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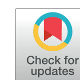
New insights into the mechanism of phthalate-induced developmental effects☆



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ABSTRACT

To investigate the biological pathways involved in phthalate-induced developmental effects, zebrafish embryos were exposed to different concentrations of di-(2-ethylhexyl) (DEHP) and di-butyl phthalate (DBP) for 96 h. Embryonic exposure to DEHP and DBP induced body length decrease, yolk sac abnormities, and immune responses (up-regulation of immune proteins and genes). The lipidomic results showed that at a concentration of 50 μg/L, DEHP and DBP significantly reduced the levels of fatty acids, triglycerides, diacylglycerol, and cholesterol. These effects are partly explained by biological pathway enrichment based on data from the transcriptional and proteomic profiles. Co-exposure to DBP and ER antagonist did not significantly relieve the toxic symptoms compared with exposure to DBP alone. This indicates that phthalate-induced developmental abnormities in zebrafish might not be mediated by the ER pathway. In conclusion, we identified the possible biological pathways that mediate phthalate-induced developmental effects and found that these effects may not be driven by estrogenic activation.

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1. Introduction

Phthalates are commonly used in a variety of industrial and consumer products, including food packaging, children's toys, and building materials. Di-(2-ethylhexyl) phthalate (DEHP) is the most commonly used phthalate plasticizer in polyvinyl chloride (PVC) formulations; DEHP accounts for 80% of phthalate production in China and approximately 18% of all plasticizers used in Western Europe (Zolfaghari et al., 2014; European Union, 2008). Di-butyl phthalate (DBP) is typically used in personal care products (Lien et al., 2015). Because phthalates are not chemically bound to the plastics, they can leach into food, medicine, water, indoor dust, and air, resulting in human and animal exposure (Zota et al., 2016; Jia et al., 2017; Net et al., 2015; Kang et al., 2012; Gaspar et al., 2014). The detected concentration of DEHP and DBP in drinking water and surface water is generally below 1 and 10 μg/L respectively (Liu et al., 2014; Zeng et al., 2008). However, hotspots of contamination occur (more than 100 μg/L) in heavily industrialised areas (Fromme et al., 2002; Yuwatini et al., 2006; Khan and Jung, 2008). As a result of widespread contamination, these chemicals can enter the human body through daily ingestion and inhalation. Phthalates are ubiquitously detected in urine samples of all age groups from all around the world (Guo et al., 2011; Gao et al., 2017; Mérida-Ortega et al., 2016; Lee et al., 2017).

Given the wide distribution of phthalates, their negative effects on humans and animals have drawn considerable attention. Most research efforts have focused on endocrine-disrupting effects, primarily the alteration of hypothalamus-pituitary-thyroid axis and hypothalamus-pituitary-gonad axis. Previous studies found that the urinary phthalate concentrations in humans are negatively associated with the levels of urinary thyroid hormones in children and serum T4 in adults (Boas et al., 2010; Huang et al., 2017). In addition, a variety of studies have shown that phthalates cause reproductive toxicity in both male and female individuals. Serum phthalates are negatively associated with serum testosterone, sex hormone-binding globulin, semen volume, semen quality, and total sperm count, whereas they are positively associated with DNA damage in the sperm of adult men (Joensen et al., 2012; Pant et al., 2008; Specht et al., 2014). In male fish, decreased plasma

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testosterone (T) concentrations, reduced ability to fertilise oocytes spawned by untreated females, decreased sperm motility and velocity, and altered spermatogenesis were observed after DEHP exposure (Uren-Webster et al., 2010; Crago and Klaper, 2012; Golshan et al., 2015). In females, urinary concentrations of DEHP metabolites are negatively associated with oocyte yield, clinical pregnancy, and live birth (Hauser et al., 2016). Ye et al. (2014) reported that female marine medaka (*Oryzias melastigma*) showed decreased egg production, increased concentrations of T and E2 and up-regulation of ldlr.

In addition to the effects on reproduction and hormone levels, the developmental effects of phthalates have been increasingly studied in recent years. Tsai et al. (2016) demonstrated that daily DEHP intake is negatively associated with height percentile, weight percentile, bone age/chronological age, and insulin-like growth factor 1 (IGF-1) levels in children. The levels of urinary phthalate metabolites are associated with delayed pubic hair development in boys and earlier breast development in girls (Zhang et al., 2015). Yamasaki et al. (2009) reported that maternal exposure to dicyclohexyl phthalate can induce a series of developmental effects in male offspring, including prolonged preputial separation, reduced ano-genital distance, increased areola/nipple retention, and decreased ventral prostate and levator ani/bulbocavernosus muscle weights. In addition, neurodevelopment has been identified as another target of phthalates. Research on humans and other mammals has shown that phthalate exposure can result in attention problems along with aggressive or rule-breaking behavior in children and induce cell apoptosis in hippocampal neurons as well as impair spatial learning and memory in rat pups (Kobrosly et al., 2014; Engel et al., 2010; Li et al., 2013; Li et al., 2009). However, the mechanism of action and pathways involved in phthalate-induced developmental effects remain uncharacterized, although these symptoms have been considered as the downstream health outcomes that resulted from the disorder of hormone, for instance lower production of estradiol or progesterone (Kay et al., 2013).

Currently, zebrafish (*Danio rerio*) embryos are an important model for toxicological investigations of hazardous chemicals (Zhang et al., 2017; Brox et al., 2016; Huang et al., 2016). Zebrafish embryos possess many advantages, including in vitro fertilization, high fecundity, rapid embryonic development, and optical transparency, which make it easy to detect morphological endpoints or observe the development process in early life stages (Mu et al., 2016). Previous studies have shown that phthalates have several negative effects on zebrafish, including steroidogenic alteration, cardiac defects, reproductive toxicity and oxidative stress (Sohn et al., 2016; Sun and Liu, 2017; Zhang et al., 2014; Corradetti et al., 2013). In this study, we applied several biological approaches with an experimental morphological investigation using zebrafish embryo as a model to identify the biological pathways that mediate phthalate-induced developmental effects.

2. Materials and methods

2.1. Zebrafish maintenance and embryo collection

Wild-type zebrafish (AB strain) were purchased from a local provider. All adult zebrafish were maintained in flow-through feeding equipment (made by Esen Corp.) at 26 °C with a photoperiod of 14/10 (light/dark). The zebrafish were fed daily with live brine shrimp (*Artemia salina*). The preparation and collection of zebrafish embryos followed the procedure described in our previous work (Mu et al., 2013).

2.2. Chemicals and reagents

Standard water was prepared in the lab according to the formula of iso-7346-3: 2 mmol L^{-1} Ca^{2+}, 0.5 mmol L^{-1} Mg^{2+}, 0.77 mmol L^{-1} Na^{+}, and 0.08 mmol L^{-1} K^{+} (ISO, 1996). Di-(2-ethylhexyl phthalate (DEHP, 99.5%, CAS: 117-81-7) and dibutyl phthalate (DBP, 99%, CAS: 84-74-2) were purchased from Sigma-Aldrich (Darmstadt, Germany). Stock solutions of DEHP and DBP for drug-exposure experiments were prepared using acetone AR. ICI-182,780 (98%, CAS 129453-61-8) was purchased from Sigma-Aldrich (Darmstadt, Germany).

2.3. Exposure and sample collection

Experiments were performed in accordance with current Chinese legislation and were approved by the independent animal ethics committee at Chinese Academy of Fishery Sciences.

2.3.1. Exposure for morphological endpoints

Test solutions of DEHP and DBP with concentrations of 0 (control), 50, and 250 μg/L (based on pre-experiment data) were made using standard water. Embryos at approximately 2 h post-fertilization (hpf) were randomly transferred into the test solutions in 24-well plates. Exposure solutions for solvent control and all treatment groups contained the same concentration of acetone (0.05 mL/L). Each treatment was replicated three times. The number of dead individuals was determined, and the dead embryos were removed daily. Embryo death was judged using the lethal toxicological endpoints proposed by Nagel (2002). The hatching and development status of embryos were checked daily. Teratogenic effects were identified and recorded using a ZEISS Vert.A1 microscope (Jena, Germany). Body length along with yolk sac width and height were measured linearly using the microscope software. See the Supplemental Information for more details.

2.3.2. Exposure for transcriptomic and proteomic analysis

Embryos were randomly transferred into test solutions (50 μg/L) in 1-L beakers at 2 hpf. Each beaker contained 500 mL of exposure solution and approximately 100 embryos, and there were three beakers in each treatment group. At 96 hpf, 75 hatched (or decorticated) larvae from each replicate (beaker) were collected and washed twice with standard water (25 for RNA extraction and 50 for protein extraction). The embryo samples were stored at −80 °C until analysis.

2.3.3. Exposure for lipidomic analysis

Embryos were randomly transferred into test solutions (50 μg/L) in 200-mL beakers at 2 hpf. Each beaker contained 100 mL of exposure solution and 40 embryos, and there were eight beakers in each treatment group. At 96 hpf, 20 embryos were collected from each beaker and washed with standard water. The embryo samples were stored at −80 °C until analysis.

2.3.4. Exposure for immune response and estrogenic activity test

Embryos were randomly transferred into test solutions (50 and 250 μg/L) in 1-L beakers at 2 hpf. 50 and 250 μg/L 17β-estradiol was used as positive control in the estrogenic activity test. Each beaker contained 500 mL of exposure solution and approximately 200 embryos, and there were three beakers in each treatment group. At 96 hpf, 120 hatched (or decorticated) larvae from each replicate were collected and washed twice with standard water (30 for mRNA extraction and 90 for the ELISA assay of hormones, ERα and immune protein levels). The embryo samples were stored at −80 °C until analysis.

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