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Interactions between three typical endocrine-disrupting chemicals (EDCs) in binary mixtures exposure on myocardial differentiation of mouse embryonic stem cell

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HIGHLIGHTS

- Binary mixtures of three EDCs mainly show additive effects on embryonic stem cell.
- Combination between BPA and PFOS shows synergistic actions on differentiation.
- Myh6 may be more efficient on combined toxicity evaluation than contract rate.

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ABSTRACT

In recent years, various kinds of endocrine-disrupting chemicals (EDCs) have been detected in human blood and urine. Thus, it was important to investigate the combined toxicity effect of EDCs. In the present study, we evaluated the individual and combined developmental toxicities of three classic EDCs: perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA), and bisphenol A (BPA) by embryonic stem cell test (EST). The similarities and differences between combination of same chemical group as well as different chemical groups were investigated in this research. Our results showed that the three compounds were all classified as weak embryotoxicity. The results of co-exposure revealed that there was synergistic action in combination of PFOS and BPA on myocardial differentiation. However, in all endpoints, the combined effects between PFOA with PFOS or BPA were both additive action. Therefore, we concluded that the additive effects were found in most different EDC mixtures whether they had similar structure or not. On the other hand, synergistic action was observed in a mixture of EDCs that belonged to a different chemical groups.

1. Introduction

Perfluorinated compounds (PFCs) are a typical group of endocrine-disrupting chemicals (EDCs). Due to their excellent stability and hydrophobic-oleophobic property, they are widely used in non-stick, oil, and grease-resistant food packaging linings (Fromme et al., 2009). PFCs have been detected in various environmental media, animals, and human. The exposure route of PFCs in human is mainly via food (Ericson et al., 2008). Among PFCs, PFOS and PFOA have the highest concentrations in human blood or urine in most of the reports (Zhang et al., 2011; Genuis et al., 2013). In recent years, many studies reported that exposure of PFOA or PFOS could cause adverse effects on embryonic development. Thibodeaux reported that rats which were exposed to 0.3 mg/kg PFOS or PFOA had offspring with developmental defects of the brain and liver (Thibodeaux et al., 2003). Prenatal exposure of mice to PFOA could cause changed the exploratory behaviors in male and female offspring as well (Onishchenko et al., 2011). PFCs exposure could also cause adverse effects on cardiac developments. Prenatal exposure to PFOS could lead to a mitochondria-mediated apoptosis in the heart of the rat's offspring (Zeng et al., 2015; Deb et al., 2016). The mitochondria-mediated apoptosis have also been found in ESCs-differentiated cardiomyocytes *in vitro* (Cheng et al., 2013).

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Bisphenol A (BPA) is a typical EDC which has structural homology with β–estradiol. It is widely used in consumer products, including food and beverage containers (Huang et al., 2012). In addition to PFCs, BPA is widely detected in the environment and organisms, and the main exposure route for humans is intake (Gao et al., 2016). Exposure to BPA could lead to birth defects in various species. Oral exposure of rats to a reference dose (50 μg/kg·bw/d) (Vandenberg et al., 2007) of BPA during the whole pregnancy period lead to a slight swelling and mitochondria ridge separation in neonatal rat heart (Jiang et al., 2014). The toxicities of BPA is also observed in an exposed concentration far lower than reference dose. Prenatal exposure to 2 μg/kg/day of BPA in female mice from gestation day 5 to day 18 resulted in motor activities hyper function in newborns, including crawling, pivoting, and so on (Hardin et al., 1981). Epidemiological studies indicated that prenatal exposure to BPA may lead to adverse effects on birth weight, male genital abnormalities, childhood behavior neurodevelopment, and so on (Rochester, 2013).

Embryonic stem cell test (EST) is one of three alternative methods for the evaluation of developmental toxicity, which had been validated by the European Center for the Validation of Alternative Methods Registry (ECVAM). Three endpoints, including the 50% inhibition of embryonic stem cells (ESCs) differentiation (ID_{50}), 50% inhibition of cells growth (IC_{50}) of 3T3, and IC_{50} of ESCs, are used to classify the toxicity of test compounds based on the EST models (Rochester, 2013; Genschow et al., 2002, 2004). The total accuracy of EST compared to *in vivo* test is 78%.For strong embryotoxic compounds, the accuracy of EST compared to *in vivo* test is 100% (Seiler and Spielmann, 2011). Compared to the *in vivo* and other *in vitro* prediction model (PM), the EST showed a short evaluation period and a similar assessment accuracy. Therefore, EST was used extensively in developmental toxicity evaluation in recent years.

Most people are widely exposed to massive kinds of EDCs, and the combined effects of EDCs exposure could not be ignored. Usually the exposure effects of compounds mixtures are judged via two non-interaction models: concentration addition (CA) and independent action (IA) model. It is generally agreed that the CA model could be used for predicting the effects of EDCs mixtures both *in vivo* and *in vitro* (Kortenkamp, 2007). Toxicological interactions in a mixture are defined as combined effects that deviate from expectation of reference non-interaction models that based on additive effects (Kim et al., 2012). If the effects are stronger than expected, it will be described as a synergistic action; if effects are weaker than expected, it will be described as antagonistic. To date, no authoritative models have been used to evaluate the interaction effects (Sun et al., 2009).

In the current study, based on the EST model, we evaluated the developmental toxic effects of individual or combined effects of PFOS, PFOA, and BPA. Using factorial designs, we analyzed the interaction effects of these three chemicals. In our results, the additive effects were found in most different EDC mixtures whether they had similar structure or not. On the other hand, synergistic action was observed in the mixture of EDCs that belonged to different chemical groups.

2. Methods

2.1. Chemicals and reagents

BPA (98% pure), PFOA (99% pure) and dimethyl sulfoxide (DMSO) were purchased from Sigma (USA). PFOS (98% pure) was purchased from Wako (Japan). All test compounds were dissolved in DMSO for further evaluation. The final concentrations of DMSO in the culture medium were 0.1%.

2.2. Cell culture

Mouse embryonic stem cells (ESCs) line R1 derived from 129 mouse strain were used in the current study. ESCs were pluripotent stem cells derived from the inner cell mass of a blastocyst, an early-stage preimplantation embryo. ESCs were cultivated in an undifferentiated state in a complete medium, which contained KnockOut™ Dulbecco's Modified Eagle Medium (Gibco, USA) supplemented with 15% qualified fetal bovine serum (Gibco, USA), 2 mM L-glutamine (Gibco, USA), 0.1 mM b-mercaptoethanol (Gibco, USA), 1% (v/v) nonessential amino acid (Invitrogen, USA), and 1000 U/mL leukemia inhibitory factor (mLIF; Millipore,USA). The media were changed every day. Mouse NIH 3T3 cell line was originally established from the primary mouse embryonic fibroblast cells. It was cultured in high glucose (4.5 g/L) Dulbecco's Modified Eagle Medium (Gibco,USA), supplemented with 10% fetal calf serum (Gibco,USA).

2.3. Myocardial differentiation

After two passages, ESCs were digested into single cells, 40 drops of 20 μl (800 cells/drop) cells in the differentiation medium with different concentrations test compounds were plated onto the lid of a 6 cm cell dish. The lid was inverted onto a dish containing 3 mL PBS to maintain humidity. On day 3, Embryoid bodies (EBs) were moved into low attachment dishes (Alpha, China) in order to cultivate in suspension. On day 5, 24 EBs were collected and plated into a 48-well plate as one EB per well. The beating area could be observed after day 7 or day 8 with a light microscopy. The contract positive rate of each well was counted, and the EB were harvested on day 10 for RNA analysis. The myocardial differentiation rate was determined by measurements of contract positive rate and the expression of myh6.Gene myh6 encode the protein a-MHC, which is the major protein comprising the cardiac muscle thick filament, and functions in cardiac muscle contraction. After that, the concentration–effect curve was fitted by the contract positive rate and expression of myh6 and the inhibition of differentiation by 50% (ID_{50}) of each compound were calculated.

The differentiation medium were high glucose (4.5 g/L) Dulbecco's Modified Eagle Medium supplemented with 20% qualified fetal bovine serum, 2 mM L-glutamine, 0.1 mMb-mercaptoethanol, 1% (v/v)nonessential amino acid, and10^{-4} M ascorbic acid (Sigma USA).

The concentration of each compound used in this experiment were as followed: PFOS or PFOA: 2.5, 5, 10, 20, 40, 80, 160 μg/mL; BPA: 0.5, 1, 2, 4, 8, 16 μg/mL.

2.4. Cell viability assessment with MTT

The cytotoxic effects of each compound on ES and 3T3 cells were determined by the alamarBlue® assay. On the first day, 500 ES cells or 1000 3T3 cells in 100 μl per well complete medium were seeded to a 96-well plate and incubated for 2 h to make cells adhesion. Then the media were replaced with complete medium supplemented with test compound, and the media were renewed on day 4. On day 7, the cells were incubated in DMEM with 10% almarBlue® for 5 h. Then, the absorbance of each well was monitored at 570 nm, using 600 nm as a reference wavelength in order to calculate the cell viability of each well.

The concentration of each compound used in this experiment were as followed: PFOS or PFOA: 10, 20, 40, 80, 160, 320 μg/mL; BPA: 2, 4, 8, 16, 32, 64 μg/mL.