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Estrogens in the wrong place at the wrong time: fetal BPA exposure and mammary cancer

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Abstract

Iatrogenic gestational exposure to diethylstilbestrol (DES) induced alterations of the genital tract and predisposed individuals to develop clear cell carcinoma of the vagina as well as breast cancer later in life. Gestational exposure of rodents to a related compound, the xenoestrogen bisphenol-A (BPA) increases the propensity to develop mammary cancer during adulthood, long after cessation of exposure. Exposure to BPA during gestation induces morphological alterations in both the stroma and the epithelium of the fetal mammary gland at 18 days of age. We postulate that the primary target of BPA is the fetal stroma, the only mammary tissue expressing estrogen receptors during fetal life. BPA would then alter the reciprocal stroma-epithelial interactions that mediate mammaryogenesis. In addition to this direct effect on the mammary gland, BPA is postulated to affect the hypothalamus and thus in turn affect the regulation of mammatropic hormones at puberty and beyond.

Keywords

fetal mammary gland; mammary gland development; xenoestrogens; endocrine disruptors; environmental exposure; prenatal exposure

1. Introduction

Over the past four decades, strong evidence indicates that fetal exposure to estrogens causes perturbations in mammary gland development leading to mammary cancer manifested during adulthood (1–4). Beside the effects reported from exposure to endogenous and pharmacological estrogens (DES), a new source of concern is fetal exposure to endocrine disrupting chemicals (EDCs), including estrogen mimics (xenoestrogens) (5–7). An EDC is

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an exogenous chemical, or a mixture of chemicals, that interferes with any aspect of hormone action (8). Among these EDCs, BPA has gained much attention because of its ubiquitous presence in the environment (9–12). The increased incidence of breast, uterine and testicular cancers observed in European populations in the past 50 years has been postulated to be due to EDC exposure during periods of increased vulnerability, such as fetal development and peri-pubertal stages (13–17). This hypothesis was tested by examining the effect of environmentally relevant doses of BPA during fetal life of experimental animals. Perinatal BPA exposure to mice and monkeys resulted in alterations in both the stroma and the epithelium of the developing mammary gland (18–20). Moreover, intraductal hyperplasias, ductal carcinoma *in situ* (DCIS) and palpable tumors in murine species were documented long after the end of exposure (21–26). What remains to be determined is whether BPA acts exclusively by directly affecting epithelial-stromal interactions in the developing mammary gland or, in addition, by interfering with the hypothalamus-pituitary-ovarian axis (HPOA). In this review, we discuss the effects of fetal exposure to environmentally relevant levels of BPA on fetal mammary gland development and examine the hypothesis that BPA, by virtue of its estrogenicity, increases the risk of developing breast cancer in adulthood by exerting its deleterious effects through the stroma of the developing mammary gland.

2. Fetal exposure to endogenous and pharmacological estrogens

Epidemiological studies suggest that changes in the endocrine-fetal milieu predispose women to diseases that are manifested during adulthood (4;27–29). For instance, non-identical twin birth is considered an indicator of high exposure to estrogen, while pre-eclampsia is regarded as an indicator of low exposure to estrogen. Dizygotic birth correlates with increased risk of breast cancer in the offspring while pre-eclampsia correlates with a lower risk (29–31).

Fetal exposure to pharmacological doses of estrogens increases breast cancer risk at the age of prevalence (28;30;31). Compelling evidence favoring this claim was gathered from the daughters of mothers who were treated with massive doses of DES between 1948 and 1971 in order to prevent spontaneous abortion (32–35). The “DES daughters”, as these women are now commonly referred to, have been diagnosed with a variety of anatomic-physiological abnormalities in the uterus, oviduct and cervix as well as with clear cell adenocarcinoma of the vagina. Recently published data suggest that the DES daughters aged 40 years and older have an increased breast cancer incidence (1;36).

3. Mode of action of xenoestrogens during fetal life

Endogenous estrogens regulate multiple aspects of reproduction and development in males and females. Estrogens bring about their action by binding to nuclear receptors, estrogen receptor alpha (37) and beta (38) as well as the membrane-bound receptor, GPR30 (39). In females, estrogens regulate the morphology and physiology of the reproductive tract at all stages of life, including puberty, pregnancy and menopause (40). In males the generation of null mutants of the estrogen receptor (ER) alpha gene revealed that these mice were infertile. Also in males, it was shown that germ cells do not require ER alpha for development and

function, but that somatic cells of the reproductive tract require ER alpha to produce sperm that are capable of fertilization (41). During adulthood, endogenous estrogens mediate biological events in the reproductive tract that, for the most part, are reversible. However, fetal development of the female genital tract is thought not to require estrogens, and excess estrogens are known to produce irreversible alterations, such as those observed in DES daughters.

Xenoestrogens bind to estrogen receptors alpha and beta, both when residing in the nucleus and when attached to the plasma membrane. Additionally, xenoestrogens are known to bind to the membrane receptors GPR30 (42–44) and the orphan receptor ERR gamma (45). Low-dose effects of xenoestrogens may be due to their acting through membrane receptors and to additive action with endogenous estrogens (46).

4. BPA as a xenoestrogens

BPA is commonly used in the manufacturing of plastics and is present in polycarbonate products and epoxy resins. BPA exposure occurs when it leaches into containers holding food, beverages, water and milk, which are later consumed. Additionally, BPA is released from water pipes, dental materials, protective coatings, adhesives, protective window glazing, compact discs, thermal paper and paper coatings (9;47). Pervasive use of BPA increases the risk of exposure to both the developing fetus indirectly through maternal exposure, and the neonate directly through ingestion of infant formula or maternal milk (11;48).

BPA is present in the urine of 95% of a representative sample of a non-institutionalized U.S. population over 6 years of age (49), including pregnant women (50). BPA has also been detected in maternal and fetal serum and placental tissue of newborn humans. The range of BPA concentrations in fetal serum ranged from 0.2 to 9.2 ng/ml, indicating that the developing human fetus and neonate are readily exposed to this chemical (11). To date, BPA is the best-studied xenoestrogen for which effects of exposure have been reported at various time points ranging from fetal to postnatal development.

5. Role of the environment during development

The influence of the environment on phenotype determination has been known since the 1880's from observations in wildlife species. For instance, August Weismann documented that the spring and summer morphs of a butterfly species could be regulated by temperature. However, the gene-centric view of development that dominated 20th century research overlooked these phenomena that revealed phenotypic plasticity (51). The theory proposing that development is the execution of a genetic program gained prevalence from studies in animal models including *Drosophila* and rodents generated under strictly regulated laboratory conditions. Under these artificial conditions, animals reproduce all year long under light, temperature and strict diet regulations, avoiding external environmental influences. As a consequence, explanations in embryology have been narrowed down to genetics and molecular pathways. However, the phenomenon called polyphenism – one genotype, many phenotypes – contradicts the theory that development is the execution of a genetic program (51). Lately, the role of the environment in phenotype determination is

regaining central stage due to its relevance to disease. For example, epidemiological studies showed that malnutrition during fetal development increased the risk to coronary and metabolic diseases during adulthood. Thus the old phenomenon of developmental plasticity is coming back under a medical disguise, namely, the “fetal origins of adult disease” hypothesis (52).

6. Stromal-epithelial interactions in fetal mammary gland development

Besides being influenced by the external environment, embryonic development is mediated by interactions at various levels of biological organization. Epithelial cells interact with neighboring epithelial cells as well as with cells in the surrounding stroma and with the extracellular matrix (ECM). These interactions take place along several spatial and temporal scales that define the shape of the organism (53). The organism imposes constraints at the local and global level via biophysical and biochemical interactions through cell proliferation, cell motility and cell adhesion. Tissue development in appendages like the mammary gland, tooth, feather and hair occur as a result of constant reciprocal interactions between epithelial cells and the surrounding ECM (54;55). Pioneering work by Kratochwil showed that stromal-epithelial interactions are crucial for mammary gland morphogenesis (56). Using an explant model and tissue recombination techniques, he separated embryonic day (E) 12 to 16 mammary epithelium from the mesenchyme, and showed that the epithelium develops only after recombination with its mesenchyme. In addition, he showed that recombination with the salivary mesenchyme induces the originally mammary epithelium to display a dichotomous branching pattern typical of the salivary gland. Later, Sakakura *et al* confirmed these findings *in vivo*, by recombining E16 mammary epithelium with E14 salivary mesenchyme under the kidney capsule (57). These results clearly pointed out the inductive role of the stroma in mammary and salivary gland morphogenesis.

7. Breast carcinogenesis: a consequence of altered tissue interactions?

Cell-based theories of carcinogenesis, such as the somatic mutation theory (SMT), consider cancer as a disease of cell proliferation and postulate that it occurs as a result of DNA mutations in genes that control cell proliferation (58). Alternatively, the tissue organization field theory (TOFT) postulates that cancer, like morphogenesis is a matter of tissue organization, and it proposes cancer as development gone awry. In fact, pathologists diagnose cancer by looking at altered tissue organization. In the context of TOFT, carcinogens interfere with cell-cell communication and cell-matrix interactions, resulting in disruption at the tissue-level that gives rise to neoplasia (59). From fetal life throughout the life time of the individual, mammary gland morphogenesis involves reciprocal interactions between the stroma and the epithelium (56;57;60;61).

A theory-neutral experimental strategy was used to determine which tissue was the target of a carcinogen. Rats from susceptible strains developed mammary gland tumors when exposed to the chemical carcinogen nitrosomethylurea (NMU). Separately, mammary gland epithelium and mammary gland stroma were exposed to NMU or vehicle and then recombined. The recombination of stroma exposed to NMU with vehicle-exposed epithelial cells resulted in neoplasms (62). The reverse combination did not. This observation showed

that the stroma, rather than individual cells in the epithelium, is the target of the carcinogen. These results also pointed to the contextuality of the neoplastic phenotype. In a complementary experimental approach, the epithelial neoplastic phenotype was reversed when epithelial cells isolated from a mammary gland carcinoma were transplanted into a “normal” unexposed stroma (63). From these results, we concluded that tissues are the targets of carcinogens and that TOFT most reliably explains carcinogenesis. Comparable evidence has been gathered for other types of neoplasias such as metastatic breast cancer (64), metastatic melanoma (65) and hepatocellular carcinoma (66) and altogether provide further support for the central role of stromal-epithelial interactions in neoplastic development. Extrapolating from this compelling experimental and clinical evidence, we hypothesized that, BPA exerts its carcinogenic effects by binding to the ERs present in the stroma of the fetal mammary gland.

8. Bisphenol-A: a disruptor of stromal-epithelial interactions?

Fetal exposure to BPA results in morphological alterations in both the stroma and the epithelium of the developing mammary gland (18;19) which can lead to neoplasia later in adulthood (24;26). During fetal development, estrogen receptors α and β are exclusively detected in the stroma of the mammary gland (20;67). The transcriptomal changes induced by BPA and ethinyl estradiol are dissimilar in the stroma from those in the epithelium of fetal exposed mammary glands. This apparently counterintuitive result is easily explained given that different estrogens directly induce slightly different patterns of gene expression in the target ER positive cells (68). The gene expression pattern in the epithelium is more similar between BPA and ethinyl estradiol treatments. This is due to the fact that only a fraction of genes induced in the stroma play a role in stromal-epithelial interactions; and those are the ones that are involved in carrying estrogenic signals to the epithelium. Thus the effects observed in the epithelium must be mediated by the stroma (Figure 1) (20). Altogether this buttresses the hypothesis that BPA exerts its deleterious effects during fetal mammary gland development through the stroma.

9. Fetal exposure to BPA results in altered mammary gland morphogenesis

Environmentally relevant levels of BPA altered the developing mammary gland in mice exposed *in utero* from gestational day (gd) 8 to 18. At gd18, the mammary glands were collected and examined to identify alterations in the epithelium and in the stroma (18;20). The stroma of BPA exposed mammary glands displayed alterations in the ECM organization as well as in its cellular components. For instance, collagen deposition was higher near the epithelium while it decreased further away in the loose connective tissue. The gene expression of biomechanical modulators of the ECM, versican and tenascin C, was down-regulated in the periductal stroma of BPA exposed mammary glands (20). Regarding cells in the stroma, BPA increased the number of adipocytes containing lipid droplets and accelerated adipocyte differentiation. BPA also increased apoptosis in the presumptive fat pad as shown by Bax staining (18). These histological changes observed in the presumptive fat pad correlated with up-regulation of adipogenesis related genes (20). The mammary epithelium was overall more developed in BPA-exposed mammary glands compared to controls, as indicated by an increased area subtended by the ducts and ductal extension. BPA

delayed ductal lumen formation, indicating that BPA had not produced a mere acceleration in development but its disruption (18). Additionally, fetal mammary glands of *in utero* BPA-exposed non-human primates showed a higher density of mammary buds and a more advanced epithelial development than the control group (Figure 2) (19).

10. Alterations in the postnatal mammary gland due to *in utero* exposure to BPA

The mammary glands of mice fetally-exposed to BPA showed increased lateral branching (Figure 3) and increased numbers of epithelial cells expressing PR at 4 months of age (69). At 6 months of age, these mice showed clear changes in their mammary gland histoarchitecture compared to controls. BPA-exposed mammary glands exhibited a more developed epithelial tree and a greater than 300% increase in the relative area of alveolar buds (Figure 4). The overall appearance of mammary tissue of these BPA-exposed virgin mice resembled that of early pregnancy. Additionally, these glands displayed an altered ratio of DNA synthesis between the stroma and the epithelium as detected by BrdU incorporation; DNA synthesis was decreased in the epithelium at 10 days of age and in the stroma at puberty and increased in the stroma at 6 months of age (Figure 5) (70). The mammary gland of male offspring also showed morphological changes due to BPA exposure (Figure 6) (71).

In terms of functionality, the adult mammary gland of BPA-exposed fetuses were more sensitive to estradiol (69) and to progesterone (72). Decreased milk production was observed in lactating F1 females that had been perinatally exposed to BPA. Their milk composition was also altered as it showed a lower content of β -casein than controls (73). We set out to investigate whether BPA could also exert its deleterious effects at the genomic level by inducing epigenetic changes in the developing mammary gland. The DNA methylation profile of mammary glands was analyzed at PND 4, 21 and 50 of WF rats exposed *in utero* to BPA. BPA-induced alterations in the DNA methylation profile were observed throughout the genome at all three time points (Figure 7). However, genome-wide changes in mRNA expression were only observed at PND 50, i.e., the time at which increased incidence of intraductal hyperplasias and DCIS are observed in this rat strain (74). Therefore, these changes appear to be a consequence of the developmental alterations caused by BPA rather than the cause of neoplastic development later in adulthood. Thus, again, this evidence validates the hypothesis that BPA-induced carcinogenesis is the result of altered stromal-epithelial interactions in the fetal mammary gland.

11. Developmental exposure to BPA increases susceptibility to mammary carcinogenesis

Animal studies showed that BPA exacerbates the effects of carcinogens such as NMU and dimethylbenzanthracene (DMBA) in the mammary gland. Rats that were exposed through lactation to BPA 250 $\mu\text{g/kg/day}$ developed significantly higher numbers of palpable tumors induced by a one-time exposure to DMBA at PND 50 compared to sesame-oil treated animals (23). Interestingly, rats exposed *in utero* to BPA 250 $\mu\text{g/kg/day}$ showed decreased tumor latency after a one-time DMBA exposure at PND 100 compared to controls (fetally

exposed to sesame oil), indicating a shift in the window of sensitivity from PND50 to PND100. Furthermore, the percentage of rats with DMBA-induced tumors and number of tumors per rat were significantly higher in prenatally exposed BPA compared to sesame-oil treated controls (21). *In utero* exposure of rats to BPA 25 µg/kg/day augmented the responsiveness of the mammary gland to a subcarcinogenic dose of NMU compared to controls. The BPA treated rats showed a significant increase in hyperplastic lesions and further, 13% of the rats developed mammary tumors compared to 0% in controls (22). Altogether, these studies demonstrate that developmental exposure to BPA is able to enhance a carcinogenic insult to the mammary gland. In addition, BPA alone is sufficient to induce mammary gland neoplasia. *In utero* exposure to BPA induced ductal hyperplasia at all doses (2.5 – 1000 µg/kg/day) and carcinoma *in situ* at the higher doses (250 and 1000 µg/kg/day) in Wistar-Furth rats (24). Moreover, perinatal exposure to environmentally relevant doses of BPA induced adenocarcinoma of the rat mammary gland at PND90 in Sprague-Dawley rats (26).

12. Is the HPOA involved?

In addition to direct effects on reproductive tissues, perinatal BPA exposure may also exert indirect effects by altering development and function of hypothalamic-pituitary axes, including the HPOA. Studies in BPA-exposed rats and mice have documented alterations in estrous cyclicity (75), ovarian follicular development and in the genital tract (76), and in circulating hormone levels (76;77) all of which could be related to alterations at the hypothalamus and/or pituitary. Changes in the ER levels in the hypothalamus and pituitary have been reported following perinatal exposure to BPA (77–79). Perinatal BPA exposure alters the size and composition of the AVPV (80;81), a brain region essential for cyclic gonadotropin release and the preovulatory LH surge. These results suggest that effects of perinatal BPA exposure at the hypothalamic/pituitary level may contribute to alterations in circulating ovarian hormones and in mammatropic hormones that could in turn influence mammary gland development. Animals exposed to BPA show alveolar development at ages where controls do not (70), suggesting a role for altered prolactin secretion.

13. Conclusions

The Environmental Protection Agency (EPA) defines a carcinogen as a chemical or physical agent capable of causing cancer (82). Under this definition, BPA should now be considered a direct mammary gland carcinogen. In the context of TOFT, a likely explanation for BPA-induced breast carcinogenesis emerges. Namely, by binding to ERs present in the primary mesenchyme, BPA affects collagen deposition and adipocyte differentiation. This modifies the mechanical properties of the ECM resulting in a denser and stiffer tissue, which is consistent with delayed lumen formation. The fact that mammographic density, due to a dense stromal compartment, is a main risk factor for breast cancer buttresses this hypothesis. The affected stroma would induce alterations in epithelial development such as increased branching and ductal growth. Finally, the altered mammary gland displays an abnormal response to natural hormones and is more susceptible to carcinogenic insults, developing intraductal hyperplasias and DCIS later in adulthood (Figure 8).

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Abbreviations

EDC	Endocrine Disrupting Chemicals
BPA	bisphenol A
DES	diethylstilbesterol
HPOA	hypothalamus-pituitary-ovarian axis
DCIS	ductal carcinoma <i>in situ</i>
GPR30	G-protein coupled receptor 30
ERR	Estrogen receptor related
ECM	extracellular matrix
NMU	nitrosomethyl urea
TOFT	Tissue Organization Field Theory
PND	postnatal day
GD	gestational day
AVPV	anteroventral periventricular nucleus
EPA	Environmental Protection Agency
DMBA	dimethylbenzanthracene

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Highlights

- The primary target of BPA is the stroma of the fetal mammary gland.
- BPA alters reciprocal stroma-epithelium interactions that regulate mammaryogenesis.
- Perinatal BPA exposure sensitizes the mammary gland to additional carcinogen hits.
- BPA is a direct mammary gland carcinogen.
- Carcinogenesis is development gone awry.

Figure 1.

Hierarchical Clustering analyses of the transcriptomal profiles show the effects of perinatal exposure to BPA, ethinyl estradiol (EE2) and vehicle on peri-ductal stroma (left panel) and epithelium (right panel) of WT and null ER α mice. Status of mouse genotype is indicated on the left (Green: WT (+/+), Black: ER knock-out (-/-)) and treatment is indicated on the right of the heat maps. Unique gene clusters observed are shown on the top of the heat maps. Originally published in Wadia et al PLOS ONE 2013 (20).

Figure 2.
Whole mounts of neonatal mammary glands of control and BPA dosed non-human primates.
(A) Control; (B) BPA dosed. Scale bar is equivalent to 500 μm . Originally published in
Tharp et al., Proc. Natl. Acad. Sci USA 2012 (19).

Figure 3.

Increased lateral branching observed in adult mammary glands of mice exposed *in utero* to BPA. (A) Number of side branches per 500 μm of ductal length in mammary glands of 4 month-old mice treated perinatally with vehicle, 25 ng BPA/kg bw/day and 250 ng BPA/kg bw/day (* $p < 0.05$). Error bars indicate SEM. (B) Whole mounts of adult mammary glands of vehicle (left panel) and 25 ng BPA/kg bw/day (right panel) treated animals. Scale bar represents 1 mm. Originally published in Munoz de Toro et al, Endocrinology (69) Copyright 2005, The Endocrine Society.

Figure 4.

Quantification of ductal and alveolar structures shows an increase in the relative area of (A) ducts, (B) terminal ducts and, (C) alveolar buds in mammary glands of 6 months-old mice exposed *in utero* to 25 or 250 ng/kg of BPA relative to control. (* $p < 0.05$) relative to control. Originally published in Markey et al. Biol. Reprod. 2001 (70).

Figure 5.

Ratio of DNA synthesis between the stroma and the epithelium. Bar graph represents the ratio of the percentage of epithelial cells that incorporated BrdU to the percentage of stromal cells that incorporated BrdU in the mammary glands of 6 months-old mice exposed *in utero* to 25 or 250 ng/kg of BPA or control. Originally published in Markey et al. Biol. Reprod. 2001 (70).

Figure 6.

Whole mount mammary glands of male mice at two different ages. These animals were exposed *in utero* to BPA. The black box indicates samples where the epithelium was small. All samples from the same age were taken at the same magnification. Scale bar represents 1 mm in all panels. Reprinted from Vandenberg et al, Reproductive Toxicology 2013 (71) with permission from Elsevier.

Figure 7.

BPA-induced changes in DNA methylation observed at PND 4, PND 21 and PND 50 in the mammary glands of perinatally exposed Wistar-Furth rats. The red graphs to the left of each chromosome indicate relative numbers of the transcriptional initiation sites found within a 1 megabase window, which corresponds to the density of promoters. Originally published in Dhimolea et al PLOS ONE 2014 (74).

Figure 8.

BPA exposure alters mammary gland development and increases the risk of mammary carcinogenesis: BPA binds to the fetal mammary gland mesenchymal ERs, and in turn affects the composition of the ECM increasing tissue rigidity. Increased rigidity delays lumen formation. BPA also induces precocious adipocyte differentiation, which in turn accelerates duct elongation and branching. These changes lead to an increased sensitivity to mammotropic hormones in adulthood. BPA also binds to ER in the hypothalamus, where it alters the control of ovarian cyclicity and the control of the secretion of mammotropic hormones. The solid arrows link effects of *in utero* exposure to BPA in rodents and non-human primates. Dashed arrows indicate hypothesized links between effects during fetal mammary gland development and mammary carcinogenesis.