

Long-Term Effects of Fetal Exposure to Low Doses of the Xenoestrogen Bisphenol-A in the Female Mouse Genital Tract¹

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ABSTRACT

Developmental exposure to estrogenic chemicals induces morphological, functional, and behavioral anomalies associated with reproduction. Humans are routinely exposed to bisphenol-A (BPA), an estrogenic compound that leaches from dental materials and plastic food and beverage containers. The aim of the present study was to determine the effects of in utero exposure to low, environmentally relevant doses of BPA on the development of female reproductive tissues in CD-1 mice. In previous publications, we have shown that this treatment alters the morphology of the mammary gland and affects estrous cyclicity. Here we report that in utero exposure to 25 and 250 ng BPA/kg of body weight per day via osmotic pumps implanted into pregnant dams at Gestational Day 9 induces alterations in the genital tract of female offspring that are revealed during adulthood. They include decreased wet weight of the vagina, decreased volume of the endometrial lamina propria, increased incorporation of bromodeoxyuridine into the DNA of endometrial gland epithelial cells, and increased expression of estrogen receptor- α (ER α) and progesterone receptor in the luminal epithelium of the endometrium and subepithelial stroma. Because ER α is known to be expressed in these estrogen-target organs at the time of BPA exposure, it is plausible that BPA may directly affect the expression of ER-controlled genes involved in the morphogenesis of these organs. In addition, BPA-induced alterations that specifically affect hypothalamic-pituitary-gonadal axis function may further contribute to the anomalies observed at 3 mo of age, long after the cessation of BPA exposure.

early development, environment, estradiol receptor, progesterone receptor, toxicology

INTRODUCTION

It is well established that the intrauterine milieu to which the human fetus is exposed is critical to neonatal health. However, recent data have suggested that perturbations in this same developmental environment may predispose individuals to disease or dysfunction (or both), such as hypertension and coronary heart disease, which become apparent only in adulthood [1]. The history of exposure to the synthetic estrogen diethylstilbestrol (DES) provides a tragic example of such a phenomenon. Administered to pregnant women to prevent miscarriage from 1948 to 1971, this potent synthetic estrogen induced severe abnormalities in the reproductive tract and gonads of daughters, condi-

tions that became apparent only after puberty or when they attempted to become pregnant [2]. Administration of DES during pregnancy was discontinued after clear cell adenocarcinoma of the vagina was diagnosed in the daughters of the DES-exposed mothers [2].

Over the last 60 yr, humans have been exposed to a plethora of synthetic hormonally active chemicals either overtly, because of their deliberate use in agriculture and medicine, inadvertently as byproducts of industrial use, or as waste released into rivers, lakes, and the atmosphere. Environmental exposure to these chemicals has coincided with an increase in endocrine-related diseases of the male reproductive system [3] and with an increase in testicular cancer [4] and breast cancer [5].

Bisphenol-A (BPA) is one hormonally active chemical that is receiving increased attention due to its high potential for human exposure. Used in the manufacture of polycarbonate plastics and epoxy resins, BPA has been shown to leach from food containers [6], beverage containers [7], and dental sealants and composites [8] under normal conditions of use [9]. These reports suggest that humans routinely ingest BPA. This chemical is also used in the manufacture of many products in addition to those mentioned above [10], which would be expected to further increase levels of human exposure to the compound. Due to a lack of extensive data on human exposure to BPA, animal studies to date have attempted to estimate potential exposure levels from available studies measuring the amount of BPA that leaches from food cans, containers, and dental sealants. These measurements have led to estimates of 2–20 μ g BPA per kilogram of body weight (BW) per day as a plausible exposure range in the population [11]. BPA has recently been measured in maternal and fetal plasma, and in placental tissue at birth in humans [12, 13]. The concentration of BPA in amniotic fluid was observed to be approximately 5-fold higher than that measured in maternal plasma. Further, the range of BPA concentrations assessed in the placenta was 1–100 ng/g with a median level of 12 ng/g, whereas in fetal plasma, levels ranged from 0.2 to 9.2 ng/ml. Recognizing that it is not feasible to generate accurate exposure levels from the existing data, we chose to administer 25 and 250 ng BPA/kg-BW per day s.c. by means of osmotic pumps to pregnant female mice beginning on Day 9 of pregnancy. We estimated that this level of BPA exposure would provide levels of BPA to the offspring that are within the range of human exposures reported to date.

An increasing number of publications suggest that perinatal exposure to BPA results in a variety of anomalies in the female reproductive system. BPA has been shown to alter estrous cyclicity, alter plasma LH levels, and increase body weight in rats exposed perinatally to this compound [14]. Reports of in utero or perinatal exposure to low doses of BPA in mice have revealed advanced puberty [15], earlier onset of vaginal opening [16], and altered estrous cy-

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clivity at 3–4 mo of age [17]. Further, we have reported previously that littermates of the animals used in the present study exhibited significant changes in ovarian morphology at 3 and 6 mo of age [17]; altered DNA synthesis in the stroma and epithelium of the mammary gland at 10 days and 6 mo of age; and increased numbers of terminal end ducts, alveolar buds, and total area of the mammary ductal tree at 6 mo of age [18]. To date, there have been no detailed studies describing the effects of fetal exposure to low, environmentally relevant doses of BPA on the development of the uterus. This is the focus of the current paper.

Although the mechanism by which BPA is able to induce developmental abnormalities in estrogen-sensitive tissues is unknown, it is reasonable to consider that estrogen receptors (ERs) may mediate BPA-induced effects because this chemical binds both ER α and ER β [19–21]. Interaction between BPA and ER may take place during fetal development, because ER α is expressed in the mesenchyme of the müllerian ducts and the stroma of the uterus by Gestational Days 12.5 and 16 in the mouse, respectively [22, 23]. Moreover, the expression of lactoferrin, known to be up-regulated by estradiol, is induced at Gestational Day 14 upon DES exposure, suggesting that the ER α is functional at this point in development [24]. ER β mRNA has not yet been detected prenatally in these tissues [23]. The knowledge that ERs (mRNA and protein) are expressed in the developing human fetus in the second trimester of gestation [25] supports the potential for BPA to have ER-mediated effects during development in humans.

The aim of the current study was to determine the long-term effects of developmental exposure to low doses of BPA administered to pregnant females for a period of 14 days beginning on Day 9 of gestation, on reproductive tract tissues in female offspring. Cell cycle dynamics (DNA synthesis, apoptosis), sex steroid receptor expression (ER α and progesterone receptor [PR]), and morphology of the uterus and vagina were examined in BPA-exposed offspring at 3 mo of age, when hypothalamic-pituitary-ovarian axis function has reached maturity.

MATERIALS AND METHODS

Animals

Sexually mature (8 wk of age) female CD-1 mice (Charles River, MA) were maintained in temperature- and light-controlled (14L:10D) conditions at the Tufts University–New England Medical Center Animal Facility. Mice were fed Purina Rodent Chow (Purina, St. Louis MO) that tested negligible for estrogenicity using the E-SCREEN assay [26], and tap water was supplied from glass bottles only. Cages and bedding also tested negative for estrogenicity using the E-SCREEN assay [26]. All experimental procedures were approved by the Tufts University–New England Medical Center Animal Research Committee in accordance with the *Guide for Care and Use of Laboratory Animals*. Female mice were mated to CD-1 males of proven fertility, and the morning on which a vaginal plug was observed was designated Day 1 of pregnancy. On Day 9 of pregnancy, mice were weighed and implanted with Alzet osmotic pumps (Alza Corp., Palo Alto, CA) designed to deliver either dimethyl sulfoxide (DMSO; vehicle control), or 25 or 250 ng BPA/kg-BW per day dissolved in DMSO (Sigma, St. Louis, MO) for a period of 14 days, which encompassed the remainder of pregnancy through Postnatal Day 4 ($n = 6$ –10 per treatment). The spent Alzet pumps remained in the mothers until the litters were weaned due to concern for disruption of maternal care. Offspring were born on Day 20 of gestation, culled to 10 pups per mother on Postnatal Day 7, and weaned on Postnatal Day 20. Female offspring were killed at 3 mo of age ($n = 6$ –10 per treatment group; one pup per litter) on the afternoon of proestrus, which was determined on the basis of daily vaginal smears for 2 consecutive weeks. Because a significant percentage of BPA-treated animals exhibit periods of disrupted cyclicity [17], only the best cyclers were used for these experiments.

Tissue Collection and Histology

One and one-half hours before animals were killed by CO₂ inhalation, 3-mo-old female offspring were weighed and injected i.p. with bromodeoxyuridine (BrdU; 1.5 mg per 100 g BW; Roche Molecular Biochemicals, Indianapolis, IN). The uterus and vagina were immediately dissected out and weighed. The uterus was blotted on paper to remove fluids and reweighed to obtain the blotted weight. These tissues were immerse-fixed in 4% formaldehyde in 0.1 M PBS for 10–16 h at room temperature and embedded with Paraplast paraffin (Fisher, Pittsburgh, PA) under vacuum. Prior to embedding, one horn of each uterus and the vagina were dissected transversely into three segments. Five-micrometer sections were cut on a Leica RM2155 Rotary Microtome (Nussloch, Germany) and mounted on Superfrost slides (Fisher). Sections of uterus and vagina were stained with hematoxylin-eosin to determine changes in tissue structure by morphometric analysis, and with anti-BrdU (an index of DNA synthesis) to assess cell proliferation. Uterine sections were also analyzed for in situ apoptosis detection using the TUNEL method, and for the expression of ER α and PR. To minimize spurious variation among experiments, each immunostaining procedure was performed in sets containing tissues from an equal number of subjects from all experimental groups (0, 25, and 250 ng BPA/kg-BW per day).

Immunolocalization of BrdU

BrdU was immunolocalized within paraffin sections of uterus and vagina by immunofluorescence. Sections were hydrated, microwaved in 10 mM citrate buffer (pH 6) for antigen retrieval [27], and nonspecific binding was blocked with 2% normal sheep serum in 0.01 M PBS. Sections were incubated with monoclonal BrdU antibody immunoglobulin G (IgG; 1:400; clone 85-2C8; Novocastra, U.K.) overnight in a humid chamber at 4°C. Biotinylated sheep anti-mouse IgG (Roche Diagnostics Corp., Indianapolis, IN) was applied to sections, and BrdU was visualized by streptavidin Alexa Fluor conjugate (Molecular Probes, Eugene, OR). Sections were counterstained with Hoechst 33258 (1:1000; Sigma) and mounted in glycerol/0.01 M PBS (1:1).

Localization of Apoptotic Cells

Apoptosis was assessed by two methods: the TUNEL method that reflects early stage apoptosis, and by histological analysis of apoptotic bodies, which reflects the later stages of apoptosis. The TUNEL technique was based on in situ detection of DNA strand breaks within paraffin sections of the uterus (apoptotic cells; ApopTag; Intergen Co., Purchase, NY). Briefly, sections were deparaffinized, rehydrated, incubated with proteinase K (5 μ g/ml; Intergen) for 10 min at 37°C, and then treated with hydrogen peroxide in PBS for 10 min at room temperature to quench endogenous peroxidase activity. Sections were then incubated with a mixture containing digoxigenin deoxynucleotide triphosphate, unlabeled deoxynucleotide triphosphate, and terminal deoxynucleotidyl transferase enzyme in a humidified chamber at 37°C for 1 h. Slides were rinsed with PBS and incubated with anti-digoxigenin-peroxidase for 30 min at room temperature, and substrate-chromogen mixture (diaminobenzidine [DAB]; Sigma) for 4 min. Samples were counterstained with Mayer hematoxylin, dehydrated, and mounted with permanent mounting medium. Involuting mouse mammary gland, collected 3 days postweaning, was included as a positive tissue control. For histological analysis of the later stages of apoptosis, paraffin sections of uteri were stained with hematoxylin-eosin.

Immunolocalization of ER α and PR

Receptors were immunolocalized within sections of the uterus using the streptavidin-biotin labeling system. Briefly, sections were hydrated through an alcohol gradient, microwaved in 10 mM citrate buffer (pH 6) for antigen retrieval, treated to suppress endogenous peroxidase, and incubated in blocking reagent. Sections were incubated with antibody against ER α (mouse monoclonal IgG, clone ER6F11, 1:80; Novocastra, or PR (mouse monoclonal IgG, 1:100; PR-AT4-14, Affinity Bioreagents, Inc., Golden, CO) overnight in a humid chamber at 4°C. Biotinylated anti-mouse IgG (1:200; MOM kit; Vector Laboratories) was then applied to sections, and nuclear localization of the receptors was visualized by incubation with streptavidin-horseradish peroxidase and DAB. Sections were lightly counterstained with Harris hematoxylin and mounted with a glass coverslip for light microscopy.

Morphometric Analyses

Digital images of uterus and vagina sections were captured with a SPOT-Real Time digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) attached to a Zeiss microscope (Carl Zeiss Inc., Germany) using a 20× objective lens. Quantitative analysis of all tissue parameters was performed using the Optimus 6.5 program (Media Cybernetics, Silver Spring, MD).

For morphometric analysis of the uterus, the relative and absolute areas of the endometrium occupied by the mucosa (luminal epithelium, glandular epithelium, and lamina propria), myometrium, uterine and glandular lumina, and blood vessels were measured in three transverse sections, sampling the entire section in two fields of view with the 10× objective. For analysis of the vagina, the height of the epithelium and number of epithelial cells present were measured from the point of the basement membrane to the lumen in three transverse sections, sampling five regions within two fields of view with the 20× objective.

Quantitative Analysis of BrdU, Apoptosis, ER α , and PR

For quantitative analysis of DNA synthesis in the uterus and vagina, the number of BrdU-positive epithelial cells was counted per 1000- μ m basement membrane of epithelium in three transverse sections, sampling four fields of view in the uterine sections, and three fields of view in the vaginal sections, with the 20× objective. For quantitative analysis of apoptotic cells, three equidistant cross sections of one horn of each uterus were assessed counting an average of 250 cells per cross section. Data were expressed as the number of DAB-stained apoptotic cells per total number of cells per sample section. For quantitative analysis of ER α and PR expression in the uterus, sections were viewed under a Zeiss microscope (Carl Zeiss Inc.) using a 100× objective lens with oil. A graticule was placed in the eyepiece to quantify the percentage of cells expressing receptors within the luminal and glandular epithelium of the uterus. The intensity of staining was divided into two categories (each cell was scored as high (++) or low (+) based on the intensity of the DAB reaction product.

Statistical Analysis

Data were assessed by one-way analysis of variance and differences between the control and BPA treatment groups were determined using a least significant difference test. In specific cases in which the data were not distributed normally or showed heterogeneity of variance, a Kruskal-Wallis test was employed and differences between the control and BPA treatment groups were assessed by Mann-Whitney *U*-tests.

RESULTS

Wet Weight of Reproductive Organs in 3-Mo-Old Mice

In 3-mo-old mice, a significant decrease in the absolute weight of the vagina (63.38 ± 2.77 mg; $P < 0.01$), and relative weight of the vagina (0.227 ± 0.011 ; $P < 0.01$) was observed in the 250 ng BPA/kg-BW per day group relative to the control group (83.11 ± 5.91 mg and 0.306 ± 0.025 , respectively; Table 1). A similar trend was observed for relative weight of the vagina in the 25 ng BPA/kg-BW per day group, although this difference did not reach significance ($P = 0.052$). The absolute and relative weights of the uterus in both the 25 and 250 ng BPA/kg-BW per day groups were decreased relative to the control group, but this decline was not statistically significant.

Uterine Changes in 3-Mo-Old Mice

Morphometry. In utero exposure to low doses of BPA did not result in statistically significant differences relative to the vehicle-treated controls in the percentage of tissue compartments within the uteri of adult mice (Table 2). In contrast, the absolute volume of the lamina propria was significantly reduced in the 250 ng BPA/kg-BW per day group ($P = 0.015$) compared with that of controls. Other compartments also showed a decrease in volume; however, these did not reach statistical significance. For example,

TABLE 1. The absolute and relative weight (mean \pm SEM) of the uterus and vagina in 3 mo-old female mice exposed perinatally to DMSO (control) or BPA (ng/kg bw/day).^a

Treatment	Absolute weight (mg)						Relative weight (% body weight)					
	n	Uterus (wet)	Uterus (blot)	n	Vagina	n	Uterus (wet)	Uterus (blot)	n	Vagina	n	Vagina
Control	8	124.42 \pm 8.45	119.77 \pm 8.37	8	83.11 \pm 5.91	8	0.421 \pm 0.031	0.411 \pm 0.029	5	0.306 \pm 0.025	5	0.306 \pm 0.025
BPA, 25 ng/kg	8	108.21 \pm 9.52	100.68 \pm 8.93	8	74.39 \pm 5.94	8	0.367 \pm 0.025	0.342 \pm 0.024	8	0.253 \pm 0.016	8	0.253 \pm 0.016
BPA, 250 ng/kg	8	100.22 \pm 3.23	93.82 \pm 4.03	8	63.38 \pm 2.77*	8	0.359 \pm 0.013	0.338 \pm 0.013	6	0.227 \pm 0.011*	8	0.227 \pm 0.011*

^a For all variables within each column, an asterisk denotes that mean values are significantly different relative to the control group ($*P < 0.01$); n represents the number of mice; ng/kg bw/day = nanogram per kilogram per body weight per day.

TABLE 2. Morphometric analysis of the uterus from 3 mo-old mice exposed perinatally to DMSO (control) or BPA (ng/kg bw/day).^a

Treatment	n	Luminal epithelium	Glandular epithelium	Uterine lumen	Glandular lumen	Lamina propria	Myometrium
Relative area (%)							
Control	8	7.86 ± 1.40	8.76 ± 0.91	9.94 ± 1.30	1.00 ± 0.09	32.89 ± 1.15	35.77 ± 2.58
BPA, 25 ng/kg	11	5.82 ± 0.52	9.03 ± 0.75	9.35 ± 1.48	1.05 ± 0.18	34.28 ± 0.68	37.33 ± 2.10
BPA, 250 ng/kg	8	5.27 ± 0.44	11.14 ± 1.07	8.17 ± 1.07	1.14 ± 0.14	30.56 ± 1.03	39.97 ± 0.46
Absolute volume (mg)							
Control	8	10.16 ± 2.38	10.77 ± 1.38	12.53 ± 2.08	1.22 ± 0.11	40.49 ± 3.39	43.06 ± 2.03
BPA, 25 ng/kg	11	5.86 ± 0.82	9.35 ± 1.46	9.85 ± 2.35	1.07 ± 0.23	34.97 ± 3.97	36.96 ± 2.59
BPA, 250 ng/kg ^b	8	4.95 ± 0.45	10.46 ± 1.12	7.76 ± 1.23	1.06 ± 0.12	28.54 ± 0.97*	37.55 ± 1.87

^a Values for each group are expressed as mean ± SEM; n represents number of mice in each group; ng/kg bw/day = nanogram per kilogram per body weight per day.

^b For all variables within each row, an asterisk denotes that mean values are significantly different relative to the control group (* $P < 0.05$).

exposure to 25 ng BPA/kg-BW per day and 250 ng BPA/kg-BW per day resulted in a 42.3% and 51.4% decrease, respectively, in the mean absolute volume of luminal epithelium relative to the control group. Moreover, the mean percentage of uterus occupied by luminal epithelium was decreased by 26.0% and 33.1% in the 25 ng and 250 ng BPA/kg-BW per day groups, respectively, relative to the control group (Table 2).

DNA synthesis. DNA synthesis was altered in the uterine epithelium in adult mice following fetal exposure to BPA. There was a significant increase in the number of glandular epithelial cells that incorporated BrdU (per 1000- μ m basement membrane) in the 250 ng BPA/kg-BW per day group (23.06 ± 1.44) relative to the control group (7.57 ± 2.46 ; $P < 0.05$; Fig. 1). BrdU incorporation did not differ significantly in the luminal epithelium.

Apoptosis. Regarding the number of apoptotic cells, no statistically significant changes were observed in the luminal and glandular epithelium of the uterus (Table 3).

Expression of ER α

The expression of ER α within the luminal epithelial cells of the adult mouse uterus was increased by approximately 4-fold in the 25 ng BPA/kg-BW per day group (69.7 ± 12.5 ; $P < 0.05$), and by approximately 5-fold in the 250 ng BPA/kg-BW per day group (82.8 ± 11.3 ; $P < 0.01$) relative to the control group (15.6 ± 4.3 ; Fig. 2, A and B). As depicted in Figure 2B, females exposed to 25 ng BPA/kg-BW per day and 250 ng BPA/kg-BW per day exhibited a 13-fold (50.0 ± 11.9 ; $P < 0.05$) and 16-fold ($61.6 \pm$

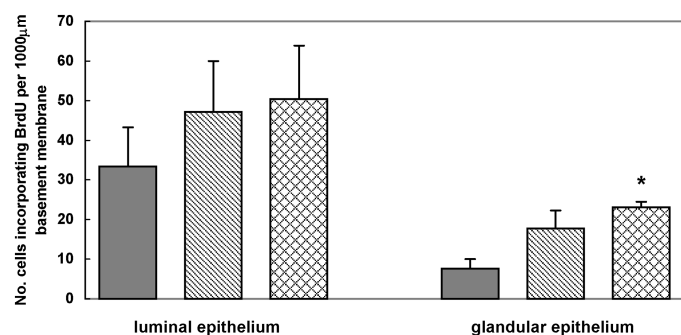


FIG. 1. The number of luminal and glandular epithelial cells incorporating BrdU per 1000- μ m basement membrane in the uterus of 3-month-old mice exposed perinatally to DMSO (control; shaded bar), 25 ng BPA/kg-BW per day (diagonal bar), and 250 ng BPA/kg-BW per day (criss-cross bar). Bars depict mean ± SEM (n = 6 per group). Asterisks denote a statistically significant difference of the BPA-treated groups relative to the vehicle-treated control (* $P < 0.05$).

10.6; $P < 0.05$) increase, respectively, in the percentage of cells showing high intensity (++) staining for ER α relative to control females (3.9 ± 1.3). The pattern of expression of ER α in the endometrial stroma was also altered, showing an increase in the number of ER α -positive stromal cells beneath the epithelium (Fig. 2A).

Expression of PR

The expression of PR in the luminal epithelial cells of the adult mouse uterus was also altered by fetal exposure to BPA (Fig. 3A). The percentage of epithelial cells expressing detectable levels of PR was increased by approximately 14-fold in the 25 ng BPA/kg-BW per day group (89.9 ± 6.9 ; $P < 0.01$), and by approximately 13-fold in the 250 ng BPA/kg-BW per day group (82.6 ± 15.6 ; $P < 0.01$) relative to the control group (6.6 ± 2.3 ; Fig. 3A). As depicted in Figure 3B, females exposed to 25 and 250 ng BPA/kg-BW per day exhibited a 125-fold (74.5 ± 12.0 ; $P < 0.01$) and 124-fold (75.0 ± 15.7 ; $P < 0.01$) increase, respectively, in the number of cells showing high intensity (++) staining for PR relative to control females (0.6 ± 0.5).

Vaginal Changes in 3-Mo-Old Mice

Morphometry. There was a significant decrease in the absolute and relative wet weights of the vagina of animals exposed in utero to 250 ng BPA/kg-BW per day compared with those of controls (Table 1). In contrast, there were no statistically significant changes in other vaginal parameters assessed. The thickness of the vaginal epithelium (measured perpendicular from the basement membrane to the lumen) increased slightly in controls from 62.45 ± 2.97 μ m to 70.94 ± 3.20 μ m, and 77.50 ± 4.97 μ m in the 25 and 250 ng BPA/kg-BW per day treatment groups, respectively; however, this was not statistically significant. Also, the number of epithelial cells in the 25 and 250 ng BPA/

TABLE 3. Apoptosis in the uterus of 3 mo-old mice exposed perinatally to DMSO (control) or BPA (ng/kg bw/day).^a

Treatment	n	Apoptotic epithelial cells (%)	
		Luminal epithelium	Glandular epithelium
Control	7	2.33 ± 0.61	2.04 ± 0.54
BPA, 25 ng/kg	8	5.63 ± 1.44	3.37 ± 0.93
BPA, 250 ng/kg	6	2.05 ± 0.32	3.46 ± 0.58

^a Values for each group are expressed as mean ± SEM; n represents number of mice in each group; ng/kg bw/day = nanogram per kilogram per body weight per day.

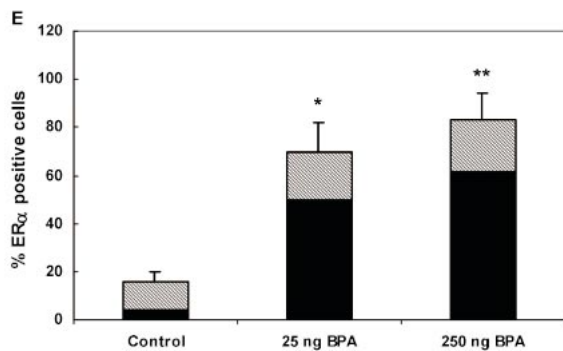
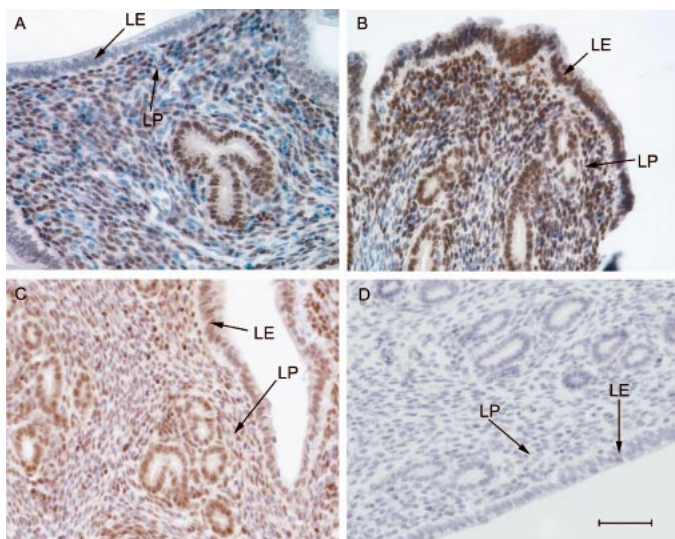


FIG. 2. Photomicrograph showing the expression of ER α in the uterus of 3-mo-old mice exposed perinatally to (A) DMSO (control), (B) 25 ng BPA/kg-BW per day, (C) 250 ng BPA/kg-BW per day, and (D) negative control. Note the increased expression of ER α in both the luminal epithelium (LE) and subepithelial compartment of the lamina propria (LP) in the BPA-treated mice relative to the control. Bar = 5 μ m. E The percentage of luminal epithelial cells showing low-intensity (diagonal bar) and high-intensity (shaded bar) staining for ER α in the uterus of 3-mo-old mice exposed perinatally to DMSO (control; n = 6), 25 ng BPA/kg-BW per day (n = 8), and 250 ng BPA/kg-BW per day (n = 6). The standard error is depicted for the percentage of cells stained positive (all intensities) for ER α . Asterisks denote a statistically significant difference in the total number of cells that stained positive for ER α in the BPA-treated groups relative to the vehicle-treated control (* P < 0.05, ** P < 0.01).

kg-BW per day groups (9.17 ± 0.56 and 9.20 ± 0.62 , respectively) remained unchanged compared with that of the control group (8.74 ± 0.27).

DNA Synthesis. DNA synthetic activity was not altered in the vaginal epithelium of adult mice following perinatal exposure to BPA. There was no difference in the number of BrdU-positive epithelial cells per 1000- μ m basement membrane within the vagina between the control (45.04 ± 14.53) and the 25 and 250 ng BPA/kg-BW per day groups (33.19 ± 7.26 and 44.83 ± 8.02 , respectively).

DISCUSSION

Due to its widespread use in food and beverage containers and dental sealants, mounting evidence suggests that humans may routinely ingest BPA. In the present study, pregnant mice were exposed to BPA at concentrations (25 or 250 ng BPA/kg-BW/day) that should produce levels of BPA exposure within the range of those reported to exist in human placental and fetal tissues [12, 13]. BPA was also

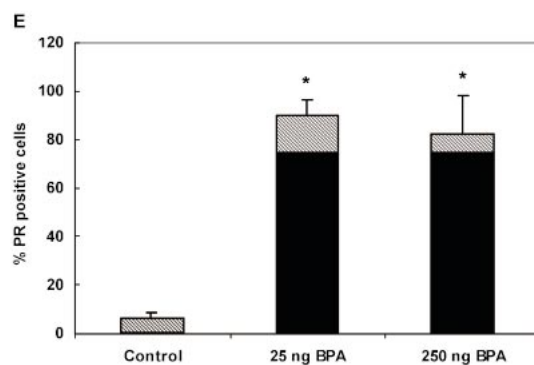
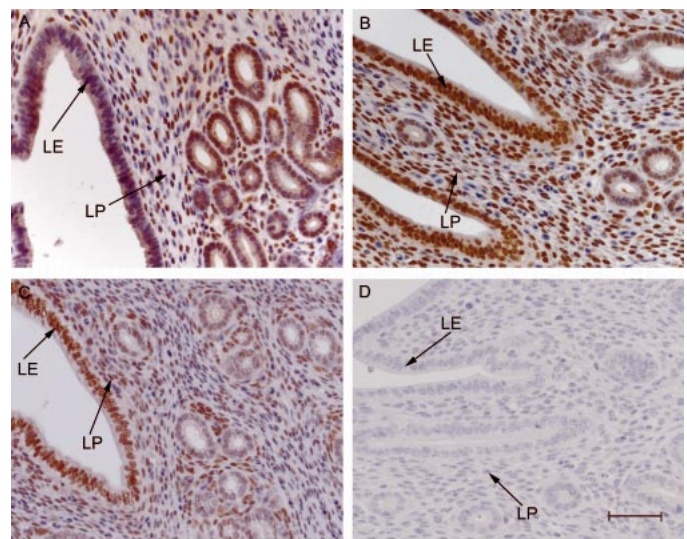


FIG. 3. Photomicrograph showing the expression of PR in the uterus of 3-mo-old mice exposed perinatally to (A) DMSO (control), (B) 25 ng BPA/kg-BW per day, (C) 250 ng BPA/kg-BW per day, and (D) negative control. Note the increased expression of PR in both the luminal epithelium (LE) and subepithelial compartment of the lamina propria (LP) in the BPA-treated mice relative to the control. Bar = 5 μ m. E The percentage of luminal epithelial cells showing low-intensity (diagonal bar) and high-intensity (shaded bar) staining for PR in the uterus of 3-mo-old mice exposed perinatally to DMSO (control; n = 5), 25 ng BPA/kg-BW per day (n = 6), and 250 ng BPA/kg-BW per day (n = 5). The standard error is depicted for the percentage of cells that stained positive (all intensities) for PR. Asterisks denote a statistically significant difference in the total number of cells stained positive for PR in the BPA-treated groups relative to the vehicle-treated control (* P < 0.01).

shown to cross the placental barrier in mice. Zalko et al. [28] reported that 24 h after s.c. administration of 25 μ g/kg tritiated BPA to pregnant mice on Day 17 of gestation, approximately 0.4% of the radioactivity administered to the dams was recovered in the uterus, 0.3% was present in the amniotic fluid, and 4.1% was present in the entire litter of fetuses.

BPA is an estrogen mimic, albeit several orders of magnitude less potent than estradiol. When administered perinatally, it produces measurable effects at doses that are 10^4 -fold to 10^6 -fold lower than those required to induce a uterotrophic effect in the prepubertal mouse [29]. This finding highlights the critical sensitivity of the developing organism to environmental estrogen exposure [29] and may be interpreted to mean that BPA acts in the fetus through mechanisms that are distinct from those that mediate estrogenicity. However, Nagel et al. have shown that the low-dose effects observed after prenatal exposure could be predicted from the relative estrogenic potency of BPA [11]. More-

over, the effects reported by this group were also observed with estradiol and DES administered at equivalent doses [30]. These effects were reproduced upon in vitro exposure and were blocked by antiestrogens [31, 32]. Therefore, based on the available evidence, it is reasonable to consider that the low-dose effects of prenatal BPA exposure would likely be mediated through the estrogen receptors.

The results of the current study demonstrate that administration of 25 or 250 ng BPA/kg-BW per day to pregnant females affected the development of the genital tract; this was documented at both the gross and cellular levels in their female offspring long after the exposure had ended. At the tissue level of organization, the absolute volume of the lamina propria of the endometrium was significantly decreased in the animals exposed in utero to 250 ng BPA/kg-BW per day, while the remaining compartments of the uterus showed a decrease that was not statistically significant. At the organ level, the wet weight of the vagina was significantly decreased. At the cellular level, a significant increase in DNA synthesis in the absence of any apoptotic changes was observed within the glandular epithelium of the uterus, suggesting an accrual of new cells. Other studies have reported morphological changes in reproductive tract tissues after exposure to significantly higher doses of estrogenic substances. For example, neonatal exposure to 2 μ g DES per pup per day (Postnatal Days 1–5) has been shown to induce hypertrophy of luminal epithelial cells, a decrease in the number of endometrial glands, and disorganization of the stroma and muscularis, associated with an overall decrease in the size of the uterus [33].

The expression of ER α is typically low in the luminal epithelium of the mouse uterus relative to the stroma [34]. The current study confirmed the former observation, and demonstrated that prenatal exposure to low doses of BPA dramatically increased the expression of both ER α and PR in the luminal epithelium at 3 mo of age. Increased expression of ER α (detected by immunohistochemistry and in situ hybridization) in the uterine epithelium of the newborn mouse has been described following prenatal exposure to high DES doses (10 and 100 μ g/kg) [34]. Increased expression of ER α mRNA has also been observed in the pituitary gland of 30-day-old male Fisher rats exposed postnatally at Days 1–5 to either DES (5 μ g per pup per day) or to much higher doses of BPA (100 or 500 μ g per pup per day) [35]. In contrast, a down-regulation of ER α mRNA was observed in the uterus of female littermates exposed to DES, and no significant change in level was reported in the uterus of female littermates exposed to BPA. That BPA affected ER α expression in the uterus of CD1 mice but not in the uterus of Fisher rats may be due to the multiple differences between the two experiments concerning species, age, and dose of exposure, and the age at which they were killed. Sensitivity to BPA varies among species and strains, a phenomenon that is well documented in the field of endocrine disruption [36]. In this regard, it is remarkable that significant effects resulted from perinatal exposure to low doses of BPA in CD-1 mice [15, 17, 18, 37], because this strain is among the least sensitive to natural estrogens [38]. However, the sensitivity to BPA does not always parallel sensitivity to estradiol. For example, Fischer 344 and Sprague-Dawley rats were similarly sensitive to estradiol regarding cell proliferation in the vaginal epithelium. However, BPA evoked a proliferative response in Fischer 344, but not in Sprague-Dawley rats. Yet the BPA dose response was similar in both strains in the early events evoked by estrogens, such as c-fos induction [36].

The BPA-induced changes in the expression of sex steroid receptors described herein may have consequences for 1) the hormonal responsiveness of the uterus to both endogenous and exogenous hormones, and 2) the potential for predisposition of the organ to disease later in life. Alworth et al. [39] have shown that prenatal exposure of mice to DES at 0.1 and 10 μ g/kg per day as well as to methoxychlor at 100 and 10 000 μ g/kg per day affected the way in which the adult uterus was able to respond to exogenously administered estrogens (i.e., lower doses induce an enhanced response while higher doses induce a dampened response). Newbold et al. have shown that DES doses lower than those used by Alworth et al. (10 ng/kg per day) administered directly to newborn mice also enhanced the response to estrogens during adulthood [40]. Regarding potential predisposition to disease, BPA-induced changes in the expression of sex steroid receptors reported here may exacerbate the response of the uterus to subsequent hormonal administration, such as that imposed by hormonal replacement therapy or the contraceptive pill. For example, studies in which neonatal hamsters are exposed to DES and subsequently to estradiol later in adulthood have shown hyperplasia and increased apoptosis of the uterine luminal epithelium, which subsequently developed neoplasms [41]. Neonatal exposure of mice (at Postnatal Days 1–5) to 2 μ g DES per pup per day induced ovary-dependent uterine adenocarcinoma in 18-mo-old mice, a pathology that did not occur in the control mice [42]. Finally, transgenic mouse models in which ER α expression is up-regulated in the uterus by approximately 25% exhibited an earlier onset and a higher incidence of uterine adenocarcinoma following exposure to DES at Postnatal Days 1–5 than wild-type mice [43]. The finding in the present study that BPA induced a subepithelial clustering of ER α -intense stromal cells within the uterus is also of interest, because Cooke et al. [44] observed that the expression of ER α in the stroma alone was a prerequisite for estradiol-mediated cell proliferation of the epithelium.

At the tissue level of organization, the absolute volume of the lamina propria of the endometrium was significantly decreased in the animals exposed in utero to 250 ng BPA/kg-BW per day, while the remaining compartments of the uterus showed a decrease that was not statistically significant. At the organ level, the wet weight of the vagina was significantly decreased. Other studies have reported morphological changes in reproductive tract tissues after exposure to significantly higher doses of estrogenic substances. For example, neonatal exposure to 2 μ g DES per pup per day (Postnatal Days 1–5) has been shown to induce hypertrophy of luminal epithelial cells, a decrease in the number of endometrial glands, and disorganization of the stroma and muscularis, associated with an overall decrease in the size of the uterus [33].

The cellular and morphological changes in the mouse uterus observed in the current study, in addition to those reported previously in the mammary gland of their littermates [18], suggest that prenatal exposure to BPA may act directly by altering the expression of patterning genes in the target organs. Others have suggested that environmental estrogens can directly alter estrogen-sensitive genes during fetal development, as evidenced by the DES-induced up-regulation of lactoferrin protein [24] and down-regulation of *Hoxa10* [45] and *Wnt7* mRNAs in the fetal mouse uterus [46]. These changes in *Hoxa10* and *Wnt7* gene expression seem to mediate the abnormal uterine morphology that characterizes the DES-exposed uterus [47].

Early BPA exposure may also exert indirect effects on reproductive tract tissue by altering the function of the hypothalamic-pituitary-gonadal axis, an effect that would become apparent after puberty. Perinatal exposure to estrogens or to androgens that can be aromatized is known to alter patterns of estrous cyclicity in rodents [48, 49]. Thus far, we have documented alterations in patterns of estrous cyclicity in BPA-exposed rats and mice [14, 17] that could affect circulating ovarian steroid levels and, in turn, contribute to alterations in reproductive tract tissue. The up-regulation of PR that was observed within the uterine epithelium of BPA-exposed mice, a response that typifies estrogen activity [50], might suggest that these mice are in a hyperestrogenized state, or that their estrogen-target tissues are more sensitive to estrogens than those of controls. Indeed, increased ductal morphogenesis of the mammary gland, an estrogen-mediated process, was observed previously in the littermates of the animals used in the present study [18].

In summary, exposure of the developing fetus during critical periods of uterine organogenesis to low, environmentally relevant, doses of BPA causes changes in DNA synthesis, increases the expression of the sex steroid receptors ER α and PR in the endometrium, decreases the volume of the lamina propria, and decreases the wet weight of the vagina of the adult mouse. These findings, coupled with those of our previous studies that described altered morphogenesis of the mammary glands and disrupted estrous cyclicity in animals exposed to these same doses of BPA, strongly suggest that this environmental chemical is of concern to human health. Further examination of the effects of this and other environmental estrogens should strengthen our understanding of the etiology of the increasing incidence of developmental and reproductive diseases in humans. In turn, this should help regulators more accurately determine the risks of the continuous use of this estrogen mimic.

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