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Perinatal exposure to the xenoestrogen bisphenol-A induces mammary intraductal hyperplasias in adult CD-1 mice

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ABSTRACT

Humans are routinely exposed to bisphenol-A (BPA), an estrogenic compound that leaches from consumer products. Given the sensitivity of the developing organism to hormones, exposure of fetuses and infants is a concern. Here, CD-1 mice were exposed to environmentally relevant doses of BPA during gestation and the lactational period (gestational day 8 through postnatal day 16). At 3, 9 and 12–15 months of age, mammary glands from exposed offspring were examined for structural changes. BPA-exposed females demonstrated altered mammary phenotypes including the appearance of alveolar buds. Additionally, intraductal hyperplasias were observed exclusively in BPA-exposed females. These lesions had the appearance of "beaded" ducts, with epithelial cells present inside the ductal lumen and increased proliferation indexes compared to normal ducts. Similar structures have also been observed following exposure to other estrogens. These results are further evidence that perinatal BPA exposure can alter the morphology of the rodent mammary gland in adulthood.

1. Introduction

Estrogens are involved in many aspects of female sexual development including organogenesis and maintenance of the reproductive tract and secondary sexual characteristics, as well as the regulation of the menstrual cycle, pregnancy and lactation. Estrogens also mediate cell proliferation in cells containing estrogen receptor (ER) and can bind to estrogen responsive elements to regulate the expression of select genes.

In humans, exposure to estrogens throughout life is the main known risk factor for breast cancer [1]. The positive correlation between increased intrauterine levels of estrogens observed in twin births and breast cancer in daughters born from such pregnancies also supports this link [2]. Additionally, women exposed therapeutically to the potent synthetic estrogen diethylstilbestrol (DES) while pregnant have a higher incidence of breast cancer [3]. Their daughters, so-called "DES daughters", are now reaching the age at which breast cancer is prevalent. A recent study reported that the incidence of breast cancer in DES daughters age 40 and older was significantly increased compared to unexposed women [4].

Bisphenol-A (BPA), a xenoestrogen used in the manufacturing of polycarbonate plastics and epoxy resins, has been shown to leach from food and beverage containers [5–7], and dental sealants and composites [8] under normal conditions of use [9]. BPA is a full agonist that binds both ERα and ERβ, although at lower affinities than estradiol [10,11]. Additional evidence suggests that BPA may be as potent as estradiol in mediating effects modulated via the membrane ER (reviewed in [12]). In 2005, a study conducted by the Centers for Disease Control and Prevention (CDC) examined 394 Americans and reported that BPA was found in 95% of urine samples [13] indicating that humans are routinely exposed to this chemical. A more recent CDC study of over 2500 Americans supports this finding with BPA detected in 92.6% of participants [14]. Urine concentrations ranged from 0.4 to 149 μg/L with a geometric mean of 2.6 μg/L and were significantly higher in children and adolescents compared to adults. Several studies have reported the presence of BPA in the serum of pregnant women, umbilical cord blood, amniotic fluid and fetal plasma [15–19]; these reports suggest that human fetuses are exposed to BPA during gestation. Additionally, BPA has been detected in human breast milk [20,21] indicating that exposure during lactation is also likely.

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Because of its widespread presence in the environment and its estrogenic activity *in vivo* and *in vitro* [22–25], the potential for adverse effects of BPA exposure on human health is a concern. Exposure to xenoestrogens such as BPA during early development may have contributed to the increased incidence of infertility, genital tract abnormalities, and breast cancer observed in European and US human populations over the last 50 years [26–28]. In fact, exposure of rodents to low doses of BPA during perinatal development has been shown to induce early vaginal opening [29], advance the onset of puberty [30], disrupt estrous cyclicity [31,32] and decrease serum levels of luteinizing hormone following ovariectomy [32]. In female rodents, perinatal BPA exposure alters the development of estrogen-sensitive organs including the brain [33], vagina [34], ovaries [31,35], uterus [31,36], and the mammary gland [28,31,37–39].

Previous work in our laboratory has focused on the effects of perinatal exposure to BPA on mammary gland development (from gestational day 8 to postnatal day 2). Effects of BPA exposure were observed in morphological endpoints in the stromal and epithelial compartments as early as embryonic day (E)18 [38]. While normal pubertal mammary gland development is characterized by expansion of the epithelial tree into the surrounding stromal tissue, *in utero* exposure to BPA caused a decreased invasion of the stromal compartment, an increased number of the highly proliferative structures known as terminal end buds and an enhanced sensitivity to estradiol [28,39]. At 4 months of age, these animals had a significant increase in lateral branching, a phenomenon regulated by progesterone [28]. By 6 months of age, BPA-exposed animals demonstrated an overall increase in epithelial structures including terminal ducts and a premature appearance of alveolar buds, normally associated with pregnancy in the mouse [37]. Several of these changes suggest an increased risk of developing mammary cancer.

The effects of prolonged exposure to BPA through lactation have not yet been determined. ER is initially expressed in the developing mammary gland at E12.5 in the mesenchyme surrounding the epithelial bud [40] and at E18 is detected predominantly in the stroma with only punctate expression in the epithelium [38] suggesting that gestational BPA exposure is likely targeting ER in the mammary gland stroma. However, ER expression is mainly localized to the epithelium at postnatal time points [41]. Therefore, exposure during gestation and the lactation period during which the pups are reliant solely on maternal milk for their nutrition (through postnatal day 16) is likely to lead to phenotypes that are not predicted by gestational exposure alone.

We have now examined the effects of exposure to BPA from gestational day 8 through postnatal day 16 on the morphology of the adult mammary gland. At 3 months of age, we observed the appearance of alveolar buds in BPA-exposed animals, a structure that is normally associated with pregnancy in the mouse. Additionally, whole-mounted mammary glands from BPA-exposed females were examined for preneoplastic and neoplastic lesions at 3, 9, and 12–15 months of age. Intraductal hyperplasias were observed exclusively in the BPA-exposed females, and these lesions were characterized in greater detail.

2. Materials and methods

2.1. Animals

Sexually mature CD-1 mice (Charles River, MA) were maintained in temperature and light controlled (14 h light, 10 h dark, lights on at 04:00 h) conditions at the Tufts Medical Center Animal Facility in accordance with the Guide for Care and Use of Laboratory Animals. The cages and bedding were extracted using methods described previously [42]. Briefly, each item was incubated at 37 °C for 1 h with 100 ml of HPLC grade methanol. The methanol was collected and dried down to completion with nitrogen gas and the residue was resuspended in sterile medium containing 5% charcoal–dextran-stripped (estrogen-free) fetal bovine serum. All tested negligible for estrogenicity using the E-SCREEN assay [42]. Food (Harlan Teklad 2018) was supplied *ad libitum* and was extracted using a method outlined in [42]. Briefly, the solid was homogenized and extracted with 10 mM sodium acetate. These extracts were cleaned by methanol and *n*-hexane extraction followed by extraction with dichloromethane which was then passed through a Sep-Pak C-18 cartridge. Xenobiotics were then eluted from the column with *n*-hexane, dried down and resuspended as described above. Estrogenicity of feed was measured at ≤20 femtomoles of estrogen equivalents per gram, a negligible amount [42]. Water was supplied by glass bottles only.

Animals were allowed to adapt to the animal facility for several days before being placed together for mating. The morning on which a vaginal plug was detected was considered pregnancy day 1. On the evening of pregnancy day 8, dams were weighed and implanted subcutaneously with Alzet osmotic pumps (Alza Corp, Palo Alto, CA, model 2004) designed to deliver 50% dimethyl sulfoxide (DMSO; vehicle control) or BPA (Sigma) in 50% DMSO. These pumps continued to release at a constant rate (0.25 μl/h) until day 16 of lactation. Exposure groups included: 0 (control), 0.25 (0.25BPA), 2.5 (2.5BPA), or 25 (25BPA) μg BPA/kg BW/day. Dams were allowed to deliver naturally and the litters were culled to eight pups per mother on the day after birth. Litters were weaned on postnatal days 22–24.

2.2. Whole-mounts and paraffin embedding

At 3, 9, and 12–15 months of age, female offspring were killed; an incision was made along the skin at the ventral midline and the fourth inguinal mammary glands were dissected from the skin. One mammary gland was immediately immersed in phosphate buffered formalin overnight and prepared for paraffin sections using the methods described previously [28]. The second mammary gland was spread on a Superfrost positive charged glass slide (Fisher) and placed in phosphate buffered formalin overnight. Whole-mounted mammary glands were processed and stained with Carmine-alum using the methods described previously [28].

2.3. Whole-mount morphometrics

Digital images of whole-mount mammary glands were visualized using a Zeiss Stemi 2000-C dissection scope using a 2× objective and images were captured at 3900 dpi with a Zeiss AxioCam HRc digital camera (Carl Zeiss, Inc., Thornwood, NY). First, the entire whole-mounted mammary gland was examined and the number and location of ducts with a beaded appearance was recorded. Second, quantitative analyses of mammary gland dimensions were performed using the Zeiss AxioVision program version 4.4. For the morphometric analysis of mammary gland whole-mounts, one image was taken of each whole-mount in the area just anterior to the central lymph node. A total of 4–20 whole-mounts were examined for each treatment and time point (see Table 4). To analyze the percentage of tissue occupied by ducts, terminal ducts, and alveolar buds (including lobuloalveolar units), a 130-point grid was superimposed on each image and the structure at each crosshair was counted. The volume fraction of each structure was calculated as the number counted at crosshairs/130 crosshairs × 100 as described previously [43]. For a randomly positioned point grid, the number of points hitting the phase of interest (i.e. ducts, alveolar buds, etc.) divided by the number hitting the whole field of view gives an unbiased estimate of volume fraction. The area of the phase of interest per unit area of the reference space is an excellent predictor of the volume of the phase of interest per unit volume [43].

2.4. Excision of ducts from whole-mounts

In some whole-mounts, epithelial ducts were excised using a scalpel with the aid of a dissection scope. Some excised ducts were placed on Superfrost slides and mounted with permanent mounting medium and glass coverslips for confocal imaging. Other excised ducts were washed with xylene, infiltrated with paraffin under vacuum, and embedded in paraffin positioned parallel or perpendicular to the tissue mold cassettes. Longitudinal and cross-sections of the excised ducts were obtained.

2.5. Confocal imaging of normal and "beaded" ducts

Because the whole-mount excisions were stained with Carmine-alum, which autofluoresces, Z-series optical sections (0.98 μm) were collected on excised ducts using a Zeiss LSM510 Confocal microscope. Ducts were visualized using a HeNe laser with an excitation wavelength of 633 nm and a reflective (detected) wavelength of 650 nm. Images were collected using a Plan-Apochromat 20× objective.

2.6. Histological staining

Sections were treated with xylene to remove paraffin and rehydrated through a series of alcohols and distilled water. Sections were then stained with hematoxylin and eosin, PAS, von Kossa, or Masson's trichrome according to standard protocols. Samples were dehydrated and mounted with a permanent mounting medium (Sigma). Sections were viewed through a Zeiss Axioskop 2 plus light microscope at 10× and 40×. Images were captured at 3900 dpi using the AxioCam HRc digital camera.