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DEHP exposure *in utero* disturbs sex determination and is potentially linked with precocious puberty in female mice

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A B S T R A C T

Human's ubiquitous exposure to di (2-ethylhexyl) phthalate (DEHP) is thought to be associated with female reproductive toxicity. Previous studies found that DEHP inhibited follicle growth and decreased estradiol levels in adult female mice. However, limited information is available on the link between *in utero* DEHP exposure and ovarian development in female mouse offspring. The present study evaluates the disturbances in regulatory genes involved in female sex determination and the ovarian outcomes in fetal and postnatal female mice treated with *in utero* DEHP exposure. Pregnant mice were exposed to DEHP by gavage, with the dosage regime beginning at human relevant exposure levels. After *in utero* DEHP exposure, increased follicular atresia was observed in the female pups at postnatal days (PND) 21. *Foxl2* expression was significantly upregulated, and *Fst* was significantly downregulated by DEHP above 2 mg/kg/d at PND 1 and 21. This suggests that lesion of granulosa cell differentiation and disturbance of follicle development in postnatal female mice. The expression of *Cyp11a1* and *Star* were significantly downregulated by *in utero* DEHP exposure, indicating effects on estradiol biosynthesis. The female sex determination pathway was disturbed in fetus by DEHP at 2 mg/kg/d and above during the critical time window of sex determination causing significant upregulation of *Foxl2*, *Wnt4*, *β-catenin* and *Fst*. Furthermore, the increased expression of *Wnt4* was supported by whole-mount *in situ* hybridization (WISH). These results suggest a possible association between *in utero* DEHP exposure and precocious puberty in the postnatal life of mice offspring, where disturbance of the sex determination regulating pathway acted as an important mechanism.

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1. Introduction

Di-(2-ethylhexyl) phthalate (DEHP) is widely used in plastic consumer products, drugs, and cosmetics as a plasticizing agent (Kay et al., 2013; Hannon et al., 2015). DEHP can leach into the environment from plastics after repeated use, heating, and cleaning, allowing the toxicant to be ingested and resulting in widespread human exposure (Andrade et al., 2006; Heudorf et al., 2007; Kay et al., 2013). DEHP has been identified as a top contaminant among detectable phthalates homologues in human tissues, and its metabolites were found in most tested human urine samples (Silva et al., 2003; Marsee et al., 2006). Furthermore, the presence of DEHP and its metabolites in amniotic fluid, cord blood, and breast milk, suggesting prenatal and neonatal exposure to DEHP (Latini et al., 2003a; Latini et al., 2003b; Hogberg et al., 2008; Huang et al., 2009; Krotz et al., 2012).

Increasingly, studies suggest that DEHP is an endocrine disrupting chemical and a reproductive toxicant (Heudorf et al., 2007; Kay et al., 2014). To date, the majority of the studies examining the reproductive toxicity of DEHP have focused on male reproduction. They have demonstrated that DEHP can adversely affect the development of the male reproductive tract with the most sensitive outcome being decreased semen quality (Kay et al., 2014). Pant et al. (2008) found that DEHP concentration in semen was associated with decreased sperm concentration, decreased motility, and increased abnormal morphology. Huang et al. (2011) also found that DEHP exposure was associated with increased DNA damage and decreased motility. In comparison, much less information is available on the adverse effects of PAEs on female reproduction and development (Lovekamp-Swan and Davis, 2003; Kay et al., 2013; Mu et al., 2015). Animal studies have provided some evidence regarding adverse effects of DEHP on hormone production and ovarian development in females. DEHP exposure in Chinese rare minnow and Japanese medaka resulted in downregulation of estradiol (E2) synthesis and inhibition of oocyte development (Kim et al., 2002; Wang et al., 2013). In rodent models, DEHP decreased serum estradiol levels; prolonged estrous cycles; decreased primordial follicles numbers; increased the primary, secondary, and antral follicles numbers; and prevented ovulations in rats and mice (Lovekamp-Swan and Davis,

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2003; Moyer and Hixon, 2012; Hannon et al., 2014). In *in vitro* culture models, most studies found that DEHP can inhibit antral follicle growth, induce atresia, and inhibit steroid hormone production (Gupta et al., 2010; Wang et al., 2012; Hannon et al., 2015).

Limited epidemiologic data indicate a possible association between PAEs exposure in humans and precocious puberty and adverse pregnancy outcomes. Both occupational and non-occupational exposure to phthalates increased the risk of pregnancy loss and caused higher rates of miscarriage (Lovekamp-Swan and Davis, 2003; Mu et al., 2015). Higher urinary concentrations of DEHP metabolites were also reported to be associated with decreased antral follicle count, oocyte yield, and live birth rate (Hauser et al., 2016; Messerlian et al., 2016). Particularly, PAEs may disturb female sexual maturity. In Puerto Rico, the country with the highest known incidence of premature thelarche, Colon et al. (2000) found that the girls with premature breast development had significantly higher levels of blood phthalates compared with the control subjects. Moreover, low-molecular-weight phthalates were positively correlated with rapid breast development, while high-molecular-weight phthalates had the opposite effect on breast development (Vo et al., 2009).

However, little is known about the molecular mechanism by which DEHP induces female reproductive toxicity. Several mechanistic toxicological studies conducted *in vitro* found that DEHP and its metabolite, mono-(2-ethylhexyl) phthalate (MEHP), increased reactive oxygen species (ROS) and activated peroxisome proliferator-activated receptors (PPARs). This suppressed aromatase in the cultured granulosa cells and antral follicles, and eventually led to decreased E2 (Lovekamp-Swan and Davis, 2003; Wang et al., 2012). StAR and CYP11a1 are the key rate-limiting enzymes in the steroidogenesis pathway, which is critical for the production of sex hormones. In addition, aromatase acted on the last step of female sex hormone biosynthesis to mediate the production of E2. A previous study indicated that DEHP suppressed the synthesis of E2 by decreasing the mRNA expression of cyp11a1 and 17β-hydroxysteroid dehydrogenase (17β-HSD) (Hannon et al., 2015). In addition, DEHP inhibited follicle growth by changing the expression of the cell cycle regulators cyclin D2, cyclin-dependant-kinase-4, cyclin A2, and cyclin B1 (Gupta et al., 2010; Hannon et al., 2015).

Ovary development initiates from the female sex determination pathway in fetal mouse (Wilhelm et al., 2007; Tevosian, 2013). However, few studies have focused on the impact of prenatal DEHP exposure, particularly, exposure during the critical period of sex determination, or on ovarian development in female offspring. It has been suggested that prenatal exposure to DEHP leads to disruption of follicle development, resulting in premature ovarian senescence (Moyer and Hixon, 2012; Niermann et al., 2015). Hannas et al. (2013) found that after *in utero* exposure of rats to a mixture of 5 PAEs from GD 8-13, the rats displayed similar reproductive tract abnormalities as those seen in women with Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome. Our previous studies have found that both high and low doses of *in utero* DEHP exposure lead to inhibition of key genes involved in the male sex determination pathway of fetal mouse (Wang et al., 2015; Wang et al., 2016). Therefore, it is vital to examine the effects of *in utero* DEHP exposure on female sex determination to elucidate the mechanisms underlying the reproductive and developmental toxicity induced by DEHP in female mice.

The present study aims to evaluate the potential link between *in utero* DEHP exposure and follicular development in female mice, and to explore the mechanisms regulating female sex-determination. The dosage regime was begun at 0.02 mg/kg/d in order to be comparable to real human exposure levels. Histopathological analysis, along with detection of *Foxl2*, a biomarker for granulosa cell differentiation, and *Fst*, a follicular development modulator, were conducted in female mice offspring on postnatal day (PND) 1 and 21. Furthermore, the sex determination signaling pathway in fetal mice was analyzed to elucidate the underlying mechanisms.

2. Materials and methods

2.1. Animals

ICR mice were purchased from the Experimental Animal Center of Dalian Medical University and were allowed to acclimate to the facility prior to dosing. The mice were housed in a controlled animal room at 20–26 °C with a relative humidity of approximately 40–70% and 12L:12D cycles, and were provided food and water *ad libitum*. Females were time-mated with males overnight. The vaginal plugs were detected at noon 0.5 days post coitum (dpc).

All animal experimental procedures were carried out in accordance with the National Institutes of Health's guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1996), and were approved by the School of Environmental Science and Technology in Dalian University of Technology.

2.2. Dose and administration of DEHP

Each pregnant female mouse was weighed, ranked, and randomly assigned to one of the following 6 DEHP treatment groups: control, 0.02, 0.2, 2, 20, and 200 mg/kg/d. Each group contained 4–6 dams at each examination stage. The dosing study for identifying disturbances in fetal sex determination consisted of 6 pregnant dams in each treated group/stage that were dosed daily from 0.5 dpc to 11.5–13.5 dpc by oral gavage. Dosing study for whole gestation period consisted of 4 pregnant dams in each treated group/stage that was dosed daily beginning from 0.5 dpc by oral gavage. The lowest dose, 0.02 mg/kg/d is equal to the reference dose (RfD) of the Environmental Protection Agency of the United States, approximately 2 orders of magnitude lower than the no observed adverse effect level (NOAEL) in a fertility evaluation for DEHP in male mice (Kavlock et al., 2006). Pregnant mice were euthanized and fetuses were removed by cesarean section on 11.5, 12.5 and 13.5 dpc. Genital ridges from the fetus mice were stored at −80 °C. The sex of the fetuses was chromosomally analyzed by a PCR assay (Wang et al., 2015). Genital ridges from the fetus mice in the same litter were pooled for Real-time quantitative PCR (RT-qPCR) analysis at 11.5 and 12.5 dpc. Genital ridges from 13.5 dpc fetus mice in the same litter were used for whole-mount *in situ* hybridization (WISH). For the postnatal pups, an individual ovary sample from each animal was analyzed separately. The ovaries from the 5 newborn pups were collected for RT-qPCR analysis at PND 1. At PND 21, ovaries of three pups from each litter per exposure group were used for histopathological analyses. For the remaining pups in the same litter, one ovary was used for Western Blot analysis, and the others were used for RT-qPCR analysis.

2.3. Real-time quantitative RT-PCR

Real-time quantitative RT-PCR was performed to measure the expression of key genes in the sex determination pathway, including *Wnt4*, *β-catenin* and *Fst*, as well as the biomarkers for granulosa cell differentiation, *Foxl2*. Furthermore, the expression of *Star* and *Cyp11a1*, encoding enzymes of sex hormone synthesis, were detected at PND 21. Total RNA was extracted from the genital ridges and gonads using RNeasy Plus mini kit (Qia-gen, Valencia, CA) according to the manufacturer's protocol, and was then reverse transcribed to complementary DNA (cDNA) using the Reverse Transcription kit (TaKaRa, Dalian, China). RT-qPCR was conducted using the StepOne real-time PCR System (Applied Biosystems, Foster City, CA, USA). Optimal PCR conditions were established for each set of primers by varying the cycle number and annealing temperature. Mice specific primers were designed for the genes of interest (Table 1) using Primer Premier 6.0 software (Palo Alto, CA, USA) and synthesized by BBI Life Science Corporation (Sangon, Shanghai, China) (Wang et al., 2015). For the pooled genital ridge samples, three replicates were run for RT-qPCR analysis after the RNA extraction and reverse transcription. For the ovary samples from

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