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Maternal di-(2-ethylhexyl) phthalate exposure during pregnancy causes fetal growth restriction in a stage-specific but gender-independent manner

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ABSTRACT

Di (2-ethylhexyl) phthalate (DEHP) is male developmental toxicant that impairs testis development with reduced anogenital distance. The present study aimed to investigate whether maternal DEHP exposure during pregnancy causes intrauterine growth restriction (IUGR) in a gender-specific manner and to identify the critical window of DEHP-induced fetal IUGR. Pregnant mice were administered with DEHP (0, 50 or 200 mg/kg) by gavage. Fetal IUGR was observed not only in males but also in females when litters were exposed to DEHP on gestational day (GD)0-GD17. Interestingly, fetal weight and crown-rump length were reduced, markedly in dams with DEHP on GD13-GD17, slightly in dams with on GD7-GD12, but not in dams with on GD0-GD6. Further analysis showed that maternal DEHP exposure on GD7-GD12 inhibited cell proliferation, lowered placental weight, and reduced blood sinusoid area in placental labyrinth layer. These results suggest that maternal DEHP exposure induces IUGR in a stage-specific but gender-independent manner.

1. Introduction

Phthalate diesters (PAEs) are a category of compounds extensively used as plasticizer around the world. Among the PAEs, di (2-ethylhexyl) phthalate (DEHP) represents almost 50% of total PAE production in the annual world market [1]. PAEs are ubiquitous in almost all environmental compartments including surface water, soil, street and indoor dusts [2–5]. PAEs have been widely used in many consumer products, including food packages, pharmaceuticals, cosmetics, pesticides, and even children's toys [6,7]. Thus, humans are exposed to PAEs via ingestion, inhalation and dermal absorption for their lifetimes. PAEs are well-known endocrine disruptors and male reproductive toxicants. The results from animal experiments demonstrated that PAE exposure reduced serum testosterone (T) level through disturbing hypothalamic-pituitary-testis axis [8]. Two in vitro studies found that the expression of T synthases was decreased in PAE-treated Leydig cells [9,10]. According to several epidemiological reports, urinary PAE metabolites were negatively associated with serum testosterone level and semen quality not only in infertile men but also in general population [11–17].

On the other hand, PAEs are developmental toxicants. The results from rodent animals showed that maternal PAE exposure during pregnancy impaired male reproductive development with reduced anogenital distance (AGD) and abnormal Leydig cell aggregation in fetal testis, similar to testicular dysgenesis syndrome in humans [18–22]. Further studies found that prenatal PAE exposure impaired development of the hippocampus and neurobehavior [23–25]. Intrauterine growth restriction (IUGR), which manifests as small for gestational age (SGA) infants, increases the risk of infant morbidity [26]. In addition, IUGR has been associated with mental disorder and metabolic diseases in adulthood [27,28]. Several epidemiological studies explored the association between maternal PAE exposure and fetal IUGR with contradictory results. A recent cohort study showed that maternal PAE exposure elevated the risk

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of low birth weight infants [29]. According to another recent report from cohort study, there was little evidence of association between maternal PAE exposure and fetal IUGR [30].

The aim of the present study was to investigate whether maternal exposure to DEHP, a representative PAE, induces fetal IUGR in a mouse model. If so, we were to identify the critical time window of DEHP-induced fetal IUGR. Our results showed that fetal IUGR was observed not only in males but also in females. Moreover, fetal weight and crown-rump length were reduced markedly in dams with DEHP at late gestational stage, slightly in dams with DEHP at middle gestational stage, but not in dams with DEHP at early gestational stage. We demonstrate that maternal DEHP exposure induces fetal IUGR in a stage-specific but gender-independent manner.

2. Materials and methods

2.1. Animals and treatments

The ICR mice (8–10 week-old; male mice: 28–30 g; female mice: 24–26 g) were purchased from Beijing Vital River whose foundation colonies were all introduced from Charles River Laboratories, Inc. The animals were allowed free access to food and water at all times and were maintained on a 12-h light/dark cycle in a controlled temperature (20–25 °C) and humidity (50 ± 5%) environment for a period of 1 week before use. For mating purposes, four females were housed overnight with two males starting at 9:00 p.m. Females were checked by 7:00 a.m. the next morning, and the presence of a vaginal plug was designated as gestational day (GD) 0. To investigate the effects of maternal DEHP exposure throughout pregnancy on fetal development, pregnant mice were divided into three groups randomly. All pregnant mice except controls were administered with DEHP (50 or 200 mg/kg, dissolved in corn oil) by gavage daily from GD0 to GD17. Controls were administered with corn oil by gavage daily from GD0 to GD17. The volume for DEHP or corn oil is 1 ml/100 g body weight. The doses of DEHP used in the present study referred to others with minor modulation [31–33]. To investigate the effects of maternal DEHP exposure at different gestational stages on fetal development, pregnant mice were divided into three groups randomly. In Group 1, pregnant mice except controls were administered with DEHP (200 mg/kg) by gavage daily in early gestational stage (GD0-GD6). Controls were administered with corn oil by gavage daily from GD0 to GD6. In Group 2, pregnant mice except controls were administered with DEHP (200 mg/kg) by gavage daily in middle gestational stage (GD7-GD12). Controls were administered with corn oil by gavage daily from GD7 to GD12. In Group 3, pregnant mice except controls were administered with DEHP (200 mg/kg) by gavage daily in late gestational stage (GD13-GD17). Controls were administered with corn oil by gavage daily from GD13 to GD17. The volume for DEHP or corn oil was 1 ml/100 g body weight. All dams were sacrificed on GD18 and gravid uterine weights were recorded. For each litter, the number of live fetuses, dead fetuses and resorption sites were counted. For live fetuses, gender was identified and weighed. Crown-rump length was measured. Placentas were collected for histopathology and immunohistochemistry.

2.2. Placental histopathology

Placentas were fixed in 4% formalin and embedded in paraffin according to the standard procedure. Paraffin embedded tissues were cut 5 μm thick and stained with hematoxylin and eosin (H & E). Placental sections were then analyzed for vascular space quantification. In each section, 5 fields were randomly selected in the labyrinthine region at magnification ×400. The images were given a color threshold to cover the internal space of maternal and fetal blood vessels in the labyrinth layer. The blood sinusoids area in the labyrinthine region was estimated from the analysis of two nonconsecutive sections in each placenta. The public domain NIH Image J Program was used to perform an image analysis. The average percentage was calculated as the ratio between the number of pixels covered by the area defined by the threshold and the overall number of pixels in the image.

Table 1
Effects of DEHP exposure from GD0 to GD17 on fetal outcomes.

	DEHP(mg/kg/d)		
	0	50	200
Number of pregnant mice(n)	17	15	12
Litters of abortion(n)	0	0	0
Litters of preterm delivery(n)	0	0	0
Litters of term delivery(n)	17	15	12
Implantation sites per litter(n)	13.00 ± 0.37	13.60 ± 0.39	12.92 ± 0.82
Resorptions per litter(n)	0.82 ± 0.27	0.53 ± 0.24	0.17 ± 0.11
Dead fetuses per litter(n)	0.24 ± 0.16	0.47 ± 0.22	0.58 ± 0.34
Live fetuses per litter(n)	11.94 ± 0.46	12.60 ± 0.48	12.17 ± 0.86
Total sex ratio (Male/Female)	0.99 (101/102)	0.87 (88/101)	1.25 (81/65)
Average sex ratio (male%)	50.86 ± 4.43	46.65 ± 3.59	53.57 ± 4.46

Quantified data are represented as means ± SEM. The results of implantation sites per litter, live fetuses per litter, dead fetuses per litter and total sex ratio were analyzed using one-way ANOVA followed by SNK. The result of resorptions per litter was analyzed using Nonparametric tests and average sex ratio was analyzed using chi-square test.

2.3. Immunohistochemistry

For immunohistochemistry, paraffin-embedded placental sections were deparaffinized and rehydrated in a graded ethanol series. After antigen retrieval and quenching of endogenous peroxidase, sections were incubated with Ki67 monoclonal antibodies (Abcam, USA, 1:200 dilutions) at 4 °C overnight. The color reaction was developed with HRP-linked polymer detection system and counterstaining with hematoxylin.

2.4. Statistical analysis

The litter was considered the unit for statistical comparison among different groups. Fetal mortality was calculated per litter and then averaged per group. For fetal weight, crown-rump length, placenta weight and placenta diameter, the means were calculated per litter and then averaged per group. Quantified data were expressed as means ± S.E.M. at each point. $P < 0.05$ was considered statistically significant. ANOVA, the Students-Newman-Keuls post hoc test and chi-square test were used to determine differences between the treated animals and the controls.

3. Results

3.1. Maternal DEHP exposure reduces fetal weight and crown-rump length in a gender-independent manner

The effects of maternal DEHP exposure throughout pregnancy on food consumption and body weight of pregnant mice were analyzed. As shown in Fig. 1A and B, maternal DEHP exposure throughout pregnancy did not affect maternal weight and weight gain of the pregnant mice. In addition, maternal DEHP exposure throughout pregnancy had no effect on food consumption (Fig. 1C). The effects of maternal DEHP exposure throughout pregnancy on pregnant outcomes were analyzed. No abortion and preterm delivery were observed (Table 1). Moreover, no dams died throughout pregnancy. No significant difference on the numbers of resorptions, dead fetuses and live fetuses per litter was observed among different groups (Table 1). The effects of maternal DEHP exposure