Environmental Pollution 236 (2018) 21–32

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

DEP and DBP induce cytotoxicity in mouse embryonic stem cells and abnormally enhance neural ectoderm development☆

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ARTICLE INFO

Article history:
Received 13 October 2017
Received in revised form
14 January 2018
Accepted 14 January 2018

Keywords:
DEP
DBP
Embryonic stem cell
Developmental neural toxicity
Phthalate

ABSTRACT

Diethyl phthalate (DEP) and dibutyl phthalate (DBP) are two typical small phthalate esters, extensively used in personal care and consumer products. Although previous studies have linked phthalate esters to several health issues, it is still unclear whether they can affects the early stages of embryonic development. In this study, we evaluated the early developmental neurotoxicity as well as the cytotoxicity of DEP and DBP, using mouse embryonic stem cells (mESCs). Our results showed that both DEP and DBP could decrease mESC viability in a dose-dependent manner. Moreover, while DBP could activate the caspase-3/7 enzymes and cause cell membrane damage as well as intracellular ROS accumulation, interestingly DEP treatment only showed stimulation of ROS production. In addition, DEP and DBP treatment at non-cytotoxic concentrations, abnormally altered the expression levels of several vitally important regulators of embryo development. For instance, neural ectoderm markers, such as *Pax6*, *Nestin*, *Sox1* and *Sox3*, were significantly up-regulated upon DEP and DBP exposure. In conclusion, our work suggests a potential developmental toxicity of DEP and DBP on mammals, especially for neural ectoderm specification. Our findings help better understand the association between health problems and DEP/DBP exposure and most significantly remind us of the importance of additional health risk tests for these two largely used chemicals.

1. Introduction

Phthalate esters are highly produced synthetic industrial chemicals and widely used as plasticizers. They have been recognized as endocrine disrupting chemicals and can cause adverse effects on the reproductive system (Lovekamp-Swan and Davis, 2003; Akingbemi et al., 2004). Although the industrial usage of phthalates is severely restricted in some countries (EC, 2005; PRC-NS, 2008), we are still widely exposed to those chemicals. For instance, their global production was about 5 million tons in 2010 (Guo et al., 2012). Diethyl phthalate (DEP, CAS No. 84-66-2) and dibutyl phthalate (DBP, CAS No. 84-74-2) are two representative small phthalate esters, extensively used in personal care and consumer products, such as cosmetics, perfumes, nail polish, food packages and medical devices (Al-Saleh and Elkhatib, 2016; Guo and Kannan, 2013; Hauser et al., 2004; Guo et al., 2012). Like most of the phthalate esters, DEP and DBP are not covalently bound to the materials, so they are easily released into the environment. Humans can be exposed to DEP and DBP through inhalation, dietary and dermal intake, etc. (Guo et al., 2012; Janjua et al., 2007; Kang et al., 2012; Weschler et al., 2015; Huang et al., 2018). Previous reports demonstrated the two chemicals had a high detection frequency in human body fluid samples (Blount et al., 2000; Zhu et al., 2006). The concentrations of monoethyl phthalate (mEP) and mono-*n*-butyl phthalate (mBP) (metabolites of DEP and DBP, respectively) in human urine samples reached as high as 3161 μg/g-creatinine and 4243 μg/g-creatinine respectively, and their mean concentrations spanned from 100 to 1000 nM (Polanska et al., 2014; Gao et al., 2017; Huang et al., 2018). In a dual placenta

☆ This paper has been recommended for acceptance by Dr. Chen Da.

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https://doi.org/10.1016/j.envpol.2018.01.035
0269-7491/

perfusion system, DEP and DBP metabolites were detected in both maternal and fetal perfusates (Mose et al., 2007). In another study, measurable concentrations of mEP and mBP were found in human amniotic fluid samples (Silva et al., 2004). Previous reports also indicated that DEP and DBP themselves may accumulate in the human body and possibly permeate the placental barrier. Moreover, several studies have identified that exposure to phthalates, including DEP and DBP, at either prenatal or postnatal stages may cause adverse effects on children health, such as asthma, learning disabilities, attention deficit disorders, and other behavior related troubles (Engel et al., 2010; Yolton et al., 2011; Huang et al., 2009; Chopra et al., 2014; Polanska et al., 2014; Lien et al., 2015; Philippat et al., 2015; Ejaredar et al., 2015; Hu et al., 2017; Li et al., 2017a; Gao et al., 2017). Noticeably, quite a number of the health issues associated with phthalate exposure involved the nervous system development, which indicates that DEP and DBP might potentially cause developmental neurotoxicity. Up to now, little is known about it.

There is a growing concern that exposure to environmental chemicals may be linked to the rising incidence of neurodevelopmental disorders worldwide (Grandjean and Landrigan, 2014). Experiments with zebrafish embryos showed that both DEP and DBP exposure inhibited acetylcholinesterase activity and altered the expression levels of neural genes (Xu et al., 2013). In a primary mouse cortical neuron culture system, DBP treatment could induce apoptosis and neurotoxicity via stimulation of caspase-3 and LDH, as well as ROS formation (Wójtowicz et al., 2017). Other cell lines (*e.g.* SH-SY5Y cells, MCF-7 cells) and animal models (*e.g.* mouse, rabbit) have been also employed to study the toxic effects of DEP and DBP (Mapuskar et al., 2007; Kim et al., 2015; Higuchi et al., 2003; Okubo et al., 2003; Kaun-Yu et al., 2004). Despite all these toxicological data and the possible mechanisms of toxicity they suggest, both the animal exposure models and mature cell culture systems cannot directly clarify the toxic effects occurring during early stages of embryonic development. In order to shed light on this topic, it is extremely urgent to dissect the developmental neurotoxicity of DEP and DBP.

Embryonic stem cells (ESCs), which are derived from the inner cell mass (ICM) of the blastocyst, were first isolated from mice in 1981 (Evans and Kaufman, 1981). When cultured in suspension in defined conditions, ESCs can spontaneously form three-dimensional cell aggregates called embryoid bodies (EBs). EBs will generate the three primary germ layers (ectoderm, endoderm and mesoderm) and thus mimic the early embryonic development process and generate various cell lineages that could develop into different somatic cells and tissues. Because of their extensive proliferation and multilineage differentiation properties, ESCs provide a promising model to test, beside cytotoxicity, embryonic toxicity, developmental toxicity, functional toxicity and reproductive toxicity (Faiola et al., 2015; Yao et al., 2016; Liu et al., 2017). Researches have successfully used stem cell technology to explore the toxicity of environment pollutants such as bisphenol A, halobenzoquinones and naphthenic acid mixtures (Fu et al., 2017; Mohseni et al., 2015; Li et al., 2015; Yin et al., 2015). In this study, we employed a mouse ESC (mESC) system to analyze not only DEP and DBP cytotoxicity, but also their potential development neurotoxicity. We showed that the cytotoxic effects of DBP on mESCs involved cell membrane integrity, intracellular ROS accumulation as well as activation of caspase-3/7, whereas DEP was only involved in ROS production. More importantly, mESC-dependent neural specification was dysregulated upon both DEP and DBP treatment. Our findings may help guide the manufacture, management, application, and disposal of phthalate esters, as well as avoid potential environmental hazards and human health risks due to exposure to those chemical compounds.

2. Materials and methods

2.1. Chemicals

DEP (Sigma, USA) and DBP (Sigma, USA) stock solutions were prepared in DMSO (Amresco, USA).

2.2. Cell culture

J1 mESCs were obtained from Cell Bank/Stem Cell Core Facility, SIBCB, CAS. Cells were cultured at 37 °C and 5% CO_2 on 0.1% gelatin-coated dishes, in KnockOut™ DMEM medium (Life Technologies, USA) supplemented with 15% fetal bovine serum (Corning, USA), 2% penicillin/streptomycin (Life Technologies, USA), 1% nucleosides (Millipore, USA), 1% non-essential amino acids (Life Technologies, USA), 10^{-4} M β-mercaptoethanol (Life Technologies, USA) and 10^{-3} U/mL leukemia inhibitory factor (LIF) (Merck Millipore, Germany).

2.3. Cell viability assay

ESCs were cultured in 96-well plates as 10000 cells per well. Cells were cultured for 24 h prior to be exposed to various concentrations of DEP and DBP. Cell viability was determined 48 h later (or otherwise specified) with the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. Briefly, cells were incubated with 1 mg/mL MTT (Molecular Probe, USA) solution in FBS-free ES medium for 4 h at 37 °C and 5% CO_2. The formazan crystals were dissolved in DMSO, and the absorbance measured at 595 nm and 655 nm. The IC_{50} value was calculated with the SPSS software (version 16.0).

To determine the cell viability of mESCs during differentiation, ESCs were cultured in 96-well plates as 500 cells per well in ES medium without LIF. ESCs cultured for 24 h after seeding (day 0) were exposed to various concentrations of DEP and DBP. Medium was refreshed every other day. Cell viability was determined on day 7 with the MTT assay as described above.

2.4. Cellular membrane integrity analysis

ESCs cultured for 24 h after seeding were exposed to DEP (0–2 mM) and DBP (0–2 mM) at 37 °C and 5% CO_2 for 6 h. LDH activity was assessed using CytoTox-ONE™ Homogeneous Membrane Integrity Assay kit (Promega, USA) according to the manufacturer's guidelines.

2.5. Intracellular ROS accumulation

2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Sigma, USA) was used to detect intracellular ROS. Briefly, ESCs cultured for 24 h after seeding were loaded with DCFH-DA (100 μM) for 30 min at 37 °C and 5% CO_2, and then exposed to (0–2 mM) DEP and DBP for 6 h. The fluorescence was assessed using a microplate reader (Varioskan Flash, Thermo Scientific, USA) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

2.6. Intracellular caspase-3/7 activity

ESCs cultured for 24 h after seeding were exposed to DEP and DBP, at concentrations from 0 to 2 mM for 6 h, at 37 °C and 5% CO_2. Activated caspase-3/7 activity was assessed using the Caspase-Glo® 3/7 Assay kit (Promega, USA) according to the manufacturer's instructions.