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***In utero* bisphenol A exposure disrupts germ cell nest breakdown and reduces fertility with age in the mouse**

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Abstract

Bisphenol A (BPA) is a known reproductive toxicant in rodents. However, the effects of *in utero* BPA exposure on early ovarian development and the consequences of such exposure on female reproduction in later reproductive life are unclear. Thus, we determined the effects of *in utero* BPA exposure during a critical developmental window on germ cell nest breakdown, a critical process for establishment of the finite primordial follicle pool, and on female reproduction. Pregnant FVB mice (F0) were orally dosed daily with tocopherol-stripped corn oil (vehicle), diethylstilbestrol (DES; 0.05 µg/kg, positive control), or BPA (0.5, 20, and 50µg/kg) from gestational day 11 until birth. Ovarian morphology and gene expression profiles then were examined in F1 female offspring on postnatal day (PND) 4 and estrous cyclicity was examined daily after weaning for 30 days. F1 females were also subjected to breeding studies with untreated males at three to nine months. The results indicate that BPA inhibits germ cell nest breakdown via altering expression of selected apoptotic factors. BPA also significantly advances the age of first estrus, shortens the time that the females remain in estrus, and increases the time the females remain in metestrus and diestrus compared to controls. Further, F1 females exposed to low doses of BPA exhibit various fertility problems and have a significantly higher percentage of dead pups compared to controls. These results indicate that *in utero* exposure to low doses of BPA during a critical ovarian developmental window interferes with early ovarian development and reduces fertility with age.

Keywords

ovary; bisphenol A; follicle; germ cell nest breakdown; apoptosis; fertility

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Introduction

Infertility is a major public health concern because it affects approximately 10% of all reproductive-aged couples. Exposure to endocrine disrupting chemicals (EDCs) on a daily basis is one of the major factors that may increase a woman's risk of infertility. Bisphenol A (BPA) is a known EDC that is widely used as a synthetic plasticizer in the manufacturing of polycarbonate plastics, including food packaging products, baby bottles, milk containers, epoxy resins of food and beverage can liners, and dental materials. Under certain conditions, such as exposure to UV light, basic and acidic solutions, and heat, BPA can leach out from plastic products into the food and beverages of consumers, causing humans to be exposed to BPA on a daily basis (Markey *et al.*, 2002). Recent epidemiological studies have shown that 95% of human urine samples have measurable levels of BPA (Calafat *et al.*, 2005). Furthermore, BPA has been found in placental tissues, amniotic fluid, umbilical cord blood, breast milk, and ovarian follicular fluid (Ikezuki *et al.*, 2002; Yamada *et al.*, 2002). More recent studies have shown that BPA is associated with reproductive dysfunction in women (Takeuchi *et al.*, 2004; Tsutsumi, 2005; Caserta *et al.*, 2011). Although the "safe" exposure limit for BPA is 50µg/kg/day (EPA, 1988), numerous studies with developmental exposure to lower doses than the "safe" dose suggest that BPA exposure causes various detrimental defects in the female reproductive system (Chen *et al.*, 2007; Susiarjo *et al.*, 2007).

Studies have also shown that BPA has estrogenic properties and it can pass from the mother to offspring through the placenta and breast milk (Ikezuki *et al.*, 2002; Schonfelder *et al.*, 2002). Animal studies have shown that developmental BPA exposure leads to advanced mammary gland development, morphological and functional alterations in the reproductive tract, early puberty onset, and irregular estrous cyclicity in adult life (Markey *et al.*, 2002; Maffini *et al.*, 2006; Richter *et al.*, 2007; Lawson *et al.*, 2011). Further, studies have shown that *in utero* BPA exposure impairs meiotic maturation of the oocyte, suggesting that the effects of BPA might be across generations (Susiarjo *et al.*, 2007; Lawson *et al.*, 2011). In addition, studies have shown that exposure to estrogenic compounds such as BPA and genistein during neonatal life can alter oocyte development and limit the number of primordial follicles available for later reproduction (Karavan and Pepling, 2012). However, to our knowledge, no studies have examined whether *in utero* BPA exposure, during a critical ovarian development window, impairs germ cell nest breakdown, a critical process for forming the finite primordial follicle pool, and the consequences of this impairment in later reproductive life.

Hormonal disturbance during critical ovarian developmental windows may predispose individuals to disease and/or dysfunction later in life. In the mouse, primordial germ cells migrate to the genital ridge around embryonic day (E) 10.5. Then, these primordial germ cells rapidly proliferate and form clusters, which will be surrounded by a single layer of proliferating somatic cells, forming germ cell nests. After birth, these germ cell nests break apart to release individual oocytes and form primordial follicles. The process of germ cell nest breakdown occurs via natural apoptosis of inter-connected germ cells and is driven by the drop in estrogen levels that happens around birth. The individual surviving oocytes become surrounded by a single layer of somatic cells (pre-granulosa cells) and eventually form primordial follicles (Pepling, 2006; Tingen *et al.*, 2009). The process of primordial

follicle assembly is mostly complete by postnatal day (PND) 6 in the mouse. The primordial follicles formed during this period represent a finite pool available for the female in her entire reproductive lifespan. Any disturbance that happens during this developmental window has the potential to affect the size of the finite primordial follicle pool and lead to early depletion of follicles, resulting in long-term effects on fertility (Pepling, 2006). Since BPA has estrogenic properties, it could adversely affect germ cell nest breakdown via interfering the apoptotic process driven by the drop in estrogen levels around birth (Chen *et al.*, 2007). Thus, this study was designed to determine the effects of *in utero* environmentally relevant low dose BPA exposure during the critical ovarian developmental window of germ cell nest breakdown and the long-term effects on adult reproductive functions such as puberty onset, estrous cyclicity, and fertility. In addition, this study was designed to evaluate whether *in utero* BPA exposure leads to early reproductive senescence by examining fertility at three, six, and nine month of age.

Material and Methods

Chemicals

BPA (99% purity) (obtained from National Institute of Environmental Health Sciences) and DES (Sigma Chemical Co.) were first dissolved in ethanol and then diluted in tocopherol-stripped corn oil to obtain the selected doses. The final ethanol concentration in tocopherol-stripped corn oil was 0.1%.

Animals

Inbred FVB mice were housed at 25°C in conventional polystyrene cages on a 12L:12D cycles. The mice were given Teklad Rodent Diet 8604 (Harlan) and high purity water (reverse osmosis filtered) provided in glass water bottles *ad libitum*. All animal procedures were approved by the University of Illinois Institutional Animal Care and Use Committee.

Study design

At 12 weeks of age, female mice (F0) were mated with fertility confirmed control males. Mating was confirmed by the presence of vaginal plug. The day the vaginal plug detected was defined as gestation day (GD) 1. Once the vaginal plug was observed, females were removed from males and individually caged. These dams (F0 generation) were observed daily and body weight gain was measured daily to further confirm pregnancy. On GD 9, confirmed pregnant females (40 dams total) were randomly assigned to 5 treatment groups (8 dams per group). On GD 11, dams were orally dosed by placing a pipet tip containing the dosing solution into the mouth once a day with tocopherol-stripped corn oil (vehicle control), diethylstilbestrol (0.05 µg/kg/day), or 0.5, 20, and 50 µg/kg/day BPA from GD 11 to birth. DES at 0.05 µg/kg/day was chosen as a positive control to ensure the animals could respond to an estrogenic compound. BPA at 0.5 µg/kg/day was selected to mimic the estimated human exposure from bottle-feeding (USFDA, 2009). BPA at 20 µg/kg/day was selected because previous studies show it disrupts oocyte meiosis and causes aneuploid eggs in later reproductive life (Hunt *et al.*, 2003). BPA 50 µg/kg/day was selected because it is the EPA referenced safe dose (EPA, 1988).

The exposure window was selected because this is the critical ovarian development window in the mouse. During the course of the exposure period, pregnant dams were observed twice per day for any adverse clinical signs or abnormal behavior that may result from toxicity. The doses were calculated and delivered in 28–32 μ l corn oil based on their body weight. The dams were weighed daily during the dosing period to adjust the administered dose for body weight changes during pregnancy. The dams were allowed to deliver naturally and the delivery day was designed as PND 0. The numbers of live births, stillbirths, female/male ratio, and average pup weights were recorded on PND 0.

After delivery, the size of each litter was standardized on PND 4 by eliminating extra pups through random selection within sex from litters with more than 10 pups to yield 10 pups, with five females and five males per litter. Natural litters with 10 or fewer pups were not standardized. On PND 4, to avoid litter effects, only one female per litter from each treatment group was euthanized by decapitation and ovaries were collected for analysis. For each female pup, the left ovary was collected and snap-frozen for gene expression analysis, whereas the right ovary was fixed in 10% neutral-buffered formalin (Sigma) overnight, and then stored in 70% ethanol until histological evaluation.

After weaning at PND 21, two F1 females from each litter were selected for examination of puberty onset and estrous cyclicity. These mice were weighed and checked for vaginal opening daily. Once vaginal opening occurred, estrous cyclicity was evaluated by examining vaginal smears daily for thirty days. The first observed estrus and the percentage of time that F1 females remained in each estrous stage were calculated and reported.

At least one F1 female from each litter was selected to examine fertility at the ages of three, six, and nine months. In each fertility test, the selected F1 females were first subjected to vaginal smears for one week to evaluate estrous cyclicity and then they were mated with fertility confirmed untreated males for two weeks or until a vaginal plug was observed. During the mating phase, all the females were weighed twice per week. Once the plug was observed, females were weighed and individually caged. Then, these females were monitored twice daily, and weighed twice per week until parturition. Litter sizes, average pup weights, sex ratios, and percent of dead pups were recorded on PND 0. After the two-week mating period, unmated females were singly housed, their estrous cyclicities were checked for another 10 days, and body weights were monitored for another two weeks. The females were considered infertile if they did not cycle and/or did not have significant body weight gain during the entire testing period.

Histological evaluation

Ovaries from F1 females on PND 4 were collected and fixed in 10% neutral-buffered formalin (Sigma) overnight and then stored in 70% ethanol. The fixed tissues were embedded in paraffin, serially sectioned (5 μ m), mounted on glass slides, and stained with Weigert's hematoxylin and picric acid-methyl blue. Every 10th section of the ovary was used to evaluate ovarian development by counting the numbers of germ cells remaining in nests, primordial follicles, and primary follicles. If two or more germ cells were in direct contact with each other and were surrounded by pre-granulosa cells, they were considered to be localized within a nest. All sections were evaluated by an observer who did not have

knowledge of the treatment groups. Counts on a subset of sections were confirmed by another observer without knowledge of the treatment groups.

Gene expression analysis

Total RNA was extracted from PND 4 ovaries using the miRNeasy Mini Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's protocol. Due to the small amount of RNA that could be extracted from the PND 4 ovary, 2–3 ovaries per treatment group were pooled to yield enough total RNA for the PCR analysis. To remove any possible genomic DNA contamination, RNA was further treated with DNase (Qiagen, Inc., Valencia, CA). Messenger RNA (mRNA; 100ng) was reverse transcribed to cDNA using the RT² first strand Kit (Qiagen, Inc., Valencia, CA) following the manufacturer's instructions.

We focused on determining the effects of *in utero* BPA exposure on the expression of various apoptotic factors because germ cell nest breakdown is a natural apoptotic process. cDNA (25ng) was first amplified using RT² Preamp Pathway Primer Mix - Mouse Apoptosis (Qiagen, Inc., Valencia, CA) and then was subjected to an apoptosis pathway specific PCR array using RT² Profiler Mouse Apoptosis PCR Array kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's protocols. The data from the PCR arrays were analyzed using Qiagen online PCR array support software.

The genes with more than 1.5 fold change and CT value > 25 in PCR array analysis (listed in Table 1) were selected to further examine their expression levels via quantitative real-time PCR (qPCR) using the CFX96 Real-Time PCR Detection System (Bio-Rad Inc.) and accompanying software (CFX Manager Software) according to the manufacturer's instructions. We specifically focused on genes from table 1 that were known to be regulators of apoptosis in the ovary. Specific qPCR primers for the genes of interest are listed in Table 2. An initial incubation of 95°C for 10 min was followed by 45 cycles of 94°C for 10 s (denaturation step), 60°C for 10 s (annealing step), and 72°C for 10 s (extension step), along with final extension at 72°C for 10 min. At the end of the each reaction, a melting curve was generated to monitor the generation of a single product. All data were normalized to β -actin was because the expression levels of this housekeeping gene were not significantly different between treatment groups. Relative fold changes were calculated as the ratio to control group level, which was set as 1.0. All samples were measured in triplicate.

Statistical analyses

For follicle counting analysis, data were expressed as means $\pm$ standard error of the means (SEM) from ovaries from at least 5 different litters. One-way ANOVA followed by Tukey's *post hoc* comparisons were used to make multiple comparisons between treatment groups. Student's t-tests were used to make comparisons between two groups. Statistical significance was assigned at $p = 0.05$ for all comparisons.

For gene expression analysis, PND 4 ovaries from eight different litters (one ovary per litter) were randomly pooled into three groups (2–3 ovaries per pool) and RNA was extracted from these pooled samples. Data were expressed as means $\pm$ SEM from the pooled samples. One-way ANOVA followed by Tukey's *post hoc* comparisons were used to make multiple

comparisons between treatment groups. Student's t-tests were used to make comparisons between two groups. Statistical significance was assigned at $p = 0.05$ for all comparisons.

For puberty onset, estrous cyclicity, body weight analysis, and fertility tests, considering the litter effects, the means of two to three females from the same litter were calculated and used as one data point. Data were expressed as means $\pm$ SEM from six to eight litters per treatment group. One-way ANOVA followed by Tukey's *post hoc* comparisons were used to make multiple comparisons between treatment groups. Student's t-tests were used to make comparisons between two groups. Statistical significance was assigned at $p = 0.05$ for all comparisons.

Results

Effect of BPA on germ cell nest breakdown

To explore the effect of *in utero* BPA exposure on germ cell nest breakdown, we performed histological evaluation and compared the ovaries between BPA treatment groups and controls on PND 4. Our data show that all selected doses of BPA cause significantly more germ cells to remain in nests and fewer primordial follicles to form compared to control ovaries (representative images shown in Figure 1A). To quantify this observation, the numbers of germ cells remaining in nests and the numbers of primordial follicles were counted and the data were reported as percent of total oocytes. As shown in Figure 1B, *in utero* BPA exposure (at all selected doses) significantly increases the percent of germ cells remaining in germ cell nests compared to controls. Accordingly, BPA exposure at 0.5 $\mu\text{g/kg/day}$ and 50 $\mu\text{g/kg/day}$ decreases the percent of primordial follicles compared to controls. Please note that percentages of germ cells remaining in nests and primordial follicles do not add up to 100% total germ cells because a small percentage of germ cells per ovary were contained in primary follicles (3–9%). The percent of primary follicles did not differ between treatment groups (data not shown).

Effects of BPA on the expression of apoptotic factors

Germ cell nest breakdown is a natural apoptotic process, which is driven by the drop in estrogen levels around birth. Thus, we compared the expression of apoptotic factors in control and BPA treated PND 4 ovaries. To select the apoptotic targets of BPA during the germ cell nest breakdown process, apoptosis PCR arrays were performed and the results were compared and summarized in Table 1. We further performed q-PCR analysis to confirm the effects of BPA on the expression of the selected genes listed in Table 2. As shown in Figure 2A, BPA increases the expression of selected anti-apoptotic factors, whereas it decreases the expression of selected pro-apoptotic factors. Specifically, the lowest dose of BPA (0.5 $\mu\text{g/kg/day}$) decreases the expression of pro-apoptotic factor, *Bax*. The middle dose of BPA (20 $\mu\text{g/kg/day}$) increases the expression of anti-apoptotic factor, *Bcl2l1*, whereas it decreases the expression of pro-apoptotic factors, *Bax* and *Bak1*. The highest dose of BPA (50 $\mu\text{g/kg/day}$) increases the expression of anti-apoptotic factors, *Bcl2* and *Bcl2l1*, but decreases the expression of pro-apoptotic factor, *Bak1*. BPA also decreases the expression of selected factors in tumor necrosis factor signaling pathways. Specifically,

BPA 0.5µg/kg/day and BPA 50µg/kg/day decreases the expression of *Tnfrsf11b*, *Tnfrsf1a*, *Tnfrsf12*, and *Ltbr*. BPA 20µg/kg/day also decrease the expression of *Tnfrsf11b* (Figure 2B).

Effect of BPA on puberty onset and estrous cyclicity

After weaning at PND 21, female mice were weighed and checked for vaginal opening daily. Once vaginal opening was observed, vaginal smears were examined daily for 30 days. In the mouse, the first estrus is a good indicator of puberty onset. BPA did not significantly change the age of vaginal opening compared to the control group (data not shown). The age of the first estrus in DES 0.05µg/kg/day treated females, however, was significantly advanced (Figure 3A). Compared to controls, the time span between vaginal opening and the first estrus observed was significantly shorter in DES and BPA 50µg/kg/day treatment groups compared to controls (Figure 3B).

To determine the effects of *in utero* BPA exposure on estrous cyclicity, we compared the time that females spent in different estrous stages among treatment groups (Figure 4). Females in the 0.5µg/kg/day BPA treatment group spent less time in proestrus and estrus, but more time in metestrus and diestrus compared to controls. Animals in the DES 0.05µg/kg/day treatment group had significantly shortened proestrus, but extended metestrus compared to controls. BPA at 20µg/kg/day caused the females to have shortened estrus compared to controls (Figure 4).

Effect of BPA on body weight

Previous animal studies and epidemiological studies have linked developmental BPA exposure to metabolic disorder and obesity (Somm *et al.*, 2009; Harley *et al.*, 2013). In this study, we also monitored and compared the body weight change of F1 females from the day of weaning to 9 months of age. As shown in Figure 5A, BPA 0.5µg/kg/day and BPA 50µg/kg/day treated body weights were significantly higher than controls at six months and nine months. We also compared body weight gain between the treatment groups and controls. We found that BPA significantly increased body weight gain compared to controls (Figure 5B). Specifically, females in the BPA (0.5µg/kg/day) treatment group gained more weight during juvenile development (from the day of weaning to three months of age) and adulthood (from six months to nine months of age). BPA at 20µg/kg/day increased body weight gain from six months to nine months of age and BPA at 50µg/kg/day increased body weight gain from three months to six months of age compared to controls.

Effect of BPA on fertility at three, six, and nine month of age

To determine the effects of *in utero* BPA exposure on fertility in later reproductive life and whether the effects would change with age, we examined and compared the fertility in controls and BPA treatment groups at three, six, and nine months of age. Different pregnancy outcomes, including litter size, average live pup weight, female/male ratios, and percent of dead pups were recorded and compared to controls. For the effects of BPA on litter size, BPA (50 µg/kg/day) reduced the litter size at six months of age (Figure 6A). At nine months in the BPA 0.5 µg/kg/day treatment group, only one female out of 5 females gave birth, but all the pups were stillborn (Figure 6A). BPA (0.5 µg/kg/day) increased the percentage of dead pups at three months of age and no live pups were born at nine months of

age (Figure 6B). As shown in Figure 6C, BPA decreased fertility at all selected levels and the fertility decline became more severe with age, especially for the BPA 0.5 µg/kg/day treatment group compared to controls.

In Table 3, we summarized the infertility problems observed during the fertility tests. Based on the phenomena and the time of the problems observed, the infertilities can be divided into three different types. The first type of infertility: females had been housed with fertility confirmed control males for three weeks, no vaginal plugs were observed. Daily vaginal smear examinations suggested that these females did not cycle. Histological evaluation of the ovaries from the three month old female in BPA 0.5µg/kg/day treatment group showed no corpora lutea, suggesting that the infertility of this female is due to ovulation problems. The second type of infertility: vaginal plugs were detected as a sign of successful mating. The pregnant females started to show significant body weight gain around GD 7 until around GD 12. After GD 12, their body weights significantly dropped and returned to pre-pregnancy weight within a few days. No pups were born at the end of gestation, suggesting the infertility of these females might be caused by mid-gestation problems. The third type of infertility group: females went through the entire pregnancy, however, no live pups were born. All the pups were found dead, suggesting that the infertility of these females might be caused by late gestation problems.

Discussion

Previous animal studies have shown that perinatal BPA exposure affects the development of the brain, liver, adipose tissue, mammary gland, and reproductive tract and adversely affects their functions (Maffini *et al.*, 2006; Palanza *et al.*, 2008; Rubin and Soto, 2009). Hunt *et al.* also showed that *in utero* BPA exposure impairs meiotic maturation in oocytes and therefore, affects the quality of oocytes for future generations (Hunt *et al.*, 2003). However, based on our best knowledge, no study focused on the effect of *in utero* BPA exposure on germ cell nest breakdown and the fertility consequences over the time in later reproductive life. Thus, this study was designed to determine if exposure to environmentally relevant levels of BPA during an embryonic ovarian developmental window has adverse effects on germ cell nest breakdown and has long lasting effects on fertility in later reproductive life.

In the present study, we found that *in utero* BPA exposure disrupts germ cell nest breakdown and reduces the size of the primordial follicle pool by altering the expression of pro- and anti- apoptotic factors. We also found that *in utero* BPA exposure advances puberty onset, disturbs estrous cyclicity, and increases body weight gain. In addition, most interestingly, *in utero* BPA exposure causes different fertility problems and these problems become more severe with age.

Previous studies have shown that *in utero* BPA exposure disrupts synapsis and recombination during meiotic prophase in the mouse and rhesus monkey, which increases the incidence of chromosomally abnormal eggs and reduces egg quality (Hunt *et al.*, 2003; Susiarjo *et al.*, 2007; Hunt *et al.*, 2012). In the current study, we chose to focus on the effect of *in utero* BPA exposure on another critical ovarian development process, germ cell nest breakdown. In rodents, germ cell nest breakdown is triggered by the drop in estrogen levels

around birth (Chen *et al.*, 2007). Further, the estrogenic properties of BPA have been documented in numerous studies (Vom Saal *et al.*, 2012). Thus, one of the purposes of the current study was to examine if BPA exposure during a critical ovarian developmental window affects the natural germ cell apoptotic process via interfering the balance between pro- and anti-apoptotic factors that are regulated by the drop in estrogen levels around birth. Our results show that *in utero* BPA exposure disrupts the balance between various pro- and anti-apoptotic factors and therefore, blocks the germ cell nest breakdown process. These data suggest that *in utero* BPA exposure has lasting effects on the early postnatal germ cell nest breakdown process.

Although our dosing window and dose levels were not the same as previous studies, our results on BPA-related advanced puberty onset (20ug/kg/day) and irregular estrous cyclicity (0.5 and 20ug/kg/day) are consistent with previous studies that used higher doses of BPA than those used in our study exposure (Nah *et al.*, 2011). Collectively, these data suggest that developing reproductive organs are very sensitive to BPA, even at environmentally relevant levels, and that developmental BPA exposure has long lasting effects on the reproductive system, confirming that BPA is a developmental and reproductive toxicant.

In addition, we found that after weaning, BPA increases body weight by accelerating body weight gain compared to controls. These findings are also consistent with other studies, which have shown that developmental BPA exposure disturbs metabolism, interferes with adipocyte proliferation and differentiation, and may be linked to obesity in later life (Vom Saal *et al.*, 2012). However, because we did not observe significant differences in body weights between BPA-treated animal and controls from birth through weaning, we speculate that *in utero* BPA exposure might reprogram the progenitor adipocytes and increase body weight significantly later in life.

The mechanisms by which developmental BPA exposure affects puberty onset, estrous cyclicity, and metabolism are still not clear. The development of the female reproductive system is strictly controlled by the development and function of hypothalamic - pituitary - ovarian axis. Previous animal studies have shown that *in utero* BPA exposure affects the proliferation and differentiation of progenitors in developing hypothalamus and pituitary, and affects hormone levels (Brannick *et al.*, 2012). Thus, it is possible that developmental BPA exposure might affect the development of hypothalamic - pituitary - ovarian axis; therefore, having lasting effects on several physiological processes such as germ cell nest breakdown and female reproduction in adult life. Future examination of hormone levels at different development stages might provide more insight into this possibility.

In rodents, folliculogenesis starts from a finite primordial follicle pool formed within the first few days of neonatal life via germ cell nest breakdown. Any disturbance of this process will reduce the size of finite primordial follicle pool available for the entire reproductive life, and might lead to premature ovarian failure or other fertility problems when the animal ages. In current study, we found that *in utero* BPA exposure causes different fertility problems such as ovulation problems and mid- and late gestation pregnancy loss. Additionally, these effects were more severe as the animal aged. Although we cannot make the direct link between the blockage of germ cell nest breakdown and the decline in fertility with age, one

of the possible explanations is that developmental BPA exposure leads to premature ovarian failure by disrupting the germ cell nest breakdown process and reducing the size of the finite primordial pool available for the entire reproductive life.

Based on the fertility problems we have observed in the present study, including ovulation and mid- and late gestation problems, we suggest that *in utero* BPA exposure might affect the development and function of multiple organs. The possible explanations might include that BPA affect the meiosis process, therefore reducing egg quality. Hunt *et al.* (2003) have shown that *in utero* BPA exposure reduces egg quality, which could cause peri-implantation loss. It is also possible that BPA might affect the development and function of the uterus, which may lead to early and late pregnancy loss. Previous studies have shown that developmental BPA exposure causes the uterus to be less responsive to hormones and leads to more fertility problems compared to controls (Varayoud *et al.*, 2008). In addition, BPA might affect the functions of the hypothalamic-pituitary-ovarian axis and disturb homeostasis, processes which are critical for maintaining normal reproductive function. Animal studies have shown that perinatal and postnatal BPA exposure disrupt the proliferation and differentiation of the progenitor cells in pituitary and alter follicle-stimulating hormone and luteinizing hormone levels and therefore may affect fertility in later reproductive life (Xi *et al.*, 2011). Further investigation will be focused on elucidating the mechanism by which *in utero* BPA exposure causes mid- and late gestation pregnancy problems and determining whether the effects of BPA on germ cell nest breakdown and reproduction are transgenerational effects.

The current study was not designed to fully characterize the effects of DES on the ovary and instead it was designed to use DES as a positive control to ensure that the mice were responsive to estrogenic chemicals. Our data indicating that DES advances puberty onset indicate that the positive control was working in our system. Interesting, *in utero* exposure to DES did not affect germ cell nest breakdown on PND 4 in our study. This is in contrast to previous studies that have shown that neonatal DES exposure causes abnormalities in germ cell nest breakdown, leading to formation of multi-oocyte follicles (Iguchi and Takasugi, 1986; Kim *et al.*, 2009). However, the current study was just a snapshot in time and does not tell us what happened to germ cell nest breakdown in response to DES before and after PND 4. Thus, future studies should be done to fully examine the effect of *in utero* DES exposure on germ cell nest breakdown.

In conclusion, the current study indicates that *in utero* BPA exposure during a critical ovarian developmental window disrupts germ cell nest breakdown and impairs female reproduction with age. Further studies focused on examining the hormone profiles, ovarian morphology at later reproductive life, and pregnancy status during mid and late gestation will be helpful in enhancing our understanding of the mechanism by which developmental BPA exposure affects female reproduction.

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Highlights

1. *In utero* BPA exposure inhibits germ cell nest breakdown in female mouse offspring
2. *In utero* BPA exposure alters expression of regulators of apoptosis in the ovaries of mouse offspring
3. *In utero* BPA exposure advances the age at first estrus and increases the time spent in metestrus and diestrus compared to controls in female mouse offspring
4. *In utero* BPA exposure causes various fertility problems in female mouse offspring and these problems get worse with age

Figure 1.

Effect of BPA on germ cell nest breakdown. Ovaries were collected from one female per each litter on PND 4 and then subjected to histological evaluation. A, Representative Weigert's methyl blue staining of PND 4 ovaries. The left panel is a control ovary and the right panel is a BPA-treated ovary (0.5 $\mu\text{g}/\text{kg}/\text{day}$). Primordial follicles are marked by stars and germ cells remaining in nests are circled. B, The percent of germ cells remaining in nests (left panel) and the percent of formed primordial follicles (right panel). Data are presented as the mean $\pm$ SEM from 5–8 different litters (one female pup per litter). Asterisks (*) indicate significant differences from the vehicle control ($p < 0.05$). Please note that percentages of germ cells remaining in nests and primordial follicles do not add up to 100% total germ cells because a small percentage of germ cells per ovary were contained in primary follicles (3–9%). The percent of primary follicles did not differ between treatment groups.

Figure 2.

Effect of BPA on the mRNA expression levels of selected apoptotic factors. Ovaries were collected from PND 4 mice and subjected to real-time PCR analysis to confirm the mRNA expression levels of potential BPA-targeted apoptotic factors, identified by an apoptosis specific q-PCR array. All values were normalized to β -actin as a loading control and reported as relative fold change compared to controls. Graph represents mean $\pm$ SEM from 6–8 ovaries. Asterisks (*) indicate significant differences from the vehicle control ($p < 0.05$).

Figure 3.

Effect of BPA on puberty onset. At least two female pups per litter were selected for examination of vaginal opening daily after weaning. Once vaginal opening was observed, the females were subjected to examinations of estrous cyclicity for 30 days. The dates of first estrus were recorded and compared using the litter as the experimental unit. A: the age at which the first estrus was observed. B: the time span between vaginal opening and the first observed estrus. Graph represents mean $\pm$ SEM from 6–8 litters. Asterisks (*) indicate significant differences from the vehicle control ($p < 0.05$). ^ indicates borderline differences from the vehicle control ($p = 0.1$).

Figure 4.

Effect of BPA on estrous cyclicity. At least two female pups per litter were selected for examination of the estrous cyclicity for 30 days. The percentages of time of the animal remained in a particular phase were reported using the litter as the experimental unit. Graph represents mean $\pm$ SEM from 6 – 8 litters. Asterisks (*) indicate significant differences from the vehicle control ($p < 0.05$).

Figure 5.

Effect of BPA on body weight. The body weight (A) and body weight gain (B) of the females selected for fertility tests were monitored and compared to controls from weaning until nine months of age. Each litter was used the experimental unit. Graph represents mean $\pm$ SEM from 5 – 8 litters. Asterisks (*) indicate a significant difference from the vehicle control ($p < 0.05$). M = month. The ^ indicates a borderline difference from the vehicle control ($0.05 < p < 0.1$).

Figure 6.

Effect of BPA on fertility in reproductive life. At least one female from each litter was selected and subjected to three fertility tests at the age of three, six, and nine months. Pregnancy outcomes, including the number of total pups per litter (A), percentage of dead pups on PND 0 (B), and percentage of fertile females (C) were recorded and compared to controls. A and B: each litter was used the experimental unit and graph represents mean $\pm$ SEM from 5 – 8 litters. Asterisks (*) indicate significant differences from the vehicle control ($p < 0.05$). BPA 0.5 μ g/kg/day did not have any live pups born and thus, was not subjected to statistical analysis.

Table 1

Selected apoptosis PCR array results

Gene symbol	BPA 0.5	BPA 20	BPA 50
<i>Akt1</i>	-1.61	-1.61	-2.81
<i>Atf5</i>	-1.73	-1.74	-3.76
<i>Bag3</i>	-1.68	-1.61	-2.86
<i>Bak1</i>	-1.46	-2.02	-3.08
<i>Bax</i>	-1.60	-1.92	-1.38
<i>Bcl2</i>	1.02	1.90	1.26
<i>Bcl2l1</i>	1.80	2.42	1.63
<i>Card10</i>	-1.23	-1.40	-2.59
<i>Casp6</i>	-6.97	-6.63	-10.04
<i>Cd40lg</i>	-1.84	-1.46	-2.95
<i>Cidea</i>	2.21	1.06	-1.34
<i>Dapk1</i>	-1.28	-1.39	-2.33
<i>Igflr</i>	-1.48	-5.14	-2.00
<i>Lhx4</i>	2.45	2.52	1.29
<i>Ltbr</i>	-1.34	-1.42	-2.27
<i>Tnf</i>	-1.44	-1.43	-2.30
<i>Tnfrsf11b</i>	-1.23	-1.55	-2.94
<i>Tnfrsf1a</i>	-1.44	-1.51	-2.17
<i>Tnfrsf12</i>	-1.56	-1.44	-2.45
<i>Trp73</i>	1.33	-1.10	-2.74

Table 2

Sequences of primer sets used for gene expression analysis

Gene Name	Abbreviation	Forward (5'→3')	Reverse (5'→3')
Bcl2-antagonist/killer 1	<i>Bak1</i>	CCAGCGTGGTTTGACCGGCT	CCCAACCGCCTCTCTGTGCG
Tumor necrosis factor receptor super family, member 11b	<i>Tnfrsf11b</i>	GTGTGGAATAGATGTCACCCTGT	TCCACCAAAACACTCAGCCA
Activating transcription factor 5	<i>Atf5</i>	AGGCAGCGTGGAAGATTCTT	TCTAATGATGCGGAAGGGGC
Lymphotoxin B receptor	<i>Ltbr</i>	AAACCAGACTTGCTGGGACC	GCTGCATACCGCAAAGACAA
Tumor necrosis factor (ligand) superfamily, member 12	<i>Tnfsf12</i>	CCCTGGGCTCATCTTAAGGC	AAGGCCCTCAGTGAAGTTG
Tumor necrosis factor receptor super family, member 1A	<i>Tnfrsf1a</i>	TGTAAGTCCATGCAGGGTT	GTGACATTTGCAAGCGGAGG
Actin, beta	<i>Actb</i>	GGGCACAGTGTGGGTGAC	CTGGCACCACCTTCTAC
B-cell lymphoma 2	<i>Bcl-2</i>	ATGCCTTTGTGGAAGTATATGGC	GGTATGCACCCAGAGTGATGC
Bcl2-associated X protein	<i>Bax</i>	TGAAGACAGGGCCTTTTGTG	AATTCGCCGAGACACTCG
Bcl2 like 1	<i>Bcl2l1</i>	AAGCGTAGACAAGGAGATGCAGGT	CTGCTGCATTGTTCCCGTAGAGAT

Table 3

Types of infertility

Type of infertility	Control	DES	BPA 0.5	BPA 20	BPA 50
Ovulation problem	0	1 (9 month)	1 (3 month) 2 (9 month)	1 (9 month)	0
Mid-gestation problem	2 (9 month)	1 (6 month) 1 (9 month)	1 (6 month) 2 (9 month)	1 (9 month)	2 (3 month) 1 (6 month) 6 (9 month)
Late-gestation problem	0	1 (6 month)	1 (9 month)	2 (6 month)	2 (6 month)