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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.

Keywords

Bisphenol-A; xenoestrogen; endocrine disruptor; mammary gland; intraductal hyperplasia; critical period; lactation; breast cancer

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

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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 [5,6,7], and dental sealants and composites [8] under normal conditions of use [9]. BPA is a full agonist that binds both ER α and ER β , 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 0.4–149 $\mu\text{g/L}$ with a geometric mean of 2.6 $\mu\text{g/L}$ 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 [15,16,17,18,19]; 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.

Because of its widespread presence in the environment and its estrogenic activity *in vivo* and *in vitro* [22,23,24,25], 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 [26,27,28]. 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 [37,31,28,38,39].

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.

Materials and Methods

Animals

Sexually mature CD-1 mice (Charles River, MA) were maintained in temperature and light controlled (14 hour light, 10 hour dark, lights on at 0400 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 hour with 100ml 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 10mM 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/hour) 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 8 pups per mother on the day after birth. Litters were weaned on postnatal days 22–24.

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].

Whole-mount morphometrics

Digital images of whole-mount mammary glands were visualized using a Zeiss Stemi 2000-C dissection scope using a 2x 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 timepoint (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 $\times$ 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].

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.

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 μ m) 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 20x objective.

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 10x and 40x. Images were captured at 3900 dpi using the AxioCam HRC digital camera.

Immunohistochemistry

Paraffin sections of whole mammary glands were treated with xylene, rehydrated, and then heated in 10 mM citrate buffer (pH 6) for antigen retrieval as described previously [28]. Antigen retrieval was not performed on sections of ducts excised from whole-mounts because of their fragility, thus requiring different concentrations of antibodies to achieve a positive signal (see Table 1). Blocking of non-specific binding and treatment with antibodies

was performed as described previously [28]. Samples were counterstained with Harris' hematoxylin, dehydrated, and mounted with a permanent mounting medium. Sections were viewed through a Zeiss Axioskop 2 plus light microscope with 5x, 10x, 40x, and 100x objectives. Images were captured at 3900 dpi using the AxioCam HRC digital camera. To quantify Ki67, ER α and progesterone receptor (PR) expression in normal ducts, all epithelial cells in three arbitrarily chosen 40x fields were counted, with at least 200 cells assessed for each animal. For each antibody, 4–6 animals were examined per treatment. To calculate the proliferation index of epithelial ducts, the number of Ki67 positive cells was divided by the total number of cells counted.

Statistics

The SPSS statistical software package 15.0 (SPSS Inc., Chicago, IL) was used for all statistical analyses. ANOVA followed by Bonferroni posthoc tests were used to assess differences between treatment groups for each exposure paradigm. A Chi Square test was performed to compare the incidence of beaded ducts in the mammary gland epithelium of control and BPA-exposed animals. To account for litter effects, one animal was randomly selected from each litter for each endpoint. For all statistical tests, results were considered significant at $p < 0.05$. All results are presented as mean $\pm$ SEM and they were collected under blind conditions.

Results

Morphometric analysis of BPA-exposed glands

Mammary glands were examined from 3-month old control, 0.25BPA, 2.5BPA, and 25BPA female mice. Significant differences were noted for the volume fraction of alveolar buds for the 0.25BPA group compared to all other treatment groups (Figure 1 and Table 2A). These animals also had a decrease in the volume fraction of ducts (Table 2A) and an increase in total epithelial structures (data not shown), although these differences were not statistically significant. There were no significant differences in body weight to account for the presence of alveolar buds in the 0.25BPA animals (controls: 41.7 ± 2.1 g; 0.25BPA: 36.8 ± 1.5 g; 2.5BPA: 40.0 ± 2.8 g; 25BPA 41.1 ± 2.3 g). There were also no qualitative differences in estrous cycles or significant differences in uterine wet weight in any group (data not shown.)

A similar analysis was performed at 9 months of age. The volume fraction of alveolar buds was significantly increased in the 2.5BPA group compared to control females (Table 2B). There was also a significant decrease in the volume fraction of ducts in the 0.25BPA group compared to controls (Table 2B). Additionally, the volume fraction of alveolar buds in the 0.25BPA group was almost twice as large as the controls, but this difference was not significant, likely because of the increased alveolar bud volume fraction in some controls compared to earlier ages, as was seen previously [31]. The volume fraction of terminal ducts did not vary, regardless of treatment (Table 2B). In these animals there was a significant difference in body weight ($p < 0.02$), but the difference was between the 25BPA group compared to all three other groups (controls: 52.4 ± 1.6 g; 0.25BPA: 51.3 ± 2.5 g; 2.5BPA: 46.8 ± 2.8 g; 25BPA: 61.2 ± 1.7 g). This indicates that the increase in volume fraction of alveolar buds was unrelated to body weight. As with the 3-month old animals, there were no differences in uterine wet weight or the quality of estrous cycles to account for the observations made in the mammary gland (data not shown.)

Finally, we examined mammary glands from 9-month old animals for alterations in cellular parameters using sections stained with Masson's Trichrome. No significant differences were detected in the width of the periductal stroma or in optical density of fibrous collagen in this region (Table 3). Additional measurements were made following immunohistochemical

analysis of Ki67, ER α and PR. No quantitative differences were observed in the percentage of cells stained positive for any of these markers (Table 3).

Presence of “beaded ducts” in adult mice

Detailed examination of the mammary gland demonstrated the presence of ducts with a beaded appearance. These ducts were present exclusively in animals exposed to BPA (Table 4). Beaded ducts were observed in only 1 animal at 3 months of age, but in multiple animals at 9 and 12–15 months of age. Of note, however, is the lack of beaded ducts in the 12–15-month old 25BPA females. It is unclear whether this finding is due to small sample size or regression of beaded ducts in this group during later life. It is worth noting that data from a previous study in rats showed a similar trend [44].

Ducts with a beaded appearance were generally found between the lymph node and the terminal edge of the gland and were rarely seen in the nipple region or in the ducts between the nipple and the lymph node (data not shown). In some animals, these beaded ducts were quite extensive and covered a significant portion of the gland (Figure 2A), while in other animals they were only found sparsely (Figure 2B). These beaded ducts were excised and some were examined by confocal imaging. In comparison to a normal duct (Figure 2C) the beaded ducts from the 3-month old animal looked as if they were filled with cells, leaving a scalloped lumen (Figure 2D). This distinct scalloped shape was more pronounced in the 9-month old animals (Figure 2E); some ducts had areas where the lumen was completely filled in by epithelial cells. These distinguishable structures were present in the oldest set of animals as well (12–15-month old, data not shown.)

Histological staining of beaded ducts

Hematoxylin and eosin staining of beaded ducts excised from whole-mounted mammary glands highlighted several aspects of their morphology. The beaded aspect of the ducts was due to the presence of epithelial cells growing inside the ductal lumen, forming bridges between the duct’s walls; mitotic figures were visible in some areas, indicating that these cells were actively proliferating. It is worth noting that proliferating cells are generally not apparent in normal adult virgin mammary ductal epithelium. These beaded ducts were classified as intraductal hyperplasias using the Annapolis criteria, i.e. an increase in cell number without cytologic atypia [45] (Figure 3A).

We also observed that many beaded ducts contained eosinophilic secretions. In some ducts, these secretions stained with two different intensities (Figure 3B). The darker secretions often appeared to push away the ductal epithelium, indicating that there may be a solid component to them. von Kossa’s stain was used to identify possible concretions and the results suggested that these denser secretions are not calcified (data not shown). Thus, the varied appearances of these secretions may be due to the “trapping” of fluid within discrete areas of the ductal lumen; differential reabsorption of some of the secretory components could have then contributed to their variable content and appearance. PAS staining indicated that there is an intact basement membrane around the epithelium of beaded ducts, even in those ducts where secretions had displaced the luminal epithelial cells (Figure 3C).

Finally, the periductal stroma of these beaded ducts was highly eosinophilic (Figure 3A–C). Masson’s trichrome staining illustrated that the periductal stroma of most beaded ducts is high in fibrous collagen (Figure 3D). Small to mid-sized blood vessels were located adjacent to most beaded ducts and occasional areas with brownish staining suggested the presence of active lysosomes, perhaps in immune cells such as macrophages in the periductal stroma surrounding beaded ducts (Figure 3D). Staining with toluidine blue also indicated the presence of mast cells around these ducts (Figure 3E).

In some sections of beaded ducts, a second phenotype was observed; longitudinal sections appeared to indicate that ducts were branching, then merging back together, and then branching again (Figure 4A). Because this type of branching pattern is unexpected in the mammary gland, additional beaded ducts were embedded perpendicular to the paraffin cassette mold and cross-sections were obtained. H&E staining of serial sections confirmed that these beaded ducts had multiple lumina (Figure 4B). Additionally, there were areas where periductal stroma was apparent between separate ducts, while in subsequent sections this periductal stroma was absent (Figure 4).

Proliferation, receptor localization and morphology of beaded ducts

To further characterize the observed beaded ducts, immunohistochemical analyses were performed for smooth muscle actin (SMA), ER α , PR, and Ki67. SMA, a marker of myoepithelial cells, was used to determine whether the beaded ducts maintained normal myoepithelial localization. We found that, in general, the myoepithelial layer remains intact around both beaded ducts and associated alveolar buds (Figure 5A). In fact, myoepithelial cells were present even in areas where luminal epithelial cells were displaced by secretions in the lumen. Interestingly, in a few beaded ducts, SMA-positive cells were also found inside some ducts and surrounding intraductal cyst-like structures (Figure 5B). Together with the abnormal branching patterns illustrated in Figure 4, these results suggest that stroma-epithelial interactions are disrupted in BPA-exposed glands and that these disruptions may initiate intraductal epithelial proliferation that manifests as beaded ducts.

ER α staining in beaded ducts (Figure 5C) was not qualitatively different compared to non-beaded ducts. However, the epithelial cells in intraductal hyperplasias were often positive for PR (Figure 5D). Because PR expression in mammary epithelium is thought to be dependent on estrogen exposure, the expression of PR in these beaded ducts suggests that they are estrogen-sensitive.

Finally, Ki67, a marker of proliferating cells, was utilized to quantify the hyperplastic quality of beaded ducts compared to normal ducts. Epithelial cells in the beaded ducts were positive for Ki67 (Figure 5E). The proliferation index was approximately 5 times higher in the beaded ducts compared to normal ducts and alveolar buds (Figure 5F). Interestingly, the epithelium closely associated with the beaded ducts, i.e. neighboring alveolar buds, also had high proliferation indices, approximately 8 times higher than those of normal ducts (Figure 5F).

Discussion

The results presented herein underscore the consequences of BPA exposure through gestation and lactation, i.e. postnatal day 16. There is an acceleration by as much as 3 months in the appearance of alveolar buds when compared to animals exposed to BPA only through postnatal day 2. More importantly, here we describe new evidence of intraductal hyperplasias exclusively in BPA exposed mice regardless of the exposure dose.

The results from the animals examined at 3 months of age indicate that exposure from gestational day 8 through lactation (postnatal day 16) alters the development of the mammary gland. Specifically, 0.25BPA females showed a significant increase in the number of alveolar buds compared to controls (Figure 1 and Table 2A). In animals exposed to BPA only through postnatal day 2, these alterations were not observed until several months later, i.e. at 6 months of age [37], suggesting that a longer exposure to BPA (through postnatal day 16) may contribute to premature mammary gland development.

We also noted here that by 9 months of age, differences in the number of lobuloalveolar structures between BPA-exposed females and controls were greatly decreased, probably due to the increased presence of alveolar buds in the controls, as was reported previously [31]. A small amount of budding of alveolar structures develops during each menstrual/estrus cycle due to the stimulus of ovarian hormones [46]. During pregnancy and lactation, the number of lobuloalveolar structures increases exponentially under the control of the pituitary hormone prolactin [47,48]. The phenotype associated with BPA exposure was an earlier appearance of alveolar buds; mammary glands of BPA-exposed animals often appeared similar to those seen in pregnant animals (Figure 1). One interpretation of these data would be that the hormonal balance in BPA-exposed animals was altered such that these females were producing high levels of ovarian or pituitary hormones, leading to excessive and premature alveolar bud development. At this time, there are no data supporting or refuting this hypothesis. However, radioimmunoassay data indicate that there are no significant differences in serum estrogen levels at proestrous between control and BPA-exposed females [28], although circulating hormone levels at other points in the estrous cycle are currently unknown in treated animals. Alternatively, it is plausible that the advanced appearance of alveolar buds is due to a heightened response to ovarian hormones leading to excessive epithelial proliferation. A recent paper implicates ER α as critically important for alveologenesi s [49]; a genetically modified mouse with targeted deletion of ER α in the epithelium had a less-developed mammary gland during late pregnancy with significantly fewer alveolar buds forming during subsequent pregnancies and lactational periods. Thus, lobuloalveolar development may be partially but directly controlled by estrogen. Therefore, an increased sensitivity to estrogen in BPA-exposed females may explain the appearance of alveolar buds and associated increased ductal density. An increased response of BPA-exposed females to estradiol following ovariectomy at puberty buttresses this hypothesis [28,39]. A third possibility is that the earlier appearance of alveolar buds in BPA-exposed females is indicative of more rapid reproductive aging in these animals (for instance, an increased number of lifetime estrous cycles). At this time, we cannot determine which of these three possibilities are responsible for the increased appearance of alveolar buds in the BPA-exposed females.

Perhaps the most novel and interesting observation reported herein is the development of intraductal hyperplasias in animals exposed perinatally to BPA (Table 4). Intraductal hyperplasias are considered the precursors of carcinomas in both rodents and humans because they can progress to palpable tumors when transplanted into hosts with the appropriate hormonal environment [50,51]. As expected, the proliferation index of areas classified as intraductal hyperplasias was significantly increased compared to normal ducts obtained from the same gland. Remarkably, the proliferation index of alveolar buds located in close proximity to beaded ducts was also significantly increased compared to normal epithelium.

Beaded ducts were previously observed in whole-mount mammary glands, but not described in histological or histochemical detail prior to this report. Specifically, beaded ducts have been noted following either prenatal or neonatal exposure to pharmacological doses of other estrogenic compounds. Bern and colleagues exposed BALB/c mice to 20 μ g 17 β -estradiol for 5 days starting on postnatal day 1 [52]. At 12 months of age, the mammary ducts of exposed animals frequently had a beaded appearance and were filled with secretions. These ducts were described as “indicative of an aberrant secretory state” [52]. Additional studies from Bern’s group demonstrated that BALB/c mice exposed to doses higher than 10⁻⁴ μ g DES for 5 days starting on postnatal day 1 also displayed beaded ducts at fifteen months of age [53]. A similar exposure protocol of CD-1 mice to 5 or 50 mg/kg/day of the phytoestrogen genistein also led to the development of beaded ducts at 9 months of age [54]. Finally, exposure to 0.5 or 10 mg/kg/day zearalenone, a mycoestrogen, from gestational day 15

through gestational day 18 led to the development of beaded ducts filled with a secreted fluid observed at 4 months of age [55]. These glands were otherwise described as undergoing growth arrest and lacked tertiary branches and alveolar buds. In fact, the mammary glands from animals exposed to estradiol, DES and genistein also lacked alveolar buds, which is in contrast with our BPA-exposed females (Figure 1). Collectively, our results and those mentioned above indicate that the appearance of beaded ducts may be a common feature following developmental exposure to natural or synthetic estrogens.

Because of the availability of genetic mutants and the relative flatness of the inguinal mammary glands, the mouse has been the preferred model for developmental mammary gland studies. However, the rat model of carcinogenesis mimics human breast cancer better than the available mouse models [56,57,58]. Studies in a rat model showed that prenatal BPA exposure lead to the development of preneoplastic lesions such as intraductal hyperplasias, and *carcinomas in situ* in early adulthood [44]. Similar to the results of the current study, intraductal hyperplasias persisted for a longer period of time in mammary glands of rats exposed to the lowest dose of BPA; rats exposed to higher doses of BPA had fewer intraductal hyperplasias at older ages. These types of non-monotonic responses, also observed in the morphometric data presented herein, have been observed previously in the mammary gland and other organs (reviewed in [59]).

The effects of exposure to xenoestrogens during lactation on mammary gland development remain largely unexplored, in spite of its relevance when considering exposure of humans to xenoestrogens. BPA has been detected in human breast milk [20,21], polycarbonate baby bottles [60], and in some infant formulas, likely due to the packaging materials used [61,62]. Average exposure of bottle-fed newborns to BPA was estimated at 24 µg/kg BW/day, and 15 µg/kg BW/day for infants at 3 months of age [60]. Human infants produce glucuronidases in their digestive tracts with increased production until adult levels are reached at four years of age [63] suggesting that conjugated BPA may be deconjugated and activated during the digestion process. Additionally, neonatal rodents have limited ability to conjugate BPA to an inactive form regardless of the mode of administration (subcutaneous vs. oral) [64].

We have been careful to limit exposures to exogenous estrogenic compounds other than the BPA administered in these experiments; however, the human fetus and neonate are exposed to dozens if not hundreds of chemicals with hormonal activity [18,65,66,67]. Although methods are available to determine total xenoestrogen body burden in tissue samples from adult humans, to our knowledge, these methods have not been applied to fetal or neonatal tissues [68]; thus, the total level of xenoestrogens to which human neonates are exposed is currently unknown. Several *in vitro* studies of xenoestrogen mixtures, some containing BPA, revealed either additive or synergistic effects. In other words, the estrogenic effect of the mixture was equal or greater than those expected from the individual estrogenicities of BPA and the other substances [69,70]. Another study examined mixtures of 11 xenoestrogens, including BPA, and found that the presence of these chemicals at levels below their no-observed-effect-concentrations significantly increased the effects of estradiol [71]. While very few animal studies have examined xenoestrogen mixtures, these studies may indicate that low levels of BPA may act additively with other estrogenic chemicals to have significant clinical effects [72].

We have now shown that early BPA exposure leads to the development of intraductal hyperplasia in mice at adulthood (Figure 3). Perinatal BPA exposure in these mice also resulted in: i) increased epithelial density [37], which may be equivalent to increased mammographic density, a risk factor in women [73], ii) an increased density of terminal

ducts and terminal end buds [28,37], i.e. the structures where cancers are thought to arise [74], and iii) an increased responsiveness to estradiol [28,39].

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Figure 1. Mammary gland whole-mounts from 3-month old animals

Carmine stained whole-mounted mammary glands illustrate that control females have glands composed mostly of ducts (D) and terminal ducts (TD) while a large number of alveolar buds and lobuloalveolar units (AB) were observed in 0.25BPA glands. Magnification = 20x. Insets show higher magnification.

Figure 2. Beaded ducts in whole-mounted mammary glands from BPA-exposed offspring
Glands from 9-month old females exposed to BPA during perinatal development displayed beads in a large number of ducts (A) while others had beads in localized areas (B). Beads are marked by arrowheads. Inset illustrates a higher magnification of a beaded duct. In comparison to a normal duct (C), confocal images of beaded ducts in BPA-exposed females demonstrate the presence of cells inside the ductal lumen, both at 3 months of age (D) and with a more severe phenotype at 9 months of age (E). Areas with cells inside the ductal lumen are marked by yellow arrows. All pictured mammary glands were stained with carmine. Magnification: A and B = 32x; C–E = 200x.

Figure 3. Histological examination of beaded ducts in 9-month old BPA-exposed females
A) Hematoxylin and eosin staining illustrates the presence of epithelial cells within the luminal space (arrows). Eosinophilic secretions (marked by *) are apparent in the lumen (**A** and **B**), with some secretions staining more densely than others (marked by **). **B)** Some secretions appear to displace the luminal epithelial cells (arrowhead). **C)** PAS staining highlights the presence of an intact basement membrane (arrowhead), even in areas where the epithelial cells have been completely displaced (arrows). **D)** Masson's trichrome staining highlights the collagen-rich periductal stroma. Blood vessels are often observed close to beaded ducts (arrowhead) and brown staining indicates the presence of active lysozymes

(arrows). Inset shows a higher magnification image of active lysozymes. **E)** Toluidine blue staining illustrates the presence of mast cells (red arrows) in the stroma surrounding beaded ducts. Magnification of **A–E**: 400x.

Figure 4. Serial sections of beaded ducts from BPA-exposed 9-month old females illustrate abnormal branching patterns

A) Longitudinal serial sections (a–c) of a beaded duct stained with hematoxylin and eosin illustrate areas where ducts appear to bifurcate then merge again (magnification = 50x). Enlarged insets of (c) demonstrate apparent branching from one duct to two, with periductal stroma between the two new ducts (c'), two distinct ducts separated by periductal stroma, with secretions visible in the lower duct (c''), a single duct with two beaded secretions (c'''), and abnormally organized associated alveolar buds (c''') (magnification = 1000x). **B)** Serial cross-sections of a beaded duct stained with hematoxylin and eosin illustrate the numerous lumina (L), which are occasionally separated by periductal stroma (P). Strongly stained

secretions (S) are visible in some sections and not others. Blood vessels (BV), alveolar buds (AB), and mast cells (M) are also indicated. Magnification = 1000x.

Figure 5. Immunohistochemical analysis of beaded ducts from 9-month old BPA-exposed females

While the majority of beaded ducts had normal localization of myoepithelial cells (SMA positive, marked by arrow) (A), some beaded ducts demonstrated SMA positive cells within the ductal lumen (B). Qualitative analysis of ERα (C) and PR (D) showed no gross differences in their expression or localization. E) Many epithelial cells within the beads were positive for Ki67, a marker of proliferation (arrows mark positive cells). F) Quantification of the proliferative index in normal ducts (non-beads), beaded ducts, and alveolar buds associated with beaded ducts (assoc ep), indicate an almost 5-fold increase in

proliferation in beaded ducts ($p=0.001$) and an almost 8-fold increase in associated epithelium ($p<0.001$). Magnification of **A–E** = 400x.

Table 1

Antibodies and concentrations used, with and without microwave (MW) antigen retrieval

Antibody	Company	Secondary	Block	Dilutions (primary/secondary)	
				MW	No MW
Ki67	Vector	Gt anti-Rb	Goat serum	1:3000/1:1500	1:1500/1:800
ER α	Novocastra	Gt anti-Ms	MOM*	1:200/1:600	not effective
smooth muscle actin (SMA)	Abcam	Gt anti-Rb	Goat serum	1:2400/1:1000	1:2400/1:1000
progesterone receptor (PR)	Affinity Bio Reagents	Gt anti-Ms	MOM*	1:80/1:250	not effective

* Mouse-on-Mouse blocking kit, Vector

Table 2A

Morphometric analysis of mammary glands from 3-month old females

Endpoint	control	0.25BPA	2.5BPA	25BPA
Volume fraction ducts	20.8 ± 2.3	15.8 ± 1.4	19.7 ± 0.97	19.4 ± 2.9
Volume fraction terminal ducts	6.5 ± 1.1	9.36 ± 2.1	8.1 ± 1.2	8.1 ± 1.7
Volume fraction alveolar buds	9.0 ± 4.3	27.1 ± 5.6*	7.2 ± 3.6	6.0 ± 2.4

* denotes significant difference (p<0.05) compared to control.

Table 2B

Morphometric analysis of mammary glands from 9-month old females

Endpoint	control	0.25BPA	2.5BPA	25BPA
Volume fraction ducts	21.36 ± 1.71	16.38 ± 1.47*	18.84 ± 1.54	17.31 ± 1.10
Volume fraction terminal ducts	7.27 ± 0.84	6.31 ± 0.75	9.23 ± 1.30	7.31 ± 1.00
Volume fraction alveolar buds	6.75 ± 2.25	11.42 ± 2.33 ^a	14.49 ± 3.84*	9.65 ± 1.98

* denotes significant difference (p<0.05) compared to control.

^a p=0.059.

Table 3

Histological analysis of mammary glands from 9-month old perinatally BPA-exposed females

	control	0.25BPA	25BPA
Periductal stroma width (μm)	15.0 $\pm$ 1.3	17.5 $\pm$ 1.5	16.5 $\pm$ 0.9
Density of periductal collagen (arbitrary units)	166.65 $\pm$ 3.40	160.56 $\pm$ 9.41	168.87 $\pm$ 4.39
Proliferative index (% Ki67- positive)	24.3 $\pm$ 4.2	21.2 $\pm$ 3.1	20.2 $\pm$ 3.8
% ER α - positive	10.2 $\pm$ 2.4	17.1 $\pm$ 7.5	15.7 $\pm$ 7.9
% PR- positive	25.6 $\pm$ 9.8	11.9 $\pm$ 3.7	9.6 $\pm$ 5.2

Table 4

Incidence of beaded ducts in animals based on age and dose of BPA.

Age of assessment:	3 months	9 months	12–15 months
control	0/6	0/18	0/7
0.25BPA	1/6	5/20**	4/11 ^a
2.5BPA	0/6	3/12*	1/6
25BPA	0/4	6/20***	0/10

p-value relative to respective controls (in bold)

^a
p=0.070

*
p=0.025

**
p=0.023

p=0.011