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

Ru Shen[a,b,1], Ling-Li Zhao[a,b,1], Zhen Yu[c], Cheng Zhang[a,b], Yuan-Hua Chen[a,b,d,*], Hua Wang[a,b], Zhi-Hui Zhang[a,b], De-Xiang Xu[a,b,*]

[a] Department of Toxicology, Anhui Medical University, China
[b] Laboratory of Environmental Toxicology, Anhui Medical University, China
[c] Department of Maternal, Child & Adolescent Health, Anhui Medical University, China
[d] Department of Histology and Embryology, Anhui Medical University, Hefei, 230032, China

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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

* Corresponding authors at: Department of Toxicology, Anhui Medical University, China.
E-mail addresses: yuanhuach@126.com (Y.-H. Chen), xudex@126.com (D.-X. Xu).
[1] These authors contributed equally to this work.

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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

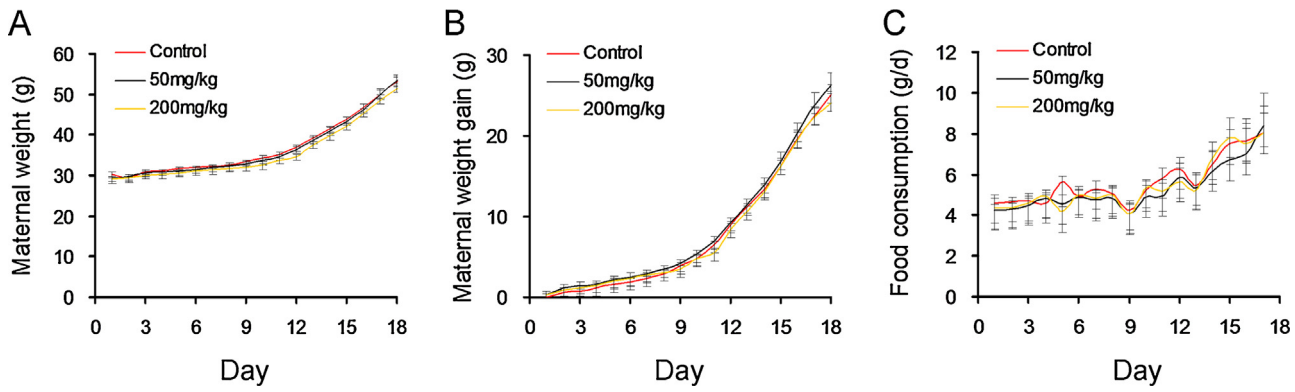

Fig. 1. Effects of maternal DEHP exposure throughout pregnancy on body weight growth and food consumption in pregnant mice. Pregnant mice except controls were administered with DEHP (50 or 200 mg/kg) by gavage daily from GD0 to GD17. Pregnant mice were inspected for food consumption and weighted daily. (A) Change of maternal body weight. (B) Maternal weight gain. (C) Food consumption. Data were expressed as means ± SEM. (N = 12–17).

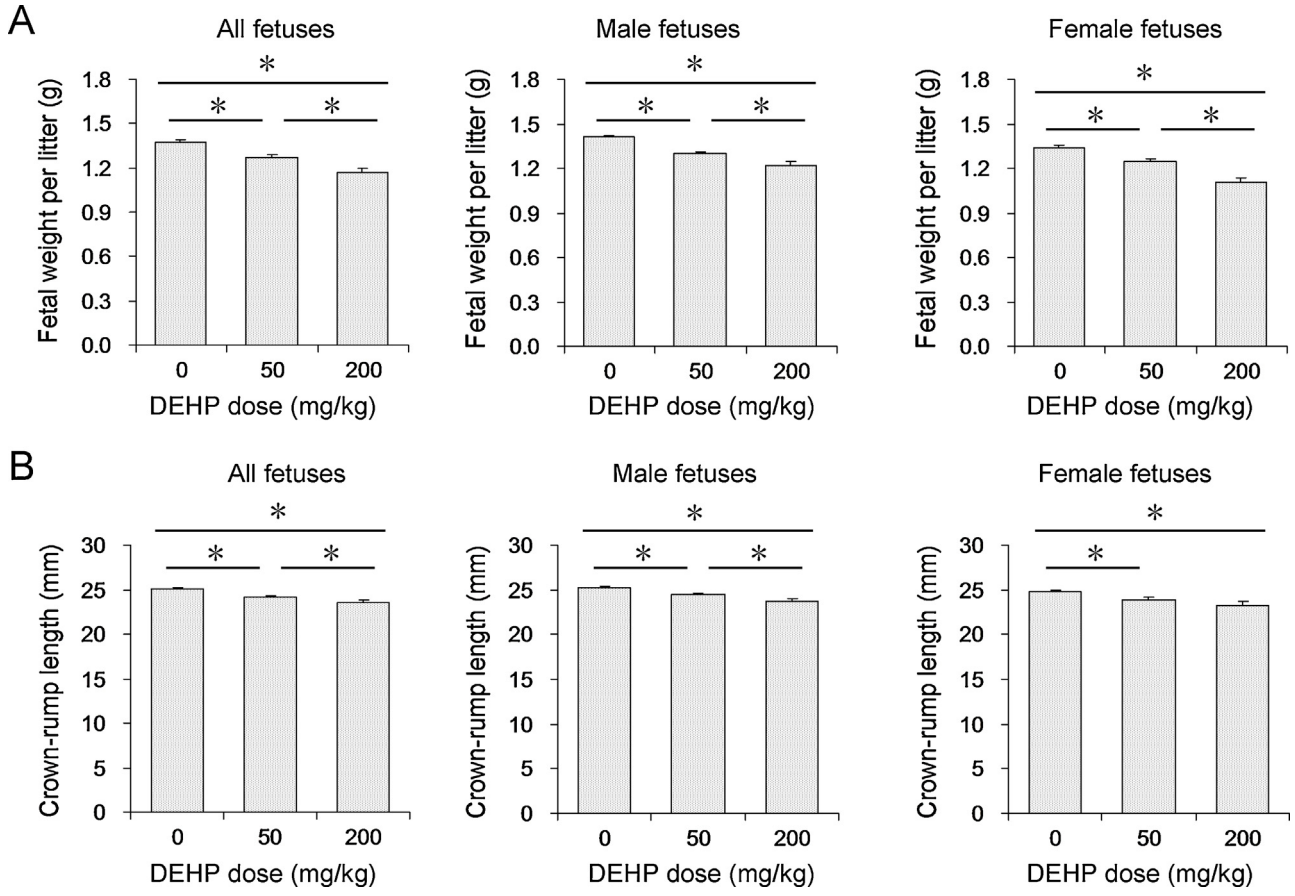

Fig. 2. Effects of maternal DEHP exposure throughout pregnancy on fetal weight and crown-rump length. Pregnant mice except controls were administered with DEHP (50 or 200 mg/kg) by gavage daily from GD0 to GD17. All dams were sacrificed on GD18. (A–C) Male and female fetuses were identified and weighed. (A) Body weight of all fetuses; (B) Body weight of male fetuses; (C) Body weight of female fetuses. (D-F) Crown-rump length was measured. (D) Crown-rump length of all fetuses; (E) Crown-rump length of male fetuses; (F) Crown-rump length of female fetuses. All data were expressed as means ± S.E.M. (N = 12–17). *$P < 0.05$.

throughout pregnancy on fetal weight and crown-rump length are presented in Fig. 2A and D. As expected, maternal DEHP exposure throughout pregnancy markedly reduced fetal weight and crown-rump length in a dose-dependent manner. The effects of maternal DEHP exposure throughout pregnancy on body weight and crown-rump length of male and female fetuses were further analyzed. Body weight and crown-rump length of male fetuses were significantly decreased in DEHP-exposed mice (Fig. 2B and E). Interestingly, body weight and crown-rump length of female fetuses were significantly reduced in DEHP-treated mice (Fig. 2C and F).

3.2. *Identification of critical window for DEHP-induced fetal growth restriction*

To identify the critical time window of DEHP-induced fetal IUGR, the present study investigated the effects of maternal DEHP exposure at different gestational stages on fetal development. No matter what gestational stages pregnant mice were exposed to DEHP, there

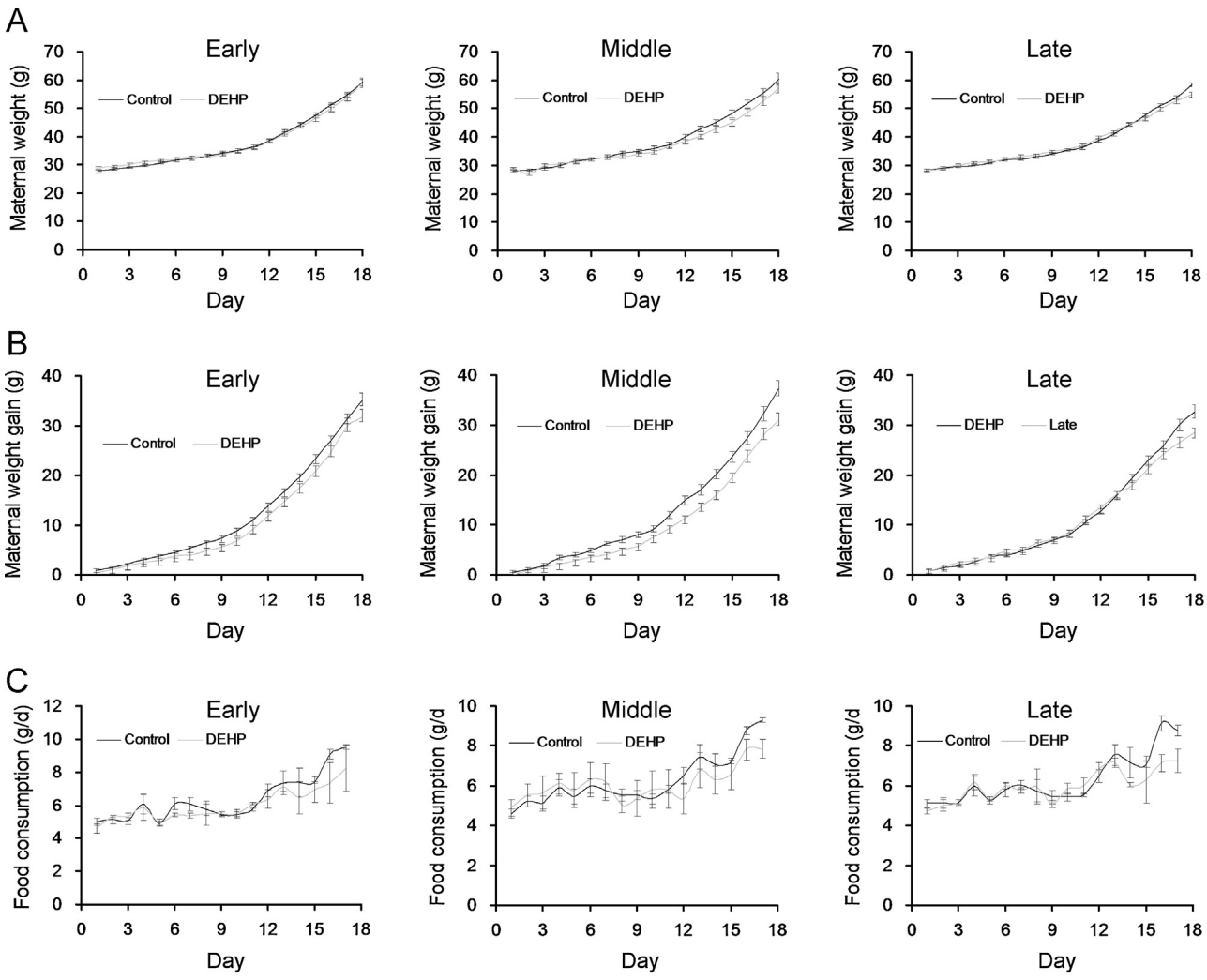

Fig. 3. Effects of maternal DEHP exposure at different gestational stages on body weight growth and food consumption in pregnant mice. Pregnant mice except controls were administered with DEHP (200 mg/kg) by gavage daily at early (from GD0 to GD6) or middle (from GD7 to GD12) or late (from GD13 to GD17) gestational stage. Pregnant mice were daily inspected for food consumption and weighted. (A) Change of maternal body weight. (B) Maternal weight gain. (C) Food consumption. Data were expressed as means ± SEM. (N = 11–14).

Table 2
Effects of DEHP exposure at different gestational stages on fetal outcomes.

	Early (GD0-GD6)		Middle (GD7-GD12)		Late (GD13-GD17)	
	Control	DEHP (200 mg/kg/d)	Control	DEHP (200 mg/kg/d)	Control	DEHP (200 mg/kg/d)
Number of pregnant mice(n)	14	11	11	11	11	11
Litters of abortion(n)	0	0	0	0	0	0
Litters of preterm delivery(n)	0	0	0	0	0	0
Litters of term delivery(n)	14	11	11	11	11	11
Implantation sites per litter(n)	14.29 ± 0.51	14.27 ± 0.94	14.00 ± 0.47	14.00 ± 0.66	14.09 ± 0.69	14.36 ± 0.41
Resorptions per litter(n)	0.64 ± 0.23	0.36 ± 0.15	0.46 ± 0.25	0.36 ± 0.15	0.36 ± 0.20	0.91 ± 0.25
Dead fetuses per litter(n)	0.29 ± 0.13	0.36 ± 0.20	0.36 ± 0.15	0.18 ± 0.12	0.27 ± 0.20	0.09 ± 0.09
Live fetuses per litter(n)	13.36 ± 0.45	13.55 ± 1.01	13.18 ± 0.40	13.45 ± 0.62	13.45 ± 0.64	13.36 ± 0.39

Quantified data are represented as means ± SEM. The results of implantation sites per litter, resorptions per litter, dead fetuses per litter, and live fetuses per litter were analyzed using Nonparametric tests.

was no significant difference on food consumption and weight gain of the pregnant mice (Fig. 3). Moreover, no significant difference on the numbers of resorptions, dead fetuses and live fetuses per litter was observed among different groups (Table 2). The effects of maternal DEHP exposure at different gestational stages on fetal weight and crown-rump length were analyzed. As shown in Fig. 4A, maternal DEHP exposure at early gestational stage did not affect fetal weight. Interestingly, maternal DEHP exposure at middle gestational stage slightly reduced fetal weight and crown-rump length. Moreover, maternal DEHP exposure at late gestational stage obviously reduced fetal weight and crown-rump length. The effects of maternal DEHP exposure at different gestational stages on the weight and crown-rump length of male and female fetuses were further analyzed. As shown in Fig. 4B and E, the weight and

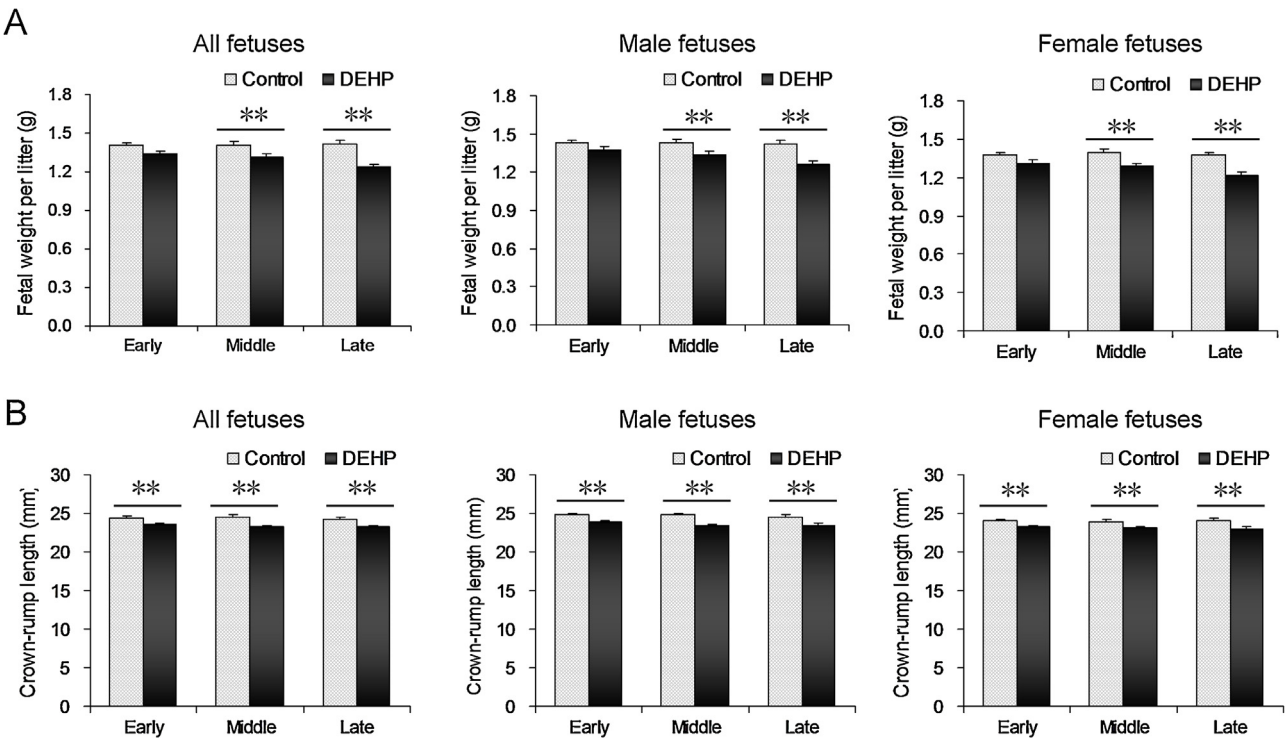

Fig. 4. Effects of maternal DEHP exposure at different gestational stages on fetal weight and crown-rump length. Pregnant mice except controls were administered with DEHP (200 mg/kg) by gavage daily at early (from GD0 to GD6) or middle (from GD7 to GD12) or late (from GD13 to GD17) gestational stage. All dams were sacrificed on GD18. Male and female fetuses were identified. (A) Body weight of all fetuses (left), male fetuses (middle) and female fetuses (right) was weighed. (B) Crown-rump length of all fetuses (left), male fetuses (middle) and female fetuses (right) was measured. All data were expressed as means ± S.E.M. (N = 11–14). $^{*}P < 0.05$; $^{**}P < 0.01$.

crown-rump length of male fetuses were decreased, slightly in mice treated with DEHP at middle gestational stage and markedly in mice treated with DEHP at late gestational stage. Correspondingly, the weight and crown-rump length of female fetuses were reduced, slightly in mice treated with DEHP at middle gestational stage and markedly in mice treated with DEHP at late gestational stage (Fig. 4C and F).

3.3. Maternal DEHP exposure impairs placental development in a stage-dependent manner

The effects of maternal DEHP exposure in different gestational stages on placental weight were analyzed. Although there was no statistically significant difference on placenta weight in mice exposed to DEHP in early (GD0-GD6) or late (GD13-GD17) gestational stage, placenta weight was significantly reduced in mice exposed to DEHP in middle gestational stage (GD7-GD12). The effects of maternal DEHP exposure in different gestational stages on placental diameter of all fetuses were evaluated. As shown in Fig. 5B, no statistically significant difference on placenta diameter was observed among groups. The effects of maternal DEHP exposure at different gestational stages on the placental weight of male and female fetuses were then analyzed. Although no significant difference on placental diameter of female fetuses was observed, placenta diameter of male fetuses was markedly reduced in mice exposed to DEHP from GD7 to GD12 (Fig. 5B, middle). To investigate whether maternal DEHP exposure at different gestational stages impairs placental vascular space, a computerized morphometry was used to analyze cross-sectional areas of blood sinusoids in placental labyrinthine region (Fig. 5C and D). As shown in Fig. 5D, there was a downward trend on blood sinusoid area in mice exposed to DEHP at early and late gestational stages. Interestingly, blood sinusoid area in the placental labyrinth layer was markedly reduced in mice exposed to DEHP at middle gestational stage (Fig. 5D).

3.4. Maternal DEHP exposure inhibits cell proliferation in placenta

Cell proliferation was determined by staining with Ki67. A few Ki67-positive cells were observed in trophoblast region (data not shown). Interestingly, there were numerous Ki67-positive cells in labyrinthine region (Fig. 6A). The effects of maternal DEHP exposure at different gestational stages on cell proliferation were then analyzed in mouse placenta. Although maternal DEHP exposure at early and late stages did not cause statistically significant decrease in the rate of Ki67-positive nuclei, there was a downstream trend on the number of placental Ki67-positive cells in labyrinthine region of mice exposed to DEHP at early or late gestational stage. Interestingly, the rate of Ki67-positive nuclei in labyrinthine region was significantly reduced in placenta of mice exposed to DEHP at middle gestational stage (Fig. 6).

4. Discussion

Increasing evidence has demonstrated that maternal PAE exposure disturbs fetal development in a gender-specific manner. Numerous animal experiments showed that maternal PAE exposure during pregnancy caused testicular dysgenesis syndrome in male pups [34–36]. A recent study found that prenatal DEHP exposure impaired spatial learning and memory among younger males but not females [25]. According to recent results from a case-control study, prenatal PAE exposure was associated with increased risk of IUGR and male newborns were more sensitive to PAEs than females [37]. The present study aimed to investigate whether maternal DEHP exposure during pregnancy causes IUGR in a gender-specific manner. Our results showed that fetal weight and crown-rump length were obviously reduced in dams exposed to DEHP throughout pregnancy. Further analysis found that fetal weight and crown-rump length were obviously reduced not only

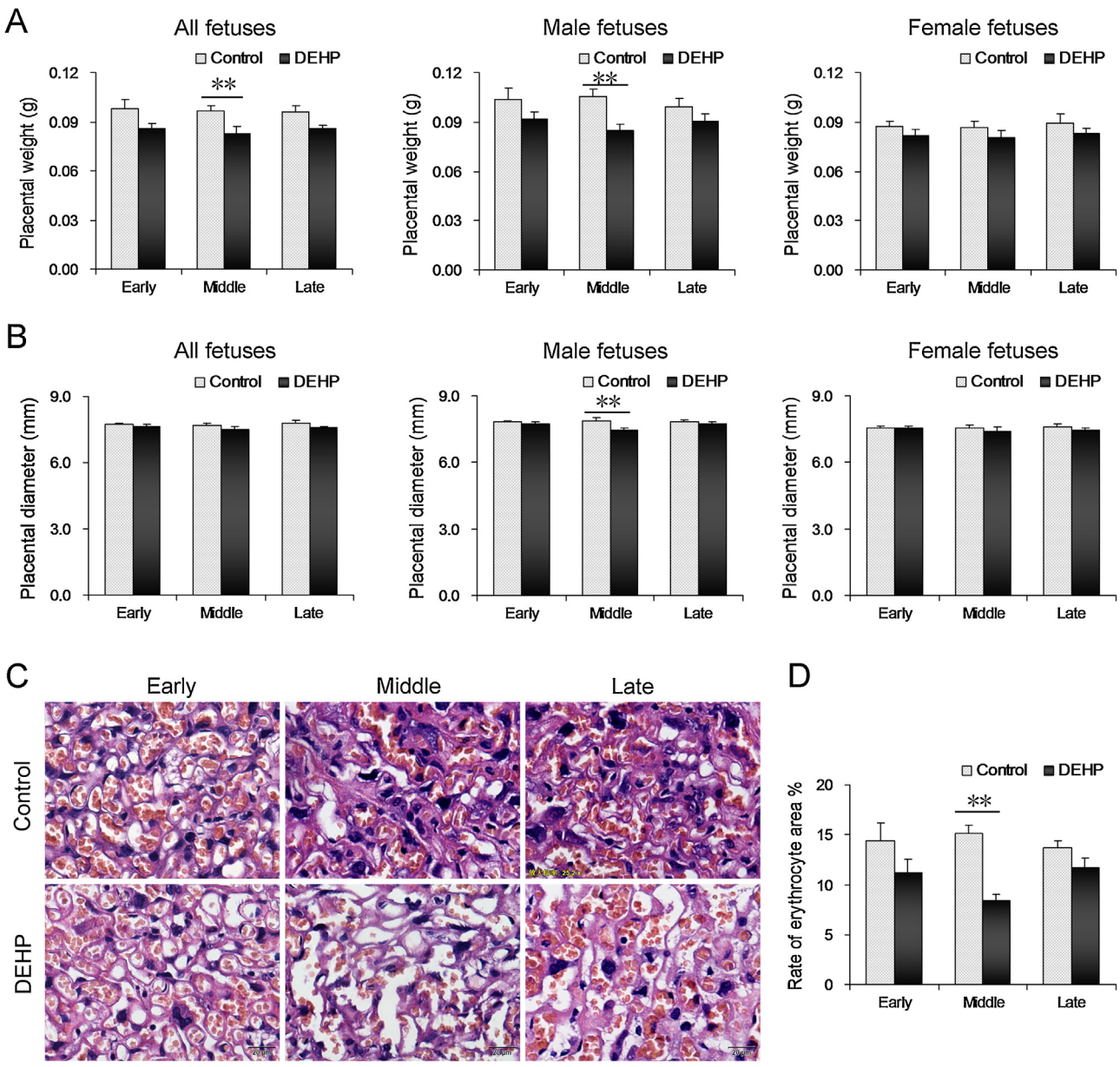

Fig. 5. Effects of maternal DEHP exposure at different gestational stages on placental development. Pregnant mice except controls were administered with DEHP (200 mg/kg) by gavage daily at early (from GD0 to GD6) or middle (from GD7 to GD12) or late (from GD13 to GD17) gestational stage. All dams were sacrificed on GD18. Male and female fetuses were identified. (A) Placenta weight of all fetuses (left), male fetuses (middle) and female fetuses (right) was weighed. (B) Placenta diameter of all fetuses (left), male fetuses (right) and female fetuses was measured. (C) Placental cross sections were stained with H&E. Original magnification: 400 ×. (D) Vascular area in the labyrinthine region was estimated from two nonconsecutive sections in each placenta using the public domain NIH Image J Program. All data were expressed as means ± S.E.M. (N = 11–14). $^{**}P < 0.01$.

in males but also in females. These results suggest that maternal DEHP exposure causes fetal IUGR in a gender-independent manner.

There are differential critical time windows for PAE-induced abnormal male reproductive development in different animals. In rats, reduced AGD and abnormal testicular and epididymal development were mainly observed in fetuses of dams with PAE at late gestational stage (GD16-18) [38]. Several studies found that mice were less sensitive than rats, but potentially more relevant to estimate effects in humans [39]. According to a recent report, several key genes for sex determination were down-regulated only in male genital ridges from mouse fetuses of dams with DEHP during the critical time window of the sex determination (GD10-11) [14]. These results indicate that critical time window for PAE-induced abnormal reproductive development in mice was at middle gestational stage. To identify the critical window of DEHP-induced fetal IUGR in mice, the present study investigated the effects of maternal DEHP exposure at different gestational stages on fetal IUGR. Unexpectedly, fetal weight and crown-rump length of male fetuses were reduced only slightly in dams with DEHP from GD7 to GD12, the critical window of the sex determination. Interestingly, our results showed that fetal weight and crown-rump length of male fetuses were reduced markedly in dams with DEHP from GD13 to GD17. Similarly, fetal weight and crown-rump length of female fetuses were reduced markedly in dams with DEHP from GD13 to GD17 but only slightly in dams with DEHP from GD7 to GD12. Taken together, these results suggest that there is a broad time window for DEHP-induced fetal IUGR, during which maternal DEHP exposure reduces fetal weight and crown-rump length.

The mechanism through which maternal DEHP exposure induces fetal IUGR remains obscure. The placenta is essential for

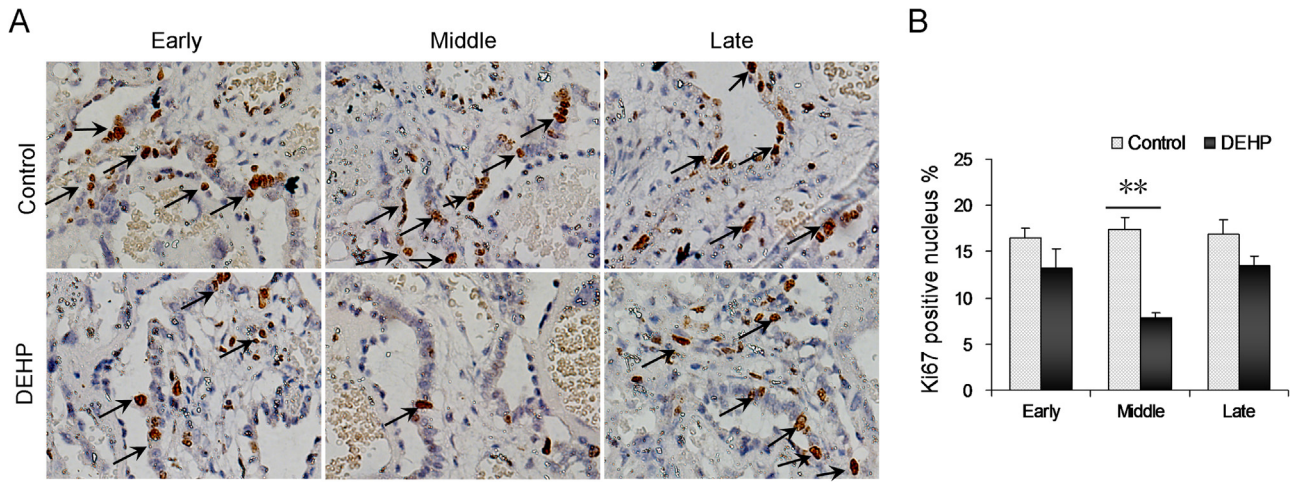

Fig. 6. Effects of maternal DEHP exposure at different gestational stages on cell proliferation. Pregnant mice except controls were administered with DEHP (200 mg/kg) by gavage daily at early (from GD0 to GD6) or middle (from GD7 to GD12) or late (from GD13 to GD17) gestational stages. All dams were sacrificed and placentas were collected on GD18. (A) Placenta section was immunohistochemically stained with Ki67. Sections were counterstained with hematoxylin. Arrows indicate Ki67-positive cells in labyrinthine region. Original magnification: 400 ×. (B) Rate of Ki67-positive cells in labyrinthine region was counted. All data were expressed as means ± S.E.M. (N = 11–14). $^{**}P < 0.01$.

sustaining the growth and development of fetuses during pregnancy [40,41]. Increasing evidence demonstrates that defects in placenta development and functional insufficiency result in fetal IUGR [42–45]. Several animal experiments indicated that some toxicants caused fetal IUGR through disturbing placental development [46,47]. A recent report showed that maternal DEHP exposure at early and middle gestational stages impaired placental development [48]. The present study investigated the effects of maternal DEHP exposure at different gestational stages on placental development. Our results showed that placenta weight was markedly reduced only in dams exposed to DEHP from GD7 to GD12. Correspondingly, the number of Ki67-positive cells in labyrinthine region, a marker of cell proliferation, was reduced in placentas of dams exposed to DEHP from GD7 to GD12. In addition, the internal space of maternal and fetal blood vessels in the labyrinth layer was reduced in placenta of dams with DEHP from GD7 to GD12. Indeed, the labyrinth layer is the site of oxygen and nutrient exchange between the mother and the fetus. Therefore, it is reasonable to assume that DEHP-induced IUGR may be at least partially attributed to defects in placental development and reduction in placental transport capacity.

The aim of the present study was to explore whether maternal DEHP exposure induced fetal IUGR in a gender-specific manner and to identify the critical time window of DEHP-induced fetal IUGR. However, the present study has several limitations. First, the present study had not investigated the effects of maternal exposure to low doses of DEHP on placental and fetal development. Actually, the doses used in the present study were 50 and 200 mg/kg which were much higher than those of human exposure. Second, the present study did not clarify the mechanism by which maternal DEHP exposure disturbed placental development. Third, the present study did not explore the effects of maternal DEHP exposure on placental function. Indeed, the present study showed that maternal DEHP exposure from GD13 to GD17 had little effect on placental development. These results could not explain why maternal DEHP exposure from GD7 to GD12 markedly reduced fetal weight and crown-rump length. Thus, additional research is required to explore the effects of maternal exposure to a low dose of DEHP on fetal development. In addition, further study is necessary to elucidate whether maternal DEHP exposure causes placental insufficiency.

In summary, the present study investigated the effects of maternal DEHP exposure at different gestational stages on fetal IUGR. Our results showed that fetal weight and crown-rump length were reduced 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.

Conflict of interest

The authors declared that they have no conflicts of interest to this work.

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