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Preimplantation exposure to bisphenol A (BPA) affects embryo transport, preimplantation embryo development, and uterine receptivity in mice

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ABSTRACT

To investigate the effects of bisphenol A (BPA) on embryo and uterine factors in embryo implantation, timed pregnant C57BL6 females were treated subcutaneously with 0, 0.025, 0.5, 10, 40, and 100 mg/kg/day BPA from gestation days 0.5–3.5. In 100 mg/kg/day BPA-treated females, no implantation sites were detected on day 4.5 but retention of embryos in the oviduct and delayed embryo development were detected on day 3.5. When untreated healthy embryos were transferred to pseudopregnant females treated with 100 mg/kg/day BPA, no implantation sites were detected on day 4.5. In 40 mg/kg/day BPA-treated females, delayed implantation and increased perinatal lethality of their offspring were observed. Implantation seemed normal in the rest BPA-treated groups or the female offspring from 40 mg/kg/day BPA-treated group. These data demonstrate the adverse effects of high doses of BPA on processes critical for embryo implantation: embryo transport, preimplantation embryo development, and establishment of uterine receptivity.

1. Introduction

Bisphenol A (BPA) is an organic compound with two phenol functional groups. It has been widely used as a monomer in manufacturing polycarbonate plastics and epoxy resins. BPA can leach from products made with these materials, such as food/liquid containers and medical devices. The general human population can be exposed to BPA mainly via ingestion, inhalation and skin contact at micrograms per kilogram of body weight daily [1–3]. BPA is detectable in the urine (0.4–149 μg/L) and serum (2.84 μg/L) of the general human population [4–6], as well as in amniotic fluid, placental tissue, and breast milk [7]. Studies on human populations have correlated higher BPA exposure with disorders such as cardiovascular diseases, diabetes, liver dysfunction, and male sexual dysfunction [1,2,8,9]. Laboratory studies on animals have demonstrated multiple adverse effects of BPA, such as on development, behavior, reproduction, the immune system, and occurrence of cancer [7,10–13]. However, it may also be argued that low doses of BPA could have adverse effects on human reproductive and developmental health [14,15].

BPA is classified as an endocrine disruptor with weak estrogenicity [3]. Its estrogenic potency was estimated to be 10,000-fold less than that of 17β-estradiol (E2) [16,17], which may reflect the affinity of BPA for the classical nuclear estrogen receptors (ERs) [18–20]. However, numerous studies demonstrate that BPA at concentrations that are too low to efficiently activate nuclear ERs also has cellular effects [15]. One mechanism postulated for the low-dose effects of BPA is a nongenomic response, e.g., BPA binding to membrane ERs other than nuclear ERs [3,21]. Non-classical nuclear receptors such as estrogen-related receptor gamma (ERRγ) may also be involved in the estrogenic effects of BPA [22]. Epigenetic mechanisms, such as DNA methylation of ER target genes, have also been postulated [23].

The reproductive system is a main target of endocrine disruptors. Extensive laboratory studies have revealed multiple adverse effects of BPA on the reproductive system. In the male reproductive system, effects of BPA include decreased sperm motility, impaired spermatogenesis, and decreased fertility of male offspring [24–26]. In the female reproductive system, BPA may target the mammary gland, the ovary, the oviduct, the uterus, and the placenta [22,27–36]. A recent study demonstrates that CD-1 mice exposed to environmentally relevant BPA levels (subcutaneously via osmotic pumps, 0.025, 0.25, and 25 μg/kg) during the perinatal period (gestation day 8–postnatal day 16) show decreased

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reproductive capacity, although the causes of such a decrease have not been determined [37]. Various BPA-induced effects in the uterus have been reported, such as increased uterine wet weight and luminal epithelium height, uterine cell proliferation, and induced expression of genes such as lactoferrin and *c-fos* [38–41]. In utero BPA exposure (5 mg/kg intraperitoneal injection) can alter DNA methylation of the Hox10 gene [23], which has been implicated in uterine development and decidualization [42].

One important function of the uterus is to accept an embryo for implantation. Embryo implantation is a hormonally controlled process involving synchronized readiness of an embryo and a receptive uterus [42,43]. It was reported that BPA exposure (10.125 mg/mouse/day, ~400 mg/kg/day) during gestation days 1.5–4.5 (it was expressed as day 1–day 4 in this referred study when the day that a vaginal plug was detected was defined as day 0) led to fewer implantation sites [44]. However, it is not known whether the fewer number of implantation sites is due to any adverse effects of BPA on the embryos and/or the uterus. The objective of this study was to examine the effects of preimplantation BPA exposure on embryonic and uterine factors critical for embryo implantation in mice.

2. Materials and methods

2.1. Animal husbandry

C57BL6 mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). The mice were housed in polypropylene cages with free access to food (rodent diet 5053, Purina Mills LabDiet) and water on a 12 h light/dark cycle (6:00 AM–6:00 PM) at 23 ± 1 °C with 30–50 relative humidity. All methods used in this study were approved by the University of Georgia IACUC Committee (Institutional Animal Care and Use Committee) and conform to National Institutes of Health guidelines and public law.

2.2. Animal treatment and detection of implantation sites

Young virgin females (2–3 months old) were mated naturally with untreated young stud males. The animals were checked each morning and when a vaginal plug was seen, that day was designated as gestation day 0.5. The plugged females were randomly distributed into seven treatment groups with five to fourteen females in each group. A subcutaneous (s.c.) exposure was used in this study in order to do comparisons with two other studies on BPA in embryo implantation, which were either to test the estrogenicity of BPA using a delayed implantation model [17] or to determine the consequence of peri-implantation BPA exposure on embryo implantation [44]. The plugged females were s.c. injected daily (between 9:00 AM and 10:00 AM) with 0, 0.025, 0.5, 10, 40, and 100 mg/kg/day (~0, 0.000625, 0.0125, 0.25, 1, 2.5 mg/mouse/day, respectively) of BPA (Sigma–Aldrich, St. Louis, MO, USA); or with 0.01 mg/kg/day E2 (Sigma–Aldrich) in 100 μl sesame oil (Sigma–Aldrich) from gestation days 0.5–3.5. The estrogenicity of 0.01 mg/kg/day E2 was assumed to be equivalent to 100 mg/kg/day of BPA based on the estimation that the estrogenic potency of BPA was ~10,000-fold less than that of E2 [16,17]. Implantation normally initiates at about gestation day 4.0 in mice when the mating night is defined as gestation day 0. At gestation day 4.5 or day 5.5, the mice were anesthetized with isoflurane (Webster Veterinary, Devens, MA, USA) by inhalation and intravenously (i.v.) injected with Evans blue dye (Alfa Aesar, Ward Hill, MA, USA) to visualize the implantation sites as previously described [45]. The number and position of implantation sites were recorded and analyzed. If no implantation sites were detected on day 4.5, the uterine horns were flushed with 1× PBS to determine the presence of embryos and thus the status of pregnancy. Uterine tissues were snap frozen and kept at −80 °C for immunohistochemistry.

2.3. Embryo transport and development

Pregnant mice were treated with 0 and 100 mg/kg/day BPA from gestation days 0.5–3.5 as described above. Uteri and oviducts were flushed with PBS to detect the presence of embryos and the stages of embryo development.

2.4. Embryo transfer

Young virgin females (2–3 months old) were superovulated with intraperitoneal (i.p.) injections of 5 IU equine chorionic gonadotropin (Sigma–Aldrich) and 48 h later with 5 IU human chorionic gonadotropin (Sigma–Aldrich). They were subsequently mated with stud males. Meanwhile, pseudopregnant females were prepared by mating with vasectomized males. The following day was designated as gestation day 0.5 when a vaginal plug was identified. The pseudopregnant females were s.c. injected daily with 0 or 100 mg/kg/day of BPA in 100 μl sesame oil between 9:00 AM and 10:00 AM from gestation days 0.5–3.5. At gestation day 3.5 between 12:00 PM and 1:00 PM, blastocysts were harvested from superovulated females and transferred to the uteri of day 3.5 pseudopregnant females. Resultant implantation sites were detected using blue dye injection at day 4.5. If no implantation sites were detected at day 4.5, the uterine horns were flushed with 1× PBS to determine the presence of transferred blastocysts. Since treatment with 100 mg/kg/day of BPA adversely affected preimplantation embryo development and embryo transport, the reverse embryo transfer (BPA-treated gestation day 3.5 embryos transferred to the uteri of untreated gestation day 3.5 pseudopregnant females) study was not performed.

2.5. Gestation period, litter size, postnatal survival rate, gender ratio, postnatal growth, and embryo implantation in the offspring females

To determine the consequences of delayed implantation in 40 mg/kg/day BPA-treated females, plugged females were treated with 0 or 40 mg/kg/day BPA as described above from gestation days 0.5–3.5. The date of birth was recorded to determine gestation period. At birth (postnatal day 1), the number of pups from each female was counted to determine the litter size. The body weight of each pup was recorded each week until 9 weeks old. The gender ratios were determined on postnatal day 21 (weaning time). The offspring females (8–12 weeks old) were also mated and examined for embryo implantation as previously described [45].

2.6. Immunohistochemistry

To determine the presence and location of progesterone receptor (PR), frozen uterine sections (10 μm) were fixed in 4% paraformaldehyde (EMD Millipore, Darmstadt, Germany) in PBS for 10 min at room temperature, washed in PBS, and subjected to antigen retrieval in 0.01 M sodium citrate buffer, pH 6.0, for 20 min. Endogenous peroxidase was inactivated with 3% H_2O_2 (Fisher Scientific Co., Fairlawn, NJ, USA). Non-specific staining was blocked using 10% goat serum. Sections were then incubated with primary rabbit-anti-progesterone receptor (PR) antibody (1:200, Dako, Denmark) at 4 °C for overnight; washed in PBS and incubated with biotinylated goat anti-rabbit secondary antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min at room temperature. PBS washed sections were incubated with ABComplex/HRP (Santa Cruz Biotechnology), washed in PBS, incubated with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Bio Basic Inc., Ontario, Canada) for 10 min, counterstained with hematoxylin (Sigma–Aldrich), and mounted for imaging. The negative control was processed exactly the same way except that the primary antibody was replaced with non-immune rabbit IgG (Santa Cruz Biotechnology).

2.7. Statistical analysis

One-way ANOVA with Dunnett's *t*-test was used to compare the number of implantation sites among different groups. Two-tail unequal variance Student's *t*-tests were used to compare the gestation periods and litter sizes. Pregnancy rate, implantation rate, rate of mice with embryo retention in the oviduct, rate of mice with delayed embryo development, rate of embryos in delayed developmental stages, and survival rate of pups were initially analyzed by the χ^2 test and if a significant difference was observed, a Fisher's exact test was performed. $P \leq 0.05$ was considered significant.

3. Results

3.1. Preimplantation 100 mg/kg/day s.c. BPA treatment inhibited embryo implantation

The BPA exposure regimen designed in this study was focused on the embryo implantation process but not the ovulation and fertilization processes. Since ovulation and fertilization happen during the dark cycle before 5:00 AM on gestation day 0.5 [46], the BPA exposure regimen in this study, which started 9:00 AM–10:00 AM of gestation day 0.5, should not affect the ovulation and fertilization processes.

Comparable implantation rates were observed among 0, 0.025, 0.5, 10, and 40 mg/kg/day BPA-treated groups on gestation day 4.5 (Fig. 1A). There was also no significant difference in the numbers of implantation sites among these five groups (Fig. 1B). None of the nine females treated with 100 mg/kg/day BPA or the five females treated with 0.01 mg/kg/day E2 (as a positive control) showed any implantation sites. The implantation rates in these two groups were significantly lower than that in the control group (Fig. 1A).

The pregnancy status of the females without implantation sites was determined by flushing their reproductive tract for the presence of embryos. None of these females in the 0, 0.025,