Published in final edited form as:

Reprod Toxicol. 2014 November ; 49: 48–54. doi:10.1016/j.reprotox.2014.07.072.

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

Fei Zhao^{a,b}, Jun Zhou^{a,b}, Ahmed E. El Zowalaty^{a,b}, Rong Li^{a,b}, Elizabeth A. Dudley^{a,b}, and Xiaoqin Ye^{a,b,*}

^aDepartment of Physiology and Pharmacology, College of Veterinary Medicine; Athens, GA 30602, USA

^bInterdisciplinary Toxicology Program, University of Georgia, Athens, GA 30602, USA

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.

Keywords

Estrogenic endocrine disrupting chemical (EEDC); diethylstilbestrol (DES); peripubertal exposure; postmating exposure; corpus luteum; embryo transport; embryo implantation; early pregnancy

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.,

*Corresponding author: Xiaoqin Ye, M.D., Ph.D., 501 DW Brooks Dr., Department of Physiology and Pharmacology, College of Veterinary Medicine; Interdisciplinary Toxicology Program, University of Georgia, Athens, GA 30602, USA (Tel.: 1-706-542-6745; fax: 1-706-542-3015; ye@uga.edu).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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 early pregnancy events, which include ovulation, fertilization, embryo transport, preimplantation embryo development, establishment of uterine receptivity, and embryo implantation. Adverse effects on any of these early pregnancy 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 have examined the sensitivity of different postweaning periods to EEDC exposure on early pregnancy. In addition, it is unknown whether the animals 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.

Materials and Methods

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 12h/12h (0600 h~1800 h) at $23 \pm 1^\circ\text{C}$ 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.

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 previous described [7, 15, 16]. If no implantation site was observed, the uterine horns and oviducts were flushed with 1× phosphate-buffered saline (PBS) to determine the presence of embryo(s), an indication of pregnancy. At least 10 females were included in each group.

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 this uterus.

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.

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 $p < 0.05$.

Results

Dose-response

Newly-weaned 3 weeks old CD-1 females were treated with 0, 5, 20 and 50 ppb DES diets. Significant lower body weights were observed in 20 ppb DES-treated group from 6 to 8 weeks old and 50 DES-treated group at 8 weeks old. No significant difference in body weight was observed in 5 ppb DES-treated group (Fig. 2A).

Age at vaginal opening showed a DES dose-dependent decrease. Since vaginal opening occurred before 5 weeks old, groups with the same treatment between weaning (3 weeks old) and 5 weeks old were combined: A1/B1, B3 and B4 on 0 ppb DES diet; A4/C1, B2, C2, C3, and C4 on 50 ppb DES diet (Fig. 1) excluding 7 mice each in A1/B1 and A4/C groups that were not examined for vaginal opening. All mice in 50 and 20 ppb DES groups had vaginal opening within 4 days of treatment, while those in 5 and 0 ppb DES groups were within 6 and 10 days of treatment, respectively (Fig. 2B). The average ages at vaginal opening were: 25.6 ± 2.8 days old ($N=24$) for 0 ppb; 24.0 ± 1.5 days old ($N=12$) for 5 ppb, 23.3 ± 0.8 days old ($N=12$) for 20 ppb, and 22.6 ± 0.9 days old ($N=44$) for 50 ppb DES-treated females, indicating a dose-dependent decrease of age at vaginal opening, although significant difference was only observed in 50 ppb DES-treated females (Fig. 2C).

DES dose-dependent effects were also seen in early pregnancy rate (% of plugged females with embryos and/or implantation sites) and embryo implantation rate (% of plugged females with implantation sites) detected on D4.5 (Fig. 3A). Significantly decreased pregnancy rate and implantation rate were observed in both A3 (20 ppb) and A4 (50 ppb) groups compared with A1 (vehicle control) group (Fig. 3A). In A4 group, the pregnancy rate (20%) was higher than the implantation rate (0%), although no significant difference was detected due to small sample size; while in A1–A3 groups, the pregnancy rate and implantation rate were the same (Fig. 3A). There was a DES dose-dependent trend of reduced number of implantation sites ($P=0.067$) (Fig. 3B) and no implantation site was detected in any of the females in the A4 group (Fig. 3B). None of the uteri in A1 and A2 (5 ppb) groups had a distended appearance (Fig. 3C); 9 out of 12 females in A3 group had uteri similar to those in A1 and A2 groups (Fig. 3C), and the remaining 3 females exhibited distension, in which two were less severe and the most severe one had a similar appearance as the uterus shown in the 50 ppb DES A4 group (Fig. 3C); and all uteri in A4 group had distended appearance (Fig. 3C), which is an indication of estrogenic effect.

Exposure timing on embryo implantation

To determine exposure timing on embryo implantation, three different periods were examined: 3–5 weeks old (B2, peripubertal), 5–7 weeks old (B3, postpubertal), and D0.5 to D4.5 (B4, postmating) (Fig. 1). Although exposure to 50 ppb DES during these periods did not significantly affect pregnancy rate, the pregnancy rate in B4 postmating group was 76.9%, which compared to the B1 vehicle control group pregnancy rate of 100% suggested that there might be some adverse effect (Fig. 4A). Indeed, the implantation rate was significantly decreased in this B4 postmating group (Fig. 4A). Peripubertal two weeks of exposure (B2 group) significantly reduced the number of implantation sites compared with B1 vehicle control group (Fig. 4B). B4 postmating group had the lowest number of implantation sites, which was significantly lower than the other three groups (Fig. 4B). B3 postpubertal group had the number of implantation sites in between those in B1 vehicle control and B2 peripubertal groups but didn't differ significantly from them (Fig. 4B).

Ovary histology from D4.5 females revealed comparable numbers of primordial and primary, growing, and antral follicles among the B1–B4 groups. However, significantly reduced numbers of corpora lutea were observed in B2 group compared with B1 and B4 groups (Figs. 4C, 4D), although both B2 and B4 groups had reduced numbers of implantation sites on D4.5 (Fig. 4B). No significant difference was observed for B3 group compared with the remaining three groups (Fig. 4B). The relative numbers of corpora lutea correlated with the relative numbers of implantation sites among B1, B2, and B3 groups ($R^2=0.9882$) but not that in B4 group (Figs. 4B, 4C, 4D).

All pregnant females, with or without implantation sites, in B4 group showed distended uterine horns (Fig. 4E). Retained expression of progesterone receptor in the uterine luminal epithelium of pregnant B4 uterus without implantation sites also indicated that embryo implantation had not occurred yet (Fig. 4F) [17]. Among the 7 females without implantation sites in B4 group, 3 females did not have embryos flushed from the reproductive tract, indicating they were not pregnant; the remaining 4 females had 2, 4, 4 and 7 embryos flushed from their reproductive tracts, respectively. The average number of embryos (4.3 ± 2.1 , $P=0.012$) flushed from the entire reproductive tract of these 4 females and the average number of implantation sites (2.5 ± 2.1 , $P<0.001$) in both uterine horns of the 6 females with implantation site(s) in B4 group were significantly lower than the average number of corpora lutea per section in one ovary in B4 group (8.8 ± 3.4 corpora lutea/ovary). In B1–B3 groups, however, the numbers of implantation sites in both uterine horns were higher than the numbers of corpora lutea per section in one ovary (Figs. 4B, 4D). These observations indicate impaired postovulation process(es) in the B4 group. Among the 4 pregnant females without implantation sites in B4 group, 2 females had 2 and 4 blastocysts, respectively, flushed from the uterus (Fig. 4G) but not oviduct; one female had 3 blastocysts in the uterus and 1 morula in the oviduct; and the fourth one had 2 blastocysts in the uterus and 5 blastocysts in the oviduct (Fig. 4H). These results indicate delayed embryo transport, potentially delayed embryo development, and impaired uterine receptivity in these pregnant females.

Partial recovery from a 5-day pre mating break

Our previous study on ZEA-treated C57BL/6J females implied potential recovery in females with delayed mating activity: females plugged within 3 days of cohabitation with stud males without treatment had an implantation rate significantly lower than that in the control, while those plugged after 5 days of cohabitation had a comparable implantation rate with the control [7]. In this set of experiments, all mice in the C1, C2, C3, and C4 groups were exposed to 50 ppb DES from weaning to 8 weeks old. The differences among these four groups began after 8 weeks old. To determine potential recovery from DES treatment, a 5-day cessation of DES exposure was introduced prior to mating in C2 (pre mating + 5-day rest + post mating) and C4 (pre mating + 5-day rest) groups, while C1 (pre mating + post mating) and C3 (pre mating) groups were mated immediately after DES exposure from week 3 to week 8 (Fig. 1). Therefore, the only difference in the exposure regimens between C1 and C2 groups or C3 and C4 groups was the 5-day break from DES exposure prior to mating (Fig. 1).

Although no significant difference in the implantation rate between C1 (0/10=0%) and C2 (2/11=18.2%) groups was detected, the pregnancy rate was significantly increased in C2 group (10/11=90.9%, $P=0.002$) compared with C1 group (2/10=20%) (Fig. 5A). The two pregnant females in C1 group had 2 and 3 blastocysts in the uterus, respectively. The two pregnant females with implantation sites in C2 group had 2 and 11 implantation sites, respectively (Fig. 5B); among the remaining 8 pregnant females without implantation sites in C2 group, 3 had embryos recovered in the uterus only, 4 had embryos recovered in the oviduct only, and the 8th one had embryos flushed from both uterus and oviduct. Among the 45 embryos flushed from the reproductive tracts of these pregnant females, 40 were hatched blastocysts, 3 were unhatched blastocyst, 1 was hatched morula, and 1 was unhatched morula. C1 and C2 groups had comparable numbers of follicles and corpora lutea (Figs. 5C, 5D). The numbers of corpora lutea in both C1 and C2 groups were significantly lower than that in the B1 vehicle control group but comparable with that in the B2 group (Fig. 4D).

C3 and C4 groups did not have DES treatment after 8 weeks old (Fig. 1). Although C4 group, which had a 5-day break between DES treatment and mating (Fig. 1), appeared to have higher pregnancy rate and implantation rate (Fig. 5A) and more implantation sites (Fig. 5B) than those in C3 group, there was no significant difference in all three parameters between these two groups. However, when compared with A1/B1 vehicle control group, C3 group had significantly decreased pregnancy rate (8/13 vs. 15/15, $P=0.013$), implantation rate (8/13 vs. 15/15, $P=0.013$), and number of implantation sites (10.1 ± 2.9 vs. 13.3 ± 1.5 , $P=0.009$) on D4.5, whereas C4 group only had significantly reduced number of implantation sites (11.9 ± 2.1 vs. 13.3 ± 1.5 , $P=0.04$) on D4.5 (Figs. 1, 4A, 4B, 5A, 5B). These data suggest less severe adverse effects in C4 group that had a 5-day pre mating rest from DES exposure. Meanwhile, the results also suggest that mice with the 5-day pre mating rest could not fully recover from 50 ppb DES exposure during week 3 and week 8.

The difference in the exposure regimens between C1 and C3 groups or C2 and C4 groups was the 4-day post mating exposure to DES (Fig. 1). The pregnancy rate was marginally higher in C3 group (8/13=61.5%, $P=0.09$) compared with C1 group (2/10=20%). The pregnancy rates between C2 and C4 groups were comparable (Fig. 5A). However, the

implantation rates were significantly higher in both C3 and C4 groups compared with C1 and C2 groups, respectively (Fig. 5A), consistently demonstrating the sensitivity of D0.5–D4.5 postmating period to endocrine disruption.

Discussion

Estrogen signaling plays important roles in pubertal development [19] and early pregnancy [20]. It is not surprising that EEDCs could interfere with these processes. Mammals begin direct dietary exposure to EEDCs from weaning, yet very limited studies have been focused on the sensitivity of mammals during the postweaning period, which encompasses peripubertal development, to EEDC exposure on early pregnancy. This study focuses on postweaning dietary exposure to DES (as a test EEDC) to provide guidance on sensitive periods to EEDC exposure and potential recovery from EEDC exposure on early pregnancy with embryo implantation as the endpoint.

The adverse effect from 50 ppb DES exposure on embryo implantation was most severe from postmating (D0.5–D4.5) exposure, less severe from peripubertal (3–5 weeks old) exposure, and least severe (no significant adverse effects) during postpubertal (5–7 weeks old) exposure. It might be expected that peripubertal exposure has less adverse effect than postpubertal exposure because the former has longer DES elimination time before mating. However, peripubertal exposure but not postpubertal exposure significantly reduced the number of implantation sites. Therefore, the data clearly indicate that peripubertal was more sensitive to endocrine disruption with DES. Peripubertal DES exposure also significantly reduced the age at vaginal opening, supporting peripubertal sensitivity to endocrine disruption.

Although postmating exposure had more severe adverse effects than peripubertal exposure on embryo implantation, it was hard to directly compare the sensitivity to endocrine disruption between these two exposure periods for the following two reasons: 1) there was over three weeks of break from DES exposure between peripubertal exposure and embryo implantation, while postmating exposure was immediately prior to embryo implantation; and 2) only the treated females were at risk during peripubertal exposure, while both the treated females and the embryos were at risk during postmating exposure. Indeed, the mechanisms for the adverse effects of peripubertal DES exposure and postmating exposure on embryo implantation were different.

Peripubertal DES exposure significantly reduced the number of corpora lutea without significantly reducing the number of follicles, indicating inhibition of ovulation, an effect that has also been reported in rats that were prepubertally treated with EEDCs polychlorinated dibenzo-p-dioxins [21]. The reduction of corpora lutea correlated with the reduction of implantation sites, indicating that peripubertal DES exposure affected the number of implantation sites via its effect on ovulation. The formation of corpora lutea depends on preovulatory surge of luteinizing hormone (LH) [22]. It has been reported that neonatal exposure to DES could reduce LH producing cells and LH secretion in mice [23, 24]. It is possible that peripubertal DES exposure in this study could also affect LH levels leading to reduced ovulation.

However, postmating DES exposure did not affect ovarian function, including follicle development and ovulation, indicated by comparable numbers of follicles and corpora lutea between DES-treated group (B4) and the vehicle control (B1). Instead, postmating DES exposure interfered with fertilization, indicated by the reduced number of embryos either as hatched but un-implanted blastocysts or implanted embryos in the reproductive tract; embryo transport, indicated by the retention of blastocysts in the oviduct on D4.5; potentially preimplantation embryo development, indicated by the occasional presence of morula on D4.5; and embryo implantation (including uterine receptivity), indicated by the presence of hatched and healthy-looking but un-implanted blastocysts in the pregnant females without implantation sites. These adverse effects on early pregnancy were also seen in mice treated during postmating with high levels of other EEDCs, such as BPA [13] and ZEA [7], suggesting that these adverse effects on early pregnancy might be common for EEDCs. Regardless of whether or not there was DES exposure during premating, and whether or not there was a 5-day premating break from DES exposure, postmating exposure aggravated the adverse effects of DES on early pregnancy (B4 vs. B1; C3 vs. C1; C4 vs. C2) (Figs. 4, 5; Table 1).

The significant reduction of numbers of corpora lutea and implantation sites indicated persistent adverse effects of peripubertal DES exposure, while our previous study on EEDC ZEA suggested that there was potential recovery when the untreated mating period was prolonged in mice previously treated with ZEA [7]. Indeed, when a 5-day premating break from DES treatment was introduced prior to mating in C2 group or C4 group in this study, there was a significant increase in the pregnancy rate in C2 group although the remaining parameters showed recovery but didn't reach significant difference (C2 vs. C1), or the adverse effects from weeks 3–8 DES treatment was general less severe in C4 group (significantly reduced number of implantation sites) than in C3 group (significantly reduced pregnancy rate, implantation rate, and number of implantation sites) when they were compared with B1 vehicle group (Figs. 4, 5; Table 1). About 90% of a single dose of DES can be quickly metabolized and excreted within 24 hours in mice [25], thus a 5-day premating break from DES exposure in C2 and C4 groups could provide sufficient time to eliminate the majority of DES from the body. Therefore, factors leading to embryo implantation, such as oocyte quality for fertilization as well as reproductive tract environment for fertilization, preimplantation embryo development, embryo transport, and uterine receptivity, could have the potential to recover from premating DES insult.

This study provides three important messages: 1) peripuberty is a sensitive period for endocrine disruption with DES and there could be persistent adverse effects; 2) early pregnancy is an extremely sensitive period for endocrine disruption with DES, which could potentially interfere with multiple early pregnancy events; and 3) avoiding significant exposure to EEDCs before getting pregnant could be beneficial to the outcome of the pregnancy.

Acknowledgements

The authors thank the Department of Pathology in the College of Veterinary Medicine, University of Georgia for an access to the imaging system; and the financial support from Interdisciplinary Toxicology Program, and Department of Physiology and Pharmacology at University of Georgia, and National Institutes of Health.

Funding Information

This work was supported by the National Institutes of Health [R15HD066301 and R01HD065939 to X.Y.]

References

1. Marques-Pinto A, Carvalho D. Human infertility: are endocrine disruptors to blame? *Endocrine connections*. 2013; 2:R15–R29. [PubMed: 23985363]
2. Zinedine A, Soriano JM, Molto JC, Manes J. Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: an oestrogenic mycotoxin. *Food Chem Toxicol*. 2007; 45:1–18. [PubMed: 17045381]
3. Janssen S, Fujimoto VY, Giudice LC. Endocrine Disruption and Reproductive Outcomes in Women. *Contemp Endocrinol S*. 2007:203–223.
4. Adgent MA, Daniels JL, Rogan WJ, Adair L, Edwards LJ, Westreich D, et al. Early-life soy exposure and age at menarche. *Paediatr Perinat Epidemiol*. 2012; 26:163–175. [PubMed: 22324503]
5. Ehrlich S, Williams PL, Missmer SA, Flaws JA, Berry KF, Calafat AM, et al. Urinary bisphenol A concentrations and implantation failure among women undergoing in vitro fertilization. *Environ Health Perspect*. 2012; 120:978–983. [PubMed: 22484414]
6. Li R, Zhao F, Diao H, Xiao S, Ye X. Postweaning dietary genistein exposure advances puberty without significantly affecting early pregnancy in C57BL/6J female mice. *Reprod Toxicol*. 2014; 44:85–92. [PubMed: 24365114]
7. Zhao F, Li R, Xiao S, Diao H, Viveiros MM, Song X, et al. Postweaning exposure to dietary zearalenone, a mycotoxin, promotes premature onset of puberty and disrupts early pregnancy events in female mice. *Toxicol Sci*. 2013; 132:431–442. [PubMed: 23291560]
8. McLachlan JA, Newbold RR, Shah HC, Hogan MD, Dixon RL. Reduced fertility in female mice exposed transplacentally to diethylstilbestrol (DES). *Fertil Steril*. 1982; 38:364–371. [PubMed: 7117561]
9. Jefferson WN, Padilla-Banks E, Phelps JY, Gerrish KE, Williams CJ. Permanent oviduct posteriorization after neonatal exposure to the phytoestrogen genistein. *Environ Health Perspect*. 2011; 119:1575–1582. [PubMed: 21810550]
10. Jefferson WN, Padilla-Banks E, Phelps JY, Cantor AM, Williams CJ. Neonatal phytoestrogen exposure alters oviduct mucosal immune response to pregnancy and affects preimplantation embryo development in the mouse. *Biol Reprod*. 2012; 87:10. 1-. [PubMed: 22553218]
11. Jefferson WN, Padilla-Banks E, Goulding EH, Lao SP, Newbold RR, Williams CJ. Neonatal exposure to genistein disrupts ability of female mouse reproductive tract to support preimplantation embryo development and implantation. *Biol Reprod*. 2009; 80:425–431. [PubMed: 19005167]
12. Varayoud J, Ramos JG, Bosquiaz VL, Lower M, Munoz-de-Toro M, Luque EH. Neonatal exposure to bisphenol A alters rat uterine implantation-associated gene expression and reduces the number of implantation sites. *Endocrinology*. 2011; 152:1101–1111. [PubMed: 21285323]
13. Xiao S, Diao H, Smith MA, Song X, Ye X. Preimplantation exposure to bisphenol A (BPA) affects embryo transport, preimplantation embryo development, and uterine receptivity in mice. *Reprod Toxicol*. 2011; 32:434–441. [PubMed: 21907787]
14. Haddad R, Kasneci A, Mephram K, Sebag IA, Chalifour LE. Gestational exposure to diethylstilbestrol alters cardiac structure/function, protein expression and DNA methylation in adult male mice progeny. *Toxicol Appl Pharmacol*. 2013; 266:27–37. [PubMed: 23142472]
15. Diao H, Xiao S, Howerth EW, Zhao F, Li R, Ard MB, et al. Broad gap junction blocker carbenoxolone disrupts uterine preparation for embryo implantation in mice. *Biol Reprod*. 2013; 89:31. [PubMed: 23843229]
16. Ye X, Hama K, Contos JJ, Anliker B, Inoue A, Skinner MK, et al. LPA3-mediated lysophosphatidic acid signalling in embryo implantation and spacing. *Nature*. 2005; 435:104–108. [PubMed: 15875025]

17. Diao H, Paria BC, Xiao S, Ye X. Temporal expression pattern of progesterone receptor in the uterine luminal epithelium suggests its requirement during early events of implantation. *Fertil Steril*. 2011; 95:2087–2093. [PubMed: 21371703]
18. Pedersen T, Peters H. Proposal for a classification of oocytes and follicles in the mouse ovary. *J Reprod Fertil*. 1968; 17:555–557. [PubMed: 5715685]
19. Kugelberg E. Reproductive endocrinology: ESR1 mutation causes estrogen resistance and puberty delay in women. *Nat Rev Endocrinol*. 2013; 9:565. [PubMed: 23897169]
20. Wang H, Dey SK. Roadmap to embryo implantation: clues from mouse models. *Nat Rev Genet*. 2006; 7:185–199. [PubMed: 16485018]
21. Gao X, Son DS, Terranova PF, Rozman KK. Toxic equivalency factors of polychlorinated dibenzo-p-dioxins in an ovulation model: validation of the toxic equivalency concept for one aspect of endocrine disruption. *Toxicol Appl Pharmacol*. 1999; 157:107–116. [PubMed: 10366543]
22. Stocco C, Telleria C, Gibori G. The molecular control of corpus luteum formation, function, and regression. *Endocrine reviews*. 2007; 28:117–149. [PubMed: 17077191]
23. Ishikawa M, Murai E, Hashiguchi Y, Iguchi T, Sato T. Effects of diethylstilbestrol on luteinizing hormone-producing cells in the mouse anterior pituitary. *Experimental biology and medicine*. 2014; 239:311–319. [PubMed: 24521563]
24. Halling A. Alterations in hypothalamic and pituitary hormone levels induced by neonatal treatment of female mice with diethylstilbestrol. *Reprod Toxicol*. 1992; 6:335–346. [PubMed: 1521007]
25. Metzler M. The metabolism of diethylstilbestrol. *CRC critical reviews in biochemistry*. 1981; 10:171–212. [PubMed: 6163591]

Research highlights

1. Accelerated pubertal onset by postweaning dietary DES exposure.
 2. Dose-dependent adverse effect of DES on embryo implantation.
 3. Most severe adverse effects upon postmating DES exposure.
 4. Sensitive peripubertal period for endocrine disruption with DES.
- Partial recovery upon DES exposure cessation.

Figure 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.

Figure 2.

Postweaning DES exposure on body weight and vaginal opening. A. Body weight during treatment from 3-week to 8-week old. N=12–27. * $P < 0.05$, 20 ppb group compared with 0 ppb control group; # $P < 0.05$, 50 ppb group compared with 0 ppb control group. B. Cumulative percentages of mice with vaginal opening during DES treatment. N=12–44. C. Average age at vaginal opening upon different doses of DES treatment. N=12–44; error bar, standard deviation; * $P < 0.05$ compared with 0 ppb DES control group.

Figure 3.

Dose-response effects of DES exposure on early pregnancy. A. Pregnancy rate and implantation rate. N=10–15; * $P < 0.05$ compared with A1 control group. B. Average number of implantation sites on D4.5. Only mice with implantation sites were included. N=5–15 in A1–A3 groups; N=0 in A4 group due to 0% implantation rate (A); error bar, standard deviation. C. Representative uterine images. Red arrows, implantation sites.

Figure 4.

DES exposure timing on early pregnancy. B1, vehicle control; B2, peripubertal exposure, 3–5 weeks old; B3, postpubertal exposure, 5–7 weeks old; and B4, postmating exposure, D0.5–D4.5. A. Pregnancy rate and implantation rate. N=12–15; [#] P<0.05 compared with B1, B2, and B3 groups. B. Average numbers of implantation sites. N=6–15. ^{*} P<0.05 compared with B1 control group; [#] P<0.05 compared with B1, B2, and B3 groups. C. Ovary histology. H & E staining; blue #, corpus luteum; scale bar, 500 μ m. D. Average numbers of follicles and corpora lutea per section. Pri, primordial and primary follicles; Gro, growing

follicles; Ant, antral follicles; CL, corpora lutea; * $P < 0.05$ compared with B1 control group; error bar, standard deviation; N=10–11. E. Uterine images of pregnant females in B4 group with implantation sites and without implantation site. Red arrows, implantation sites. F. Immunohistochemistry detection of progesterone receptor (brown staining) in the D4.5 pregnant uterus from B1 (with implantation site) and B4 (without implantation) groups. Red star, embryo; Dec, decidual zone; LE, uterine luminal epithelium; Str, stroma; scale bar, 100 μm . G & H. Representative embryos flushed from the oviduct (G) or the uterus (H) of pregnant females in B4 group without implantation sites.

Figure 5.

Effect of a pre-mating 5-day non-exposure on recovery from DES exposure. C1, weeks 3–8 + postmating DES exposure; C2, C1 + 5-day pre-mating break from DES exposure; C3, weeks 3–8 DES exposure; C4, C3 + 5-day pre-mating break from DES exposure. A. Pregnancy rate and implantation rate. N=10–13. * $P < 0.05$ compared with corresponding parameter C1 group; # $P < 0.05$ compared with C2 group. B. Average numbers of implantation sites. N=0 in C1 group, N=2 in C2 group, N=8 in C3 group and N=10 in C4 group. C. Ovary histology in C1 and C2 groups. H & E staining; # corpus luteum; scale bar, 500 μ m. D. Average numbers

of follicles and corpora lutea per section in C1 and C2 groups. N=8–9; error bar, standard deviation.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1

Summary of statistical significance among B and C groups.

		Pregnancy rate	Implantation rate	Number of implantation sites
B1	B2	-	-	+
	B3	-	-	-
	B4	-	+	+
	C1	+	+	**
C1	C2	-	+	**
	C3	+	+	+
	C4	-	-	+
	C2	+	-	**
C2	C3	* -	+	**
	C4	-	+	**
C3	C4	-	-	-

* P=0.09; +, P<0.05; -, P>0.05;

** C1 had 100% implantation failure (N=0) and C2 had only two females (N=2) with implantation sites thus preventing statistical analysis on the number of implantation sites; B1, vehicle control; B2, peripubertal (3–5 weeks old) DES exposure; B3, postpubertal (5–7 weeks old) DES exposure; B4, postmating (D0.5–D4.5) DES exposure; C1, weeks 3–8 + postmating DES exposure; C2, C1 + 5-day prematuring break from DES exposure; C3, weeks 3–8 DES exposure; C4, C3 + 5-day prematuring break from DES exposure (Fig. 1).