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Reproductive Toxicology

Timing and recovery of postweaning exposure to diethylstilbestrol on early pregnancy in CD-1 mice

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ABSTRACT

Exposure timing could play an important role in the effects of estrogenic endocrine disrupting chemicals (EEDCs) on early pregnancy. This study examined the sensitivity of different exposure periods from weaning to gestation day 4.5 (D4.5) to 50 ppb diethylstilbestrol (DES, a test EEDC) diet on embryo implantation and potential recovery upon temporary cessation of DES exposure in CD-1 mice. Peripubertal (3–5 weeks old) DES exposure reduced the numbers of corpora lutea and implantation sites. Postpubertal (5–7 weeks old) DES exposure did not have significant effects on early pregnancy. Postmating (D0.5–D4.5) DES exposure affected postovulation events leading to impaired embryo implantation. A 5-day premating rest from 5-week DES exposure (3–8 weeks old) resulted in recovery of early pregnancy rate. These data demonstrate that peripubertal and postmating periods are sensitive windows to endocrine disruption of early pregnancy and temporary cessation of exposure could partially alleviate adverse effects of DES on early pregnancy.

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1. Introduction

Estrogenic endocrine disrupting chemicals (EEDCs) are exogenous estrogenic chemicals that include man-made chemicals, such as plasticizers (e.g., bisphenol A (BPA)), pesticides (e.g., methoxychlor), and drugs (e.g., diethylstilbestrol (DES)), as well as naturally-occurring compounds, such as phytoestrogens (e.g., genistein) and mycotoxins (e.g., zearalenone (ZEA)) [1,2]. EEDCs can interfere with the endocrine system to potentially disrupt estrogen-regulated physiological processes, including puberty and pregnancy. Indeed, EEDCs have been associated with precocious puberty in girls and decreased fertility in women [3–5].

Toxicological studies in rodents have demonstrated that exposure to EEDCs can affect the age at vaginal opening, which is an indication of pubertal onset [6,7], as well as events that are critical for early pregnancy, such as ovulation, fertilization, embryo transport, preimplantation embryo development, establishment of uterine receptivity, and embryo implantation. Adverse effects on any of these events can lead to impaired embryo implantation. In utero exposure to DES decreased ovulation capability and caused structural abnormalities in the female reproductive tract, which contributed to DES-induced infertility in CD-1 mice [8]. Neonatal exposure to genistein (via subcutaneous (s.c.) injection) increased preimplantation embryo loss and decreased uterine receptivity leading to impaired embryo implantation in CD-1 mice [9–11]. Neonatal exposure to BPA or DES decreased the number of implantation sites in rats [12]. Postmating exposure to BPA (via s.c. injection) or ZEA (via diet) could also interfere with early pregnancy events leading to impaired embryo implantation in C57BL6 mice [7,13]. These studies demonstrate that besides dose, route, and duration, exposure timing is another important factor for potential effects of EEDCs on early pregnancy.

A common route of exposure to EEDCs in mammals is via the diet, especially direct dietary exposure starting from weaning. However, limited animal studies have been focused on postweaning dietary exposure to EEDCs on early pregnancy and no studies
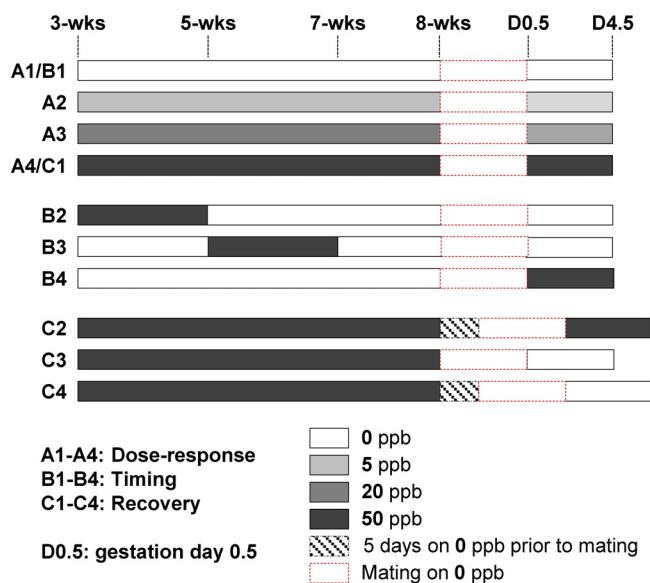
Fig. 1. Treatment regimen. Red dotted period, mating with untreated stud males on control vehicle diet; black shaded period, 5 days on control vehicle diet prior to mating; A1/B1, vehicle control group for set (A) (dose–response) and set (B) (timing); A4/C1, the highest dose group in set A and the group to be compared in set (C) (recovery). At least 10 mice were included in each group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

have examined the sensitivity of different postweaning periods to EEDC exposure on early pregnancy. In addition, it is unknown whether the animals could recover upon cessation of EEDC exposure. It was hypothesized that different exposure timing after weaning could modulate effects of EEDCs on early pregnancy and some effects could regress upon cessation of exposure. This study was designed to achieve two goals using DES as a test EEDC [14] in CD-1 mouse model: (1) to determine the sensitivity of different postweaning periods to DES on embryo implantation; and (2) to determine potential recovery from postweaning exposure to DES on embryo implantation. Embryo implantation was chosen as the end point because it is a collective point for all successful early pregnancy events.

2. Materials and methods

2.1. Animals

CD-1 mice (8 weeks old females and males) were purchased from Charles River Laboratories. They were mated 10 days after arrival at Coverdell Rodent Vivarium to produce offspring for this study. They were housed in polypropylene cages with free access to water (in polypropylene bottles) and regular rodent diet. The Coverdell Rodent Vivarium at the University of Georgia was maintained on a light/dark cycle 12 h/12 h (0600 h–1800 h) at $23 \pm 1\,^{\circ}$C with 30–50% relative humidity. All methods used in this study were approved by the Animal Subjects Programs of the University of Georgia and conform to National Institutes of Health guidelines and public law.

2.2. DES treatment

Diets containing 0, 5, 20, and 50 ppb DES were homemade using AIN-93G powder (Bio-Serv, Frenchtown, NJ) and DES (Sigma-Aldrich, USA) as previously described [7]. Newly-weaned littermate females (3 weeks old) were randomly assigned into A1/B1, A2, A3, A4/C1, B2, B3, B4, C2, C3, and C4 groups (Fig. 1). Set A was designed

to determine DES dose–response effect on embryo implantation: 0 (A1/B1), 5 (A2), 20 (A3) and 50 (A4/C1) ppb DES diets (Fig. 1). After 5 weeks of exposure, females in all groups were set up for mating at 8 weeks old with untreated CD-1 young stud males. There was no DES exposure during mating in all groups in this study to exclude any potential effect of DES on male fertility. Females were checked for a vaginal plug each morning. The morning with plug identification was designated as gestation day 0.5 (D0.5), and the plugged females resumed their premating DES diets (Fig. 1). Body weight was measured weekly from 3 to 8 weeks old. Set B was designed to determine exposure timing to 50 ppb DES diet on embryo implantation: B1 (vehicle control, same as A1), B2 (3–5 weeks old), B3 (5–7 weeks old), and B4 (D0.5 to D4.5) (Fig. 1). Set C was designed to determine recovery from 50 ppb DES exposure: C1 (same as A4, DES exposure: 3–8 weeks old + D0.5–D4.5), C2 (DES exposure: 3–8 weeks old + D0.5–D4.5, with a 5-day rest prior to mating), C3 (DES exposure: 3–8 weeks old), and C4 (DES exposure: 3–8 weeks old, with a 5-day rest prior to mating) (Fig. 1). Vaginal opening was monitored daily from weaning till it was detected except for 7 females each in A1/B1 group and A4/C1 group at the beginning of the study. All mice were dissected on D4.5 to determine implantation sites as previously described [7,15,16]. If no implantation site was observed, the uterine horns and oviducts were flushed with $1\times$ phosphate-buffered saline (PBS) to determine the presence of embryo(s), an indication of pregnancy. At least 10 females were included in each group.

2.3. Immunohistochemistry

Cross sections (10 μm) of representative D4.5 uteri from B1 and B4 groups were immunostained to detect progesterone receptor (PR) expression as previously described [6,7,17]. PR has distinct spatiotemporal expression patterns in the uterus during periimplantation [17]. Its expression pattern in the D4.5 uterus can reflect the implantation status. For example, if PR remains highly expressed in the D4.5 uterine luminal epithelium, it indicates that embryo implantation has not occurred yet in the uterus.

2.4. Ovary histology

One ovary per animal in the B1, B2, B3, B4, C1, and C2 groups was fixed in formalin, dehydrated, and embedded longitudinally in paraffin as previously described [6]. Sections were cut at 5 μm. Every 10th section was collected (about 20–40 sections per ovary) and stained with H & E. The numbers of follicles and corpora lutea were counted in 5 random sections from the center 1/3 of each ovary (e.g., sections No. 10–20 for an ovary with 30 10th sections). Three categories of follicles were counted: primordial and primary (types 1–3b), growing (types 4–5b), and antral (types 6–8) [18]. The average numbers of follicles at these three stages and corpora lutea from 5 sections were recorded for each ovary.

2.5. Statistical analyses

All the data were analyzed using SigmaPlot 12.0. Two-Way repeatedly measured ANOVA followed by Dunnett's test was used for postweaning body weight. One-Way ANOVA followed by SNK test or One-Way ANOVA on ranks (if data failed normality test or equal variance test) was used for the numbers of implantation sites, follicles, and corpora lutea. Fisher's exact test was used to analyze implantation rate and pregnancy rate. The significance level was set at $P < 0.05$.