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Prenatal exposure to di-n-butyl phthalate induces anorectal malformations in male rat offspring

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ABSTRACT

The objectives of this study were to investigate the dysplasia, histological malformations, and genetic abnormalities in male rats induced by maternal exposure to di-n-butyl phthalate (DBP). Here we report novel findings concerning developmental abnormalities resulting from prenatal exposure to DBP, which leads to significant anorectal malformations (ARMs) in male rat offspring. The incidence of ARMs was 39.5% in male offspring and all abnormal pups were complicated with secondary megacolon. General images, histological analysis and anatomy examination confirmed the malformation. The development abnormalities such as decreased bodyweight (BW) and anogenital distance (AGD), shortened body lengths (with tail removed), as well as increased abdominal circumference were observed at different developmental stages of ARMs in male rat. The developmental abnormalities in both solid organs (brain, heart, liver, spleen, lung and kidney) and reproductive organs (testes and epididymis) of abnormal pubs on PND35 were also investigated. In addition, the serum testosterone (T) level of ARMs in male rats on PND1 was significantly lower than that of controls with accompanying reduced expression of androgen receptor (AR), sonic hedgehog (Shh) and bone morphogenetic protein 4 (Bmp4) mRNA from tissues of the terminal rectum. These results conclusively demonstrate for the first time that *in utero* exposure to DBP leads to an increased likelihood for the development of ARMs and subsequent complicating megacolon in male rat offspring.

1. Introduction

Anorectal malformations (ARMs) are defects that can occur during postnatal development, which may involve the distal anus and rectum, as well as the urinary and genital tracts. The occurrence is approximately 1 in 5000 live births (Levitt and Pena, 2007). Associated anomalies of ARMs may include anal atresia and megacolon with a poor functional prognosis. However, the etiopathogenesis of ARMs remains unclear and is hypothesized to result from the combined effects of genetic factors and exposure to endocrine-disrupting compounds (Falcone et al., 2007).

For the last decade, there have been considerable concerns surrounding a group of chemicals known as phthalate esters (Silva et al., 2004; Wang and Baskin, 2008). Di-n-butyl phthalate (DBP), one of phthalate esters commonly used in plasticizers, has well defined anti-androgenic effects by interfering with the production and functional activity of testosterone (Patisaul and Adewale, 2009; Fisher et al., 2003; Zhang et al., 2004). Previous studies have revealed that prenatal exposure to high doses of DBP in mammals resulted in severe reproductive developmental disorders, including reduced anogenital distance, hypospadias, cryptorchidism, and spermatogenesis dysfunction (Baskin et al., 2001; Steinhardt, 2004; Foster, 2006; Jiang et al., 2007; Hsieh et al., 2008; Kalfa et al., 2009).

In our previous studies, we have established a reproducible hypospadiac model in rats by maternal exposure to DBP (Jiang et al.,

Abbreviations: DBP, di-n-butyl phthalate; ARMs, anorectal malformations; GD, gestation day; PND, postnatal day; BW, bodyweight; AGD, anogenital distance; T, testosterone; AR, androgen receptor; Shh, sonic hedgehog; Bmp4, bone morphogenetic protein; PAEs, phthalic acid esters; EEDs, environmental endocrine disruptors; GT, genital tubercle; S.E.M., standard errors; Tab, table; Fig, figure.

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Table 1
Primer sets for real-time quantitative PCR analyses.

Gene	Forward primer	Reverse primer
β-Actin	GCCCCTCTGAACCCTAAG	GAGGCATACAGGGACAACA
AR	CATGGGTTGGCGGTCCTTCACTAA	CCTCATCCTCACGCACTGGCTGTA
Shh	TTATCCCCAACGTAGCCGAGAAGA	CGGGGTTGTAATTGGGGGTGAGTT
Bmp4	CACAGCGCTCCAGGAAGAAGAATA	CCGCCAGTGGAAAGGGACAG

Rat-specific primers were designed using Primer Premier software.

2007; Zhu et al., 2009). After evaluation of the DBP induced developmental abnormalities in hypospadiac male rats, we found that in addition to reproductive organs (prostate, testes, epididymis, and pituitary gland) some of the solid organs (liver and kidney) were affected by DBP as well. Furthermore, our results revealed that DBP directly interacted with genes necessary for genital tubercle (GT) formation, which improved our understanding of the mechanisms underlying DBP-induced hypospadias (Zhu et al., 2009).

Although some studies have reported the deleterious influence of DBP on the male reproductive system (Norgil et al., 2002; NTP-CERHR, 2003), the effects of DBP on anorectal development have yet to be fully defined. In the present study, pregnant Sprague-Dawley rats were gavaged with DBP at a dose of 850 mg/kg during late gestation (GD12–18) to investigate DBP's effects on anorectal development. For the first time, we have demonstrated that prenatal exposure to DBP can induce ARMs in male offspring. Secondly, we characterized the histological and anatomical features of the malformation of these ARMs in male offspring. In addition, the research on possible molecular mechanisms of DBP's activity was carried out. Our findings suggest that maternal exposure to common environmental anti-androgens such as DBP may increase the risk of male anorectal system abnormalities.

2. Materials and methods

2.1. Animals

This study was conducted in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals. Sprague-Dawley rats (Shanghai SIPPR-BK Experimental Animal Co. Ltd., Shanghai, China) were used throughout 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 rats, weighing 250 ± 15 g, were mated over night with proven-fertile male rats. The day when sperm was detected in the vaginal smear was then documented as GD0 for that specimen. Successfully mated females were distributed on a random basis into 2 groups of 10 rats each and housed individually. The body weight of female rats was recorded daily.

2.2. Chemicals and dosing

DBP (99.5% pure, Sigma Chemical Co., St. Louis, MO, USA) was administered to pregnant rats once daily by gastric intubation at doses of 850 mg/kg BW/day during late gestation (GD12–18). The DBP for administration was dissolved in corn oil (99.5% pure, Shanghai Solvent Factory, Shanghai, China). The volume of each dose was adjusted to 5 ml/kg according to daily body weight. Control rats received only corn oil. All drug solutions were prepared daily before administration. The dosage levels of DBP were based on our previous study, which included the teratogenic range of DBP on rats. The DBP exposure period was associated with the prenatal period of embryonic development for male rats (Jiang et al., 2007; Zhu et al., 2009).

2.3. Filial generations

On the day of delivery (PND1), live pups were counted and their sex was determined. The male pups were weighed and subsequently examined for ARMs. On PND7 and 35 the existence secondary megacolon in rats with ARMs was checked and recorded. Gross images of malformations were photographed using a digital camera on PND1 and 35. In addition, the AGD of ARMs for male pups was measured by a digital micrometer on PND1. On PND35 the BW, abdominal circumference and the body length (removed tail) were also measured.

2.4. Histology

Histological analysis of the terminal rectum from ARMs in male rats on PND7 was carried out under light microscopy to confirm abnormalities. On PND7, the discharge of feces caused the abdomen of ARMs in male rats to become obviously swollen. Before examination, samples were fixed in 4% neutral-buffered formalin for 12 h, transferred to 70% ethanol, embedded in paraffin, then serial sectioned (5 μm) in the transverse plane and stained with hematoxylin and eosin (H&E).

2.5. Necropsy

On PND35 six abnormal male rats were selected randomly from the pool of ARM male rats and were weighed. After being sacrificed via carbon dioxide anesthesia, both general and anatomical features were examined and collected for study using a digital camera. The organ/body weight ratios of the solid organs (brain, heart, liver, spleen, lung, and kidney) and reproductive organs (testes, epididymis) were then calculated. The same number of control male rats were measured and analyzed under the same procedures.

2.6. Real-time quantitative PCR

On PND1, six ARM male fetuses were randomly selected from the ARM male rats with the same number of control male fetuses also collected. The pups were sacrificed via carbon dioxide anesthesia and blood was collected. The terminal rectum was dissected under magnification and frozen in liquid nitrogen. Real-time PCR (RT-PCR) was performed with an ABI PRISM 7300 Sequence Detection System using SYBR Green PCR according to the manufacturer's instructions for quantification of gene expression (Applied Biosystems). β-Actin was used as an on-plate internal calibration standard for all RT-PCRs. RT-PCR was performed in triplicate on each of the pups. Rat-specific primers were designed for the genes of interest (Table 1) using Primer Express software (Applied Biosystems). The experimental system for RT-PCR used here has been previously demonstrated in our prior publications (Zhu et al., 2009).

2.7. Radioimmunoassay

On PND1, the serum T concentration of ARMs in male offspring was determined using commercial direct radioimmunoassay kits (Rat Testosterone RIAkit; Immunotech, Marseille, France).

2.8. Statistical analysis

All statistical values are presented as means ± standard errors (S.E.M.). Data were analyzed using Stata 7.0 software for Microsoft Windows©. Treatment was identical within each experiment. An independent-samples *t*-test was performed

Table 2
Dysplastic findings of ARMs in male rats induced by maternal exposure to DBP.

	Control	ARMs
BW of male fetuses on PND1 (g)[a]	6.365 ± 0.085	4.876 ± 0.067*
AGD of male fetuses (mm)[a]	2.993 ± 0.403	2.401 ± 0.341*
BW of male fetuses on PND35 (g)[a]	103.431 ± 1.011	102.525 ± 1.318
Abdominal circumference of male fetuses on PND35 (cm)[a]	11.993 ± 0.339	20.021 ± 0.416*
Body length (removed tail) of male fetuses on PND35 (cm)[a]	14.943 ± 0.171	12.925 ± 0.158*
Incidence of ARMs in male fetuses (%)	0	39.5*

[a] Values are means ± S.E.M.
* Significantly different from control ($P < 0.05$).