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The effects of *in utero* bisphenol A exposure on the ovaries in multiple generations of mice

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ABSTRACT

Bisphenol A is used in polycarbonate plastics and epoxy resins. Previous studies show that *in utero* BPA exposure inhibits germ cell nest breakdown in the F1 generation of mice, but its effects on germ cell nest breakdown and on the ovary in the F2–F3 generations were unknown. Thus, we tested the hypothesis that BPA has transgenerational effects on the ovary. Mice were exposed to BPA *in utero* (BPA 0.5, 20, or 50 μg/kg/day), and ovaries were collected at postnatal days (PND) 4 and 21 from the F1–F3 generations and subjected to histological evaluation and gene expression analyses. *In utero* BPA exposure did not have transgenerational effects on germ cell nest breakdown and gene expression on PND 4, but it caused transgenerational changes in expression in multiple genes on PND 21. Collectively, these data indicate that *in utero* BPA exposure has some transgenerational effects in mice.

1. Introduction

Bisphenol A (BPA) is a synthetic compound used as a plasticizer in the manufacturing process of polycarbonate plastics and epoxy resins. It can be found in a variety of different products, such as plastic food and beverage containers, baby bottles and formula packaging, and the linings of canned food containers [1]. The potential threat to humans from use of these products stems from the ability of BPA to leach out of plastics and into the food and beverages they contain, under conditions such as ultraviolet light, heat, acidic conditions, and microwave use. Humans are commonly subjected to oral exposure of this chemical, with studies showing BPA is present in human blood, urine, ovarian follicular fluid, breast milk, and the placenta [2–4].

Previous studies have shown that *in utero* exposure to BPA has a negative impact on ovarian development and function in mice, and that it leads to reduced fertility [5–7]. One mechanism by which BPA may impair fertility is by disruption of the normal process of germ cell nest breakdown. In mice, germ cells migrate to the genital ridge around embryonic day 10.5, where they proliferate quickly and form clusters. A layer of proliferating somatic cells surrounds these clusters and helps form germ cell nests. After birth, these nests are broken apart, resulting in individual oocytes. These oocytes are then surrounded by a layer of somatic cells to form primordial follicles. This is generally finished by postnatal days (PND) 4–6 in mice, and leaves a finite number of primordial follicles available to the female for the remainder of her life [8]. This process of germ cell nest breakdown is controlled by the drop in estrogen at birth that results from the pups leaving the mother who had high levels of estrogen [9]. Additionally, germ cell nest breakdown depends on the occurrence of apoptosis, a type of programmed cell death [10]. Since BPA is an estrogenic compound, exposure during this critical stage inhibits the natural apoptosis needed to release oocytes from their germ cell nests [5].

Many previous studies have shown that BPA has overall negative effects on the reproductive development and function of ovaries [7,11,12], but the extent of these effects on future generations is unclear. Specifically, the effect of *in utero* BPA exposure on the process of germ cell nest breakdown in multiple generations has yet to

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be explored. Further, the mechanisms by which BPA affects germ cell nest breakdown are relatively unknown. Our previous study showed that *in utero* BPA exposure increases expression of anti-apoptotic factors and decreases expression of pro-apoptotic factors and that these gene expression changes coincide with decreases in germ cell nest breakdown in the F1 generation [5]. Thus, in the current study, we examined whether *in utero* BPA exposure causes changes in gene expression of apoptotic factors (i.e., B cell leukemia/lymphoma 2 (*Bcl2*)) and (Bcl2-associated X protein (*Bax*)) in the F2–F3 generations. Further, we explored whether BPA exposure alters expression of other ovarian genes that regulate oxidative stress and autophagy in the F1-F3 generations.

We focused on genes that regulate oxidative stress because oxidative stress is thought to play a role in apoptosis and this might help regulate germ cell nest breakdown [8]. Oxidative stress occurs when an over-abundance of reactive oxygen species (ROS) is present in the body. Under normal conditions, ROS play an integral role in cell signaling and homeostasis, as they are formed as a natural by-product of oxygen metabolism. In response, the body produces anti-oxidant enzymes (i.e., superoxide dismutase 1 (*Sod1*), catalase (*Cat*), and glutathione peroxidase (*Gpx*)) to clear the ROS. However, an imbalance in this process can lead to oxidative stress-related damage in vital tissues in the body. In the case of the ovary, oxidative stress can interfere with apoptosis, which could affect germ cell nest breakdown and reproductive function [13].

We also focused on genes that regulate autophagy because autophagy plays an important role in regulating cell growth and development, especially during times of environmental stress [14]. Depending on the circumstances, autophagy can lead to cell death or can help to promote cell survival [15]. During the process of germ cell nest breakdown, autophagy helps to maintain energy homeostasis, utilizing both autophagy related 7 *(Atg7)* and beclin 1, autophagy related (*Becn1)* to initiate the process. In this case, autophagy has a pro-survival role in the regulation of germ cell numbers before the formation of the primordial follicle pool [16]. Autophagy also can have protective effects against cell death due to oxidative stress [17]. One study correlates an increase in *Bcl2* with the disruption of the autophagy related 12 - autophagy related 3 (*Atg12-Atg3*) complex in embryonic fibroblast cells of mice, causing a resistance to cell death by mitochondrial pathways [18], whereas another study finds that *Atg12* alone has pro-apoptotic functions in human embryonic kidney cells [19].

Our previous study did not examine the effects of *in utero* BPA exposure on ovarian morphology at time points later than PND 4. After the breakdown of germ cell nests shortly following birth and the creation of the primordial follicle pool, BPA can continue to have effects on follicle type distribution in the ovary. In certain cases, toxicants can increase or decrease follicle recruitment into more advanced stages of development. With this in mind, we determined if *in utero* BPA exposure affects follicle numbers on PND 21, which is a later time-point than PND 4 and is just prior to puberty. We examined changes in gene expression on PND 21 in response to BPA exposure as well. In addition to the apoptotic factors and oxidative stress genes examined on PND 4, we also examined genes in the insulin-like growth factor (*Igf)* family, hormone receptors, and steroidogenesis-related genes.

We focused on whether BPA exposure affects members of the *Igf* family because they are involved in the regulation of the cell cycle and apoptosis in mice and thus, play a role in regulating folliculogenesis [20]. Insulin-like growth factor 1 *(Igf1)* is required for follicular development, and mice lacking this factor have immature ovaries with follicles arrested at the preantral and early antral stages, which causes infertility. It is only produced at low levels during embryonic development so it plays a larger role during postnatal growth and development. Insulin-like growth factor binding protein 2 (*Igfbp2)* is responsible for transporting *Igfs* in the bloodstream, and hence increases the half-life of *Igf* in the serum and prevents overstimulation of cell growth and excessive apoptosis [20].

We focused on whether BPA affects hormone receptors in the ovary because BPA is a known endocrine disrupter. Although it has been identified as having weak estrogenic activity and interfering with estrogen receptor alpha *(Esr1),* it also can have anti-androgenic activity as well. It has been shown to affect multiple steps of the activation and function of *Ar*, hence inhibiting the ability of natural androgens to bind to their receptor [21]. Further, ovaries lacking the androgen receptor have reduced fertility and abnormal ovarian function, likely because of the importance of *Ar* and androgens in enhancing follicle-stimulating hormone receptor (*Fshr*) action and the development of the proper cellular components and receptor activity needed for healthy follicles [22].

Finally, we focused on whether BPA exposure alters expression of genes that regulate steroidogenesis because the production of sex steroid hormones such as estrogens and androgens is needed for proper development and growth of follicles [23], and several steroids play an important role in their synthesis. Steroidogenic acute regulatory protein (*Star*) is responsible for the transport of cholesterol, which is the starting material for the production of steroids. Cytochrome P450, family 17, subfamily a, polypeptide 1 (*Cyp17a1*), and hydroxysteroid (17-beta) dehydrogenase 1 *(Hsd17b1)* are responsible for the conversion of progesterone and testosterone, respectively. After the stimulation of FSH, androstenedione can be converted to estrone and testosterone can be converted to 17β-estradiol [24]. BPA has been shown to affect expression of these genes *in vitro* [25]. Thus, we examined whether it alters expression of these genes in the current study.

2. Materials and methods

2.1. Chemicals

Bisphenol A (99% purity, National Institute for Environmental Health Sciences) was dissolved into ethanol and then diluted in tocopherol-stripped corn oil. The final ethanol concentration in the tocopherol-stripped corn oil was less than 0.1%.

2.2. Animals

Inbred FVB mice were housed in conventional polysulfone cages at 25 °C on 12 L: 12 D cycles. They had access to Teklad Rodent Diet 8604 (Harlan) and high purity water (reverse osmosis filtered) in glass bottles *ad libitum*. All animal procedures were approved by the University of Illinois Institutional Animal Care and Use Committee, and abide by the guidelines set forth by the National Institutes of Health for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

2.3. Study design

Female mice (F0) were mated with control male mice at 12 weeks of age. Pregnancy was confirmed by the appearance of a vaginal sperm plug, and marked as gestational day (GD) 1. Once a vaginal sperm plug was present, females were removed from the males and individually caged. They were observed daily and their body weight gain was recorded to further confirm pregnancies. At GD 9, each pregnant female was randomly assigned to a treatment group. From GD 11 to birth, female mice were orally dosed once per day with tocopherol-stripped corn oil or BPA (0.5, 20, or 50 μg/kg/day) by placing a pipette tip with the dosing solution in the corner of their mouth, as described in a previous study [5].