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Effect of perfluorooctane sulfonate on pluripotency and differentiation factors in mouse embryoid bodies

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ABSTRACT

Perfluorooctane sulfonate (PFOS) poses potential risks to early development, but the molecular mechanisms how PFOS affects embryonic development are still unclear. Mouse embryoid bodies (mEBs) provide ideal models for testing safety or toxicity of chemicals *in vitro*. In this study, mEBs were exposed to PFOS up to 6 days and then their pluripotency and differentiation markers were evaluated. Our data showed that the mRNA and protein levels of pluripotency markers (Oct4, Sox2, Nanog) in mEBs were significantly increased following exposure to PFOS. Meanwhile, the expressions of *miR-134, miR-145, miR-490-3p* were decreased accordingly. PFOS reduced the mRNA levels of endodermal markers (Sox17, FOXA2), mesodermal markers (SMA, Brachyury) and ectodermal markers (Nestin, Fgf5) in mEBs. Meanwhile, PFOS increased the mRNA and protein levels of polycomb group (PcG) family members (Cbx4, Cbx7, Ezh2). Overall, our results showed that PFOS could increase the expression levels of pluripotency factors and decrease the differentiation markers.

1. Introduction

Perfluorooctane sulfonate (PFOS) is one of the perfluorocompounds (PFCs). As it repels both water and oil, it is widely used as lubricants, paints, waterproofing agents and fire-fighting foams (Lindstrom et al., 2011). As an environmental health hazard, it can be found in air (Olsen et al., 2007), lake, runoff water (Cai et al., 2012) and sewage sludge (Navarro et al., 2011 Llorca et al., 2011). PFOS also exists in human nail, hair, urine (Li et al., 2013), semen (Raymer et al., 2012), maternal serum (Stein et al., 2012), umbilical cord blood (Arbuckle et al., 2013), amniotic fluid (Croes et al., 2012) and breast milk (Croes et al., 2012). In addition, PFOS can bind to serum proteins (Jones et al., 2003) and it is well absorbed, poorly metabolized and excreted. The serum elimination half-life of PFOS is as long as 5.4 years in humans (Olsen et al., 2007), while 1–2 months in the rodent species; in addition, the serum elimination half-life approximated 4 months in monkeys (Chang et al., 2012).

Therefore, there is a great concern about the health effects of PFOS.

The impact of PFOS on health is controversial (Olsen et al., 2009). Some investigators have advocated that there was no statistically significant associations between PFOS and some parameters of fetal growth (Fei et al., 2007). However, others suggested that PFOS poses potential risks to development. Human studies have indicated that PFOS exposure was associated with low birth weight (La Rocca et al., 2011) and size of offspring (Apelberg et al., 2007). PFOS exposure can also induce growth retardation, neonatal death in mice (Lampe, 2003; Fuentes et al., 2007) and rats (Case et al., 2001), and alter the expression of many putative markers in the fetal liver and lung of timed-pregnant CD-1 mice (Rosen et al., 2009). PFOS significantly retarded development of zebrafish embryos and resulted in abnormalities as well as lethality of developing embryos (Li et al., 2014). It could alter the early thyroid development genes, androgens and estrogens genes in zebrafish embryos (Shi et al., 2008). *In vitro* studies showed that PFOS induced the spontaneous differentiation of neural stem cells (Wan et al., 2013). PFOS also altered the

Abbreviations: PFOS, perfluorooctane sulfonate; mEBs, mouse embryoid bodies; PcG, polycomb group; MiR, microRNA.

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expression of crucial genes, reduced ATP production, induced ROS in embryonic stem cells test (EST), which might result in poor developmental outcomes (Cheng et al., 2013). Therefore, it is of great importance to determine the relationship between PFOS and embryonic development by embryoid bodies.

Mouse embryonic stem cells (mESCs) are characterized by two distinguishing attributes: pluripotency and unlimited self-renewal. Upon withdrawal of LIF in the suspension cultures, mESCs spontaneously differentiate and form embryoid bodies (EBs), which consists of a broad range of differentiating cell types representative of three germ cell layers (endoderm, mesoderm, ectoderm). The formation of EBs *in vitro* mimics embryo development during stages of pre-gastrulation and early gastrulation, and can be used to study differentiation and gene expression in early development (Dvash et al., 2004).

Polycomb group (PcG) proteins and microRNAs (miRNAs) are important to maintain stem cell identity (Flora and Mehta, 2009). miRNAs are important modulators of ESCs (Marson et al., 2008). The changes of miRNA expression may affect the expression of mRNAs involved in EB formation on a genome-wide scale (Tripathi et al., 2011). PcG proteins are chromatin modifiers required for proliferation and development, and involved in the regulation of ESC pluripotency and differentiation (Boyer et al., 2006).

Herein, we first examined the hypothesis that PFOS may alter the expression of pluripotency factors, differentiation factors during the early stages of development, and related epigenetic factors (miRNAs and PcG) may also be changed by PFOS.

2. Materials and methods

2.1. Chemicals and reagents

PFOS (≥98% purity), dimethyl sulfoxide (DMSO), diethylpyrocarbonate (DEPC) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Stock solution of PFOS was dissolved in DMSO at a concentration of 200 mM and stored at −20 °C. Then, it was diluted to desired concentrations by gradient dilution in culture medium before use. The final concentration of DMSO in the culture medium was the same across all concentrations tested as 0.1%.

2.2. The formation of mEBs, PFOS treatment and morphological study

Mouse ESC line D3 [American Type Culture Collection (ATCC), Manassas, VA, USA, no. CRL-11632] was kindly provided by Stem Cell Bank, Chinese Academy of Sciences. This cell line has been widely used in many studies (Hayashi et al., 2007). The karyotype of D3 cell line was normal (Fig. S1). Previous study has described the culture conditions of mESCs in detail (Xu et al., 2013).

To induce the formation of mEBs, feeder depleted mESCs were dissociated into single-cell suspensions, and cultured in mESCs medium without LIF. Cells were plated in non-adhesive 6-well plates at a density of about 5×10^5 per well, and this day was defined as EB day 0. The mESCs were exposed to PFOS (0.2 μM, 2 μM, 20 μM, 200 μM) or 0.1% DMSO at day 0, and changed the medium at days 2 and 4. The phase contrast microscope (Olympus, CK41, Japan) was used to monitor and record the morphology of mEBs after exposure to PFOS (0.2 μM, 2 μM, 20 μM, 200 μM) or control medium (0.1% DMSO) for 2, 4, 6 days.

2.3. RNA isolation and quantitative real-time PCR assay

Total RNA was isolated using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The concentration of total RNA was determined by measuring the absorbance at 260 nm by NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE). cDNA synthesis for genes and miRNAs was performed with 1 μg of total RNA according to the manufacturer's instructions (Takara, Tokyo, Japan). mRNA levels of pluripotency markers (Oct4, Sox2, Nanog) and PcG family members (Cbx4, Cbx7, Ezh2) were measured with SYBR PCR Master Mix reagent kits (Takara). The housekeeping gene GAPDH was used as an internal control. miRNA levels of *miR-134*, *miR-145*, *miR-490-3p* and U6 were measured with SYBR. The primer sequences are shown in Appendix A Tables A1 and A2. All oligonucleotide primers were synthesized by Invitrogen (Shanghai). All real-time PCR reactions were carried out on ABI7900 Fast Real-Time System (Applied Bio systems, Foster City, CA, USA).

2.4. Western blot analysis

The total cellular proteins were extracted from cultured cells using RIPA buffer containing protease inhibitors (Roche, Basel, Switzerland). Protein concentration was determined by the BCA Protein Assay Reagent (Beyotime, China). Equal amount of proteins 122 (80 mg) were separated with solubilized sample buffer (25 mM Tris, pH 6.8, 1% SDS (w/v), 5% b-mercaptoethanol (v/v), 1 mM EDTA, 4% glycerol, and 0.01% bromophenol blue), then fractionated by electrophoresis. The antibodies for pluripotency factors (Oct4, Sox2, Nanog) were purchased from Abcam (Kendall square, MA, USA, 1:1000 dilution). Cbx4 antibody (sc-19299), Cbx7 antibody (sc-70232) and Ezh2 antibody (sc-25383) were purchased from Santa Cruz Biotechnology (CA, USA). After incubation with donkey anti-goat or goat anti-rabbit secondary antibody conjugated with horseradish peroxidase at 1:1000, the specific signals were detected by the enhanced chemiluminescence (ECL Western blotting detection reagents, Amersham Life Science Limited). The amount of GAPDH (34 kDa) in each lane was used as a control to correct the expression of Oct4 protein (45 kDa), Sox2 protein (43 kDa), Nanog protein (35 kDa), Cbx4 protein (~80 kDa), Cbx7 protein (28 kDa), and Ezh2 protein (103 kDa). Equal amount of protein loading in each lane was confirmed using GAPDH antibody. All experiments were obtained by three independent cultures, and similar results were obtained.

2.5. Data analysis

Values are shown as means ± standard error of the means (S.E.) for all experiments. Statistically significant differences between the treatments and the control were determined by one-way ANOVA, followed by Dunnett's multiple comparison test. All tests of statistical significance were two-sided, and the statistical significance was set at $p < 0.05$.

3. Results

3.1. Effects of PFOS on mEBs morphology

The mEBs were cultured by suspension method. We tested the effects of PFOS on mEBs up to 6 days according to previous study (Chen et al., 2013). The mEBs in first 2 days are equivalent to the beginning of gastrulation. The mEBs (3–5 days) are similar to embryos during gastrulation at 6.5–7.0 days post-conception, and the mEBs (6 days) are resemble to early organogenesis-stage embryos (Leahy et al., 1999). The presence of PFOS in the medium did not prevent mEBs formation. The morphology of mEBs of control (0.1% DMSO) and PFOS treated groups (0.2 μM, 2 μM, 20 μM, 200 μM) were similar after 2, 4 and 6 days of culture (Fig. 1).