

In Utero Exposure to Bisphenol A Alters the Development and Tissue Organization of the Mouse Mammary Gland¹

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ABSTRACT

Exposure to estrogens throughout a woman's life, including the period of intrauterine development, is a risk factor for the development of breast cancer. The increased incidence of breast cancer noted during the last 50 years may have been caused, in part, by exposure of women to estrogen-mimicking chemicals that are released into the environment. Here, we investigated the effects of fetal exposure to one such chemical, bisphenol A (BPA), on development of the mammary gland. CD-1 mice were exposed in utero to low, presumably environmentally relevant doses of BPA (25 and 250 $\mu\text{g}/\text{kg}$ body weight), and their mammary glands were assessed at 10 days, 1 mo, and 6 mo of age. Mammary glands of BPA-exposed mice showed differences in the rate of ductal migration into the stroma at 1 mo of age and a significant increase in the percentage of ducts, terminal ducts, terminal end buds, and alveolar buds at 6 mo of age. The percentage of cells that incorporated BrdU was significantly decreased within the epithelium at 10 days of age and increased within the stroma at 6 mo of age. These changes in histoarchitecture, coupled with an increased presence of secretory product within alveoli, resemble those of early pregnancy, and they suggest a disruption of the hypothalamic-pituitary-ovarian axis and/or misexpression of developmental genes. The altered relationship in DNA synthesis between the epithelium and stroma and the increase in terminal ducts and terminal end buds are striking, because these changes are associated with carcinogenesis in both rodents and humans.

developmental biology, environment, mammary glands, mechanisms of hormone action, toxicology

INTRODUCTION

Estrogen exposure throughout a woman's life has been identified as a major risk factor for the development of breast cancer. Results of epidemiological studies suggest that the intrauterine milieu may also have an influential role in predisposing an individual to carcinogenesis. The level of intrauterine estrogens is heightened with twin births, a phenomenon that correlates with an increased incidence of breast cancer in daughters [1–3]. This same phenomenon is

proposed to account for the increased risk of breast cancer in daughters of older mothers [4, 5], although this correlation remains controversial [2, 6]. Conversely, preeclampsia, which is characterized by high maternal blood pressure and reduced estrogen production by the placenta, correlates with a decreased incidence of breast cancer in daughters [3] and prostate cancer in sons [7]. These epidemiological studies suggesting a strong correlation between in utero estrogen exposure and cancer are supported by numerous experimental studies involving the reproductive tract and mammary glands, the results of which attest to the proliferative and carcinogenic potency of estrogens [8–10].

The incidence of breast cancer within the United States has increased by 40% during the same quarter-century that has witnessed the release of massive quantities of estrogenic chemicals into the environment [11]. This association has led some investigators to suggest that exposure to estrogenic pesticides may increase the risk of breast cancer. In fact, exposure to dieldrin significantly increases this risk and decreases the survival rate of women in a dose-dependent manner [12, 13]. The history of diethylstilbestrol (DES), a potent estrogen that was administered in high doses to pregnant women as an antiabortive from 1948 to 1971, demonstrated unequivocally that in utero exposure to exogenous estrogens induces clear cell adenocarcinoma of the vagina in daughters after puberty [14]. No more than anecdotal evidence exists, however, that daughters of women who received DES show a higher risk of breast cancer, but the relevant birth cohorts have not, as yet, been examined over the age range that is at high risk for the disease [15].

Rodent models have reproduced almost identical tissue changes in the vagina following prenatal exposure to DES [16, 17] and have suggested that the mammary glands are 10- to 100-fold more sensitive to neonatal DES exposure than the vagina and uterus [18]. Indeed, studies in rats have shown that prenatal and neonatal treatment with DES causes an increased incidence of mammary gland tumors (hyperplastic alveolar nodules, dysplasia, and neoplasia), decreased tumor latency [19], and increased sensitivity to hormones and carcinogens in adulthood [20]. Similar studies in BALB/c mice show that palpable tumors develop only in the presence of the mouse mammary gland tumor virus [21, 22], but CD-1 mice develop tumors following prenatal DES exposure in the absence of this virus [23].

Bisphenol A (BPA) is used in the manufacture of polycarbonate plastics and epoxy resins, from which a variety of products are made, including reusable milk and food storage containers, baby formula bottles, the interior lacquer-coating of food cans, and dental sealants and composites. Studies have shown that incomplete polymerization of these products during manufacture and/or depolymerization due to increased temperatures (induced either intentionally

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for sterilization purposes or unintentionally during storage in warehouses) cause BPA and its derivatives to leach into foods (4–23 $\mu\text{g}/\text{can}$) [24], beverages (7–58 $\mu\text{g}/\text{g}$) [25], and saliva (90–913 $\mu\text{g}/\text{saliva}$ collected during a 1-h period following application of dental sealant) [26] in concentrations that are sufficient to induce proliferation of estrogen-target cells in culture. These data indicate that humans are exposed to BPA; however, these figures should be considered as conservative estimates. Bisphenol A is used in the manufacture of many products in addition to those stated above [27], and to our knowledge, no data are currently available to describe the levels that may leach from these products.

In the present study, we chose BPA as a model for xenoestrogen exposure, yet humans are exposed to many other estrogenic chemicals [27]. If these chemicals act through the estrogen receptors (ERs) α and β , as is currently believed, their effects will be additive [28]. Thus, it is not the individual exposure to a single xenoestrogen but, rather, the cumulative exposure to multiple xenoestrogens that determines the intensity of the effects [29].

Recent findings have shown that in utero exposure of rodents to low, presumably environmentally relevant doses of BPA induces precocious puberty [30], disruption of estrous cyclicity [31], increased prostate weight, and decreased epididymal weight and sperm production [32, 33]. Because the potential for human exposure to BPA is high, this chemical may affect other estrogen-target tissues, such as the mammary gland, during development. We hypothesize that in utero exposure to BPA, a synthetic chemical that has been shown to mimic the effects of estrogen, induces developmental changes in the mammary glands of mice and, in turn, that these changes increase the likelihood of carcinogenesis in adulthood.

MATERIALS AND METHODS

Animals

Sexually mature, female CD-1 mice (8 wk of age; Charles River, Wilmington, MA) were maintained in temperature- and light-controlled conditions at the Tufts University–New England Medical Center Animal Facility. Mice were fed RMH 3000 rodent diet (Prolab Agway Inc., Syracuse, NY) that tested negligible for estrogenicity, and water was supplied from glass bottles only. Cages and bedding also tested negative for estrogenicity using the E-SCREEN assay [34]. 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, and the morning on which a vaginal plug was observed was designated as Day 1 of pregnancy. On Day 9 of pregnancy, which corresponded to the beginning of organogenesis in the fetus, mice were weighed and implanted with Alzet osmotic pumps (Alza Corp., Palo Alto, CA) designed to deliver either dimethyl sulfoxide (DMSO; vehicle control; Sigma, St. Louis, MO) or 25 or 250 $\mu\text{g}/\text{kg}$ body weight of BPA per day dissolved in DMSO throughout the remainder of the pregnancy ($n = 6$ –10 per treatment). The actual dose decreased as pregnancy progressed, because the weight of the mother increased during this period. Pumps were implanted s.c. in sterile conditions. Offspring were delivered on Day 20 of gestation and weaned from their mothers at 19 days of age. Female offspring were killed at 10 days, 1 mo (puberty), and 6 mo of age ($n = 6$ –10 per treatment for each age group). For those mice killed after puberty, vaginal smears were assessed daily to determine estrous cyclicity; these mice were killed on the afternoon of proestrus.

Histology

The female offspring were weighed and injected i.p. with bromodeoxyuridine (BrdU; 1.5 mg per 100 g body wt; Roche Molecular Biochemicals, Indianapolis, IN) 1.5 h before being killed by cervical dislocation. The fourth inguinal mammary glands were dissected out bilaterally. The right mammary gland was spread onto a glass slide, fixed with 4% (v/v) formaldehyde in 0.1 M PBS overnight, stained with Carmine Alum (Sig-

ma), dehydrated, and whole mounted with Permount (Fisher, Pittsburgh, PA).

The left mammary gland was fixed with 4% (v/v) formaldehyde in 0.1 M PBS for 8–16 h at room temperature and embedded with Paraplast paraffin (Fisher) under vacuum. Five-micrometer sections were cut on a Sorvall JB-4 microtome (DuPont, Wilmington, DE), mounted on Superfrost slides (Fisher), and stained with either hematoxylin and eosin (H&E) or with anti-BrdU as an index of DNA synthesis.

Immunohistochemistry

The BrdU was immunolocalized within paraffin sections of mammary glands using the streptavidin-biotin labeling system according to a modification of the technique described by Kass et al. [35]. Sections were hydrated through a series of alcohols, microwaved in 10 mM citrate buffer (pH 6) for antigen retrieval, subjected to acid hydrolysis for DNA denaturation, and treated for both endogenous peroxidase and nonspecific binding with methanol/ H_2O_2 and 2% (v/v) normal goat serum in 0.01 M PBS, respectively. Sections were incubated with monoclonal BrdU antibody IgG (1:400; clone 85-2C8, Novocastra, Burlingame, CA) overnight in a humid chamber at 4°C. Biotinylated goat anti-rabbit IgG (Roche Diagnostics Corp., Indianapolis, IN) was applied and visualized by streptavidin-horse radish peroxidase and diaminobenzidine (Sigma) to assess cellular incorporation of BrdU. Sections were lightly counterstained with Harris hematoxylin and mounted with a glass coverslip for light microscopy.

Morphometric Analysis

Digital images of whole-mounted mammary glands were captured with a SPOT-Real Time digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) attached to a dissecting microscope (Carl Zeiss, Inc., Thornwood, NY) using a 2.5 \times objective for the mammary glands of 10-day-old and 1-mo-old mice and a 4 \times objective for the mammary glands of 6-mo-old mice. Quantitative analyses of mammary gland dimensions in mice aged 10 days, 1 mo, and 6 mo were performed using the Optimus 6.5 program (Media Cybernetics, Silver Spring, MD). In 10-day-old mice, the total length of the mammary gland ductal tree was measured in its longitudinal axis. In 1-mo-old mice, the distance between the outermost edge of the lymph node and the furthest extension of the terminal end buds beyond the node was measured. For analysis of the mammary glands of 6-mo-old mice, a 120-point grid (www.mediacy.com) was generated within the Optimus program and superimposed on the digital images (magnified to 72 \times); the percentage of tissue occupied by ducts, terminal ducts, terminal end buds, and alveolar buds was then determined.

The incorporation of BrdU in mammary glands from mice aged 10 days, 1 mo, and 6 mo was determined quantitatively by viewing sections under a Zeiss microscope using a 100 \times objective with oil. A graticule was placed in the eyepiece to quantitate the percentage of BrdU-stained cells within epithelial structures (ducts, terminal ducts, terminal end buds, and alveolar buds) and stromal structures (adipocytes, fibroblasts, and endothelial cells) per total number of cells in approximately 50 randomly chosen fields per mammary gland.

The H&E-stained paraffin sections of mammary glands from 6-mo-old mice were assessed quantitatively to determine the percentage of ducts and alveoli that contained secretory product within their lumen.

Statistical Analysis

Data collected from whole-mounted mammary glands of 10-day-old and 1-mo-old mice were analyzed by one-way ANOVA. Differences between treatment groups were determined using a least-significant difference test. Data collected from whole-mounted mammary glands of 6-mo-old mice were analyzed by one-way ANOVA using a General Linear Model, taking into account differences due to litter. In this case, differences between treatment groups were determined using *t*-tests. To determine any interactions between age and BPA treatment for BrdU incorporation in the epithelium and stroma, a two-way ANOVA was performed on the data. The effects of age and treatment on BrdU incorporation in both tissue compartments were then assessed independently by one-way ANOVA, taking into account differences due to litter for the 6-mo-old mice. In this case, differences between treatment groups were determined using a least-significant difference test. Results were considered to be significant at $P < 0.05$ [36]. The statistical program SPSS (SPSS, Inc., Chicago, IL) was used for these analyses.

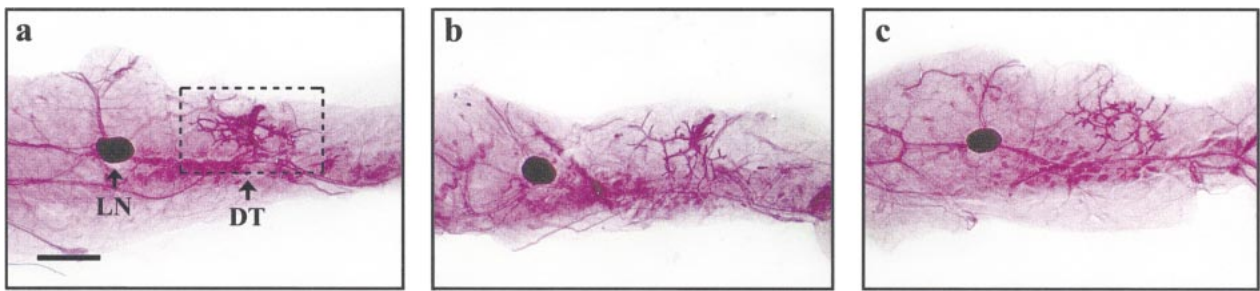


FIG. 1. Histoarchitecture of mammary glands from 10-day-old mice. A rudimentary ductal tree (DT; surrounded by box) in the area of the nipple and in close proximity to the lymph node (LN) is of similar dimensions in mice exposed to DMSO (**a**; control), 25 $\mu\text{g}/\text{kg}$ of BPA (**b**), and 250 $\mu\text{g}/\text{kg}$ of BPA (**c**) during in utero development from Days 9 to 20 of gestation. Bar = 0.95 mm.

RESULTS

Whole-Mounted Mammary Glands

Mammary glands of 10-day-old mice in both the control and BPA-treated groups comprised a rudimentary ductal tree within the vicinity of the nipple. Quantitative analysis of the total length of the ductal tree revealed no significant difference ($\pm\text{SEM}$) between the control (2.34 ± 0.12 mm), 25 $\mu\text{g}/\text{kg}$ of BPA (2.57 ± 0.18 mm), and 250 $\mu\text{g}/\text{kg}$ of

BPA (2.51 ± 0.26 mm) groups (Fig. 1). At 1 mo of age, the period that corresponds to the onset of puberty in CD-1 mice, the mammary glands had just extended beyond the edge of the lymph node. This penetration of predominantly ductal structures into the fat pad of the mammary gland was led by a front of large, characteristically bulbous terminal end buds (Fig. 2A). Quantitative analysis revealed that mice exposed in utero to 25 $\mu\text{g}/\text{kg}$ of BPA showed a greater ductal elongation beyond the edge of the lymph

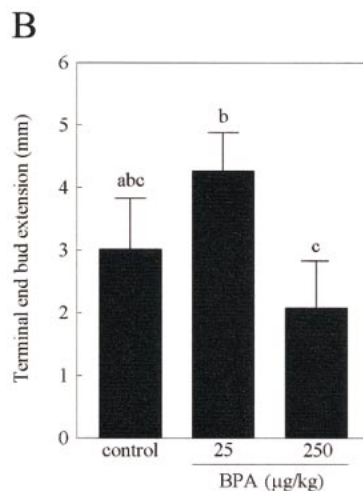
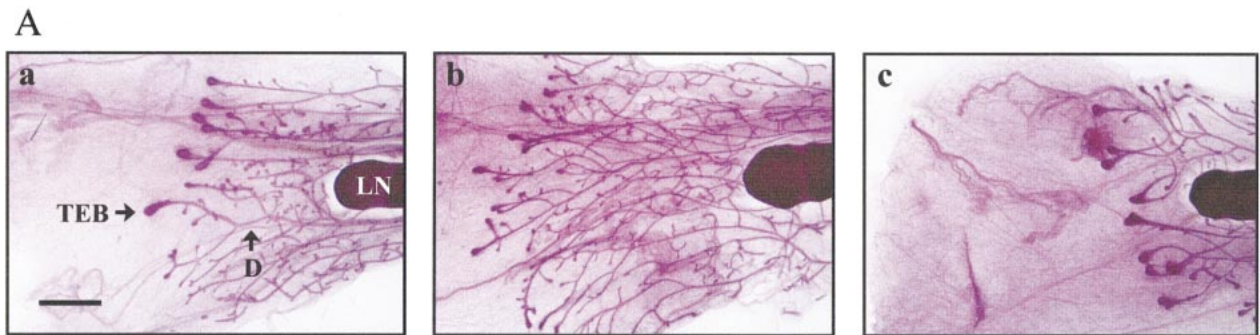


FIG. 2. Histoarchitecture of mammary glands from 1-mo-old mice. **A**) Changes in the extent of duct (D) elongation, via stromal invasion by terminal end buds (TEB), are shown in whole-mounted mammary glands from mice exposed to DMSO (**a**; control), 25 $\mu\text{g}/\text{kg}$ of BPA (**b**), and 250 $\mu\text{g}/\text{kg}$ of BPA (**c**) during in utero development from Days 9 to 20 of gestation. Ductal elongation was measured relative to the lymph node (LN). Bar = 0.95 mm. **B**) Histograms representing tissue changes in whole-mounted mammary glands. The length of ductal extension beyond the lymph node is shown in the mammary glands of mice exposed in utero to DMSO (control) or to 25 or 250 $\mu\text{g}/\text{kg}$ of BPA. Different letters represent a statistically significant difference ($P < 0.05$) between treatments.

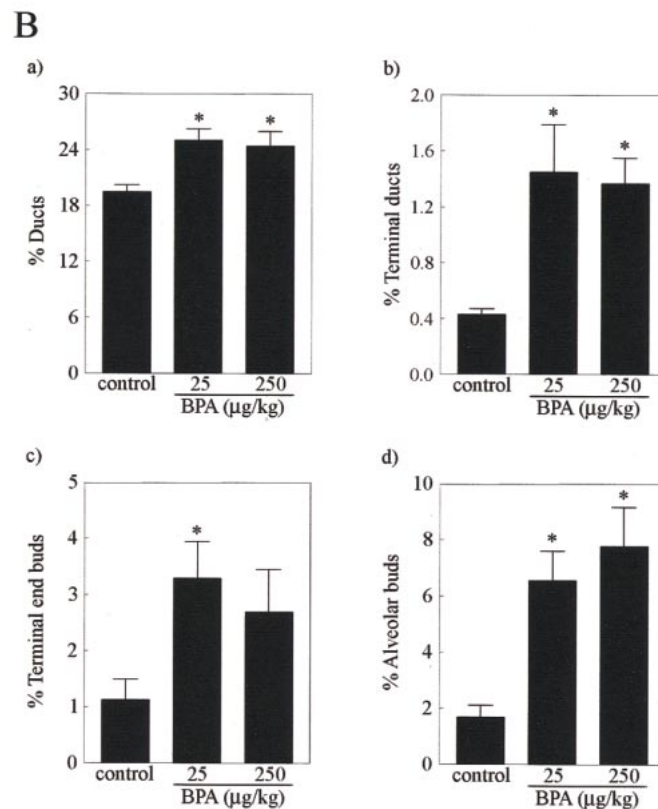
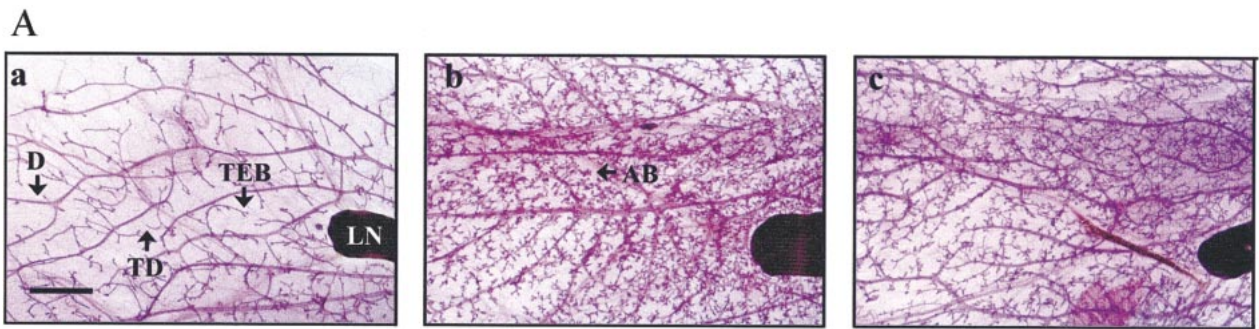


FIG. 3. Histoarchitecture of mammary glands from 6-mo-old mice. **A**) Differences are apparent in whole-mounted mammary glands from mice exposed to DMSO (**a**; control), 25 µg/kg of BPA (**b**), and 250 µg/kg of BPA (**c**) during in utero development from Days 9 to 20 of gestation. Note the proliferation of ducts (D), terminal ducts (TD), and terminal end buds (TEB) indicated in **a**, and of alveolar buds (AB) indicated in **b**, which are apparent in both BPA-treated groups. Bar = 1.49 mm. **B**) Histograms representing tissue changes in whole-mounted mammary glands. Quantitation of ductal-alveolar structures reveals increases in the relative area of ducts (**a**), terminal ducts (**b**), terminal end buds (**c**), and alveolar buds (**d**) in mammary glands of mice exposed in utero to 25 or 250 µg/kg of BPA relative to the DMSO control. The asterisk denotes a statistically significant difference ($P < 0.05$) relative to the DMSO control.

node (4.27 ± 0.61 mm), whereas those exposed to 250 µg/kg of BPA showed retarded growth (2.08 ± 0.45 mm) relative to the control group (3.02 ± 0.81 mm) (Fig. 2B). Although no statistically significant difference was found between the control and either of the BPA groups for this parameter, a significant difference was found between the 25 and 250 µg/kg of BPA groups ($P < 0.05$) (Fig. 2B).

By 6 mo of age, the mouse mammary glands showed a dramatic expansion of the ductal network, such that it filled the entire fat pad. Typically, virgin CD-1 mice of this age develop a ductal tree that comprises terminal ducts, terminal end buds (though far less bulbous than those seen during the pubertal period), and very few alveolar buds. Quan-

titative analysis of these epithelial structures revealed that in utero exposure of mice to both 25 and 250 µg/kg of BPA resulted in a significant increase of all ductal and alveolar structures relative to the control group (Fig. 3A). In response to exposure to 25 and 250 µg/kg of BPA, the mammary glands exhibited a 29% ($P < 0.01$) and a 25% ($P < 0.05$) increase, respectively, in the relative area of ducts; a 237% ($P < 0.05$) and a 219% ($P < 0.05$) increase, respectively, in the relative area of terminal ducts; a 192% ($P < 0.05$) and a 139% increase, respectively, in the relative area of terminal end buds; and a 288% ($P < 0.01$) and a 361% ($P < 0.01$) increase, respectively, in the relative area of alveolar buds (Fig. 3B).

BrdU Incorporation (DNA Synthesis)

The developmental pattern of BrdU incorporation within the epithelium of the mammary gland was affected by BPA exposure. In control mice, a significant difference was found between the percentage of cells that incorporated BrdU from 10 days through to 6 mo of age ($P < 0.05$), with puberty (1 mo of age) being the most active period of DNA synthesis (Fig. 4A). In utero exposure to BPA dampened these age-related differences in BrdU incorporation, such that no significant difference was found for this variable between 10 days, 1 mo, and 6 mo of age (Fig. 4, A and C). At 10 days of age, the percentage of epithelial cells that incorporated BrdU was decreased by 52% in the 25 $\mu\text{g}/\text{kg}$ of BPA group ($P < 0.01$) and by 36% in the 250 $\mu\text{g}/\text{kg}$ of BPA group ($P < 0.05$) (Fig. 4A).

The developmental pattern of BrdU incorporation within the stroma of the mammary gland was also affected by BPA exposure. In all treatment groups, a significant difference was found between the percentage of cells that incorporated BrdU from 10 days through to 6 mo of age ($P < 0.05$) (Fig. 4B). In the control group, 10 days of age was the most active period of DNA synthesis; however, in utero exposure to BPA increased DNA synthesis at 6 mo of age as well. The percentage of stromal cells that incorporated BrdU was decreased by 32% ($P < 0.05$) in the 250 $\mu\text{g}/\text{kg}$ of BPA group at 1 mo of age, but it was increased by 56% in the 25 $\mu\text{g}/\text{kg}$ of BPA group ($P < 0.05$) and by 95% in the 250 $\mu\text{g}/\text{kg}$ of BPA group ($P < 0.05$) at 6 mo of age (Fig. 4B).

A comparison of the ratio of cells that incorporated BrdU between the epithelium and stroma at the different developmental ages revealed a difference in pattern due to BPA exposure (Fig. 4D). This was most apparent in the 1-mo-old mice, in which the ratio of BrdU-positive epithelial to stromal cells was approximately 4:1 in the control group yet approximately 2:1 in the 25 $\mu\text{g}/\text{kg}$ of BPA group and 6:1 in the 250 $\mu\text{g}/\text{kg}$ of BPA group.

Presence of Secretory Product

Analysis of mammary glands from 6-mo-old mice revealed a difference between treatments in the presence of secretory product within the lumina of epithelial structures (Fig. 5A). The mammary glands of mice exposed in utero to 25 $\mu\text{g}/\text{kg}$ of BPA showed a 60% increase ($P < 0.05$) in the percentage of alveoli that contained secretory product; no significant change was found in this parameter in the mice exposed in utero to 250 $\mu\text{g}/\text{kg}$ of BPA (Fig. 5B). The percentage of ducts (comprising the ducts, terminal ducts, and terminal end buds) that contained secretory product within their lumina was not significantly different between the control and BPA-treated groups.

DISCUSSION

Studies regarding the effects of estrogen exposure during perinatal development on the mouse mammary gland have focused primarily on estradiol and DES. These studies have used pharmacological doses and described effects at discrete periods in the developmental process [21, 37–40]. The present study is novel, to our knowledge, in that it describes the effects of prenatal exposure to low doses of BPA during neonatal, pubertal, and adult periods of development. It is noteworthy that the observed dysgenesis was induced in CD-1 mice, a strain that demonstrates particular resistance to the effects of estradiol (determined on the basis of tes-

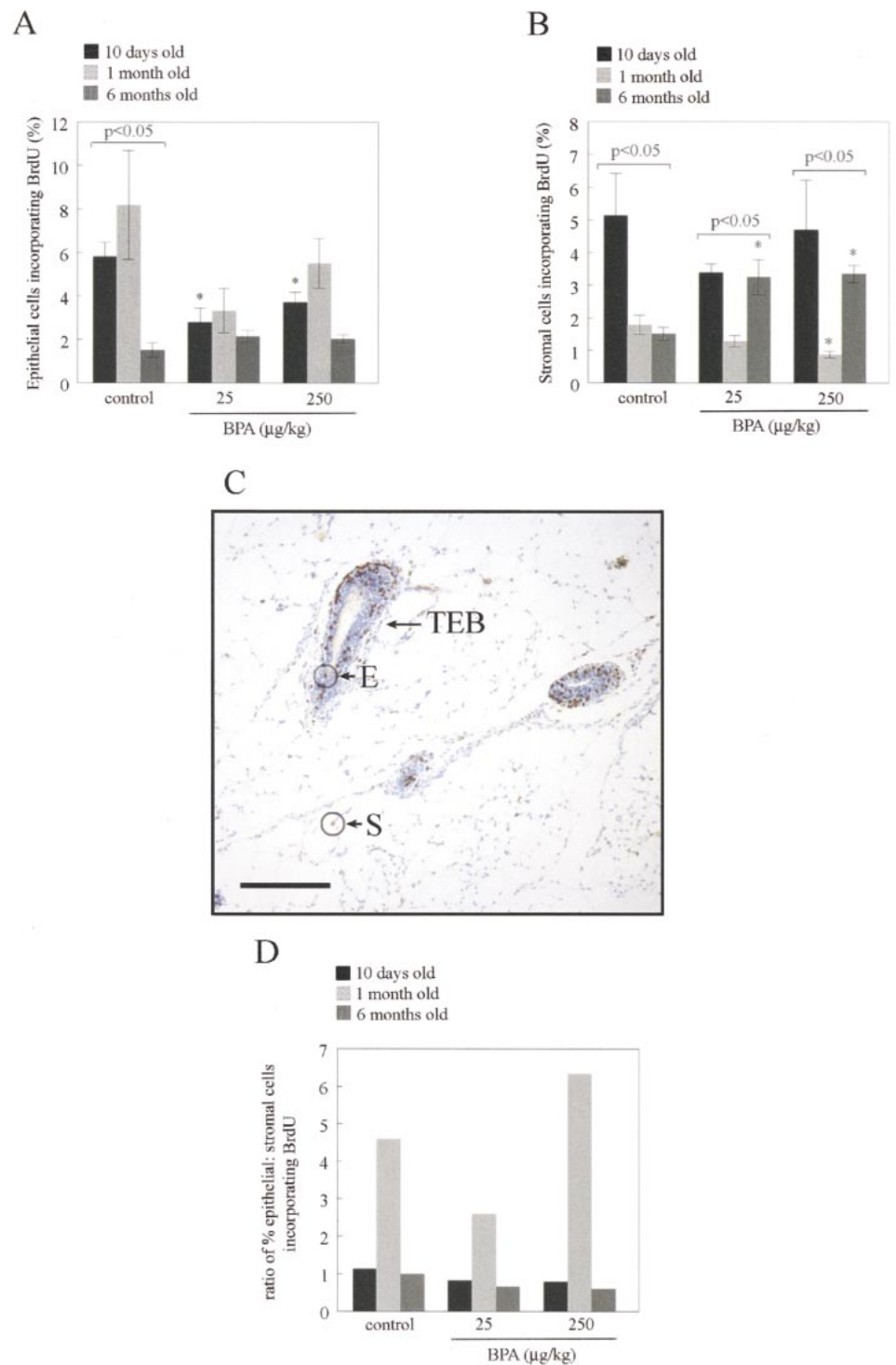
ticular parameters) [41]. We have demonstrated that changes in the mammary gland occurred at a BPA dose that is 1/4000 lower than that detected by the standard assay for estrogen activity, the uterotrophic assay [42].

In utero exposure to BPA induced changes in the timing of developmental events within the epithelium and stroma of the mammary gland, resulting in a tissue resembling that of early pregnancy. These changes were observed at two levels of complexity, the biochemical level (DNA synthesis) and the morphological level (histoarchitecture). In the control mice, DNA synthesis was comparable between the epithelium and stroma (ratio, 1:1.1) at 10 days of age. At puberty, however, this ratio increased by more than fourfold, reflecting the rapid invasion of the ductal network into the stroma. By 6 mo of age, both tissue compartments showed comparable DNA synthesis once again. In utero exposure to BPA altered this relationship throughout development. In both groups of BPA-exposed mice, DNA synthesis was decreased in the epithelium at 10 days of age and in the stroma at puberty (250 $\mu\text{g}/\text{kg}$ of BPA only), yet increased in the stroma at 6 mo of age. This phenomenon resulted in an altered ratio of DNA synthesis between the epithelium and stroma throughout development, and it portends the disruption of other molecular and biochemical signals that are critical to the normal growth and function of the mammary gland [43].

Exposure to BPA induced changes in the histoarchitecture of the mammary gland that were most apparent at 6 mo of age. Mice exhibited a significant increase in all epithelial structures, including a greater than 300% increase in the relative area of alveolar buds. Coupled with the increased presence of secretory product in alveoli, this tissue from virgin mice resembled that of early pregnancy [43], a period when extensive morphological and biochemical changes occur under the influence of increasing levels of estrogen, progesterone, and prolactin [44]. In the BPA-treated mice, changes in epithelial cell DNA synthesis, which registered at developmental ages earlier than 6 mo, did not always correlate with a morphological counterpart. Also, contrary to our expectations, no differences were found in epithelial DNA synthesis between the control and BPA-treated mice at 6 mo of age, despite a significant increase in ductal/alveolar structures in the latter group. This suggests that either increased cell proliferation or decreased cell death occurred in the epithelial compartment before this age. The increased DNA synthesis within the stroma at this age is puzzling given the critical role that it plays in signaling development of the epithelial ductal network.

The pharmacokinetics and pharmacodynamics of BPA that act during fetal development are important to our understanding of how BPA induced the observed morphological changes in the mammary gland. Neither of these subjects has been established in this model. In contrast, several studies have addressed these issues in rats using BPA at doses that are orders of magnitude higher than those used in the present study. Pottenger et al. [45] reported that the time to nonquantifiable levels measured in plasma of adult, nonpregnant female rats was 120 h (10 mg/kg of BPA) and 168 h (100 mg/kg of BPA) after s.c. administration. Takahashi et al. [46] found that 1 g/kg of BPA administered orally rapidly crossed the placenta and was more bioavailable in the fetus' than in the mother's blood, as indicated by differences in values for the area under the concentration-time curve and the mean residence time. Estrogen receptors α and β are first detected in the mouse mammary gland primordium at Day 12.5 of gestation [47], and al-

FIG. 4. Developmental changes in BrdU incorporation within mammary glands. **A)** Histograms representing immunolocalization of BrdU in the epithelium of mammary glands in mice at 10 day, 1 mo, and 6 mo of age following in utero exposure to DMSO (control) or 25 or 250 $\mu\text{g}/\text{kg}$ of BPA. For each treatment, $P < 0.05$ denotes a significant difference in BrdU incorporation at different developmental ages of the mice. The asterisk denotes a statistically significant difference for the BPA-treated groups ($P < 0.05$) relative to the DMSO control. **B)** Histograms representing immunolocalization of BrdU in the stroma of mammary glands in mice at 10 days, 1 mo, and 6 mo of age following in utero exposure to DMSO (control) or 25 or 250 $\mu\text{g}/\text{kg}$ of BPA. For each treatment, $P < 0.05$ denotes a significant difference in BrdU incorporation at different developmental ages of the mice. The asterisk denotes a statistically significant difference for the BPA-treated groups ($P < 0.05$) relative to the DMSO control. **C)** Photomicrograph showing immunolocalization of BrdU within cells of the epithelium (E) and stroma (S) in a mammary gland from a mouse exposed in utero to 250 $\mu\text{g}/\text{kg}$ of BPA. Note the high incorporation of BrdU within the terminal end bud (TEB). Monoclonal antibody BrdU (clone 85-2C8); stained with 3,3' diaminobenzidine and counterstained with Harris hematoxylin. Bar = 0.2 mm. **D)** Histogram representing the ratio of the percentage of epithelial cells incorporating BrdU to the percentage of stromal cells incorporating BrdU in the mammary glands of mice exposed in utero to DMSO (control) or 25 or 250 $\mu\text{g}/\text{kg}$ of BPA.



though BPA binds both receptors [48, 49], its binding affinity to α -fetoprotein is negligible [50]. This protein acts as a sink for endogenous estrogens during critical stages of fetal and neonatal development [51]. Thus, the potential for BPA to readily bind ER and exert its effects may be greater than that for 17β -estradiol. Despite the early ontogeny of ER in the mouse mammary gland, the tissue typically does not show a proliferative response to estradiol until 3 wk of age [52]. This is consistent with the present results, in which changes in the histoarchitecture of the ductal network were not apparent until mice were 1 mo of age, but it does not rule out the possibility that BPA induced mal-

formations in the mammary gland primordium. Indeed, BPA-exposed mice showed significant changes in BrdU incorporation within the mammary gland epithelium by 10 days of age.

Morphological changes have also been described in fetal rodents following in utero exposure to pharmacological doses of estrogen [53, 54]. The pattern of ductal retardation at puberty followed by extensive growth in adulthood, as was observed in the present study after in utero exposure to 250 $\mu\text{g}/\text{kg}$ of BPA, is consistent with findings in mice exposed neonatally (Days 1–5 postpartum) to high doses of estradiol and DES [37, 38, 55].

A

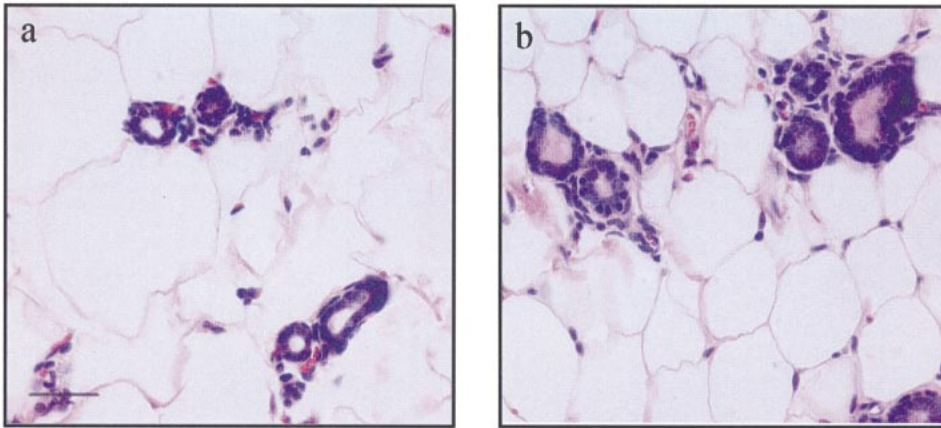
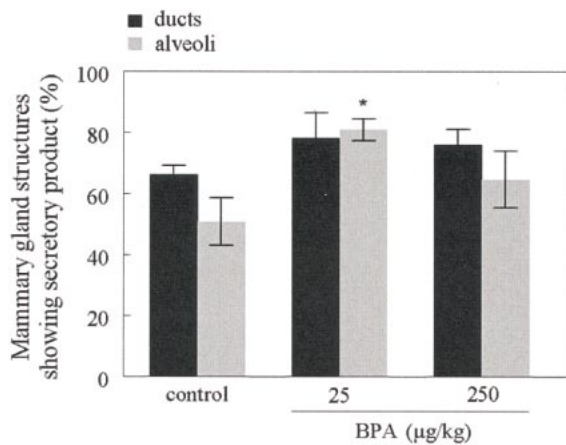


FIG. 5. Presence of secretory product in the mammary glands of 6-mo-old mice. **A)** Photomicrograph showing accumulation of secretory product within the ducts and alveoli of mammary glands from mice exposed in utero to DMSO (**a**; control) or 25 µg/kg of BPA (**b**). H&E stain. Bar = 0.06 mm. **B)** Histogram representing changes in the presence of secretory product within ducts and alveoli of mammary glands from mice exposed in utero to either DMSO (control) or 25 or 250 µg/kg of BPA. The asterisk denotes a statistically significant difference ($P < 0.05$) relative to the DMSO control.

B



The mechanisms by which BPA affects the morphology and secretory function of the mammary gland long after the period of exposure are largely unknown. One pathway may involve the direct action of BPA on genes that regulate mesenchymal-epithelial interactions [56] and differentiation/expansion of the tubuloalveolar network in the mammary gland [57]. Misexpression of these genes is associated with mammary gland dysgenesis and carcinogenesis [58]. Estradiol regulates certain homeobox genes [58–60]; thus, BPA may also have this effect. Prenatal exposure to DES down-regulates the expression of Wnt7a in the uterus [61] and AbdB Hoxa in the müllerian duct [62], and this correlates with structural abnormalities of these organs. An additional pathway by which BPA may affect mammary gland development is indirect, by disrupting the hypothalamic-pituitary-ovarian axis. This would alter the secretory patterns of pituitary and ovarian hormones, which are important in postnatal mammary gland morphogenesis. The observations that perinatal exposure of rats to low-dose BPA reduces serum LH levels in adulthood [31] and alters the behavior of animals, suggesting a change in sexual differentiation of the brain [63], are consistent with this hypothesis.

The association between fetal exposure to BPA and induction of mammary gland carcinogenesis is speculative at this stage. In the present study, two findings may suggest a predisposition of the BPA-exposed mammary gland to neoplastic change. The altered relationship in DNA synthesis between the epithelium and stroma that was observed at all stages of development is striking, because disruption of the communication between these tissue compartments is acknowledged as being critical to the development of neoplasia within both human breast and rodent mammary gland [64, 65]. In addition, the significant increase in terminal ducts and terminal end buds in the 6-mo-old mice is also remarkable, because an increase in these structures in rats [66] and humans [67] correlates positively with the incidence of carcinomas that arise specifically from such sites.

In summary, in utero exposure to low, presumably environmentally relevant doses of BPA changes the timing of DNA synthesis in the epithelium and stroma of the mammary gland, resulting in an histoarchitecture that is atypical for a virgin mouse. These changes, which are apparent long after the period of exposure is over, strengthen the hypothesis that in utero exposure to environmental estrogens may

predispose the developing fetus to mammary gland carcinogenesis in adulthood.

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