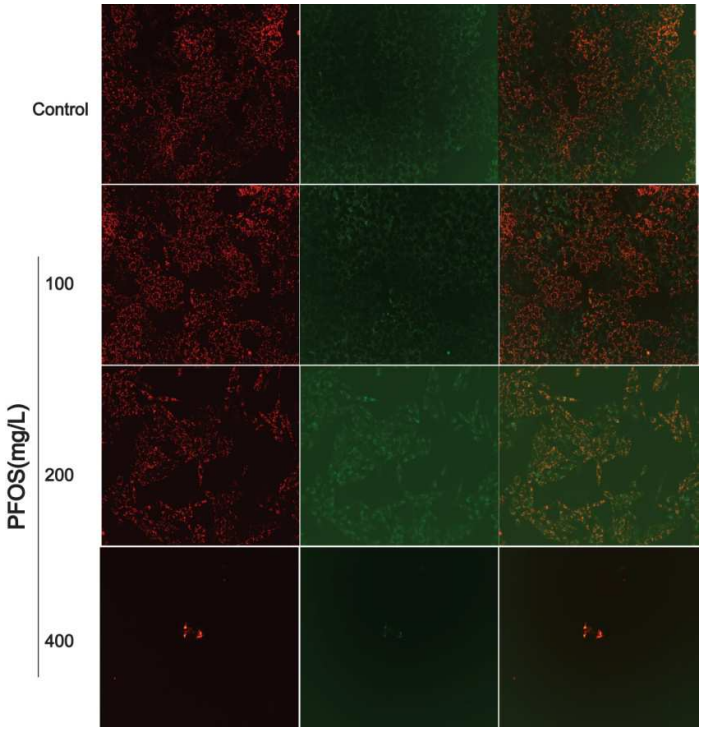
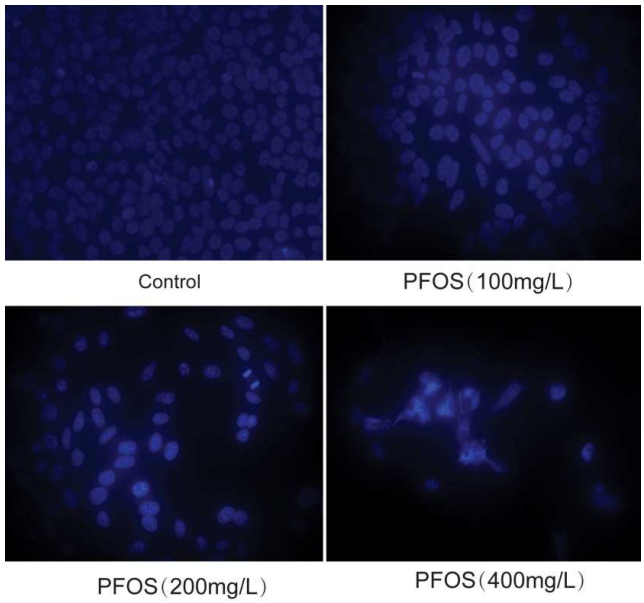


Fig 3 Effects of PFOS on MMP in HepG$_2$ cells

2.4 Effects of PFOS on HepG$_2$cell morphology

Cells in the control group exhibited homogenous blue fluorescence after stained with Hoechst 33258. In all PFOS groups (100, 200 and 400mg/L), chromatin condensation could be observed, blue fluorescence was increased, and nuclear shrinkage and fracture were indicated by patchy or punctuate fluorescence (Fig 4).



Note: A blank group, B 100mg/L, C 200 mg/L and D 400 mg/L

Fig 4 Effects of PFOS on HepG$_2$ cell morphology

2.5 Effects of PFOS on the mRNA expression levels of the apoptosis-related factors cytochrome C, caspase-3, AIF and Bax in $HepG_2$ cells

Compared to the control group, with increasing PFOS concentration, the expression levels of cytochrome C, caspase-3, AIF and Bax all increased (Fig 5).

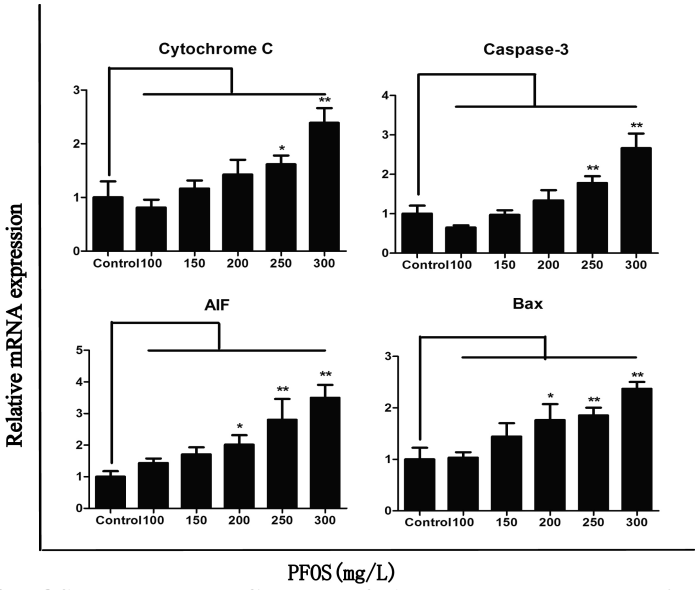


Fig 5 Effects of PFOS on cytochrome C, caspase-3, AIF and Bax gene expression in $HepG_2$ cells

3. DISCUSSION

Cell apoptosis follows many pathways. External chemical substances can interact with the tumor necrosis factor receptor (TNFR), Fas and death receptors on cell membranes to further activate the caspase family, the mitogen-activated protein kinase (MAPK) signaling pathway, or the nuclear factor-κB (NF-κB) signaling pathway to induce cell apoptosis. Chemical substances can also induce cell apoptosis through the mitochondrial pathway. These substances first induce a reduction in the MMP to cause cytochrome C to release into the cytosol from mitochondria and to interact with apoptotic protease activating factor-1 (APAF-1) to activate downstream caspase-3 and -9 to induce cell apoptosis. In addition, the Bcl-2 family is also involved in the regulation of cell apoptosis. Bax can induce cytochrome C release by increasing the permeability of the mitochondrial membrane to induce cell apoptosis, while Bcl-2 can inhibit cell apoptosis through the inhibition of Bax function. The release of a large amount of reactive oxygen species (ROS) can also lead to cell apoptosis. Large amounts of ROS function in mitochondria to increase the permeability of the mitochondrial membrane and can damage the respiratory chain to produce more ROS, thus leading to cytochrome C release into the cytosol and the induction of cell apoptosis [12].

PFOS is one type of novel persistent environmental pollutant, after polychlorinated biphenyl, 1,4-dioxin and organochloride [13]. Its damage to humans cannot be ignored. Studies have indicated that PFOS can induce increased cytochrome C expression in the lung tissue cells of rats to activate downstream caspase-3, -8 and -9, thus resulting in cell apoptosis [14].

This study selected the human hepatoma $HepG_2$ cell line as the cell model. The results of the cell proliferation inhibition experiments indicated that PFOS could inhibit $HepG_2$ proliferation. In addition, PFOS treatment and cell survival rates had dose- and time-effect relationships. After $HepG_2$ cells were treated with PFOS for 48h, FCM was performed, revealing that PFOS could induce cell apoptosis. Hoechst staining results indicated that high doses of PFOS treatment in cells induced nuclear condensation and altered cellular morphology. In addition, the MMP gradually decreased with the increase of the dose of PFOS treatment, indicating that PFOS could induce the reduction of the MMP in $HepG_2$ cells. Real-time fluorescence quantitative PCR results showed that cytochrome C, caspase-3, AIF and Bax gene expression levels increased. Based on the above results, it was speculated that the induction of $HepG_2$ cell apoptosis by PFOS might be achieved through the mitochondrial damage mechanism. First, PFOS treatment altered the MMP to cause its reduction, which resulted in the release of cytochrome C and AIF into the cytosol. Cytochrome C further activated downstream caspase-3 to eventually induce cell apoptosis. AIF was released from the mitochondria into the cytosol and then translocated into the cell nucleus to cause chromatin condensation and the breakage of large DNA fragments, which might constitute another important reason for inducing cell apoptosis.

This study describes *in vitro* experiments that were performed to confirm that PFOS could induce apoptosis in the human hepatoma $HepG_2$ cell line, the mechanism of which might be achieved through mitochondrial damage. These results provided a certain theoretical basis for subsequent studies on PFOS toxicity. Humans can be exposed to PFOS in many ways. Furthermore, the PFOS load in the human body in China shows an increasing trend year by year, which also suggests that relevant departments should take good preventive measures against PFOS accumulation to protect human health.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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