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Di (2-ethylhexyl) phthalate exposure during pregnancy disturbs temporal sex determination regulation in mice offspring

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ABSTRACT

Animal researches and clinical studies have supported the relevance between phthalates exposure and testicular dysgenesis syndrome (TDS). These disorders may comprise common origin in fetal life, especially during sex determination and differentiation, where the mechanism remains unclear. The present study evaluated the disturbances in gene regulatory networks of sex determination in fetal mouse by *in utero* Di (2-ethylhexyl) phthalate (DEHP) exposure. Temporal expression of key sex determination genes were examined during the critical narrow time window, using whole-mount in situ hybridization and quantitative-PCR. DEHP exposure resulted in significant reduction in mRNA of *Sry* during sex determination from gestation day (GD) 11.0 to 11.5 in male fetal mice, and the increasing of *Sry* expression to threshold level on GD 11.5 was delayed. Meanwhile, *Gadd45g* and *Gata4*, the upstream genes of *Sry*, and downstream gene *Sox9* were also significantly downregulated in expression. In fetal females, the expression of *Wnt4* and *beta-catenin* were up-regulated by DEHP exposure. Taken together, the results suggest that the potential mechanism of gonadal development disorder by DEHP may origin from repression of important male sex determination signaling pathway, involving *Gadd45g* → *Gata4* → *Sry* → *Sox9*. The results would promote a better understanding of the association between phthalate esters (PAEs) exposure and the reductive disorder.

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1. Introduction

Approximately 1 of every 250 newborns has some abnormality of genital and/or gonadal development (Hughes et al., 2006; Lourenco et al., 2011). It has been hypothesized that the sex differentiation disorders may comprise a testicular dysgenesis syndrome (TDS) with a common origin in fetal life (Sharpe and Skakkebaek, 2008). Animal studies suggested that fetal exposure to phthalate esters (PAEs) induced TDS (Fisher et al., 2003; Parks et al., 2000). Furthermore, clinical diagnosis found that the focal dysgenesis induced by fetal PAEs exposure in rats resemble the testicular dysgenesis syndrome in humans (Nistal et al., 2006; Sharpe and Skakkebaek, 2008).

Among the PAEs, di (2-ethylhexyl) phthalate (DEHP) represented nearly 50% of the total phthalate production in the annual world market (Tran et al., 2015), and more than two million tons of DEHP were used each year worldwide (Wang et al., 2015). DEHP is widely used in many consumer products, including food packages, pharmaceuticals, cosmetics, pesticides, and children's toys (Yuan et al., 2002). Moreover, DEHP is more difficult to degrade than other PAEs (Howdeshell et al., 2008). DEHP has been detected in various environmental media, including soil, water, and air (Peijnenburg and Struijs, 2006; Zeng et al., 2008). Thus, humans are exposed to DEHP via ingestion, inhalation, and dermal absorption for their lifetimes. Thus, the occurrence of DEHP and their metabolites in the serum and urine of both children and adults has been reported in different countries (Colon et al., 2000; Guo et al., 2011). Particularly, the presence of DEHP in the amniotic samples and breastmilk suggests the prenatal and neonatal exposure to DEHP (Silva et al., 2004; Zhu et al., 2006).

Toxicological studies using animals indicate that, DEHP is an endocrine-disrupting chemical (EDC) (Blystone et al., 2010; Gray et al., 2009; Hannas et al., 2011; Sharpe, 2006; Swan, 2008). Maternal DEHP exposure has been associated with impaired gonadal development and fertility in male rat offspring including reduced anogenital distance (AGD), hypospadias, cryptorchism, and declined semen quantity, density, mobility (Liu et al., 2008; Sharpe, 2006; Sharpe and Skakkebaek, 2008; Swan, 2008). In rats, the mechanism underlying the reproductive toxicity of DEHP is referred to an anti-androgenic activity via suppression of

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steroidogenesis (David, 2006; Johnson et al., 2012; Kay et al., 2014). However, the mode of action of DEHP in mice seems to be different from that in rats. Several studies found that mouse testosterone production was unaffected by phthalate exposure (Johnson et al., 2011; van den Driesche et al., 2012). However, some researchers found that the reproductive disorder of fetal mouse were induced by *in utero* phthalate exposure, including induced multinucleated germ cells (MNGs), increased seminiferous cord diameters, and altered expression of thousands of fetal testis genes (Gaido et al., 2007; Johnson et al., 2011; Lehraiki et al., 2009). A recent study found that dipentyl phthalate (DPeP) significantly reduced testosterone production by 25–30% at a dose level that did not induce any maternal or fetal toxicity in mice, but the 50% effective dose for this effect was about fourfold higher than in the rat (Furr et al., 2014). Therefore, mice appear resistant to *in utero* phthalate-induced sex hormone disruption compared with rats. The mechanism underlying reproductive toxicity induced by *in utero* phthalate exposure in mice is not supposed to be associated with decreased testicular testosterone and may involve other pathways (Gaido et al., 2007; Johnson et al., 2012; Lehraiki et al., 2009; Liu et al., 2005). Similarly, study on the reproductive toxicity of PAEs using human fetal testis xenografts suggests that the human fetal testes are also resistant to phthalate-induced endocrine disruption (Heger et al., 2012; Mitchell et al., 2012). Therefore, it is of significance to investigate the mechanism of the reproductive toxicity of DEHP in mice, which may promote a better understanding of association between PAEs exposure and reproductive disorder in humans.

Recent advances in the understanding of molecular sex determination pathway in mammals prompted investigation of the interaction between EDCs and genes involved in mammalian sex differentiation and gonadal development (Biason-Lauber, 2010). In non-mammalian vertebrates, the sexual development process is vulnerable to EDCs exposure and other environmental factors (Fernandez et al., 2007), whereas in mammals, sex determination is highly conservative in a gene–regulated way. In mammalian embryo, testis development depends on the expression of *Sry* in Y chromosome, prompting bipotential gonadal anlage toward the male (XY) fate and suppressing the female (XX) program (Kashimada and Koopman, 2010). The expression of *Sry* is transcriptionally regulated by *Wt1*, *Sf1*, *Gata4*, *Fog2* and *Cbx2* (Barbara et al., 2001; Katoh-Fukui et al., 2012; Tevosian et al., 2002). Most recently, researches demonstrated the requirement of *Gadd45g* in promoting Map3k4-mediated activation of p38 MAPK, phosphorylating GATA-4, finally leading to elevated expression of *Sry* (Gierl et al., 2012; Warr et al., 2012). Following expression of *Sry*, *Sry*-related high-mobility group box 9 (*Sox9*), the main Sertoli cell marker, is activated in autosome, which is proved to be critical for directing the testicular architecture and initiating a series of male-specific molecular pathways, including activating *Fgf9* and *Ptgds*, and work with *Sf1* to activate *AMH* (Ono and Harley, 2013). Sexual dimorphism in the XX genital ridge is triggered by *Rspo1* and *Foxl2*. Additionally, *Wnt4* and *β-catenin* also act in a gender-specific manner, preventing differentiation of testis by repressing *Sox9* expression (Ono and Harley, 2013).

Moreover, sex determination is regulated in a precise spatio-temporal fashion in the mouse (Warr et al., 2012). *Sry* has to function within a narrow 6 h. time window (gestation day, GD11.0-11.25), reaching the threshold on GD 11.5, to up-regulate *Sox9* in the mouse, otherwise the gene is repressed and ovary develops (Hiramatsu et al., 2009). Most studies concerning the adverse responses in sexual development induced by DEHP exposure dosed the animals during fetal period of sexual differentiation (GD14-18) or perinatal period (GD14-PND3), when the reproductive system differentiates (Lyche et al., 2009). However, little information is available about the effects of embryo DEHP exposure during the critical time window of the sex determination (GD 10.5–11.5), which is critical to understand the underlying mechanism of DEHP-induced reproductive toxicity.

The present study aims to evaluate the effects of DEHP on signaling pathways of sex determination, by quantifying expression level and describing temporal pattern of the key genes during the critical stages of gonadal development in mice. The selected molecules regulate sex determination process with a higher degree of cellular and temporal specificity of expression, including testis determination factor *Sry*, and its upstream cascade regulatory genes *Gadd45g*, *Gata4 and Sf1*. Downstream activated genes of *Sry*, including *Sox9*, *AMH* and *Fgf9*. *Rspo1*, *Foxl2*, *Wnt4*, *β-catenin*, were also examined, which suppress *Sox9* expression and initiate female sex determination pathways. The employment of whole-mount in situ hybridization (WISH) facilitates the three-dimensional overview of gene expression pattern and offers particular advantages for the mouse embryo. RT-qPCR was used to further confirm the disorder of the sex determination signaling pathways induced by DEHP exposure.

2. Materials and methods

2.1. Animals

ICR mice were acclimatized to the laboratory for one week and maintained in a humidity (30–40%) and temperature (24 °C)-controlled room in a 12: 12 h light and dark cycle. The animals were given access to food and water ad libitum. Females were time-mated with males overnight. Noon of the day of vaginal plug detection was considered as embryonic day 0.5 postcoitum (dpc).

All animal experimental procedures were carried out in accordance with the National Institutes of Health 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, Dalian University of Technology.

2.2. Dose and administration of DEHP

Dam mice were weight ranked and randomly assigned to dose groups to minimize differences in means and variance among groups. Each group contains 6 dams. Pregnant dams were dosed daily from gestation day (GD) 1 to GD 10–12 by oral gavage with vehicle (corn oil) or 350, 700 mg/kg/day DEHP. The exposure concentrations were designed based on the studies concerning the reproductive toxicity of DEHP in rats and mice, where the selected doses induced significant changes in genes expression in the male offspring without maternal mortality and overt toxicity (Blystone et al., 2010; Gray et al., 2000; Kavlock et al., 2002; Wilson et al., 2007). The dams were dissected at 10.5, 11.5 and 12.5 dpc, fetal mice collected, and the gonads were obtained. All necropsies were conducted within a 3 h time frame between 9:00 and 12:00 a.m., to avoid any potential effects on fetal growth or time of day on the fetal endpoints. Embryos sex was chromosomally analyzed by a PCR assay (Svingen et al., 2007). The collected gonads were stored at the temperature of −80 °C. Female and male gonads were analyzed for the corresponding sex-specific determination regulation pathway. Each group contains 4–6 samples (litters). One gonad from each fetus mouse was used for WISH, and another was pooled with those from the mice in the same litter for gene analyses.

2.3. Real-time quantitative RT-PCR

Total RNA was extracted from 10.5 to 12.5 dpc genital ridges using RNeasy Plus mini kit (Qia-gen, Valencia, CA, USA).

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