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Reduced Fgf10/Fgfr2 and androgen receptor (AR) in anorectal malformations male rats induced by di-*n*-butyl phthalate (DBP): A study on the local and systemic toxicology of DBP

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ABSTRACT

Previous study have demonstrated that not only the anorectal development but also the general conditions of anorectal malformations (ARMs) male rats are severely affected by di-*n*-butyl phthalate (DBP) maternal exposure. However, the mechanisms underlying DBP-induced congenital defects remain elusive. Reportedly, Fgf10/Fgfr2 and androgen receptor (AR) are pivotal for the development of multiple organs. In this study, we therefore investigated the expression of Fgf10/Fgfr2 together with AR in the terminal rectum and multiple organs of ARM male rats induced by *in utero* exposure to DBP. DBP was administered to pregnant rats to establish the model and the incidence of ARMs in male offspring was 39.5%. On postnatal day(PND)1, the gross photograph and histopathological staining confirmed the abnormal manifestations in these organs of newborn ARMs. Decreased anogenital distance, body weight and serum testosterone level were observed in ARM male offspring. The reduced expression of Fgf10/Fgfr2 mRNA and protein was seen in terminal rectum and kidney, spleen, liver, heart in ARM male rats, whereas the reduced expression of AR was only observed in the kidney and terminal rectum. Our findings suggest the potential involvement of altered Fgf10/Fgfr2 signaling and AR in pathogenesis of local and systemic development defects in ARMs male rats induce by DBP.

1. Introduction

Anorectal malformations (ARMs) are congenital anomalies characterized by the obstruction of the anal opening with an incidence of approximately 1 in 5000 live births worldwide. There is an obvious male predominance in ARM cases (Brantberg et al., 2006; Wijers et al., 2013). ARMs are associated with other local and systemic congenital malformations in 40–70% of cases, such as those present in vertebral, cardiac, trachea-esophageal, renal and limb anomalies (Brantberg et al., 2006; Herman and Teitelbaum, 2012; Moore, 2013; Wijers et al., 2013; Huibregtse et al., 2014). After urgent surgical treatment during the neonatal period, these anomalies can be corrected (Iwai and Fumino, 2013). Unfortunately, problems persisting after the treatment such as fecal incontinence continue to be a challenge in the management of these patients and therefore put a heavy economic burden on the family and seriously affect their quality of life (Santulli et al., 1971; Hendren, 1998). The etiopathogenesis of ARMs is hypothesized to result from the combined effects of environmental endocrine-disrupting compounds (EEDs) and genetic factors (Falcone et al., 2007), however still unknown is how the exposure to EEDs cause ARMs complicated with other organs congenital malformations.

Abbreviations: AGD, anogenital distance; AR, androgen receptor; ARMs, anorectal malformations; BW, body weight; DBP, di-*n*-butyl phthalate; EEDs, environmental endocrine-disrupting compounds; Fgf10, fibroblast growth factor 10; Fgfr2, fibroblast growth factor receptor 2; GD, gestation day; PND, postnatal day; Real-time PCR, real-time polymerase chain reaction; SD, standard deviation; i.p., intramuscular injection.

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Our recent studies have firstly indicated the direct link between EEDs and ARMs in neonates (Jiang et al., 2011; Liu et al., 2014). We also showed that the generalized condition of the ARM male offspring was severely affected by the di-*n*-butyl phthalate (DBP) through anatomical analysis (Jiang et al., 2011; Liu et al., 2014). DBP is known as a typical EEDs and is widely applied as a plasticizer used in a variety of industrial applications from creating plastic coatings to producing cosmetics. DBP can easily migrate from plastic products into the environment under certain usages (Heudorf et al., 2007). Human exposure to DBP can occur *via* ingestion, inhalation, intravenous or dermal contact. The average daily intake of DBP for humans is estimated to be 2.7 μg/kg body weight (BW) per day (Franco et al., 2007). Reportedly, 20–40 years old women receive more exposure to DBP than others and women of child-bearing age are the target population for the teratogenic effects of DBP (Blount et al., 2000). Moreover, Chen et al. (2008) reported detection of DBP in Chongqing women undergoing parturition. Previous studies show that exposure to DBP can affect the male reproductive system, resulting in severe developmental disorders including hypospadias, cryptorchidism, spermatogenesis dysfunction and shortened anogenital distance (Foster, 2006; Jiang et al., 2007; Hsieh et al., 2008; Kalfa et al., 2009; Zhou et al., 2011; Giribabu et al., 2014; Kalfa et al., 2015). In fact, the defects induced by DBP in male offspring rats have remarkable similarities with the symptoms seen in the human testicular dysgenesis syndrome (TDS), a condition characterized by cryptorchidism, hypospadias and low sperm counts (Fisher et al., 2003). Although some studies have reported the deleterious influence of DBP on the male reproductive system, the mechanism of DBP's local and systemic toxicology leading to such defects in ARMs male rats have yet to be fully defined.

Fibroblast growth factor 10 (Fgf10) and its receptor Fgfr2 have been associated with instructive mesenchymal–epithelial interactions, such as those that occur during branching morphogenesis. Mice deficient for Fgf10 and Fgfr2 show multiple organ defects (Min et al., 1998; Sekine et al., 1999; De Moerlooze et al., 2000; Ohuchi et al., 2000; Marguerie et al., 2006; Mailleux et al., 2002). In the rectum, Fgf10 is expressed at the time when anorectal continuity is established. Fgf10 invalidation results in a genetically reproducible anorectal malformation phenotype (Fairbanks et al., 2004). In the kidney, Fgf10, mainly secreted by the metanephric mesenchyme, bind to Fgfr2b of the ureteric bud and induce branching (Trueb et al., 2013). In the lung, Fgf10 and its receptor Fgfr2 is required for branching lung morphogenesis (Arman et al., 1999). Delayed Fgf10 gene expression during the critical period of separation of the trachea may affect lung bud formation leading to trachea malformations (Fairbanks et al., 2004). In the spleen, Fgf10 is expressed in the ventral region of the splanchnic mesodermal plate (SMP), which has a role in the formation of the spleen (Hecksher-Sorensen et al., 2004). In the liver, Fgf10 (−/−) and Fgfr2b(−/−) mouse embryonic livers are smaller than wild-type livers. Fgf10(−/−) livers exhibit diminished proliferation of hepatoblasts (Berg et al., 2007). In the heart, inactivation of Fgf10/Fgfr2 signaling pathway results in decreased myocardial proliferation and a resulting smaller thin-walled heart (Vega-Hernandez et al., 2011). The transcription of Fgf10 and Fgfr2 requires androgen receptor (AR) signaling, and that the Fgfr2 promoter contains a stereotypic androgen response element (ARE) sequence. Fgfr2 gene is a transcriptional target of AR (Petiot et al., 2005; Memarzadeh et al., 2007). So, the role of Fgf10/Fgfr2 and AR in DBP induced ARMs attracted our attention.

To address these unresolved questions concerning DBP exposure, an ARM animal model was developed based on our previous studies (Zhu et al., 2009; Jiang et al., 2011). In neonatal period, structural dysplasia and serum testosterone concentration were tested, as well as the expression of Fgf10/Fgfr2 signaling with AR in the terminal rectum and multiple solid organs such as kidney, lung, spleen, liver, heart of ARM male rats induced by DBP, so as to provide insights into the understanding of the mechanisms underlying the assortment of ARMs induced by DBP and the local and systemic toxicology of DBP.

2. Materials and methods

2.1. Animals

Sprague-Dawley rats (Shanghai Laboratory Animal Center, Chinese Academy of Sciences, Shanghai, China) were used this study. Animals were housed in an air-conditioned room maintained on a 12 h light–dark cycle at approximately 18–24 °C with a relative humidity of 40–70%. Rodent feed and tap water were provided *ad libitum*. On the evening of proestrus, virgin female were mated with proven-fertile male rats overnight. Vaginal discharge was collected with a saline wet swab and followed by smear test to examine sperms under microscope. The day when sperm was found in the vaginal smear was considered as gestation day (GD) 0. Successfully mated females were distributed on a random basis into 2 groups of ten rats each and housed individually. The body weight (BW) of female rats was recorded daily. All animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). At the end of experiments, animals were euthanized with carbon dioxide (70%/30%, CO_2/O_2). All experimental studies were approved by the Shanghai Experimental Animal Ethics Committee and complied with Regulations on the Care and Use of Laboratory Animals promulgated by The Ministry of Science and Technology of China.

2.2. Chemicals and dosing

All drug solutions were prepared daily before administration. DBP (99.5% pure, Sigma Chemical Co., St. Louis, MO, USA) was dissolved in corn oil (99.5% pure, Shanghai Solvent Factory, Shanghai, China). Timed-pregnant rats had DBP administered once daily by gastric intubation at doses of 850 mg/kg BW/day from GD 12 to 18. Control rats received only corn oil. The exposure period and dosage levels of DBP were based on our previous study for establishment of an ARM rat model (Jiang et al., 2011). The timing of DBP exposure was chosen to be administered during a period of embryonic development for rats (Jiang et al., 2007; Zhu et al., 2009).

2.3. Filial generations date and tissue collection

On PND1, live pups were counted and their sex was determined. We determined the sex of offspring by measuring the AGD. The results of AGD were confirmed by the examination of the testes during autopsy. The male pups were examined for ARMs by checking whether anal atresia and the accumulated meconium were observable and had their BW and anogenital distance (AGD) recorded. Relative AGD was calculated. The relative AGD as the metric is the ratio of AGD to the cube root of BW according to the Gallavan's study (Gallavan et al., 1999). Gross images were photographed using a digital camera.

On PND1, Six rats were selected randomly from the pool of ARM male rats came from a total of 10 litters using random number table. The blood samples and tissues such as terminal rectum as well kidney, lung, spleen, liver and heart were collected from these pubs. Blood samples were collected from heart. Serum was separated from the blood by centrifugation at 3000 rpm for 15 min at 4 °C. Tissues were harvested from the terminal rectum, kidney, lung, spleen, liver and heart. Control male rats were measured and analyzed using the same parameters as above.