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Effects of maternal exposure to di-*n*-butyl phthalate during pregnancy and breastfeeding on ovarian development and function of F1 female rats

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ABSTRACT

This study aimed to investigate the effects of maternal exposure to di-*n*-butyl phthalate (DBP) during pregnancy and breastfeeding on F1 ovarian development and function. A rat model of maternal exposure to DBP during pregnancy and breastfeeding was established by gavage feeding female Sprague Dawley rats with 0, 10, 100, or 600 mg/kg/day DBP from gestational day (GD) 12 to postnatal day (PND) 21. F1 offspring were weaned on PND21 and were not exposed to DBP afterward. The age of vaginal opening and estrus onset, estrous cyclicity, c-Kit-ligand expression on ovarian granulosa cells, and the weight of ovaries and uterus of F1 female offspring were not affected, whereas serum levels of estradiol and progesterone were increased significantly by maternal exposure to 10 mg/kg/day DBP from GD12 to PND21. Although F1 ovarian function may not be adversely affected by maternal exposure to DBP, the increased reproductive hormone levels may interfere in F1 rat fertility.

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1. Introduction

Phthalic acid esters (PAEs), such as di-*n*-butyl-phthalate (DBP), are used ubiquitously in many common consumer products, including plastics, cosmetics, adhesives, solvents, lubricants, and medical devices (Lyche et al., 2009). Thus, humans are unavoidably exposed to PAEs in daily life. In fact, PAEs and/or their metabolites have been detected in human saliva, urine, amniotic fluid, cord blood, breast milk, and urine of neonates in intensive care units (Silva et al., 2005; Guo et al., 2011; Jensen et al., 2012; Latini et al., 2006, 2009; Green et al., 2005).

Accumulating evidence support that excessive exposure to PAEs can adversely affect both male and female reproductive organ development in animal models (Martino-Andrade and Chahoud, 2010; Wine et al., 1997; Davis et al., 1994a). Wine and colleagues used a continuous breeding protocol to investigate the reproductive toxicity of DBP and found that administration of DBP (650–750 mg/kg) with food intake to Sprague-Dawley rats decreased sperm count and increased the incidence of male reproductive tract malformation in F1 males (Wine et al., 1997). DBP (1000 mg/kg) treatment can also result in polycystic ovaries in female rats (Davis et al., 1994a). Fetus and newborns may be even more susceptible than adults to the reproductive toxicity of PAEs.

Recent epidemiological and preclinical studies have suggested that maternal exposure to PAEs during pregnancy and breastfeeding may adversely affect fetal growth and reproductive organ development (Huang et al., 2014; Su et al., 2015; Saillenfait et al., 2013). Huang and colleagues found that the levels of PAEs, including DBP, in woman cord blood was significantly associated with a reduction in gestational age and preterm delivery (Huang et al., 2014). Su et al. demonstrated that increasing exposure to PAE during pregnancy of mothers was significantly associated with reduced uterus size in daughters at age of 12 years, whereas ovarian volume and testicle size were not affected by prenatal exposure to PAEs (Su et al., 2015).

Thus, maternal exposure to DBP during pregnancy and breastfeeding may cause female reproductive developmental disorder in offspring. Studies to test this hypothesis under physiological level of exposure to DBP are sparse, although numerous previous investigations that use high-dose direct exposure to DBP have confirmed the reproductive toxicity of DBP. Here, we used a rat model to examine the reproductive toxicity of maternal exposure to relatively low-dose DBP during pregnancy and breastfeeding on F1 females and explore the molecular mechanism underlying the effects. This study may increase the awareness of the potential adverse effects of

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environmental contaminants on female reproductive development in fetus and thus advocate women to avoid DBP during pregnancy and breastfeeding.

2. Materials and methods

2.1. Animals

The protocol for animal handling and experiment was approved by the Institutional Review Board for Animal Care and Usage of Wenzhou Medical University. A total of 32 healthy Sprague Dawley (SD) virgin female rats (10-week old and weighted 215–230 g) and eight male SD rats (14-week old and weighed 380–420 g) were purchased from the Laboratory Animal Center of Wenzhou Medical University. Rats were maintained in clean rooms at $23 \pm 1\,^{\circ}C$ with a humidity of 50–60% under a lighting cycle of 12-h light and 12-h dark and allowed for free access to food and water. Virgin female rats were mated with male rats in a mating cage. Successful mating was determined by the detection of vaginal plug displacement in the morning after mating. The day, on which vaginal plug displacement was detected, was considered as gestational day 0 (GD0). After becoming successfully pregnant, the 32 female rats were randomized to the following four groups (eight per group): control (vehicle, corn oil), DBP-10 (10 mg/kg/day DBP), DBP-100 (100 mg/kg/day DBP), or DBP-600 (600 mg/kg/day DBP). The date of giving birth was considered as postnatal day 0 (PND0).

2.2. Exposure to DBP

DBP (purity > 99.5%) was purchase from Sigma (St. Louis, USA) and dissolved in corn oil (Aladdin Company, USA). The volume of corn oil used for dissolving DBP was adjusted according to DBP dose and rat body weight. DBP solutions were freshly prepared daily. Pregnant rats were administered with DBP by gavage at the dosage of 10, 100, or 600 mg/kg once daily from GD12 to PND21. The volume of each dose was adjusted to 4 mL/kg body weight based on daily body weight. Rats in control group were administered with corn oil in the same manner. Rat female reproductive organs, including mammary glands, ovaries, and uterus, start to develop on embryonic age day 12, and the weaning age of rat offspring is postnatal day 21. Thus, the period from gestation day 12 to postnatal day 21 appears to be the time window when female reproductive system development is sensitive to maternal exposure to DBP. The majority of previous studies investigating the reproductive toxicity of phthalates include high-dose exposure to DBP (500–1000 mg/kg/day) (Hirosawa et al., 2006; Gray et al., 2006). However, in the real world, human and animal are usually exposed to low concentration of phthalates, which are released from plastic products. Thus, to better represent the real-world exposure to DBP, we included low-dose exposure (10 mg/kg/day) in addition to the median- (100 mg/kg/day) and high-dose (600 mg/kg/day) exposure to DBP.

2.3. Monitoring vaginal opening and estrous cyclicity

F1 female offspring were weaned on PND21 and caged separately from male pups and the mother, and then the F1 female offspring were checked for vaginal opening daily. The day when vagina opened was recorded. A normal rat estrous cycle is usually 4–5 days. Vaginal smear was conducted daily on the F1 female offspring at 8:00-9:00 A.M. for 15 days starting on the day of vaginal opening. Estrous cyclicity was determined by examination of the vaginal smear cytology. Vaginal smear samples were prepared by inserting a cotton tip moisturized with 0.9% saline into rat vagina for a length of 2.0 cm and wiping the vaginal wall to collect vaginal discharge, and then the vaginal discharge on the cotton tip was spread on a microscope slide. The vaginal smear was then stained with hematoxylin & eosin (Sigma-Aldrich, USA) and viewed under light microscopy at 100× magnification. Vaginal smear was reviewed and estrous cycle phase was determined by two researchers separately. In cases of disagreement, a third researcher joined to determine the estrous cycle phase. Rat estrous cycle phase was determined according to the following cytological criteria: proestrus: predominance of nucleated epithelial cells; estrus: predominance of cornified epithelia cells; metestrus: presence of cornified and nucleated epithelial cells and leukocytes; diestrus: predominance of leukocytes. Representative hematoxylin and eosin staining images of virginal smear for each estrous phase are displayed in Fig. 1.

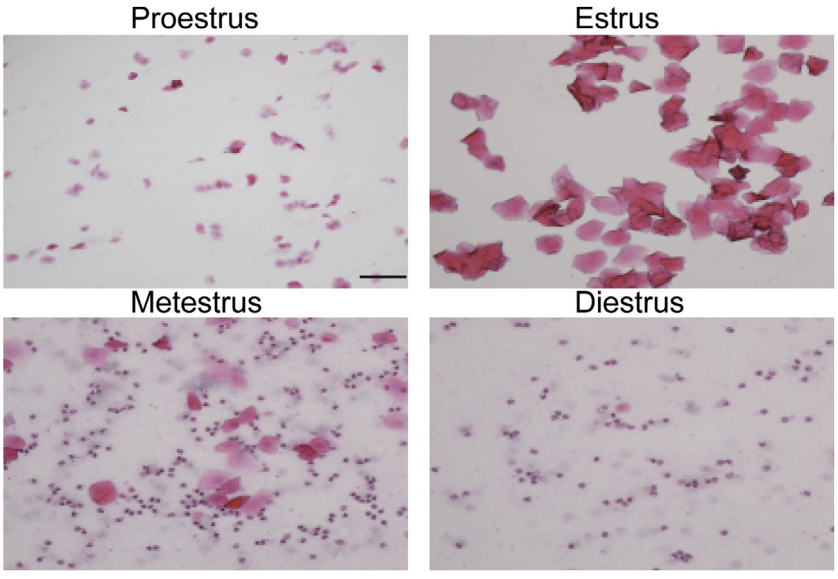

Fig. 1. Hematoxylin & eosin staining images representing different estrous phase.
Proestrus is characterized by predominant nucleated epithelial cells. Estrus shows predominant cornified epithelia cells. Metestrus is characterized by the presence of cornified epithelial cells, nucleated epithelial cells, and leukocytes. Diestrus shows predominant leukocytes. Vaginal smear was stained with hematoxylin & eosin and viewed under light microscopy at 100× magnification. Estrous cycle phase was determined by two researchers separately. The scale bar represents 16 μm.

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