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Bisphenol A exposure inhibits germ cell nest breakdown by reducing apoptosis in cultured neonatal mouse ovaries

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Abstract

Bisphenol A is a known endocrine disrupting chemical and reproductive toxicant. Previous studies indicate that *in utero* BPA exposure increases the percentage of germ cells in nests and decreases the percentage of primordial follicles. However, the mechanism by which BPA affects germ cell nest breakdown is unknown. Thus, we hypothesized that BPA inhibits germ cell nest breakdown by interfering with oxidative stress and apoptosis pathways. To test our hypothesis, ovaries from newborn mice were collected and cultured with vehicle (dimethyl sulfoxide, DMSO) or different doses of BPA (0.1, 1, 5, and 10 μg/mL). Ovaries then were subjected to histological evaluation of germ cell nests and primordial follicles or to measurements of factors that regulate oxidative stress and apoptosis. Our results indicate that *in vitro* BPA exposure significantly inhibits germ cell nest breakdown by altering the expression of key ovarian apoptotic genes, but not by interfering with the oxidative stress pathway.

Keywords

Bisphenol A; Neonatal ovaries; Mouse; Oxidative stress; Apoptosis; Germ cell nest breakdown

1. Introduction

Humans are exposed to endocrine disrupting chemicals (EDCs) on a daily basis [1,2]. This is of concern because EDC exposure has been suggested as a potential key element in human subfertility and infertility [3,4]. One of the most common EDCs is bisphenol A (BPA). BPA was first synthesized in 1891, and the production is estimated to be at 4 billion kg each year globally [1]. BPA is widely used as a backbone for epoxy resins, durable clear polycarbonate plastics such as reusable plastic food containers, food and beverage can liners, infant formula cans, baby bottles, and dental sealants. When these products are exposed to UV light, basic or acidic solutions, or heat, BPA can leach out from these products into the food and beverages, resulting in human exposure [5]. Several studies

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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indicate that BPA is present in human tissues and fluids, including placental tissues, amniotic fluid, umbilical cord blood, breast milk, and ovarian follicular fluid [6,7].

Previous studies indicate that BPA is an ovarian toxicant in animal models [8]. Specifically, studies indicate that prenatal exposure to BPA disrupts prophase events in the fetal ovary and causes meiotic aneuploidy in mouse oocytes [9], increases the incidence of multi-oocyte follicles in lambs [10], and increases the number of unenclosed oocytes and decreases the number of primordial follicles in a dose-dependent manner in CD-1 mice [11]. Further, prenatal BPA exposure causes fertility problems in the F1 generation of mice, and these problems become more severe with age [12]. Recently, prenatal exposure to BPA has also been shown to disrupt germ cell nest breakdown [12]. Specifically, prenatal exposure to BPA from gestation day (GD) 11.5 to birth increases number of germ cells that remain in nests and decreases number of primordial follicles present on postnatal day (PND) 4 [12].

The mechanism by which BPA disrupts germ cell nest breakdown is not clear. Wang et al. [12] showed that prenatal BPA exposure may inhibit factors in the apoptotic pathway, which in turn could affect the germ cell nest breakdown process. However, this study was conducted *in vivo* and thus, it is unclear if BPA disrupts germ cell nest breakdown *via* direct effects on the ovary or by indirect effects on hypothalamus and/or pituitary. Further, the previous study did not examine if prenatal BPA exposure directly induced apoptosis or oxidative stress in the ovary. It is important to determine the effects of BPA exposure on oxidative stress and apoptosis because germ cell nest breakdown is a natural apoptotic process and oxidative stress, in part, regulates apoptosis [13,14].

Oxidative stress represents a common mechanism in EDC-induced reproductive dysfunction [15–17]. If the amount of reactive oxygen species (ROS) present in a tissue exceeds the ability of the cells to defend themselves from increased ROS, oxidative stress occurs in the tissue and can cause damage to DNA and proteins. Tissues, including the ovary, contain anti-oxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), and glutathione reductase (GSR). These enzymes serve to protect the ovary from ROS. ROS are a two-edged sword: they serve as key signaling molecules in various physiological processes, but also play roles in pathological processes in female reproductive organs [18]. In the ovary, ROS play a regulatory role in oocyte maturation, folliculogenesis, ovarian steroidogenesis and luteolysis [18]. Thus, this current study utilized an *in vitro* neonatal ovary culture system to focus on the direct effects of BPA on germ cell nest breakdown, as well as the roles of oxidative stress and apoptosis in mediating any effects of BPA on germ cell nest breakdown. Specifically, the study tested the hypothesis that BPA exposure directly inhibits germ cell nest breakdown by inhibiting oxidative stress and/or apoptotic pathways.

2. Materials and methods

2.1. Chemicals

BPA powder (99%) was purchased from Sigma–Aldrich (St Louis, MO). A stock solution of BPA was dissolved and diluted in dimethyl sulfoxide (DMSO) (Sigma–Aldrich) to achieve the selected BPA treatment concentrations (0.13, 1.3, 6.65, and 13.3 mg/ml) for final

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working concentrations of 0.1, 1.0, 5.0, and 10 μg of BPA per ml of culture media (0.44, 4.4, 22, and 44 μM BPA, respectively). Using these treatment concentrations allowed each working concentration to contain the same volume of chemical and vehicle (0.75 μl of BPA/DMSO solution per ml of culture media).

The BPA concentrations were chosen based on concentrations used in previous studies [19–25]. BPA concentrations were also chosen based on studies showing the effects of BPA on ovarian cells [23,26]. For example, BPA exposure between 100 fM and 100 μM for 24–72 h increases apoptosis and causes G2-to-M arrest in cultured mouse ovarian granulosa cells [23]. BPA exposure between 10 and 100 μg/ml for 120 h decreases antral follicle growth, steroidogenesis, and expression of steroidogenic enzymes *in vitro* [26]. The lowest observable adverse effect level (LOAEL) is 50 mg/kg/day (rat, 103-week chronic dietary bioassay). This equates to approximately 210.6 μM. The doses used in the experiments were 0.44, 4.4, 22, and 44 μM (or 0.1, 1.0, 5.0 and 10 μg/ml), below the LOAEL concentration.

2.2. Animals

Adult, cycling female CD-1 mice were purchased from Jackson Laboratory (Bar Harbor, ME) and allowed to acclimate to the facility for at least 2 days before use. The mice were housed at the University of Illinois at Urbana-Champaign, Veterinary Medicine Animal Facility. Food (Harlan Teklad 8626/8604) and water were provided for *ad libitum* consumption. Temperature was maintained at 22 ± 1 °C, and animals were subjected to 12-h light-dark cycles. The Institutional Animal Use and Care Committee at the University of Illinois at Urbana-Champaign approved all procedures involving animal care, euthanasia, and tissue collection.

2.3. *In vitro* ovarian cultures

Ovaries were collected from post-natal day (PND) 0 female CD-1 mice and cultured as described by Devine et al. [27]. Pups from the same litters were randomly assigned to different treatment groups so that each treatment group contained ovaries from different pups from different litters. Briefly, PND 0 female CD-1 pups were euthanized and their ovaries were removed, trimmed of oviduct and other excess tissues, and placed onto a Millicell-CM membrane floating on 500 μl of DMEM/Ham's F12 medium containing 1 mg/ml BSA, 1 mg/ml Albumax, 50 μg/ml ascorbic acid, 5 U/ml penicillin, and 27.5 μg/ml transferrin per well in a four well plate previously equilibrated to 37 °C. A drop of medium was placed on top of each ovary to prevent it from drying. Ovaries were treated with vehicle control (0.075% DMSO) or BPA (0.1, 1.0, 5.0 and 10 μg/ml), and maintained at 37 °C and 5% CO_2 for 1–8 days. Media were changed every day, and ovaries were collected at the end of the culture.

2.4. Histological evaluation of follicle numbers

Following *in vitro* culture, one ovary from each pup was aseptically collected, placed in 10% neutral buffered formalin solution overnight, and then transferred to 70% ethanol the next day. Ovaries then were embedded in histogel and paraffin and serially sectioned (5 μM). All sections were mounted on glass slides, and stained with hematoxylin and eosin (ovaries collected on PND 1, 2, and 4) or Weigert's hematoxylin and picric acid–methyl

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blue (ovaries collected on PND 8). The numbers of oocyte containing germ cells and follicles were counted in every 5th serial section using a light microscope, and the percentages of germ cells and each follicle type were calculated by dividing the numbers of germ cells or follicles of each specific type by the total number of germ cells and follicles counted in every 5th serial section. Stage of follicular development was assessed using previously defined criteria [28,29]. Briefly, structures were classified as germ cells if they were present in clusters of at least two oocytes, with an absence of intervening somatic cells. Primordial follicles contained an oocyte surrounded by a full single layer of squamous granulosa cells, primary follicles contained an oocyte surrounded by a single layer of cuboidal granulosa cells, preantral follicles contained an oocyte surrounded by at least two layers of cuboidal granulosa cells and theca cell layers, and antral follicles contained an oocyte surrounded by multiple layers of cuboidal granulosa cells with a fluid-filled antral space and theca cell layers. All germ cells, primordial and primary follicles with oocytes, regardless of nuclear material in the oocytes, were counted, whereas only preantral and antral follicles with visible nuclear material in the oocyte were counted to avoid the risk of double counting follicles large enough to span serial sections.

2.5. Gene expression analysis

Gene expression analyses were conducted on both oxidative stress pathway-related enzymes and apoptotic factors. We focused on determining the effects of *in vitro* BPA exposure on the expression of various oxidative stress enzymes and apoptotic factors because germ cell nest breakdown is a natural apoptotic process, and oxidative stress, in part, regulates apoptosis [13,14].

For qPCR analyses, one ovary/well was collected, snap-frozen in liquid nitrogen, and stored at −80 °C until RNA extraction. Total RNA was then isolated from ovaries (n = 4–6 ovaries per pool) using an RNeasy Mini kit (Qiagen, Inc., Valencia, CA) following the manufacturer's instructions. RNA was eluted in 14 μl of RNase-free water and the concentration was determined using a NanoDrop (λ = 260/280 nm; ND 1000; Nanodrop Technologies, Inc., Wilmington, DE). Total RNA (100 ng) was reverse transcribed to complementary DNA (cDNA) using the iScript RT kit (Bio-Rad Laboratories, Inc., Hercules, CA) according to the manufacturer's protocol. Analysis of qPCR was conducted using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA) and accompanying CFX Manager Software according to the manufacturer's protocol. The machine quantifies the amount of PCR product generated by measuring SsoFastEvaGreen dye (Bio-Rad Laboratories, Inc., Hercules, CA) that fluoresces when bound to double-stranded DNA. Specific qPCR primers for all the genes of interest are listed in Table 1. All qPCR reactions were done in triplicate using 1 μl cDNA, forward and reverse primers (5 pmol) for β-actin (*Actb*), superoxide dismutase 1 (*Sod1*), catalase (*Cat*), glutathione peroxidase (*Gpx*), glutathione reductase (*Gsr*), Fas cell surface death receptor (*Fas*), TNF receptor-associated factor 3 (*Traf3*), caspase8 (*Casp8*), B cell leukemia/lymphoma 2 (*Bcl2*), Bcl2-like 1 (*Bclxl*), Bcl2-associated X protein (*Bax*), Bcl2-related ovarian killer protein (*Bok*), or Bcl2-associated agonist of cell death (*Bad*), in addition to an SsoFastEvaGreen Supermix qPCR kit for a final reaction volume of 10 μl. The qPCR program consisted of an enzyme activation step (95 °C for 1 min), an amplification and

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quantification program (40 cycles of 95 °C for 10 s, 60 °C for 10 s, single fluorescence reading), a step of 72 °C for 5 min, a melt curve (65–95 °C heating, 0.5 °C/s with continuous fluorescence readings), and a final step at 72 °C for 5 min per the manufacturer's protocol. *Actb* mRNA expression was used as a housekeeping gene, and all the gene expression data were normalized to *Actb*. Relative fold changes were calculated as the ratio to DMSO group level, which was set as 1.0.

2.6. *In vitro* ROS/RNS assay

Ovaries were collected after 2, 4, and 8 days of culture (PND 2, 4, and 8), and subjected to ROS/RNS assays for measurement of total free radicals, including reactive oxygen species (ROS) and reactive nitrogen species (RNS) using an OxiSelect *In vitro* ROS/RNS Assay Kit (Cell Biolabs, Inc., San Diego, CA) according to the manufacturer's instructions and as previously described by Wang et al. [30]. Data were normalized to protein level (measured by BCA Protein Assay Kit, Thermo Scientific, Rockford, IL). All samples were run in duplicate ($n = 3$, each n has eight ovaries per pool).

2.7. Statistical analysis

All data were analyzed using SPSS statistical software (SPSS, Inc., Chicago, IL). Data were expressed as means ± SEM from at least three separate experiments. Multiple comparisons between normally distributed experimental groups were made using one-way analysis of variance (ANOVA) followed by Dunnett *post hoc* comparison, if equal variances were assumed, or Games-Howell *post hoc* comparisons if equal variances were not assumed. If data were not normally distributed, comparison between two groups was done using Kruskal–Wallis H test for several independent samples, and Mann–Whitney U two-independent sample tests. Statistical significance was assigned at $p < 0.05$.

3. Results

3.1. Effects of BPA on germ cell nest breakdown

To examine the effects of BPA exposure on germ cell nest breakdown, neonatal mouse ovaries were cultured for 2, 4, and 8 days (PND 0 to PND 2, 4, and 8). Cultured ovaries were collected for histological evaluation and compared between controls and BPA treatment groups. The results show that BPA treatment inhibited germ cell nest breakdown compared to control (representative images are shown in Fig. 1). To quantify this observation, germ cells, primordial follicles, and primary follicles were counted and the results are presented as the percentage of germ cells, primordial follicles, or primary follicles of the total oocytes (Fig. 2). At PND 2, BPA treatment did not affect the percentages of germ cells, primordial follicles and primary follicles compared to control (Fig. 2A; $n = 4–5$, $p > 0.1$). At PND 4, BPA treatment at all doses (0.1, 1, 5, and 10 μg/ml) significantly increased the percentage of germ cells and decreased the percentage of primordial follicles compared to control (Fig. 2B, $n = 4–6$, $p < 0.05$). This inhibition of germ cell nest breakdown by BPA treatment continued to be observed on PND 8, when *in vitro* BPA exposure (1, 5, and 10 μg/ml) significantly increased the percentage of germ cells, but decreased the percentage of primordial and primary follicles compared to controls (Fig. 2C,

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$n = 3$, $p < 0.05$). For all the time points tested, BPA exposure did not affect the total oocyte counts when compared to control ($n = 3–6$, $p > 0.05$).

3.2. Effects of BPA on gene expression of key anti-oxidant enzymes

Germ cell nest breakdown is a process that may be controlled by multiple pathways, especially the apoptosis pathway [13]. Oxidative stress is a major factor that triggers the apoptosis pathway (reviewed in [14]), and endocrine disrupting chemicals have been shown to cause oxidative stress in ovaries [17,30]. Thus, we examined whether BPA treatment affects expression of major antioxidant enzymes (*Sod1*, *Cat*, *Gpx*, and *Gsr*). BPA treatment did not affect expression of *Sod1* at any time point compared to control (data not shown, $n = 3–4$, $p > 0.05$). On PND 1, however, BPA treatment (5 and 10 μg/ml) significantly increased the expression of *Cat* compared to control (Fig. 3A, $n = 3–6$, $p < 0.05$). On PND 2, only the highest dose of BPA (10 μg/ml) significantly increased the level of *Cat* expression, whereas other doses of BPA did not affect the expression of *Cat* compared to control (Fig. 3B, $n = 3–6$, $p > 0.05$). On PND 4, BPA treatment did not affect *Cat* expression at any dose compared to controls (Fig. 3C, $n = 3–6$, $p > 0.05$).

On PND 1, BPA (1 μg/ml) significantly decreased the expression of *Gpx* compared to controls (Fig. 4A, $n = 3–6$, $p < 0.05$). On PND 2, BPA treatment did not affect *Gpx* expression at any doses compared to controls (Fig. 4B, $n = 3–6$, $p > 0.05$). On PND 4, however, BPA (1 and 5 μg/ml) increased the expression of *Gpx* compared to controls (Fig. 4C, $n = 3–6$, $p < 0.05$).

On PND 1, BPA treatment did not affect the expression of *Gsr* at any doses compared to controls (Fig. 5A, $n = 3–6$, $p > 0.05$). However on PND 2 and 4, BPA 5 μg/ml and BPA 10 μg/ml significantly increased the expression of *Gsr* compared to controls (Fig. 5B and C, $n = 3–6$, $p < 0.05$).

3.3. Effects of BPA on ROS levels

Increased ROS levels are a direct indicator of elevated oxidative stress in biological systems [31]. Therefore, we examined the effects of BPA on ROS levels in neonatal ovaries using highly fluorescent 2′,7′-dichlorodihydrofluorescein (DCF) to indicate the ROS levels. Our results show that on PND 2 and PND 4, BPA treatment did not affect ROS levels compared to controls (Fig. 6A and B, $n = 3$, $p > 0.05$). On PND 8, however, BPA 5 μg/ml significantly increased ROS levels in the neonatal ovaries compared to controls (Fig. 6C, $n = 3$, $p < 0.05$).

3.4. Effect of BPA on expression of apoptotic genes

Because germ cell nest breakdown is a natural process requiring apoptosis [13], we compared the gene expression of apoptotic factors in BPA treated ovaries with control on PND 1, 2, and 4. We chose to focus on the Bcl2 family, Fas, Casp8, and Traf3 because they are important factors in regulating apoptosis in the ovary [14,32,33]. For the Bcl2 family, we chose to examine the expression of pro-apoptotic (*Bcl2*, *Bclxl*) and anti-apoptotic (*Bax*, *Bad* and *Bok*) genes. BPA treatment did not affect the expression of pro-apoptotic *Bax* and *Bok* at any time points (Fig. 7B, C, E, F, H, and I, $n = 3–4$, $p > 0.05$). BPA 1 and 10 μg/ml significantly increased the expression of *Bad* on PND 2 (Fig. 7D, $n = 3–4$, $p < 0.05$), but it

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did not affect the expression of *Bad* on PND 1 or 4 compared to controls (Fig. 7A and G, n = 3–4, $p > 0.05$).

BPA treatment significantly increased the expression of anti-apoptotic *Bcl2* and *Bclxl* compared to control. Specifically, BPA treatment significantly increased *Bcl2* expression at all the time points: BPA 1 and 5 μg/ml increased *Bcl2* expression on PND 1 (Fig. 8A, n = 3–4, $p < 0.05$), BPA 5 and 10 μg/ml increased *Bcl2* expression on PND 2 (Fig. 8B, n = 3–4, $p < 0.05$), and BPA 10 μg/ml increased *Bcl2* expression on PND 4 (Fig. 8C, n = 3–4, $p < 0.05$) compared to controls. Further, BPA treatment (0.1 and 1 μg/ml) significantly increased the expression of *Bclxl* on PND 2 (Fig. 8E, n = 3–4, $p < 0.05$), but not on PND 1 and PND 4 compared to controls (Fig. 8D and F, n = 3–4, $p > 0.05$).

When the ratios of anti-apoptotic to pro-apoptotic gene expression were compared among treatment groups, as early as on PND 1, BPA treatment (1, 5, and 10 μg/ml) significantly increased the ratio of *Bcl2/Bax* (Fig. 9A, n = 3–4, $p < 0.05$) and *Bcl2/Bad* compared to control (Fig. 9B, n = 3–4, $p < 0.05$), but no effect was observed with *Bcl2/Bok* (Fig. 9C, n = 3–4, $p > 0.05$) compared to controls. On PND 2, BPA at 5 μg/ml significantly increased the ratio of *Bcl2/Bok* (Fig. 9F, n = 3–4, $p < 0.05$), but BPA did not affect the ratio of *Bcl2/Bax* and *Bcl2/Bad* (Fig. 9D and E, n = 3–4, $p > 0.05$) compared to controls. On PND 4, BPA 10 μg/ml significantly increased the ratio of *Bcl2/Bax*, *Bcl2/Bad*, and *Bcl2/Bok* compared to controls (Fig. 9G, H and I, n = 3–4, $p < 0.05$).

In addition to examining the effects of BPA on the mitochondrial-related apoptosis pathway that is regulated by the Bcl2 family, we also examined the effects of BPA on the expression of several key factors in the extrinsic apoptosis pathway (*Fas*, *Casp8*, and *Traf3*). Our results indicate that on PND 1, BPA treatment (0.1 and 10 μg/ml) significantly decreased the expression of *Fas* (Fig. 10A, n = 3, $p < 0.05$), but it did not affect the expression of *Fas* on PND 2 and 4 compared to controls (Fig. 10B and C, n = 3, $p < 0.05$).

On PND 1, BPA treatment (5 and 10 μg/ml) significantly decreased the expression of *Casp8* compared to controls (Fig. 11An = 3, $p < 0.05$). On PND 2, only BPA 5 μg/ml significantly increased the expression of *Casp8* (Fig. 11Bn = 3, $p < 0.05$), but several BPA treatments (0.1, 5, and 10 μg/ml) significantly increased the expression of *Casp8* on PND 4 compared to controls (Fig. 11Cn = 3, $p < 0.05$). BPA did not affect the expression of *Traf3* at any doses on any time points tested (data not shown, n = 3, $p > 0.1$).

4. Discussion

In mammals, females are born with a finite pool of primordial follicles, and this primordial pool represents the entire reproductive lifespan of the females [34]. To form this finite pool of primordial follicles, germ cells need to go through mitosis, formation of germ cell nests, meiosis, and germ cell nest breakdown [13]. Any disruption in the formation of the primordial follicle pool can affect the ability of females to reproduce. Although the timing of germ cell nest breakdown for mice and humans is different (for mouse the germ cell nest breakdown starts from 2 days before birth and peaks around birth, for humans, the germ cell nest breakdown happens during fetal life and peaks during the second trimester), the mechanism that controls germ cell nest breakdown is highly conserved among species, and

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it is thought that apoptotic pathways are the key players in regulating germ cell nest breakdown in both mice and humans [35]. Many studies have shown that estrogenic compounds, such as synthetic estrogens (diethylstilbestrol, ethinyl estradiol), genistein, bisphenol A, and di-(2-ethylhexyl) phthalate, have adverse effects on the germ cell nest breakdown process [36–39]. A recent mouse study found that *in utero* BPA exposure (0.5, 20, and 50 μg/kg) significantly disrupts germ cell nest breakdown and reduces the size of primordial follicle pool as well as causes various reproductive and developmental problems [12]. However, the dosing window in this *in utero* study was from gestational day 11.5 until birth, which not only covers the timing of the prenatal germ cell nest breakdown process, but also covers germ cell mitosis, formation of germ cell nests, and meiosis before germ cell nest breakdown. Further, the previous study did not examine whether the adverse effects caused by BPA exposure are due to direct effects of BPA on the ovary or due to secondary effects of a BPA-disrupted hypothalamus–pituitary–gonadal axis. Thus, this study was specifically designed to focus on the direct effects of BPA treatment on the germ cell nest breakdown process by isolating the ovaries from the pups and directly exposing the ovaries to BPA in the culture media.

In the present study, we found that BPA treatment directly inhibits the germ cell nest breakdown process, resulting in higher percentages of germ cells and lower percentages of primordial follicles, and subsequently lower percentages of primary follicles in the ovaries compared to control. Many endocrine disrupting chemicals have been shown to cause oxidative stress in mouse ovaries [17,30], and germ cell nest breakdown is a process that is associated with apoptosis [40]. Thus, the present study also examined whether the ability of BPA to inhibit germ cell nest breakdown was due to effects on the oxidative stress and apoptosis pathways. Our results show that the BPA-induced inhibition of germ cell nest breakdown is the result of a disrupted balance between anti-apoptotic factors and pro-apoptotic factors, but not the result of disrupted oxidative stress in the ovaries.

Our results suggest that direct BPA exposure inhibits germ cell nest breakdown, even at the lowest dose (0.1 μg/ml) tested compared to control. Our results are similar to the findings of the study conducted by Zhang et al. who showed that after 3 days of neonatal ovary culture, BPA treatment significantly increased the percentages of oocytes remaining in germ cell nests, and decreased the percentages of oocytes in follicles [39]. However, the doses of BPA treatment used in our study are much lower than those used by Zhang et al. [39]. We used a series of environmentally relevant doses of BPA (0.44, 4.4, 22, and 44 μM), whereas Zhang et al. only used two doses (10 and 100 μM BPA).

BPA is a plasticizer that has been shown to directly cause oxidative stress in animal models (reviewed in [8]). Oxidative stress is caused by an imbalance in the levels of antioxidants and the levels of reactive oxygen species (ROS) [41]. Thus, in our study, we examined both the antioxidant enzyme gene expression levels and ROS levels to determine if BPA induced inhibition of germ cell nest breakdown is the result of disrupted oxidative stress. The antioxidant enzymes (SOD1, CAT, GPX, and GSR) play essential roles in the survival of organs and their health: SOD1 catalyzes the dismutation of the highly reactive superoxide anion to oxygen (O_2) and the less reactive species hydrogen peroxide (H_2O_2). Then, this less reactive peroxide is further reduced to water and O_2 by CAT and GPX coupled with the

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glutathione cycle. GSR is responsible for reducing GPX oxidized glutathione so it can be further recruited to reduce peroxide [42]. Our results suggest that BPA treatment does not significantly affect the expression of antioxidant enzymes. The random fluctuations in the expression levels of antioxidant enzymes indicate that BPA is unlikely to interfere with oxidative stress pathway based on the gene expression results. Future studies focusing on the enzyme activities are needed to confirm this conclusion. Our results also suggest that BPA does not affect ROS levels at PND 2 and 4, but it (5 μg/ml) increases ROS levels at PND 8 (Fig. 6). The increased ROS levels in the BPA (5 μg/ml) treatment group happened after the germ cell nest breakdown process, so it is unlikely to be the cause of BPA-induced inhibition of germ cell nest breakdown. These data suggest that BPA treatment does not cause oxidative stress in cultured neonatal ovaries, and thus, it is unlikely that the mechanism by which BPA inhibiting germ cell nest breakdown involves oxidative stress.

The process of germ cell nest breakdown and primordial follicle assembly has been shown to be regulated by various mechanisms (reviewed in [43]), and programmed cell death has long been associated with oocyte loss during this process (reviewed in [13]). Apoptosis occurs by two pathways: the intrinsic mitochondria-initiated pathway and the extrinsic signal-initiated pathway. In our study, we examined several key factors in both pathways. The BCL2 family proteins are the crucial regulators in the intrinsic apoptosis pathway and they have been shown to be the key factors that regulate apoptosis in the mammalian ovaries throughout life (reviewed in [44]). In this study, we examined the expression of five key factors in the BCL2 family, including two anti-apoptotic factors (*Bcl2* and *Bclxl*) and three pro-apoptotic factors (*Bax*, *Bad*, and *Bok*). Our results showed that BPA does not alter the expression of pro-apoptotic factors. However, BPA significantly increased the expression levels of anti-apoptotic factors. Several studies indicate that the ratio of anti- to pro-apoptotic factors regulate cell death in tissues [13,44]. Thus, we further calculated the ratio of the major anti-apoptotic factor (*Bcl2*) to pro-apoptotic factors (*Bax* and *Bad*). Our results suggest that BPA treatment significantly inhibits apoptosis by increasing the ratio of anti- to pro-apoptotic factor expression levels. In the extrinsic apoptosis pathway, we also found that BPA significantly decreases the expression of both *Fas* and *Casp8*. Collectively, these data indicate that BPA significantly disrupts the balance between several anti- to pro-apoptotic factors, and this may lead to a decrease in apoptosis and inhibition of the germ cell nest breakdown process.

Germ cell nest breakdown *in vivo* occurs because of a drop in maternal progesterone and 17β-estradiol (estradiol) levels (reviewed in [13,43,45]), and *in vitro* neonatal ovary culture studies have shown that estradiol and progesterone treatment can inhibit germ cell nest breakdown [38,46]. In our study, we observed similar effects of BPA on germ cell nest breakdown. However, BPA likely acts through different mechanisms than estradiol and progesterone. Estradiol, progesterone, and synthetic estrogens may act through suppressing the expression and signaling of activin, rather than preventing cellular apoptosis to inhibit germ cell nest breakdown [45], whereas our findings show that BPA may inhibit germ cell nest breakdown by reducing apoptosis. It is not known whether BPA can affect activin. Thus, future studies on the effect of BPA on activin should be conducted to determine

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whether BPA can affect germ cell nest breakdown through alteration in activin signaling, similar to estrogenic compounds.

In our study, we also observed that BPA increased the percentage of germ cells and decreased percentage of primordial follicles on PND 4, but it did not affect percentage of primary follicles. On PND 8, we observed that BPA decreased the percentage of primordial follicles as well as primary follicles. We speculate that the BPA-induced decreased percentage of primary follicles on PND 8 is related to the reduced primordial follicle pool that was observed on PND 4. However, it is also possible that BPA can directly affect primordial follicle recruitment or cause atresia in the more advanced follicles. Thus, future studies are needed to examine the effect of BPA on primordial follicle recruitment and atresia of advanced follicle types.

In a study reviewing EDC exposure and early menopause, exposure to 15 chemicals (including polychlorinated biphenyls, pesticides, dioxin/furan and phthalates) was associated with an earlier age at menopause (1.9–3.9 years earlier) [47]. In our study, we also observed a reduced primordial follicle pool after BPA exposure, but whether this smaller oocyte pool is related to the earlier depletion of the oocytes is unknown. Future studies are needed to address this question, and may be helpful to shed some light on the cause of earlier menopause that is associated with EDCs exposure.

In conclusion, this study indicates that direct exposure to environmentally relevant BPA doses can disrupt the germ cell nest breakdown process, resulting in a smaller primordial follicle pool. This BPA-induced disruption is the result of an oxidative stress independent increase in the ratio of anti- and pro-apoptotic factor expression. Future studies focusing on the later reproductive outcomes of BPA exposure-induced inhibition in germ cell nest breakdown will be helpful to increase our understanding of the effects of short term exposure to BPA on long term reproductive abilities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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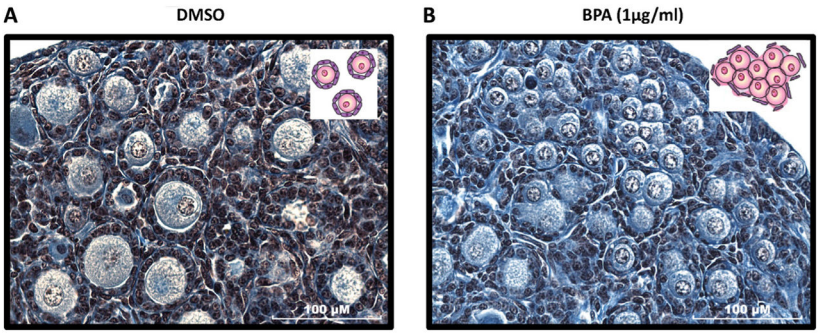

Fig. 1.

Effects of BPA on germ cell nest breakdown. Representative images of neonatal ovaries collected at postnatal day 0 (PND 0) and cultured for 8 days (PND 8) are pictured. Panel A is a control ovary and panel B is a BPA-treated ovary (1 μg/ml). Schematics on the top right of each panel illustrate the structure of primordial follicles (A) and germ cell nests (B).

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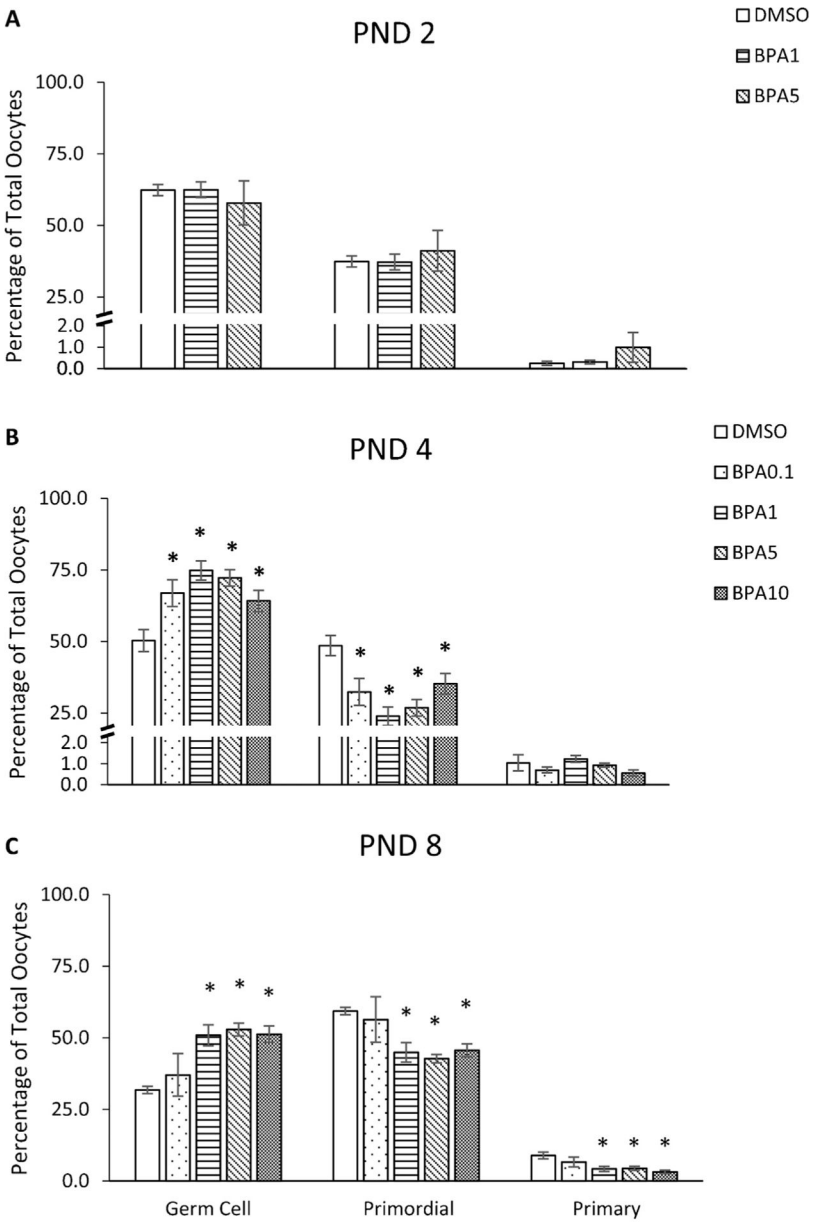

Fig. 2.

Effects of BPA on percentage of germ cells and follicles in the ovary. Neonatal ovaries were collected on postnatal day (PND) 0 and cultured for 2, 4, and 8 days with DMSO or BPA (0.1, 1, 5, and 10 μg/ml), and subjected to histological evaluation of germ cell and primordial follicle formation. Percentages of germ cells, primordial follicles, and primary follicles on PND 2 (panel A), PND 4 (panel B), and PND 8 (panel C) were calculated. Data represent means ± SEM from 3 to 6 separate ovaries. Asterisks (*) indicate significant differences from the vehicle control ($p < 0.05$).

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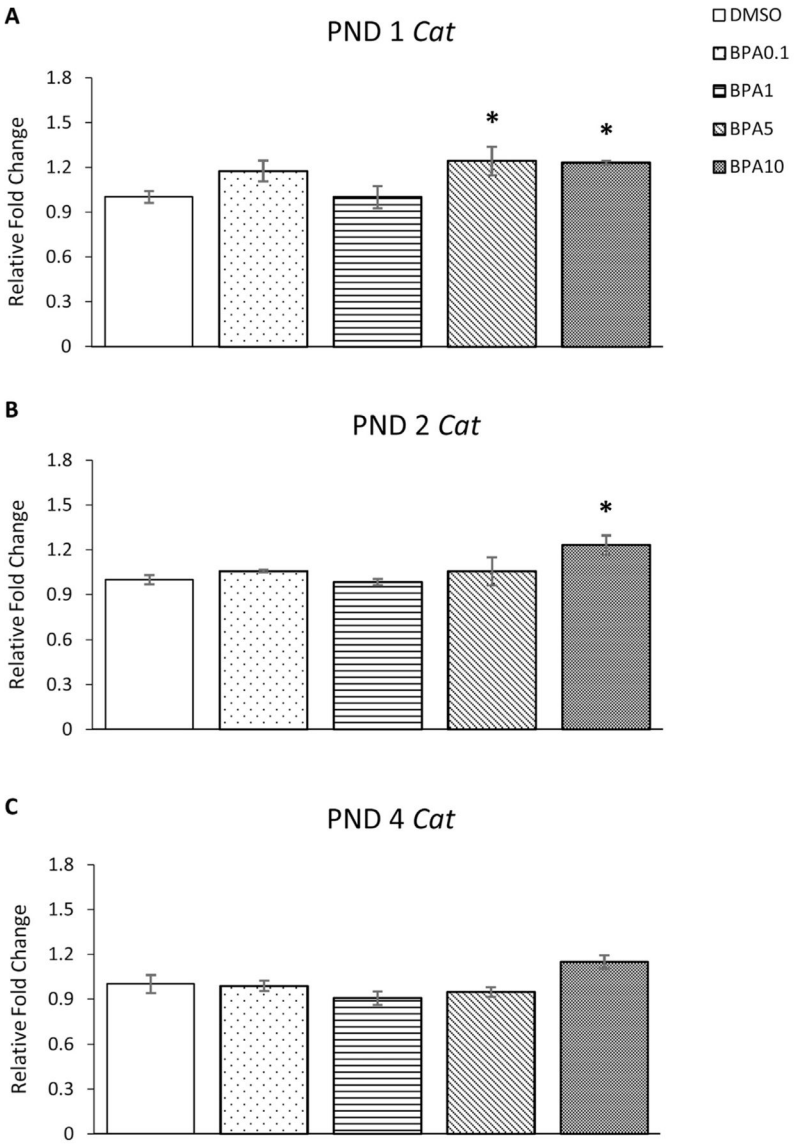

Fig. 3.

Effects of BPA on the mRNA expression level of *catalase* (*Cat*). Neonatal ovaries were collected on postnatal day (PND) 0 and cultured for 1, 2, and 4 days with DMSO or BPA (0.1, 1, 5, and 10 μg/ml). *Cat* expression was measured in the cultured neonatal ovaries using qPCR. Relative fold changes of *Cat* normalized to *Actb* are shown for (A) PND 1, (B) PND 2, and (C) PND 4. The graphs represent means ± SEM from 3 to 6 separate experiments (n = 4–6 ovaries/treatment/experiment). Asterisks (*) indicate significant differences from the vehicle control ($p < 0.05$).

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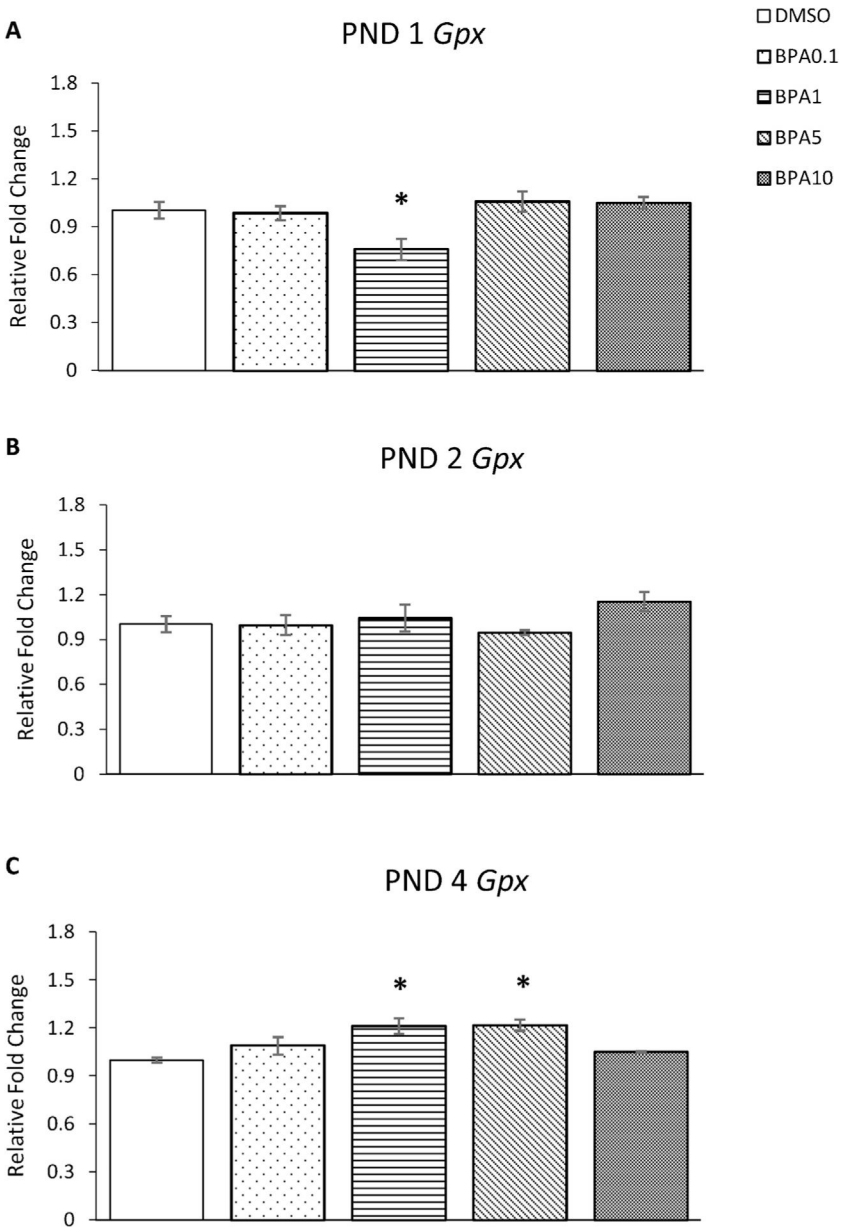

Fig. 4.

Effects of BPA on the mRNA expression level of *glutathione peroxidase* (*Gpx*). Neonatal ovaries were collected on postnatal day (PND) 0 and cultured for 1, 2, and 4 days with DMSO or BPA (0.1, 1, 5, and 10 μg/ml). *Gpx* expression was measured in the cultured neonatal ovaries using qPCR. Relative fold changes of *Gpx* normalized to *Actb* are shown for (A) PND 1, (B) PND 2, and (C) PND 4. The graphs represent means ± SEM from 3 to 6 separate experiments (n = 4–6 ovaries/treatment/experiment). Asterisks (*) indicate significant differences from the vehicle control ($p < 0.05$).

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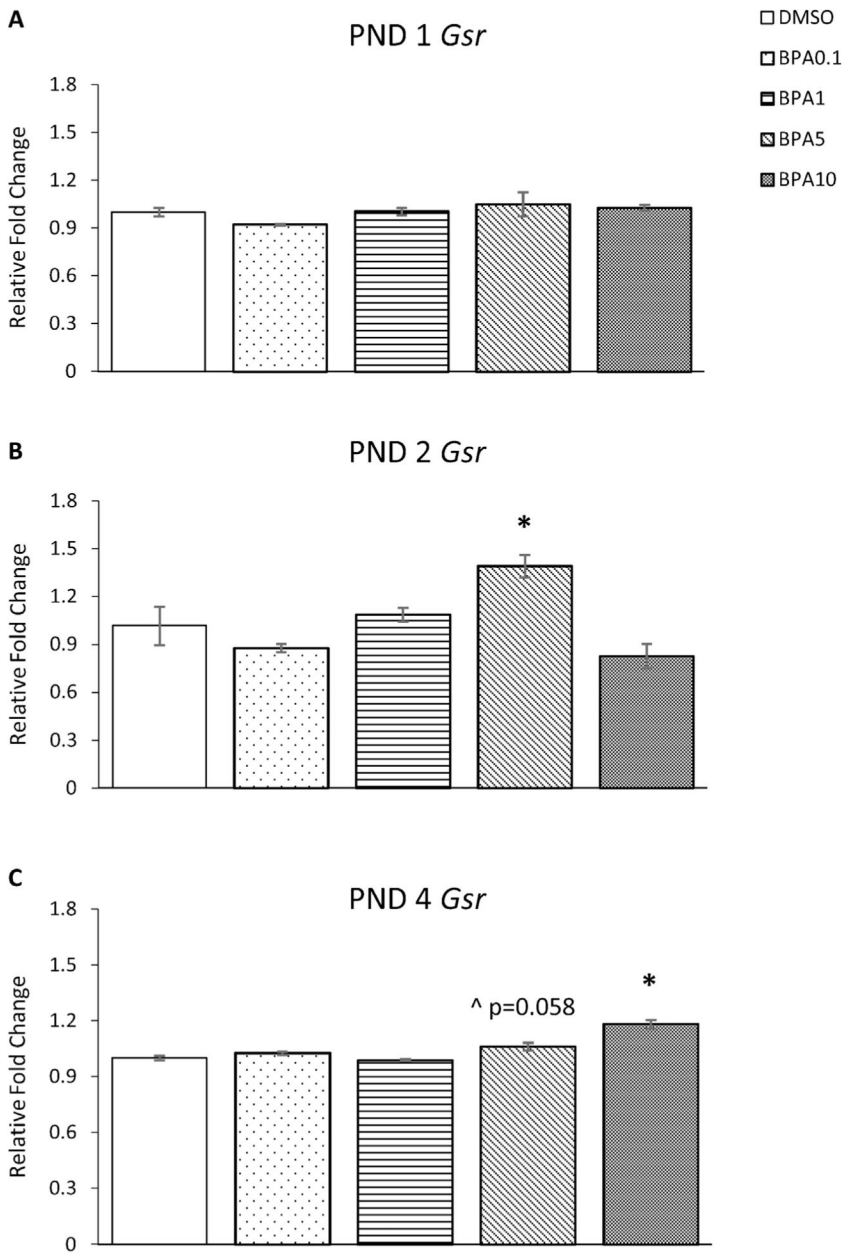

Fig. 5.

Effects of BPA on the mRNA expression level of *glutathione reductase* (*Gsr*). Neonatal ovaries were collected on postnatal day (PND) 0 and cultured for 1, 2, and 4 days with DMSO or BPA (0.1, 1, 5, and 10 μg/ml). *Gsr* expression was measured in the cultured neonatal ovaries using qPCR. Relative fold changes of *Gsr* normalized to *Actb* are graphed for (A) PND 1, (B) PND 2, and (C) PND 4. The graphs represent means ± SEM from 3 to 6 separate experiments (n = 4–6 ovaries/treatment/experiment). Asterisks (*) indicate significant differences from the vehicle control ($p < 0.05$). The ∧ indicates a borderline significant difference from the vehicle control ($p = 0.06$).

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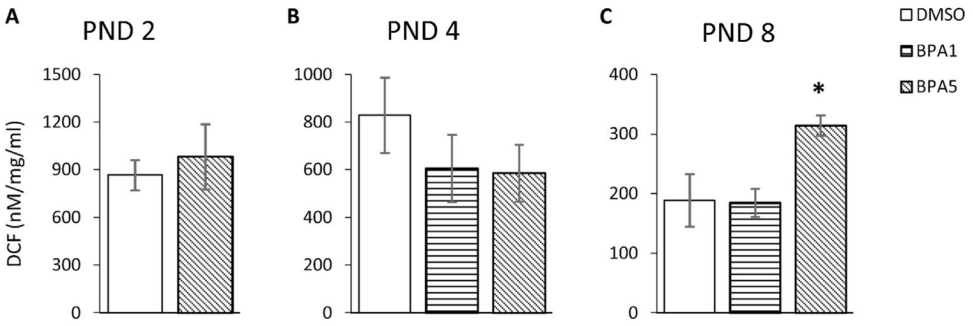

Fig. 6.

Effects of BPA on ROS levels in cultured neonatal ovaries. Neonatal ovaries were collected on postnatal day (PND) 0 and cultured with DMSO or BPA (0.1, 1, 5, and 10 μg/ml) for 2, 4, and 8 days and then subjected to *in vitro* ROS assays to measure ROS levels. Highly fluorescent 2′,7′-dichlorodihydrofluorescein (DCF) was used to indicate ROS levels. The levels of ROS were normalized to protein level in each sample and are shown as absolute concentrations on (A) PND 2, (B) PND 4, and (C) PND 8. All data represent the means ± SEM from three independent experiments (n = 8 ovaries/treatment/experiment). Asterisks (*) indicate significant differences from the vehicle control ($p < 0.05$).

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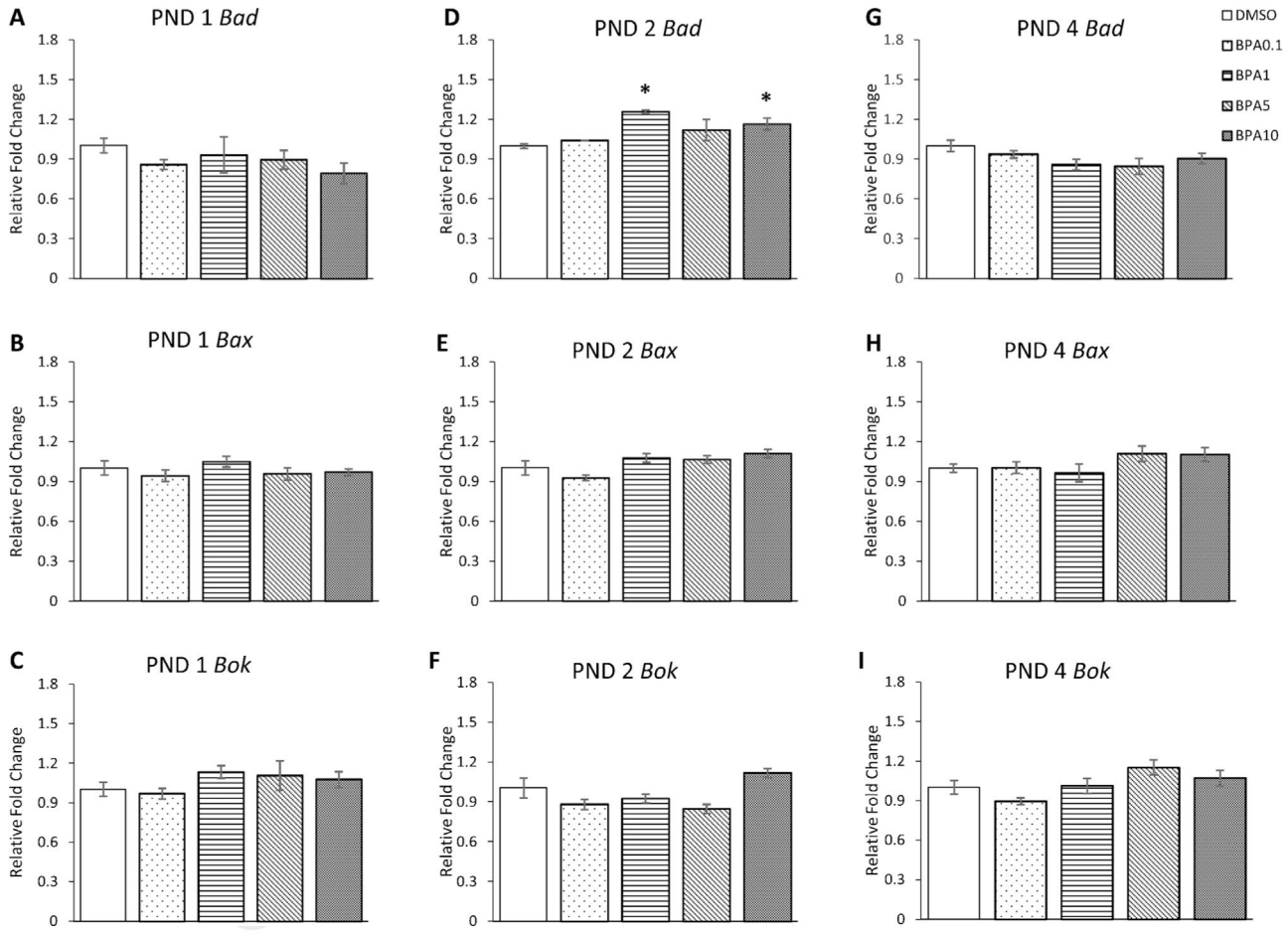

Fig. 7.

Effects of BPA on the mRNA expression level of *B cell leukemia/lymphoma 2* (*Bcl2*) and *Bcl2-like 1* (*Bclxl*). Neonatal ovaries were collected on postnatal day (PND) 0 and cultured for 1, 2, and 4 days with DMSO or BPA (0.1, 1, 5, and 10 μg/ml). *Bcl2* and *Bclxl* expression were measured in cultured neonatal ovaries using qPCR. Relative fold changes normalized to *Actb* are shown for *Bcl2* on (A) PND 1, (B) PND 2, and (C) PND 4, and for *Bclxl* on (D) PND 1, (E) PND 2, and (F) PND 4. The graphs represent means ± SEM from 3 to 4 separate experiments (n = 4–6 ovaries/treatment/experiment). Asterisks (*) indicate significant differences from the vehicle control ($p < 0.05$).

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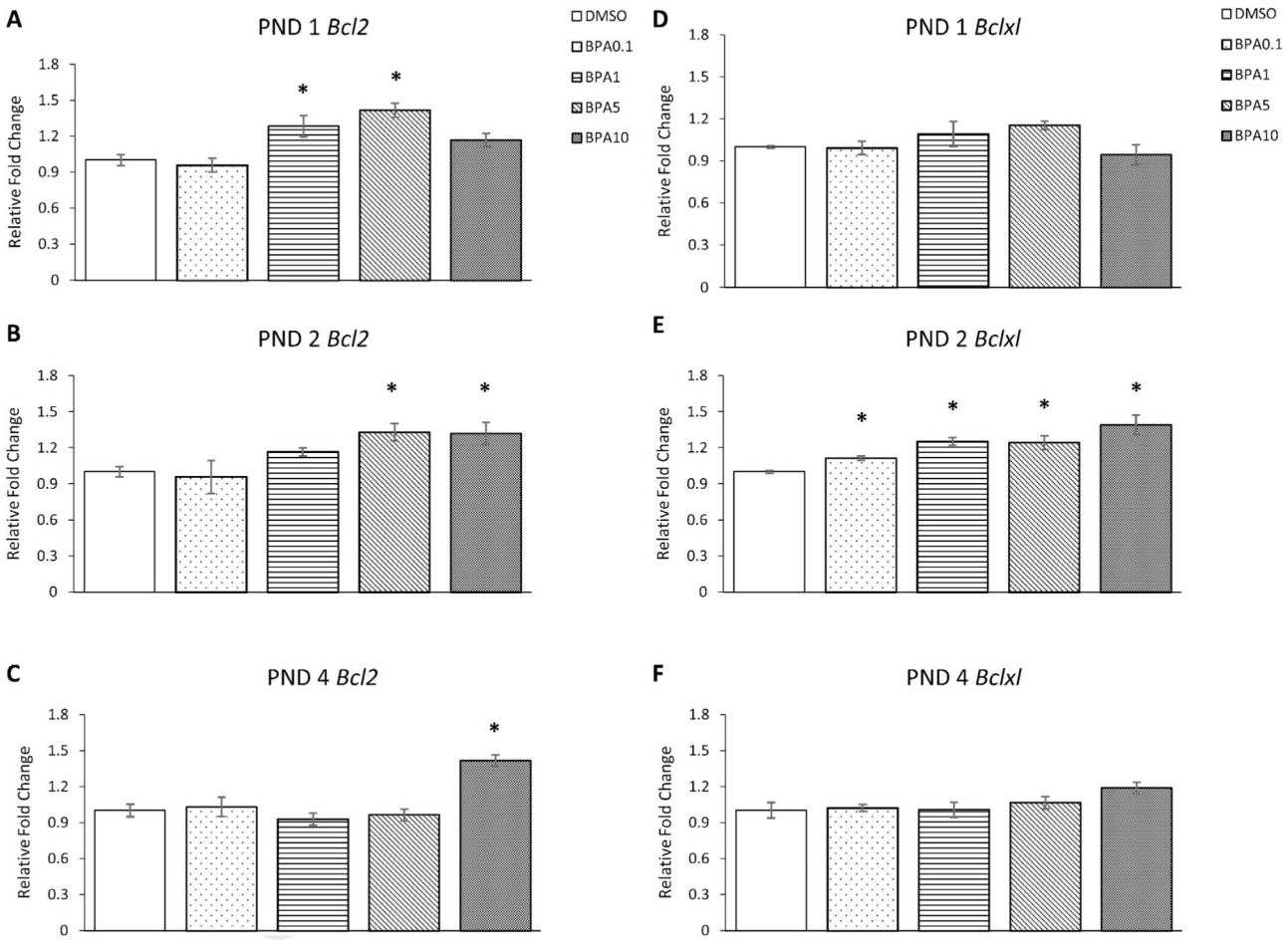

Fig. 8.

Effects of BPA on the ratios of *Bcl2* family members. Neonatal ovaries were collected on postnatal day (PND) 0 and cultured for 1 day with DMSO or BPA (0.1, 1, 5, and 10 μg/ml). *Bcl2*, *Bax*, and *Bad* expression were measured using qPCR. Ratios of the gene expression were calculated and are shown for (A) PND 1 *Bcl2/Bax*, (B) PND 1 *Bcl2/Bad*, (C) PND 1 *Bcl2/Bok*, (D) PND 2 *Bcl2/Bax*, (E) PND 2 *Bcl2/Bad*, (F) PND 2 *Bcl2/Bok*, (G) PND 4 *Bcl2/Bax*, (H) PND 4 *Bcl2/Bad*, and (I) PND 4 *Bcl2/Bok*. The graphs represent means ± SEM from 3 to 4 separate experiments (n = 4–6 ovaries/treatment/experiment). Asterisks (*) indicate significant differences from the vehicle control ($p < 0.05$).

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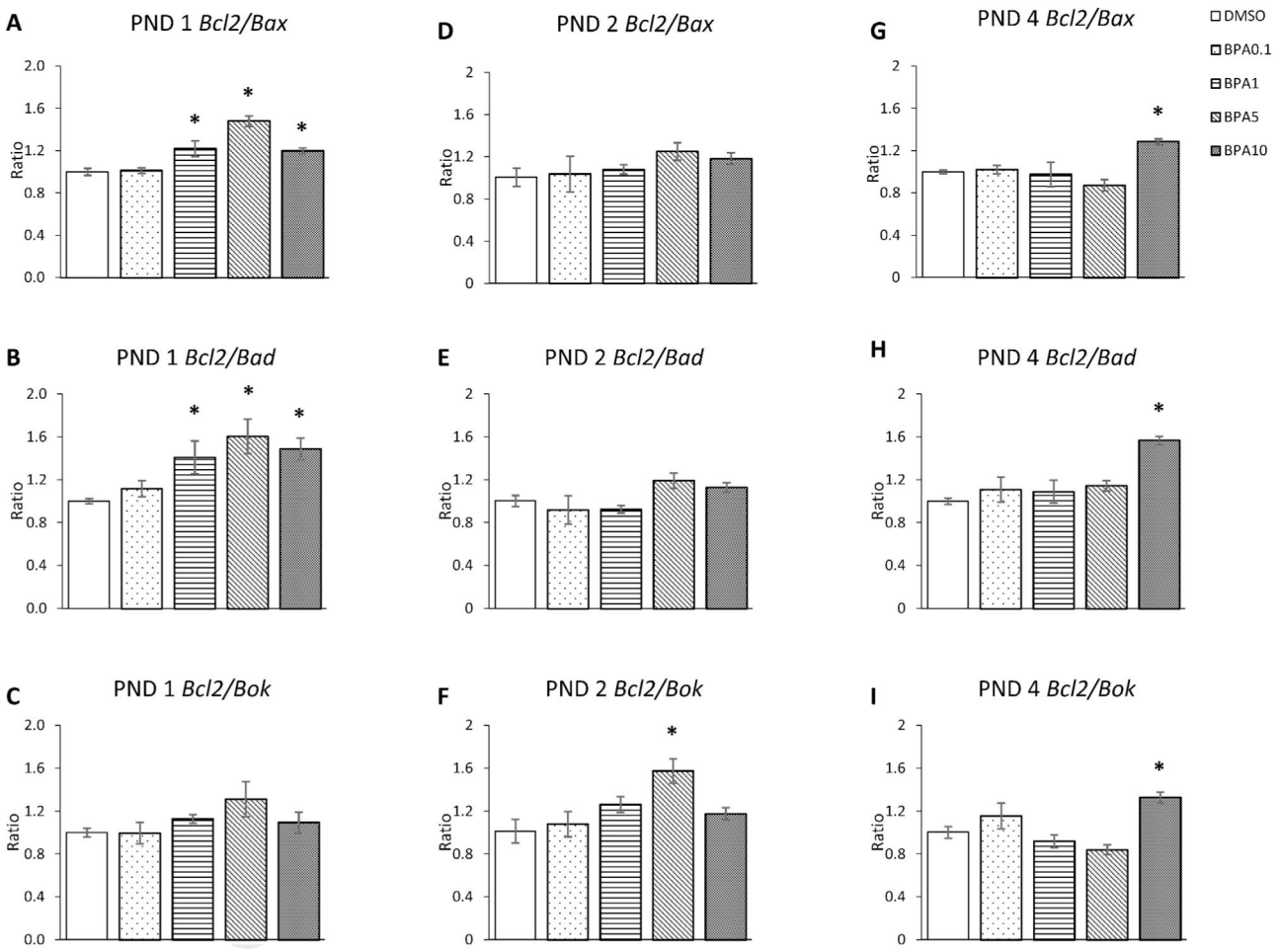

Fig. 9.

Effects of BPA on the mRNA expression level of Fas cell surface death receptor (*Fas*). Neonatal ovaries were collected on postnatal day (PND) 0 and cultured for 1, 2, and 4 days with DMSO or BPA (0.1, 1, 5, and 10 μg/ml). *Fas* expression was measured in using qPCR. Relative fold changes of *Fas* normalized to *Actb* are shown for (A) PND 1, (B) PND 2, and (C) PND 4. The graphs represent means ± SEM from three separate experiments (n = 4–6 ovaries/treatment/experiment). Asterisks (*) indicate significant differences from the vehicle control ($p < 0.05$).

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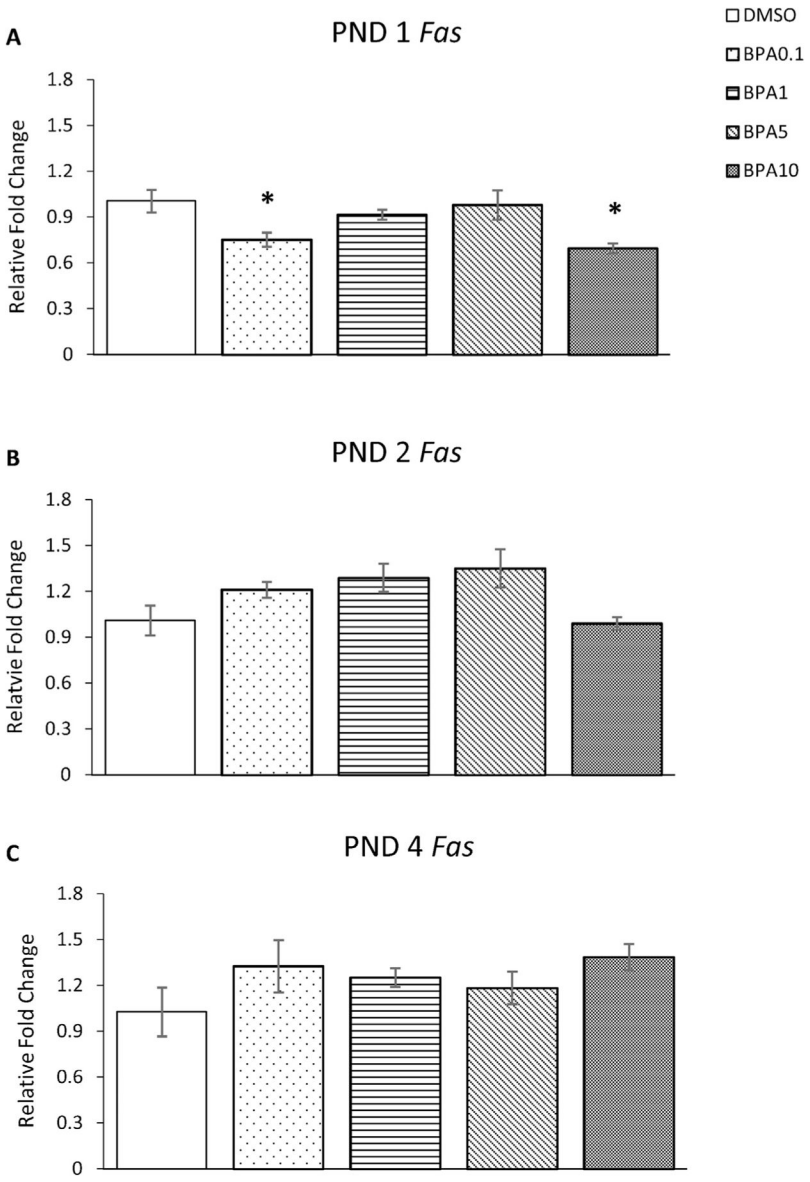

Fig. 10.

Effects of BPA on the mRNA expression level of *caspase8* (*Casp8*). Neonatal ovaries were collected on postnatal day (PND) 0 and cultured for 1, 2, and 4 days with DMSO or BPA (0.1, 1, 5, and 10 μg/ml). *Casp8* expression was measured using qPCR. Relative fold changes of *Casp8* normalized to *Actb* are shown for (A) PND 1, (B) PND 2, and (C) PND 4. The graphs represent means ± SEM from three separate experiments (n = 4–6 ovaries/treatment/experiment). Asterisks (*) indicate significant differences from the vehicle control ($p < 0.05$).

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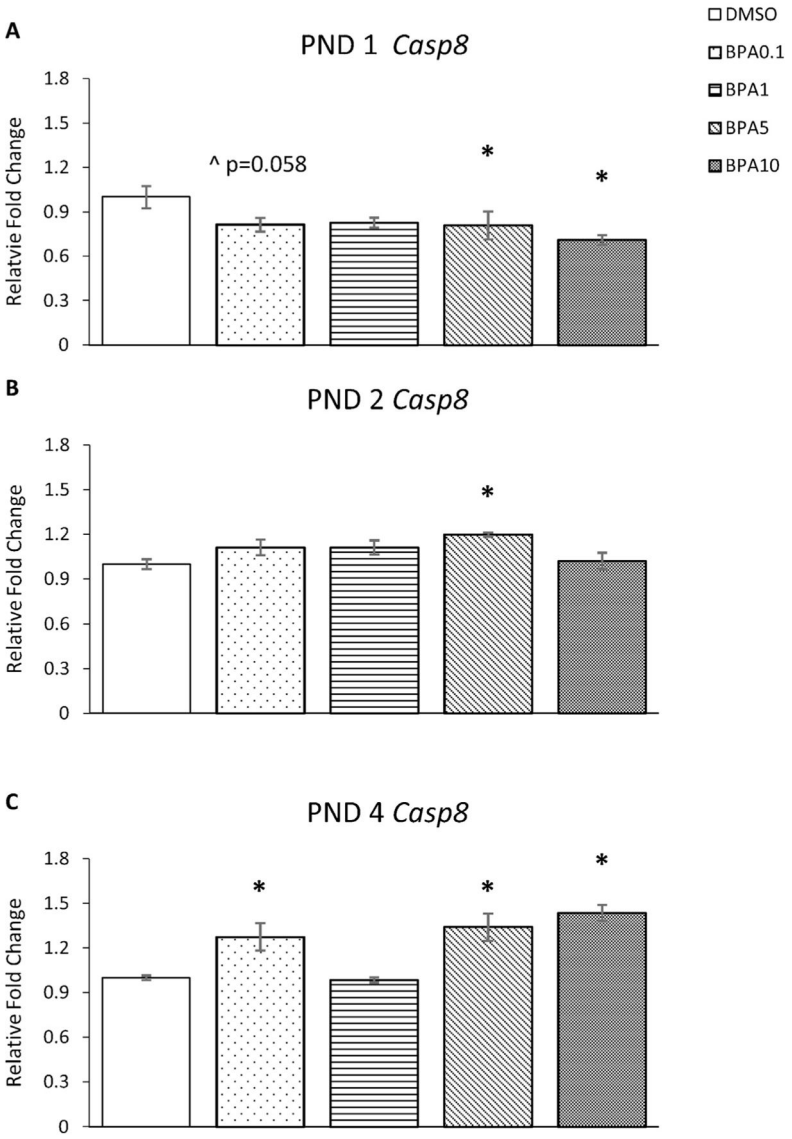

Fig. 11.

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Table 1

Sequences of primer sets used for gene expression analysis.

Gene name	Symbol	Forward primer	Reverse primer
Actin, beta	*Actb*	5′-AGCACAGCTTCTTTGCAGCTCCTT-3′	5′-CAGCGCAGCGATATCGTCATCCAT-3′
Superoxide dismutase 1	*Sod1*	5′-AACCATCCACTTCGAGCAGAAGGCAA-3′	5′-CATACTGATGGACGTGGAACCCAT-3′
Catalase	*Cat*	5′-GCAGATACCTGTGAACTGTC-3′	5′-GTAGAATGTCCGCACCTGAG-3′
Glutathione peroxidase	*Gpx*	5′-TTCGGACACCAGGAGAATGG-3′	5′-TAAAGAGCGGGTGAGCCTTC-3′
Glutathione reductase	*Gsr*	5′-CAGTTGGCATGTCATCAAGCA-3′	5′-CGAATGTTGCATAGCCGTGG-3′
Fas cell surface death receptor	*Fas*	5′-ATGCACACTCTGCGATGAAGAGCA-3′	5′-GTTCACACGAGGCGCAGCGAA-3′
TNF receptor-associated factor 3	*Traf3*	5′-AGGAGTGAGTTGAGTGCACACTTG-3′	5′-TACCGCGGAGCTGGCCTCAT-3′
Caspase8	*Casp8*	5′-GTGAGCCGGCGTGGAACAGG-3′	5′-AGAGCTGTAACCTGTGGCCGAGT-3′
B cell leukemia/lymphoma 2	*Bcl2*	5′-ATGCCTTTGTGGAACTATATGGC-3′	5′-GGTATGCACCCAGAGTGATGC-3′
Bcl2-like 1	*Bclxl*	5′-AAGCGTAGACAAGGAGATGCAGGT-3′	5′-CTGCTGCATTGTTCCCGTAGAGAT-3′
Bcl2-associated X protein	*Bax*	5′-TGAAGACAGGGGCCTTTTTG-3′	5′-AATTCGCCGGAGACACTCG-3′
Bcl2-related ovarian killer protein	*Bok*	5′-CTGCCCCTGGAGGACGCTTG-3′	5′-CCGTCACCACAGGCTCCGAC-3′
Bcl2-associated agonist of cell death	*Bad*	5′-AAGTCCGATCCCGGAATCC-3′	5′-GCTCACTCGGCTCAAACTCT-3′