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Induction of mammary gland ductal hyperplasias and carcinoma *in situ* following fetal bisphenol A exposure

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Abstract

Exposure of the fetus to excess estrogen is believed to increase the risk of developing breast cancer during adult life. Fetal exposure to low doses of the xenoestrogen bisphenol A resulted in long-lasting effects in the mouse mammary gland that were manifested during adult life. It enhanced sensitivity to estradiol, decreased apoptosis, increased the number of progesterone receptor-positive epithelial cells at puberty and increased lateral branching at 4 months of age. We now report that fetal exposure to 2.5, 25, 250 and 1000 μg bisphenol A/kg body weight/day induces the development of ductal hyperplasias and carcinoma *in situ* at postnatal day 50 and 95 in rats. These highly proliferative lesions have an increased number of estrogen receptor-α positive cells. Thus, fetal bisphenol A exposure is sufficient to induce the development of preneoplastic and neoplastic lesions in the mammary gland in the absence of any additional treatment aimed at increasing tumor development.

Keywords: Bisphenol A; Xenoestrogen; Mammary gland; Rat; Carcinogenesis; Carcinoma *in situ*

1. Introduction

Recent data have suggested that perturbations in the fetal environment may predispose individuals to disease and/or organ dysfunction, which become apparent in adulthood [1,2]. This new emphasis on the fetal origins of adult diseases has prompted scientists to hypothesize that fetal exposure to environmental estrogens may be the underlying cause of the increased incidence of uterine leiomyoma, testicular cancer and breast cancer observed in European and US populations over the last 50 years [3–5].

Epidemiological studies suggest that fluctuating estrogen levels in the fetal environment have long-term consequences regarding the risk of developing breast cancer during adult life [6–8]. Given the long latency period between exposure and effect, epidemiological studies designed to explore this hypothesis have used prenatal and perinatal markers of *in utero* estrogen exposure because direct estrogen measurements are not available from birth records. For instance, twin pregnancy was used as an indicator of high estrogen exposure and pre-eclampsia for low estrogen exposure. Increased risk of breast cancer correlated with twin dizygotic birth and pre-eclampsia was associated with lowered risk in several studies [7,9].

Direct evidence of prenatal estrogen exposure and breast cancer risk is being gathered from the cohort of women born to mothers treated with diethylstilbestrol (DES) during pregnancy. This potent synthetic estrogen was administered to women as an anti-abortive therapy between the years 1948 and 1971 in the US, Europe and Australia. Tragically, this therapy was continued long after it was shown to be ineffective, and was finally stopped in the early 1970s when a rare pathology, clear cell adenocarcinoma of the vagina [10,11], as well as other abnormalities of the uterus, oviduct and cervix were diagnosed in young women that had been exposed to DES *in utero* [12,13]. These women are now reaching the age at which breast cancer becomes more prevalent. It has been reported that in the group of women exposed *in utero* to DES, aged 40 years and older, there is a 2.5-fold increase in the incidence of breast cancer compared to unexposed women of the same age [14], suggesting that indeed, prenatal exposure to synthetic estrogens

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may play an important role in the development of breast neoplasms.

Consistent with this observation, experiments in rats showed that prenatal exposure to DES resulted in increased mammary cancer incidence during adulthood [15]. These experiments illustrated that rats exposed prenatally to DES and challenged with the chemical carcinogen dimethylbenzanthracene (DMBA) at puberty had a significantly greater incidence of palpable mammary tumors at 10 months of age than animals exposed prenatally to vehicle. In addition, the tumor latency period was shorter in the DES-exposed compared to the vehicle-exposed group. Both the epidemiological and experimental data are consistent with the hypothesis that excessive estrogen exposure during development may increase the risk of developing breast cancer.

In utero exposure to tamoxifen, an estrogen antagonist and partial agonist, has also been shown to increase the incidence of mammary tumors when the exposed offspring are challenged with DMBA at puberty. Eighteen weeks after the challenge, 95% of the tamoxifen-exposed animals developed tumors compared to 50% of the vehicle-treated rats [16]. However, in the above-mentioned studies, both DES and tamoxifen were administered at high pharmacological doses to reflect the medical use of these agents, while the effects of twinning and pre-eclampsia represent a physiological range of endogenous hormone levels to which developing fetuses are exposed. Yet there is a third type of exposure that needs to be addressed, i.e., the inadvertent and continuous exposure of fetuses to environmentally active xenoestrogens.

Among these compounds, bisphenol A (BPA) is receiving increased attention due to its ubiquitous presence in the environment and chronic human exposure. BPA is used in the manufacture of polycarbonate plastics and epoxy resins, and leaches from food containers [17], beverage containers [18] and dental sealants and composites [19] under normal conditions of use [5]. BPA has been measured in maternal and fetal plasma and placental tissue at birth in humans [20,21]. A recent study of 394 Americans reported that BPA was found in 95% of urine samples [22]. From these data, the mean exposure was estimated to be 40 ng/kg body weight (BW)/day and the 95th percentile was 230 ng/kg BW/day assuming that 70% of the daily dose was excreted into the urine. A smaller study reported a mean daily urinary excretion of BPA at levels of 1.2 μg and estimated the maximum daily intake of BPA to be 0.23 μg/kg BW [23]. Alternatively, daily intake can be estimated from the amount of BPA leached from food containers, beverage containers and dental materials. Using this approach, a probable exposure range of 2–20 μg BPA/kg BW/day was calculated [24]. It is worth noting that the US Environmental Protection Agency estimates the lowest observable adverse effects level to be 50 mg BPA/kg BW/day and from this the safe dose was calculated to be 50 μg BPA/kg BW/day [25].

Exposure of rodents to low doses of BPA during fetal development has been shown to alter a variety of biological endpoints including early vaginal opening [26], early onset of puberty [27], disrupted estrous cyclicity [28,29], and decreased levels of luteinizing hormone following ovariectomy [29].

Our previous work has focused on the effects of perinatal BPA exposure on mouse mammary gland development. In these mice, BPA caused a decreased invasion of the stromal compartment, increased number of terminal end buds (TEBs) relative to the ductal area, decreased apoptosis and increased numbers of cells expressing progesterone receptor in the pubertal mammary gland [30]. At 4 months of age, these animals had a significant increase in lateral branching [30]. By 6 months of age, we observed an overall increase in epithelial structures including terminal ends and a premature appearance of alveolar buds, normally associated with pregnancy in the mouse [31]. More importantly, BPA exposed mice that were ovariectomized prepubertally showed an enhanced sensitivity to estradiol demonstrated by an increase in the number of TEBs, TEB area, TEB density and ductal extension [30,32]. It has also been observed that increased ductal density, estimated from mammographic density, is associated with increased risk for developing breast cancer [33]. Based on all these findings, we hypothesize that perinatal exposure to environmental levels of BPA increases the risk of developing mammary cancer. To explore this hypothesis, we have chosen to use a rat model because it mimics the human disease regarding hormone factors and histopathology more closely [34,35] than the available mouse models [36].

In the rat model, treatment with chemical carcinogens such as DMBA and *N*-nitrosomethylurea (NMU) results in the development of intraductal hyperplasias, intraductal carcinomas *in situ* (CIS) and adenocarcinomas. Intraductal hyperplasias are believed to be the precursor lesion for both CIS and adenocarcinomas [37]. Additionally, transplantation studies have revealed that intraductal hyperplasias give rise to palpable tumors [38], and thus are considered preneoplastic lesions. The aim of the present work was to examine whether fetal BPA exposure is sufficient to induce the development of preneoplastic lesions in the mammary gland in the absence of any additional treatment aimed at increasing tumor development (i.e., chemical carcinogen, pharmacological hormone treatment, etc.).

2. Materials and methods

2.1. Fetal exposure to BPA

Sexually mature female Wistar-Furth rats (8-week-old; Harlan, Indianapolis, IN) were maintained in temperature-controlled and light-controlled (14-h light, 10-h dark cycle) conditions in the Tufts University School of Medicine animal facility. 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. Cages and bedding tested negligible for estrogenicity by the E-SCREEN assay [39]; water was supplied from glass bottles only. Food (Harlan Teklad 2018) was supplied *ad libitum*. Estrogenicity of the feed was measured at 20 femtomoles of estrogen equivalents per gram, a negligible amount [39]. Female rats were mated with Wistar-Furth males of proven fertility and the morning on which sperm was observed in vaginal smears was designated embryonic day (E)1. On E9, the rats were weighed and implanted with Alzet osmotic pumps (Alza Corp., Mountain View, CA) designed to deliver the following doses of BPA/kg BW/day: 2.5 μg, 25 μg, 250 μg or 1000 μg. For convenience, these will be subsequently referred to as BPA2.5, BPA25, BPA250 and BPA1000, respectively. The control animals were implanted with a pump delivering 50% dimethyl sulfoxide (vehicle control; Sigma Chemical Co., St. Louis, MO). Thus, the fetuses were exposed to BPA or vehicle from E9 until postnatal day (PND) 1. A wide range of BPA doses were