Bisphenol A Inhibits Follicle Growth and Induces Atresia in Cultured Mouse Antral Follicles Independently of the Genomic Estrogenic Pathway[1]

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ABSTRACT

Bisphenol A (BPA) is an estrogenic chemical used to manufacture many commonly used plastic and epoxy resin-based products. BPA ubiquitously binds to estrogen receptors throughout the body, including estrogen receptor alpha (ESR1) in the ovary. Few studies have investigated the effects of BPA on ovarian antral follicles. Thus, we tested the hypothesis that BPA alters cell cycle regulators and induces atresia in antral follicles via the genomic estrogenic pathway, inhibiting follicle growth. To test this hypothesis, we isolated antral follicles from 32- to 35-day-old control and *Esr1*-overexpressing mice and cultured them with vehicle control (dimethylsulfoxide [DMSO]) or BPA (1–100 μg/ml). Additionally, antral follicles were isolated from 32- to 35-day-old FVB mice and cultured with DMSO, BPA (1–100 μg/ml), estradiol (10 nM), ICI 182,780 (ICI; 1 μM), BPA plus ICI, or BPA plus estradiol. Follicles were measured for growth every 24 h for 96–120 h and processed either for analysis of estrogen receptor, cell cycle, and/or atresia factor mRNA expression, or for histological evaluation of atresia. Results indicate that estradiol and ICI do not protect follicles from BPA-induced growth inhibition and that estradiol does not protect follicles from BPA-induced atresia. Furthermore, overexpressing *Esr1* does not increase susceptibility of follicles to BPA-induced growth inhibition. Additionally, BPA up-regulates *Cdk4*, *Ccne1*, and *Trp53* expression, whereas it down-regulates *Ccnd2* expression. BPA also up-regulates *Bax* and *Bcl2* expression while inducing atresia in antral follicles. These data indicate that BPA abnormally regulates cell cycle and atresia factors, and this may lead to atresia and inhibited follicle growth independently of the genomic estrogenic pathway.

atresia, bisphenol A, cell cycle, follicle growth

INTRODUCTION

Bisphenol A (BPA) is a synthetic estrogen used in the manufacture of commonly used consumer products such as polycarbonate plastics and epoxy resins for food and beverage cans. Levels of human exposure to BPA have been reported in various tissues, including but not limited to adult sera (0.2–20 ng/ml), fetal sera (averaging 8.3 ng/ml), placental tissues (11.2 ng/g), human breast milk (0.28–0.97 ng/ml), human colostrum (1–7 ng/ml), and urine (1.12 ng/ml in women) [1]. BPA exposure has also been measured in Chinese men (sera, 2.84 μg/ml; median personal airborne level, 450 μg/ml) working in factories producing BPA and products containing BPA, such as

epoxy resins [2, 3]. Furthermore, the estimated daily intake of BPA is thought to range from 0.6–71.4 μg/day based on urine samples taken in the morning from human study subjects [1, 4]. In a government funded study (National Health and Nutrition Examination Survey III [5]), BPA has been found to be present in 95% of human urine samples, indicating that humans are constantly exposed to measurable levels of BPA [5].

BPA is an endocrine-disrupting chemical and can interact with various endocrine receptors in the female reproductive tract, including estrogen receptors alpha (ESR1) and beta (ESR2) [6, 7]. BPA has also been shown to interact with the membrane-bound estrogen receptor (GPR30) in human breast cancer cells [8], as well as with estrogen-related receptor gamma in competitive receptor-binding assays [9], indicating that BPA may work through genomic and nongenomic estrogen-related pathways. Previous studies indicate that BPA can affect facets of the reproductive and endocrine systems, including behavior [10, 11], sexual development in the brain [12, 13], and prostate tumor cell proliferation [14]. Also, in the ovary, BPA exposure impairs early oogenesis; induces meiotic incompetence, arrest, and aneuploidy; increases chromosomal congression failure during meiosis; and alters Ca^{2+} oscillations within oocytes isolated from antral follicles [15–18]. However, while these studies investigated the effects of BPA on oocytes isolated from antral follicles, few studies have focused on the effects of BPA on whole antral follicles. Furthermore, it is not known whether BPA works through *Esr* pathways in antral follicles. Thus, one goal of this study was to test the hypothesis that BPA inhibits antral follicle growth through *Esr* pathways.

Follicles, which are the functional units of the ovary, are responsible for growing and housing the oocyte and for producing sex steroid hormones. Follicles grow and develop from the primordial stage to the antral and preovulatory stages throughout the reproductive life of a female [19]. Each stage of follicle development is accompanied by an increase in the number of cell layers present in the follicle. For example, primordial and primary follicles have a single layer of granulosa cells surrounding the oocyte. Preantral, antral, and preovulatory follicles all have multiple layers of granulosa cells and outer cell layers called thecal layers. Follicle growth is required for the follicle to ovulate and release the oocyte for fertilization. Any perturbations in follicle growth can lead to improper oocyte development, anovulation, and abnormal sex steroid hormone levels. In turn, this can result in subfertility or infertility [20, 21]. Without proper growth, the follicle will also be marked for programmed cell death and undergo atresia, preventing ovulation [20, 21]. Over 99% of the finite follicles in the follicular pool at birth will undergo atresia, leaving 1% of follicles to properly develop and ovulate for fertilization during the reproductive lifespan [22, 23]. Exposure to reproductive toxicants that decrease the proper development of antral follicles can hinder normal reproduction.

BPA has been shown to inhibit mouse antral follicle growth in vitro [24]. Follicle growth is dependent on the proliferation of granulosa and thecal cells [25]. Like most other cells in the

body, granulosa and thecal cell proliferation is dependent upon the cell cycle [26]. The cell cycle is controlled in a directional and sequential pattern by cell cycle regulators, complexes of proteins called cyclins and cyclin-dependent kinases (CDK) [26, 27]. These cyclin:CDK complexes are responsible for promoting cell survival by protecting the cell from replicating with damaged DNA or from mutations that may lead to aberrant and uncontrolled cell growth [20]. Cyclin D2 (CCND2) and cyclin-dependent kinase 4 (CDK4) form a complex that monitors the progression of a cell through early G_1 phase. This complex directly up-regulates cyclin E1 (CCNE1), a cell cycle regulator part of another cyclin:Cdk complex that monitors the progression of a cell at the end of G_1 phase and prevents entry into S phase if necessary [20]. Each cyclin:CDK complex in the cell cycle acts as a checkpoint and can, if necessary, halt the cells' progression through the cell cycle and either arrest progression or program cells for death. Transformation-related protein 53 (TRP53) is a specific factor that controls whether the cyclin:Cdk complexes allow cells to progress through the cycle [20]. TRP53, after induction by tissue damage or release from binding inactivation, can down-regulate and inactivate the cyclin:Cdk complex, arresting cell growth or inducing cell death [20, 27]. Given the fact that BPA inhibits antral follicle growth [24] and antral follicle growth is partially controlled by cell cycle regulators [27], a second goal of this study was to test the hypothesis that BPA alters expression of cell cycle regulators in antral follicles, possibly leading to inhibition in growth.

In addition to inhibiting follicle growth by reducing expression of cell cycle regulators, it is possible that BPA inhibits follicle growth by inducing atresia. Atresia, or programmed cell death of antral follicles, is complementary to the cell cycle and follicle growth [27]. Thus, a third goal of this study was to test the hypothesis that BPA induces atresia of antral follicles.

MATERIALS AND METHODS

Chemicals

BPA powder (99%) was purchased from Sigma-Aldrich. A stock solution of BPA was dissolved and diluted in dimethylsulfoxide (DMSO; Sigma-Aldrich) to achieve various BPA treatment concentrations (1.3, 13.3, 133 mg/ml) for final working concentrations of 1, 10, and 100 μg of BPA per milliliter of culture medium. Using these treatment concentrations allowed each working concentration to contain the same volume of chemical and vehicle.

BPA concentrations were also chosen based on studies showing the effects of BPA on ovarian cells and antral follicles [24, 28]. For example, BPA exposure of between 100 fM and 100 μM for 24–72 h results in an increase in apoptosis and G_2-to-M arrest in cultured mouse ovarian granulosa cells [28]. Additionally, BPA exposure of between 10 and 100 μg/ml for 120 h results in a decrease in antral follicle growth, steroidogenesis, and expression of steroidogenic enzymes in vitro [24]. Our concentrations of BPA were relevant to current regulatory levels set for BPA. The lowest observable adverse effect level (LOAEL) is 50 mg/kg/day. This concentration equates to 50 μg/ml. Doses used in these experiments were 1, 10, and 100 μg/ml, encompassing the LOAEL concentration.

Estradiol was obtained from Sigma-Aldrich. A stock solution of estradiol was dissolved and diluted in DMSO (20 μM) for a final concentration in culture of 10 nM. ICI 182,780 (ICI), a high-affinity ESR antagonist, was obtained from Tocris-Cookson. Stock solutions of ICI were dissolved in DMSO (2 mM) for a final concentration in culture of 1 μM. Final concentrations of estradiol and ICI in culture were chosen based on previous studies in our laboratory [29].

Animals

Adult, cycling, female sensitivity to Friend leukemia virus B (FVB) strain mice were purchased from Jackson Laboratory and allowed to acclimate to the facility for at least 5 days before use. Esr1-overexpressing (Esr1 OE) and control mice used in this study were generated as previously described and were obtained from a breeding colony at the University of Illinois [30, 31].

Esr1 OE mice were validated for overexpression of the Esr1 gene and protein levels in ovaries and antral follicles and compared control mice as previously described [30]. All mice were housed at the University of Illinois at Urbana-Champaign, Veterinary Medicine Animal Facility. Food (catalog no. 8626; Harlan Teklad) and water were provided for ad libitum consumption. Temperature was maintained at $22° \pm 1°C$, and animals were subjected to 12L:12D 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.

Follicle Culture

Female FVB mice were euthanized on Postnatal Days (PND) 32–35, and their ovaries were removed using aseptic technique. Antral follicles were mechanically isolated from the ovary based on relative size (250–400 μm), cleaned of interstitial tissue by using fine watchmaker forceps [23, 32], and individually placed in wells of 96-well culture plates containing unsupplemented α-minimal essential medium (α-MEM) prior to treatment. Sufficient numbers of antral follicles for statistical power were isolated from unprimed mouse ovaries; follicles from 2–3 mice were isolated per experiment, providing approximately 20–40 antral follicles from each mouse. Each experiment contained a minimum of 8–16 follicles per treatment group. Concentrations of vehicle control (DMSO), BPA (1–100 μg/ml), estradiol (10 nM), and ICI (1 μM) were individually prepared in supplemented α-MEM. Supplemented α-MEM was prepared with 1% ITS (10 ng/ml insulin, 5.5 ng/ml transferrin, 5.5 ng/ml selenium), 100 U/ml penicillin, 100 mg/ml streptomycin, 5 IU/ml human recombinant follicle-stimulating hormone (FSH [provided by Dr. A.F. Parlow, National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA]), and 5% fetal calf serum (Atlanta Biologicals) [23, 32]. An equal volume of chemical was added for each treatment to control for the amount of vehicle in each preparation. In some cultures, antral follicles were cotreated with BPA plus estradiol. In follicle cultures with ICI, antral follicles were pretreated with ICI in supplemented α-MEM for 2 h prior to subsequent treatments with BPA, ICI, and BPA plus ICI. All antral follicles were cultured for 24–120 h in an incubator supplying 5% CO_2 at 37°C.

Follicle growth was measured at 24-h intervals beginning at 0 hr by measuring follicle diameters on a perpendicular axis with an inverted microscope equipped with a calibrated ocular micrometer. Follicle measurements were averaged among treatment groups at each time point, and data were presented as percent change in growth over time per treatment group. Images of the follicles were taken using a digital microscope camera (ProgRes CT3 model; Jenoptik) and captured using image acquisition software (ProgRes CapturePro; Jenoptik).

Analysis of Atresia

At the end of follicle culture (48 or 120 h), supplemented α-MEM was removed from each well, and Dietrick solution was added to fix the follicles. Follicles were fixed for at least 24 h in Dietrick solution and transferred in histology cassettes to 70% ethanol. Tissues were dehydrated, embedded using a methyl methacrylate-based resin kit (Technovit; Heraeus Kulzer GmbH), serially sectioned (2 μm), mounted on glass slides, and stained with Lee methylene blue-basic fuchsin stain. Each follicle section was examined for the level of atresia as shown by the presence of apoptotic bodies and reported at the highest level observed throughout the follicle. Follicle sections were rated on a scale of 1–4 for the presence of apoptotic bodies, where 1 = 0 apoptotic bodies (healthy); 2 = ≤10% apoptotic bodies (early atresia); 3 = 10%–30% apoptotic bodies (mid atresia); and 4 = ≥30% apoptotic bodies (late atresia), as previously described [32]. All analyses were conducted without knowledge of treatment group. Ratings were averaged and plotted to compare the effects of BPA treatments on atresia levels. Images of the follicles were taken at 40× magnification, using a digital microscopy camera (model DFC290; Leica Microsystems) and captured with image acquisition software (ImagePro; Leica).

Analysis of Gene Expression

Female FVB mouse antral follicles were cultured as described above for 24–96 h. At the end of culture, follicles were collected and snap frozen at –80°C for real-time quantitative PCR (qPCR) analysis. Total RNA was extracted from follicles using the RNeasy micro kit (Qiagen, Inc.) according to the manufacturer's protocol. Reverse transcriptase generation of cDNA was performed with 0.3–1 μg of total RNA, using an iScript RT kit (Bio-Rad Laboratories, Inc.). qPCR was conducted using the CFX96 real-time PCR detection system (Bio-Rad Laboratories, Inc.) and accompanying software (CFX Manager software) according to the manufacturer's instructions. The
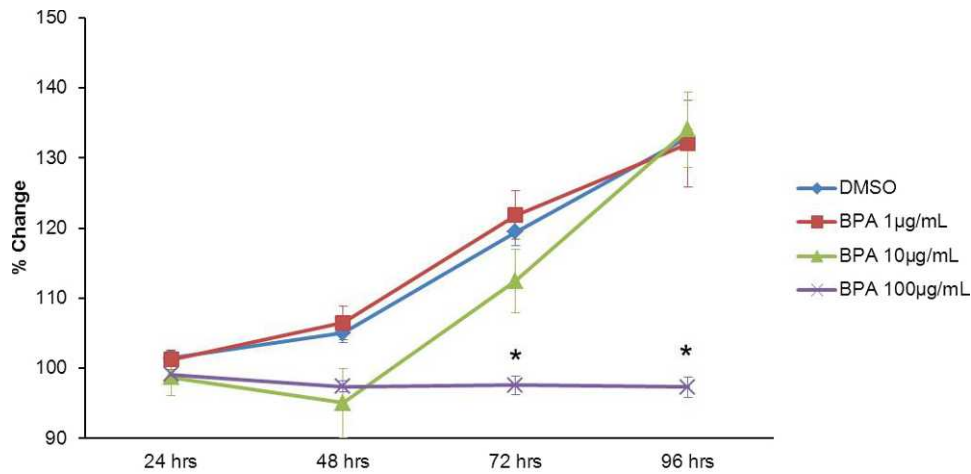
Article 63

FIG. 1. Effect of BPA on follicle growth. Antral follicles were mechanically isolated from FVB mice and exposed in vitro to DMSO or 10–100 µg/ml BPA for 24–96 h. Growth of follicles was monitored during culture and recorded in micrometers and reported as percent change. Data are means ± SEM from at least three separate experiments. Lines with asterisks (*) show data that are significantly different from those of DMSO controls (n = 8–16 follicles per treatment per experiment from at least three separate experiments; $P \leq 0.05$).

CFX96 system quantifies the amount of PCR product generated by measuring a dye (SYBR Green) that fluoresces when bound to double-stranded DNA. A standard curve was generated from five serial dilutions of one of the samples, thus allowing analysis of the amount of cDNA in the exponential phase. qPCR analysis was performed using 2 µl of cDNA, forward and reverse primers (5 pmol) for estrogen receptor alpha (*Esr1*; GenBank accession no. NM_007956), estrogen receptor beta (*Esr2*; GenBank accession no. NM_207707), cyclin-dependent kinase 4 (*Cdk4*; GenBank accession no. NM_009870.3), cyclin D2 (*Ccnd2*; GenBank accession no. NM_009829.3), cyclin E1 (*Ccne1*; GenBank accession no. NM_007633.2), transformation-related protein 53 (*Trp53*; GenBank accession no. NM_011640.3), B-cell lymphoma 2 (*Bcl2*; GenBank accession no. NM_009741.3), Bcl2-associated X protein (*Bax*; GenBank accession no. NM_007527.3), or beta-actin (*Actb*; GenBank accession no. NM_007393), in conjunction with a SsoFast EvaGreen Supermix qPCR kit (Bio-Rad Laboratories). An initial incubation of 95°C for 10 min was followed by denaturing at 94°C for 10 sec, annealing from 60°C for 10 sec, and extension at 72°C for 10 sec for 40 cycles (β-actin), followed by final extension at 72°C for 10 min. A melting curve was generated at 55°–90°C to monitor the generation of a single product. The software also generated a standard curve. The *Actb* gene was used as a reference for each sample. Final values were calculated and expressed as the ratio normalized to *Actb*. All analyses were performed in duplicate for at least three separate experiments.

Statistical Analysis

Data were expressed as means ± standard errors of the means (SEM), and multiple comparisons between experimental groups were made using general linearized model and ANOVA as applicable, followed by Tukey post hoc comparisons. At least three separate experiments were conducted for each treatment prior to data analysis. Statistical significance was assigned at a *P* value of ≤0.05.

RESULTS

Effect of BPA Treatment on Follicle Growth

Previous studies indicate that BPA inhibits follicle growth in mouse antral follicles [24]. In the current studies, we expanded on previous work by testing the hypothesis that BPA inhibits follicle growth through ESR pathways. Thus, it was first important to determine whether BPA inhibited follicle growth as was found similarly in previous studies. Exposure to BPA (100 µg/ml) significantly decreased follicle growth compared to exposure to DMSO beginning at 72 h and continuing throughout culture (Fig. 1). BPA at 1 µg/ml and BPA at 10 µg/ml did not significantly affect growth compared to DMSO at any time point.

Morphological changes in follicle growth were consistent with data indicating follicle growth is significantly inhibited after exposure to 100 µg/ml BPA compared to DMSO beginning at 72 h and continuing throughout culture (Fig. 1). In DMSO-treated follicles, follicle size steadily increased over time, suggesting that cells were proliferating, resulting in a ''fuzzy'' appearance (Fig. 2A). In 100 µg/ml BPA-treated follicles, size did not significantly change over time, suggesting no significant proliferation within the follicle (Fig. 2B).

Effects of BPA Treatment on Esr *Pathways in Antral Follicles*

To examine whether BPA used ESR pathways in antral follicles, we first examined the effect of BPA on *Esr1* and *Esr2* expression levels in antral follicles. Exposure to 1 µg/ml BPA decreased *Esr1* expression at 24 h compared to DMSO, but expression returned to control levels at 48, 72, and 96 h. Exposure to 100 µg/ml BPA significantly increased *Esr1* expression at 24 h but decreased *Esr1* expression at 72 h compared to DMSO controls (Fig. 3A). BPA at 10 µg/ml did not alter *Esr1* expression at any time point. Exposure to 10 µg/ml BPA significantly increased *Esr2* expression compared to that in DMSO controls beginning at 24 h and continuing through 72 h of culture (Fig. 3B). BPA at 1 µg/ml and BPA at 100 µg/ml did not significantly alter expression of *Esr2* at any time point.

To determine whether BPA works through the ESR pathway, we next cotreated antral follicles with BPA and estradiol to examine whether estradiol could block BPA-induced growth inhibition. Exposure to 100 µg/ml BPA significantly decreased follicle growth compared to exposure to DMSO and estradiol controls (Fig. 4A). Estradiol cotreatment did not protect antral follicles from BPA-induced inhibition of follicle growth (Fig. 4A).

Furthermore, to determine whether blocking ESRs in antral follicles could protect antral follicles from BPA-induced follicle growth inhibition, we pretreated antral follicles with the ESR antagonist ICI to examine whether ICI blocked BPA-induced growth inhibition. Exposure to BPA at 100 µg/ml significantly decreased follicle growth compared to DMSO and ICI controls (Fig. 4B). Pretreating follicles with ICI did not protect antral follicles from BPA-induced inhibition of follicle growth (Fig. 4B).
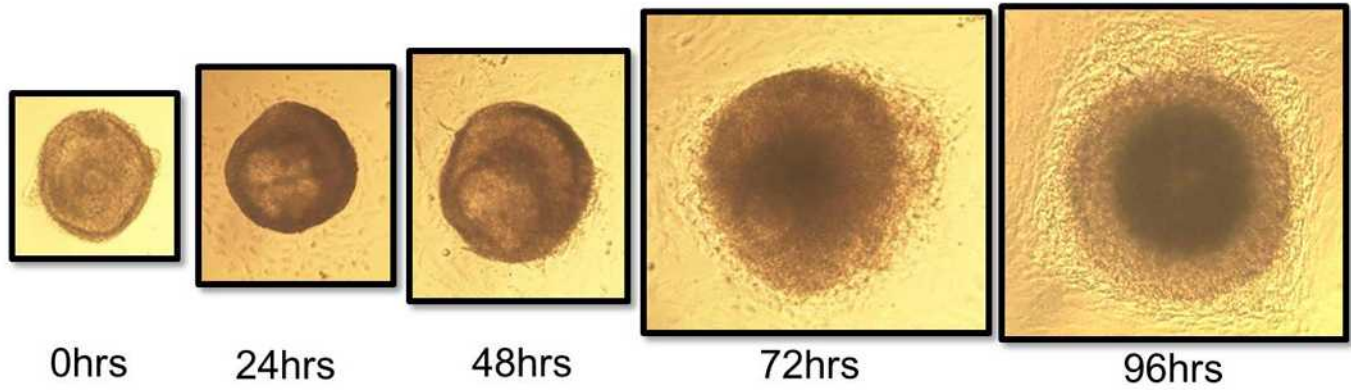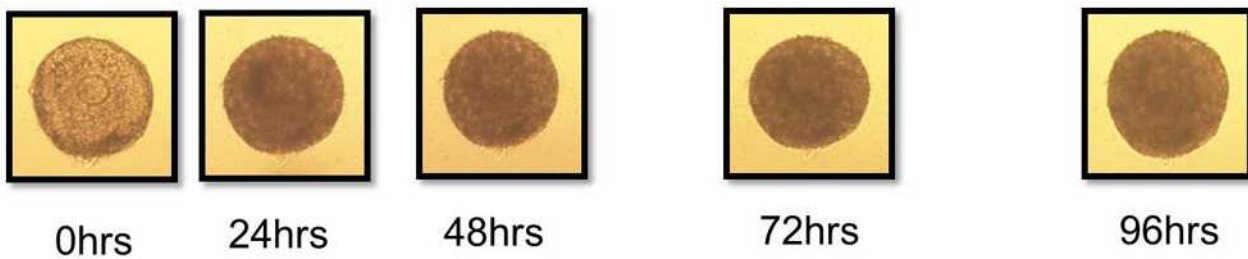
Article 63

A) DMSO

0hrs   24hrs   48hrs   72hrs   96hrs

B) BPA 100µg/mL

0hrs   24hrs   48hrs   72hrs   96hrs

FIG. 2.   Effect of BPA on morphology. Antral follicles were mechanically isolated from FVB mice and exposed in vitro to DMSO or 100 µg/ml BPA for 24–96 h. Growth of follicles was observed as described in *Materials and Methods*. Original magnification ×10.
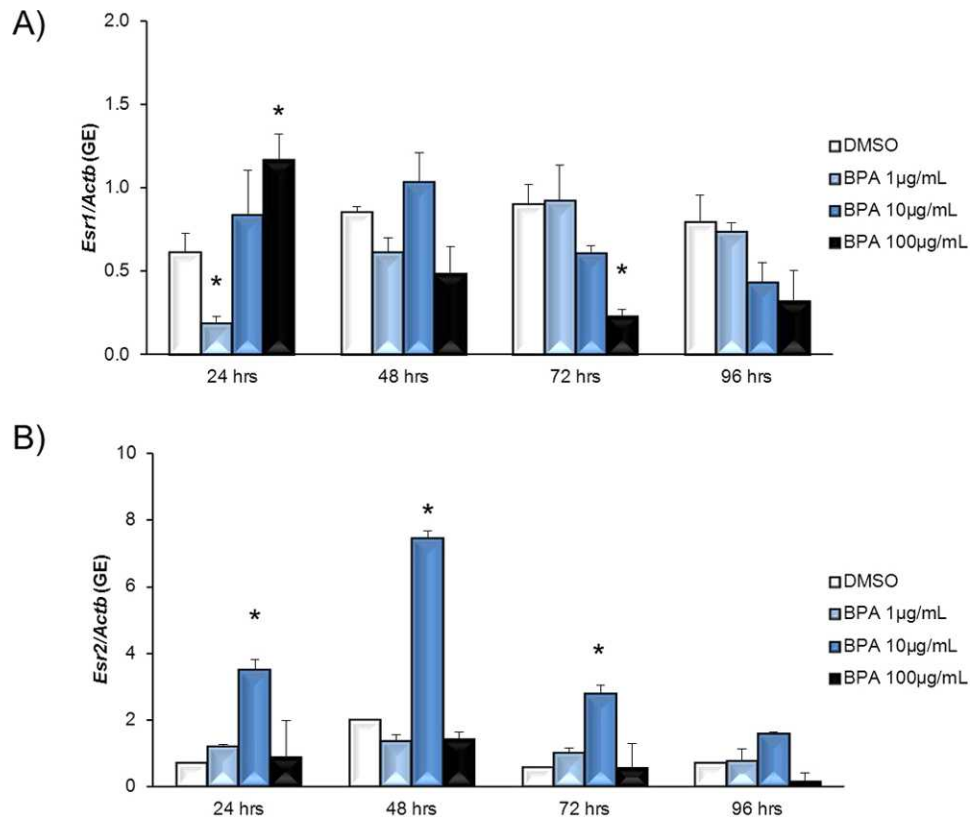

FIG. 3.   Effect of BPA exposure on *Esr1* and *Esr2* mRNA expression levels. After antral follicles were exposed to DMSO control or 1–100 µg/ml BPA for 24–96 h in vitro, the follicles were collected and subjected to qPCR analysis for *Esr1* (**A**) and *Esr2* (**B**) mRNA expression levels. All values were normalized to those of beta-actin as a loading control. Graphs show means ± SEM from at least three separate experiments. *$P \le 0.05$ from DMSO control.
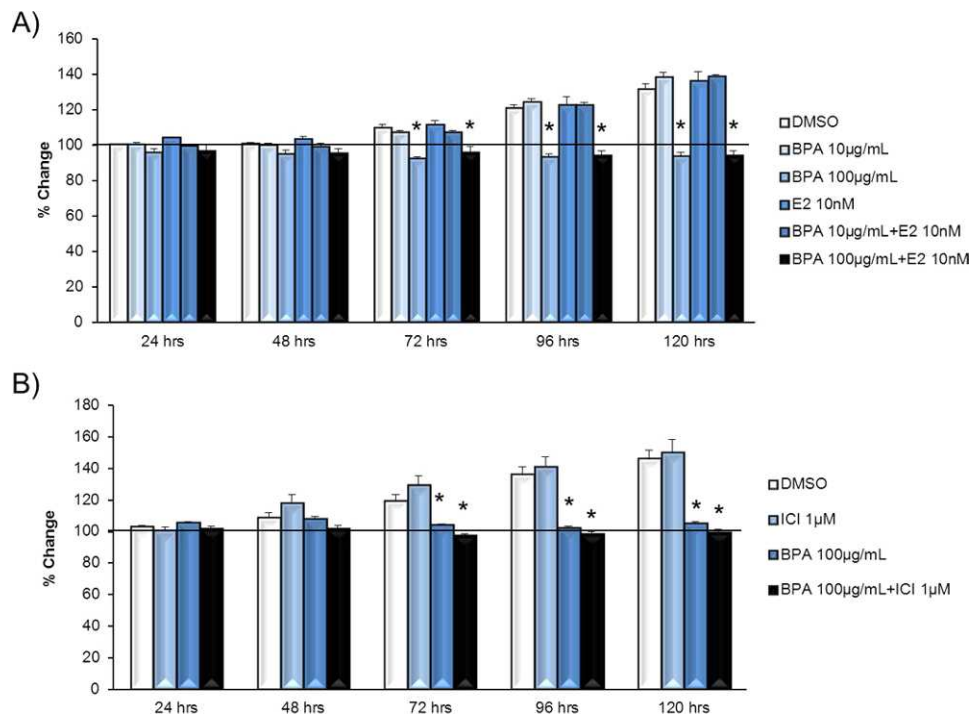
FIG. 4. Effect of estradiol and ICI on BPA-induced follicle growth inhibition. Antral follicles were mechanically isolated from FVB mice and exposed in vitro to DMSO; 10–100 µg/ml BPA; 10 nM estradiol or 10–100 µg/ml BPA plus 10 nM estradiol (**A**) or DMSO; 100 µg/ml BPA, 1 µM ICI; or 100 µg/ml BPA plus 1 µM ICI (**B**) for 24–120 h. Growth of follicles was monitored during culture, recorded in micrometers, and reported as percent change. Graphs show means ± SEM from at least three separate experiments. Lines with asterisks (*) show data that are significantly different from those of DMSO controls (n = 8–16 follicles per treatment per experiment from at least three separate experiments; $P \leq 0.05$).

Finally, because previous studies have shown that overexpressing ESR1 increases the susceptibility of follicles to growth inhibition induced by other estrogenic endocrine-disrupting chemicals [30], we treated *Esr1* OE mouse antral follicles with BPA to determine whether overexpressing *Esr1* increases the susceptibility of follicles to BPA-induced growth inhibition. Exposure to 100 µg/ml BPA significantly decreased follicle growth compared to DMSO in controls (Fig. 5A) and *Esr1* OE (Fig. 5B) follicles beginning at 72 h and continuing throughout culture. BPA at 1 µg/ml and BPA at 10 µg/ml did not significantly affect growth in either control or *Esr1* OE follicles.

Effect of BPA on Cell Cycle Regulators

Even though BPA does not work through ESR1 to inhibit follicle growth, BPA still inhibits follicle growth (Fig. 1). Thus, we next examined whether BPA alters the expression of cell cycle regulators in antral follicles, because cell cycle regulators have been shown to control granulosa cell proliferation and, thus, control follicle growth [26]. Exposure to 100 µg/ml BPA significantly increased *Cdk4* expression compared to DMSO controls beginning at 24 h and continuing throughout culture (Fig. 6A). BPA at 1 µg/ml and BPA at 10 µg/ml did not significantly affect *Cdk4* expression compared to DMSO at any time point. Exposure to 100 µg/ml BPA significantly decreased *Ccnd2* expression compared to DMSO at 48 h (Fig. 6B). BPA at 1 µg/ml and BPA at 10 µg/ml did not significantly affect *Ccnd2* expression compared to DMSO at any time point. Exposure to 100 µg/ml BPA significantly increased *Ccne1* expression compared to DMSO beginning at 24 h and continuing throughout culture (Fig. 7A). BPA at 1 µg/ml and BPA at 10 µg/ml did not significantly affect *Ccne1* expression compared to DMSO at any time point. Exposure to 100 µg/ml

BPA significantly increased *Trp53* expression compared to DMSO beginning at 24 h and continuing throughout culture (Fig. 7B). BPA at 1 µg/ml and BPA at 10 µg/ml did not significantly affect *Trp53* expression compared to DMSO at any time point.

Effect of BPA on Atresia of Antral Follicles

Another mechanism by which BPA might inhibit follicle growth may include inducing atresia. If BPA induces atresia, follicles would not be able to grow normally. Thus, we conducted studies to determine whether BPA induces atresia. Atresia was rated by quantifying the percentage of apoptotic bodies present in the follicles. These morphological changes in the follicle were used to determine whether BPA at 100 µg/ml induced atresia compared to DMSO beginning at 48 h and continuing to 120 h in culture (Fig. 8, A–D). Apoptotic bodies also were used to determine whether estradiol cotreatment does not protect follicles from BPA-induced atresia (Fig. 8, E and F). In DMSO- and estradiol-treated follicles at 48 and 120 h, granulosa cells were tightly organized in layers, and no apoptotic bodies were present within the follicle (Fig. 8, A, C, and E). In follicles treated with 100 µg/ml BPA and those treated with 100 µg/ml BPA plus estradiol (10 nM), beginning at 48 h and continuing at 120 h, granulosa cells were no longer tightly organized into multiple layers, and apoptotic bodies were present (Fig. 8, red arrows) in greater than 30% of the follicles. Furthermore, after follicles were exposed to BPA at 100 µg/ml or to BPA at 100 µg/ml plus estradiol (10 nM) at 120 h, few granulosa cells remained (Fig. 8, B, D, and F). Exposure to BPA at 100 µg/ml significantly induced atresia at 48 and 120 h compared to DMSO (Fig. 9A). Cotreatment with estradiol did not protect antral follicles from BPA-induced atresia (Fig. 9B) at any time point.
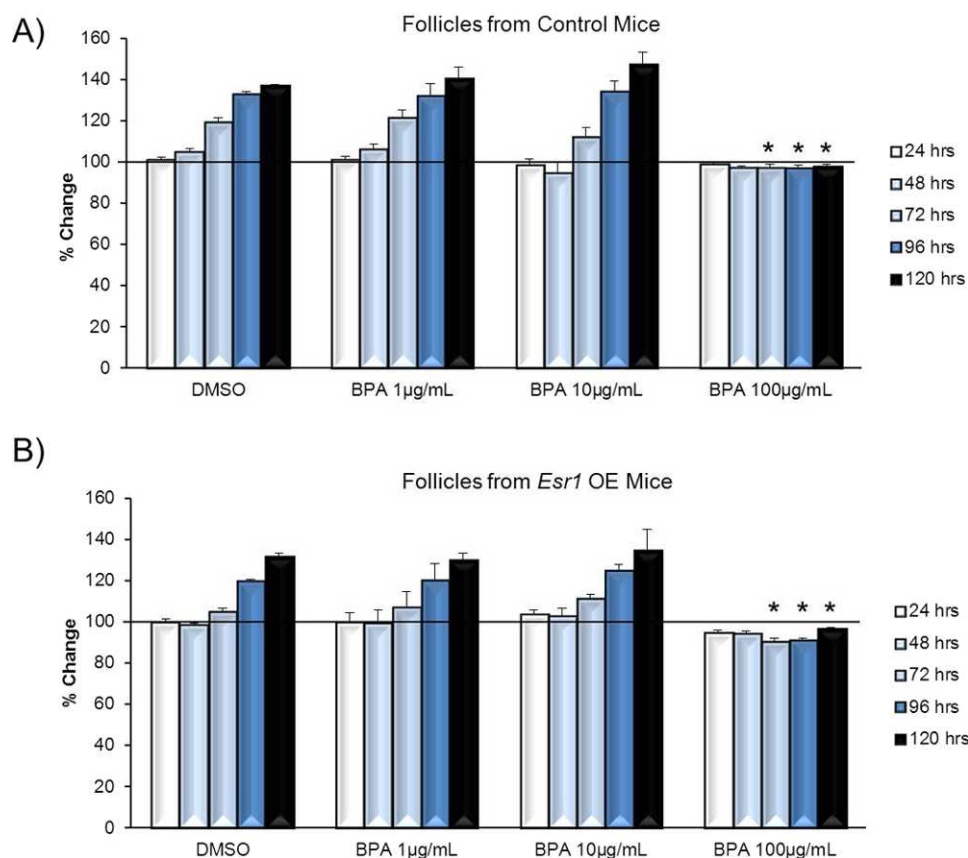
FIG. 5. Effect of overexpressing *Esr1* on susceptibility to BPA-induced follicle growth inhibition. Antral follicles were mechanically isolated from control (**A**) or *Esr1* OE (**B**) mice and exposed in vitro to DMSO or BPA at 10–100 μg/ml for 24–120 h. Growth of follicles was monitored during culture, recorded in micrometers, and reported as percent change. Graphs show means ± SEM from at least three separate experiments. Lines with asterisks (*) show data that are significantly different from those of DMSO controls (n = 8–16 follicles per treatment per experiment from at least three separate experiments; $P \leq$ 0.05).

Because BPA induces atresia in antral follicles (Fig. 9), we next determined whether BPA alters the expression of selected regulators of atresia. Exposure to BPA at 100 μg/ml significantly increased expression of *Bax*, a proatretic factor, compared to DMSO beginning at 24 h and continuing throughout culture (Fig. 10A). BPA at 1 μg/ml and BPA at 10 μg/ml did not significantly affect *Bax* expression compared to DMSO at any time point. Similarly, exposure to BPA at 100 μg/ml significantly increased expression of *Bcl2*, an antiatretic factor, compared to DMSO beginning at 24 h and continuing throughout culture (Fig. 10B). BPA at 1 μg/ml and BPA at 10 μg/ml did not significantly affect *Bcl2* expression compared to DMSO at any time point.

DISCUSSION

Consistent with this study, previous work by our group has shown that in vitro BPA exposure inhibits antral follicle growth [24]. However, the current study expands on previous studies by showing that BPA does not work through genomic estrogen receptors to inhibit follicle growth. Although BPA binds with less affinity than estradiol to ESR1 [33–35] and ESR2 [34, 34], studies have shown that BPA acts through genomic estrogen receptors in other cell types. BPA induces *Esr1* expression in fetal mouse mesenchyme cells [36], induces *Esr1* expression and estrogen responsive proteins in the female rat uterus [33], and induces activation of estrogen response elements in Hep2G cells [33] and pituitary, stimulating ESR-dependent prolactin release and cell proliferation in the pituitary [33]. In our study,

Esr1 expression was up-regulated in the follicles with inhibited growth at 24 h but then down-regulated at 72 h, indicating BPA has an effect on *Esr1* but that this effect is partially time-dependent. Furthermore, although 10 μg/ml BPA did not significantly inhibit follicle growth, exposure to 10 μg/ml BPA increased *Esr2* expression beginning at 24 h and continuing throughout culture. Granulosa cell proliferation and follicle growth are driven by ESR2 [37], thus up-regulation of *Esr2* may have protected these follicles from BPA-induced growth inhibition by promoting granulosa cell proliferation.

After identifying the fact that BPA increases expression of *Esr1* in follicles with inhibited growth, we wanted to determine whether BPA works through this receptor. Previous studies have shown that estradiol can displace BPA from ESR1 in competitive binding assays [34] and that ICI can block *Esr1* activation by BPA in Hep2G cells transfected with *Esr1* [33]. Furthermore, overexpression of *Esr1* can increase the susceptibility of follicles to growth inhibition after exposure to other endocrine-disrupting chemicals, such as methoxychlor [30]. Thus, we hypothesized that inhibition of follicle growth is mediated through *Esr1* and that addition of estradiol and ICI would protect the follicles from BPA-induced inhibition of follicle growth. Furthermore, overexpression of *Esr1* would make follicles more susceptible to BPA. However, neither cotreatment with estradiol nor pretreatment with ICI prevented BPA from inhibiting follicle growth. Additionally, overexpression of *Esr1* did not increase the sensitivity of follicles to BPA-induced follicle growth inhibition.
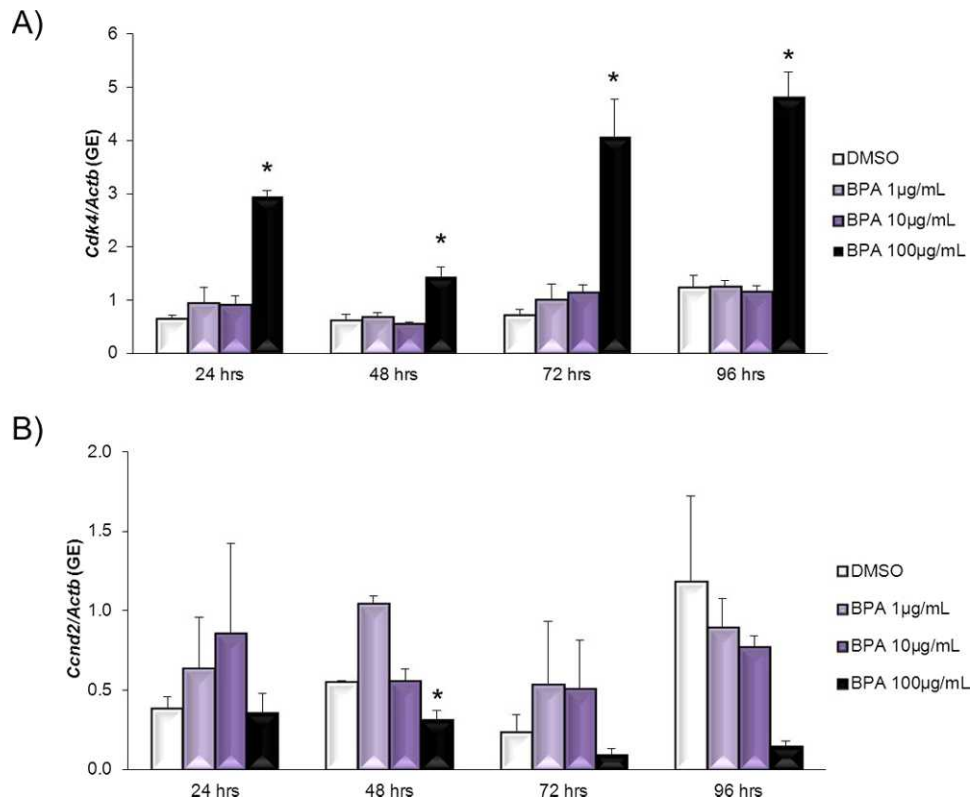
Article 63

FIG. 6. Effect of BPA on *Cdk4* and *Ccnd2* mRNA expression levels. After antral follicles were exposed to DMSO control or BPA at 1–100 μg/ml for 24–96 h in vitro, the follicles were collected and subjected to qPCR analysis for *Cdk4* (**A**) and *Ccnd2* (**B**) mRNA expression levels. All values were normalized to those of beta-actin as a loading control. Graphs show means ± SEM from at least three separate experiments. *$P \leq 0.05$ from DMSO control.
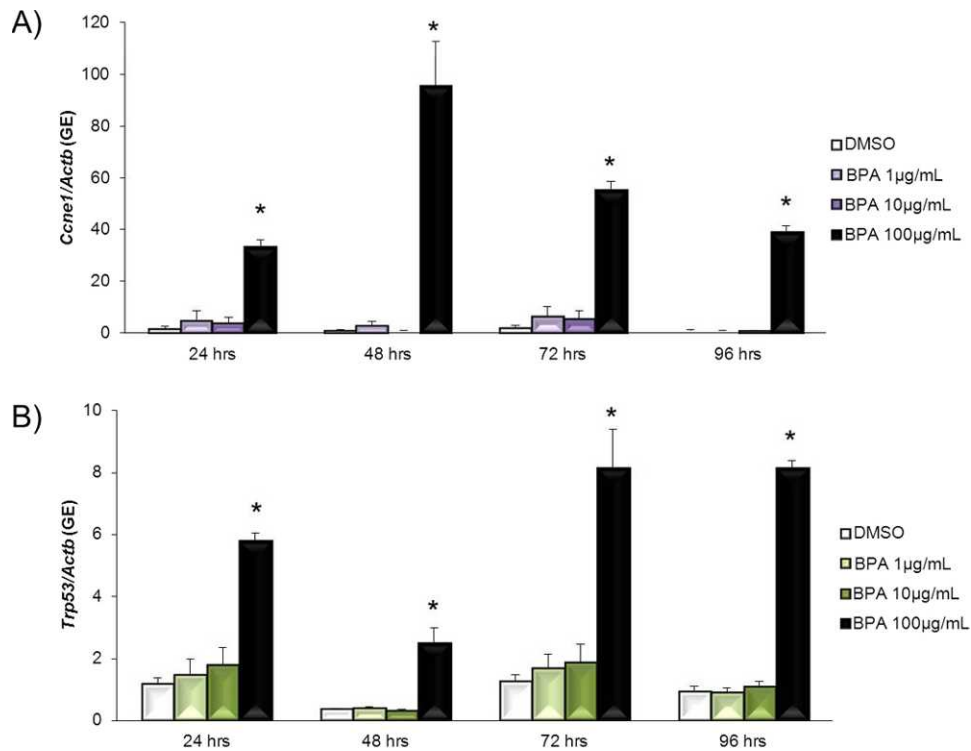

FIG. 7. Effect of BPA on *Ccne1* and *Trp53* mRNA expression levels. After antral follicles were exposed to DMSO control or BPA at 1–100 μg/ml for 24–96 h in vitro, the follicles were collected and subjected to qPCR analysis for *Ccne1* (**A**) and *Trp53* (**B**) mRNA expression levels. All values were normalized to those of beta-actin as a loading control. Graphs show means ± SEM from at least three separate experiments. *$P \leq 0.05$ from DMSO control.
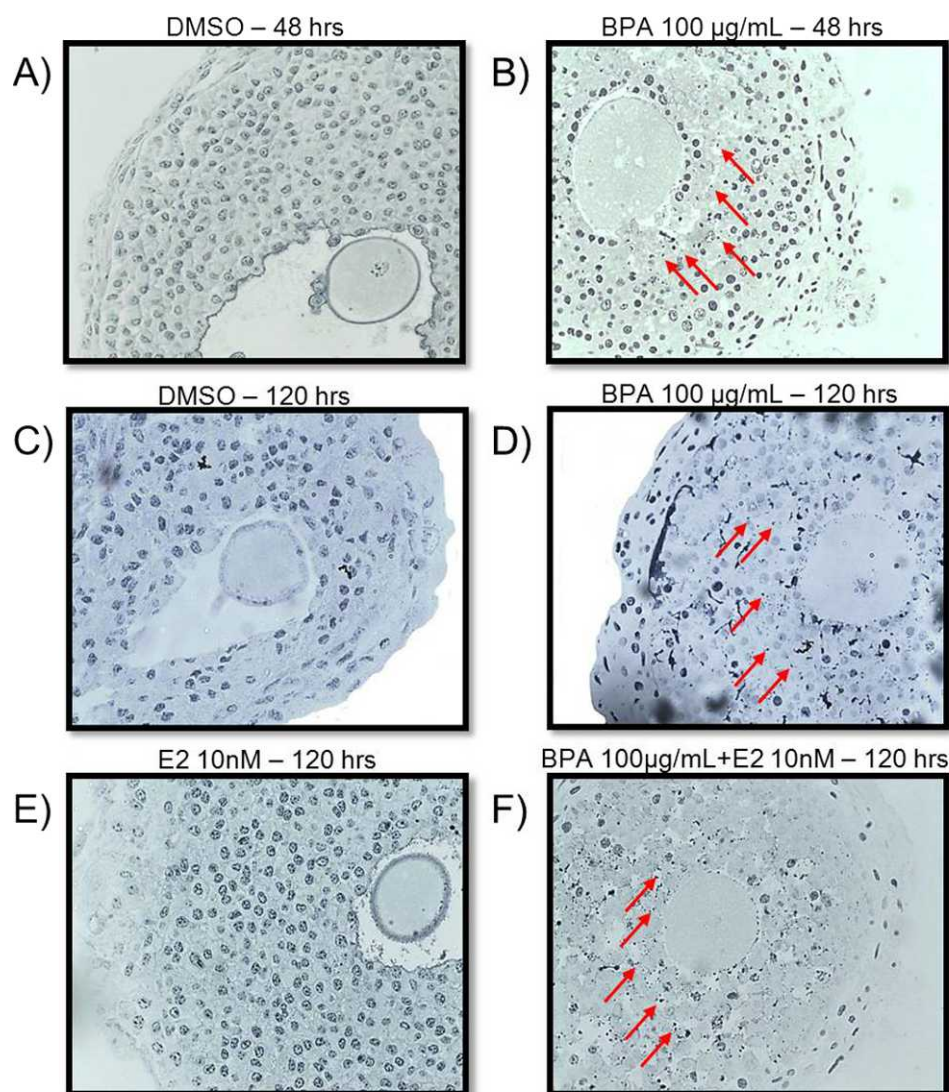
FIG. 8. Effect of BPA on morphology. After antral follicles were exposed to DMSO (**A**, **C**), BPA at 100 μg/ml (**B,D**), estradiol at 10 nM (**E**), or BPA at 100 μg/ml plus estradiol at 10 nM (**F**) for 48 and/or 120 h, the follicles were removed from culture, and atresia was observed as described in *Materials and Methods*. Red arrows indicate apoptotic bodies. *$P \leq 0.05$ from DMSO control. Original magnification ×40.

Although our data indicate that BPA does not inhibit follicle growth directly through the genomic estrogenic pathway, BPA does affect *Esr1* and *Esr2* expression and thus may mediate estrogenic effects in the follicle. Other studies have shown that BPA quickly acts through the nongenomic estrogen responsive GPR30 and ERK pathways in breast cancer cells and that similar to our results, these effects could not be prevented by ICI [8]. Therefore, we speculate that BPA may inhibit follicle growth through a nongenomic estrogenic pathway, although further studies are needed to confirm this hypothesis.

Previous studies have shown that follicle growth is not always dependent on hormone production by follicles. Specifically, previous studies indicate BPA at 10 μg/ml inhibits steroidogenesis without affecting follicle growth [24]. Thus, we examined other mechanisms by which BPA inhibits follicle growth. Morphological examination of the follicles over time indicated striking differences between control and BPA-treated follicles (Fig. 2). Control follicles readily changed in shape and expanded in size, whereas follicles treated with 100 μg/ml BPA seemed suspended in time or frozen; they did not grow or change in size or appearance outside of becoming

more opaque. Follicle growth is dependent on granulosa cell proliferation, and this is regulated, in part, by the cell cycle [26]. Therefore, we considered the possibility that this suspension/inhibition of growth by BPA was due to BPA affecting the cell cycle. The cell cycle is a dynamic process of DNA replication and cell division and is separated by growth phases [26, 27]. In these phases, cells grow in size and replicate their cytosolic contents in preparation for cell division and, thus, proliferation [26]. Estrogenic endocrine disruptors can inhibit follicle growth by down-regulating expression of cell cycle regulators during the G_1-S transition [38]. However, in this study, while BPA down-regulated *Ccnd2* expression at 48 h, BPA up-regulated the cell cycle regulators *Cdk4* and *Ccne1* and the inhibitor *Trp53* beginning at 24 h and continuing throughout culture (Fig. 11). Similar to our studies, others have shown that BPA affects the cell cycle of follicular cells. BPA inhibits cell cycle progression in oocytes [39] and reduces granulosa cell proliferation of early preantral follicles in vitro [40]. Although the up-regulation of *Cdk4* and *Ccne1* should favor cell proliferation and, therefore, these factors should be down-regulated in quiescent follicles, up-regulation of *Trp53*
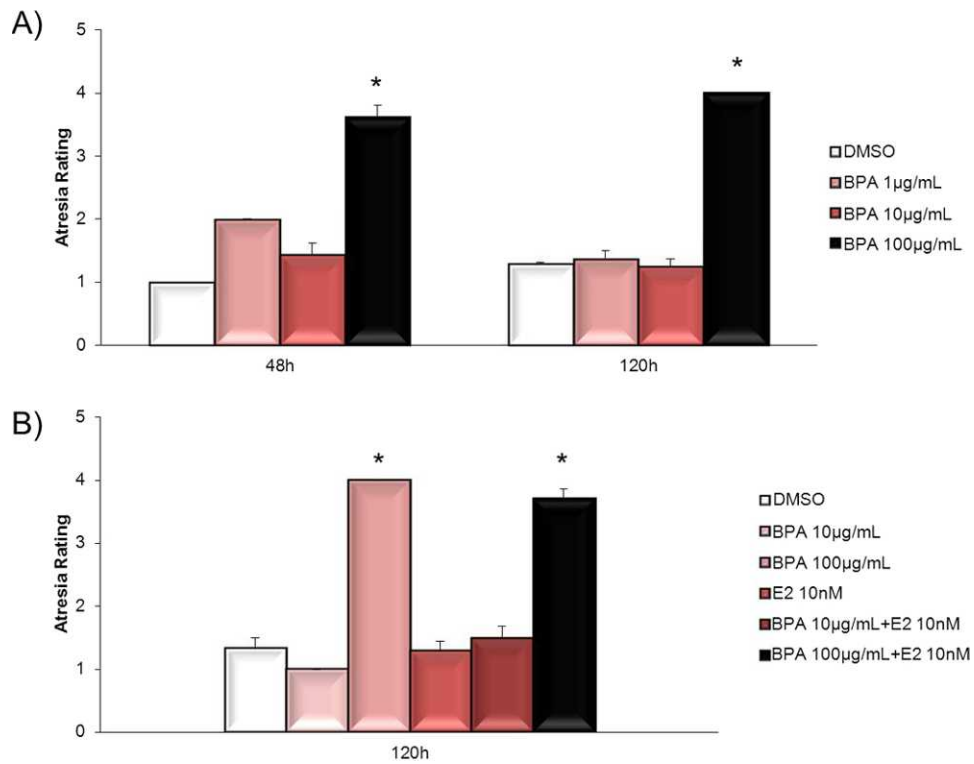
Article 63

FIG. 9. Effect of BPA on atresia. After antral follicles were exposed to DMSO or BPA at 100 µg/ml for 48–120 h (**A**) or to DMSO, BPA at 10–100 µg/ml, estradiol at 10 nM, or BPA at 10–100 µg/ml plus estradiol at 10 nM for 120 h (**B**), the follicles were removed from culture and processed for histological evaluation of atresia as described in *Materials and Methods*. Atresia of follicles was reported as a rating per treatment group. Graph shows means ± SEM from at least three separate follicles per treatment group. *$P \leq 0.05$ from DMSO control.
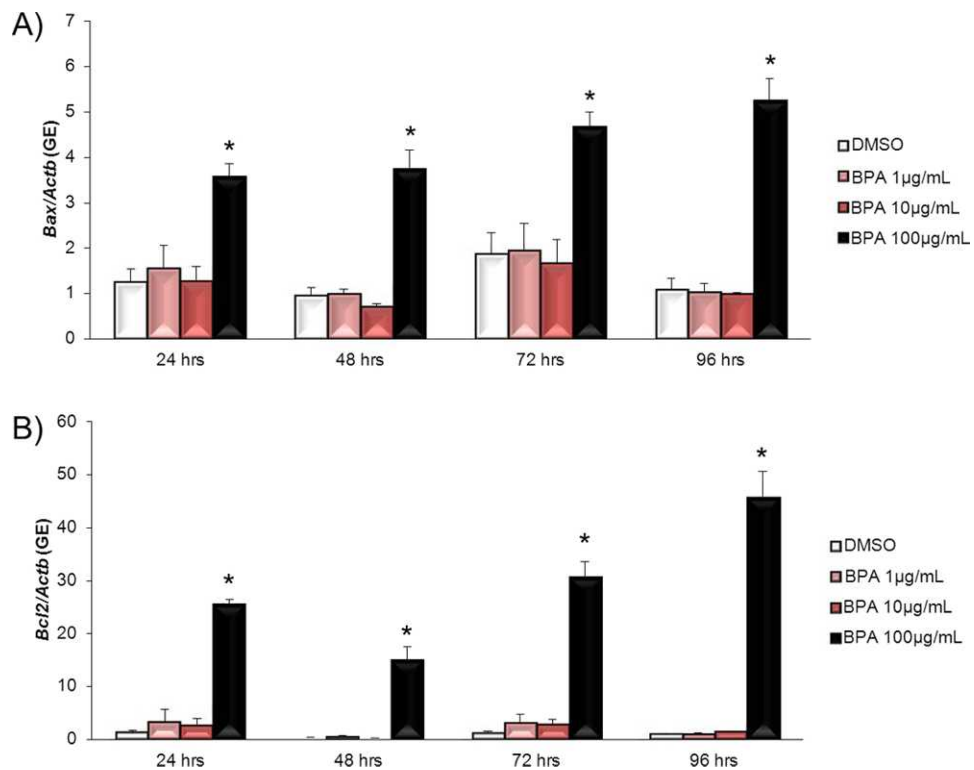

FIG. 10. Effect of BPA on *Bax* and *Bcl2* mRNA expression levels. After antral follicles were exposed to DMSO control or BPA at 1–100 µg/ml for 24–96 h in vitro, the follicles were collected and subjected to qPCR analysis for *Bax* (**A**) and *Bcl2* (**B**) mRNA expression levels. All values were normalized to those of beta-actin as a loading control. Graphs show means ± SEM from at least three separate experiments. *$P \leq 0.05$ from DMSO control.
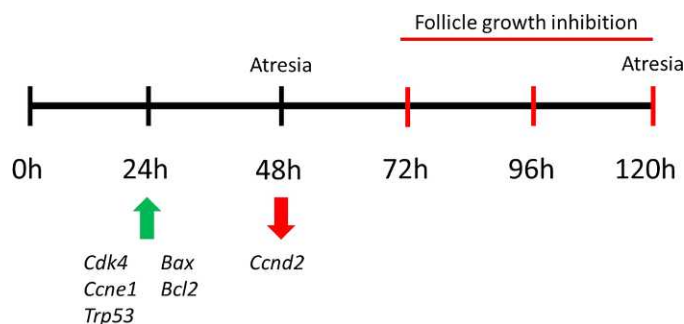
FIG. 11. Schematic of observed effects of BPA on gene expression, follicle growth inhibition, and atresia. At 24 h, BPA significantly increases expression of *Cdk4*, *Ccne1*, *Trp53*, *Bax*, and *Bcl2*. At 48 h, BPA significantly decreases *Ccnd2* and induces atresia. Between 72 and 120 h, BPA significantly inhibits follicle growth and induces atresia.

coupled with down-regulation of *Ccnd2* might be preventing cell proliferation, explaining the inhibited growth and stalled appearance in BPA treated follicles [26].

In addition to examining whether BPA inhibited follicle growth by affecting cell cycle regulators, we examined whether BPA could inhibit growth because it induced atresia. We found that BPA at 100 μg/ml induced atresia beginning at 48 h in culture, 24 h before follicle growth was inhibited, compared to DMSO controls (Fig. 11). This is the same concentration that inhibits follicle growth, suggesting that follicles are not growing because they are dying. The BCL2 family mediates atresia and helps determine whether cells within the follicle will remain healthy or undergo atresia [19, 27, 28, 41]. The family consists of numerous proteins that are either proapoptotic, such as BAX, or antiapoptotic, such as BCL2. We found that BPA at 100 μg/ml induced *Bax* beginning at 24 h and continuing throughout culture (Fig. 11). Unexpectedly, BPA at 100 μg/ml also induced *Bcl2* expression beginning at 24 h (Fig. 11). These findings correspond with those of other studies showing that BPA arrests the cell cycle and dysregulates atresia factors and induces cell death in isolated granulosa cells [28]. This increase in *Bcl2* as well as *Bax* was surprising because it is well known that a higher *Bax*:*Bcl2* ratio will promote atresia, whereas a high *Bcl2*:*Bax* ratio promotes survival because BCL2 binds to and inactivates BAX [42]. However, in this study, a higher *Bcl2*:*Bax* ratio did not protect the follicles from atresia. It is possible that follicles might up-regulate *Bcl2* to try and prevent atresia but that this rescue attempt was unsuccessful. We also found that BPA at 100 μg/ml induced *Trp53* expression, a direct transcriptional regulator of *Bax* [42], beginning at 24 h and continuing throughout culture. Therefore, BPA may trigger the atresia pathway, stimulating the up-regulation of *Trp53* and the proatresia pathway. These actions ultimately inhibit follicle growth and induce atresia despite attempts to promote survival by up-regulating *Bcl2*.

It is well understood that the oocyte and somatic cells of ovarian follicles bidirectionally regulate each other for proper oocyte maturation and somatic cell proliferation and differentiation [43]. In these studies, the effects of BPA on the oocyte were not measured. Although the effects of BPA in both the oocytes and the surrounding follicles have been investigated separately, there are no studies investigating whether the effects of BPA originate in the oocyte or the somatic cells. In isolated granulosa cells, BPA exposure within 24–72 h resulted in arrested mitosis of granulosa cells [26]. Numerous studies have shown BPA affects the oocytes, although these effects ranged from 16 h [13] to 7 days [16]. Further studies are

needed to accurately assess whether BPA effects originate in the somatic cells or in the oocyte.

The implications of our findings may be relevant to humans. Although the highest dose used in this study, 100 μg/ml BPA, is higher than some other concentrations observed in other fluids and placental tissues, it is not known how much BPA is found in antral follicles, and it is possible that concentrations could reach these levels. Antral follicles are highly vascularized structures, potentially exposed constantly to BPA released from bioaccumulation in fatty tissues surrounding the ovaries [44]. Studies have shown that levels of BPA in fatty tissues can be higher than those measured in serum [45, 46]. Furthermore, humans are constantly exposed to BPA, as indicated by the presence of BPA in 93%–96% of urine samples taken in various studies. Estimates of daily BPA intake range from 0.6 to 71.4 μg/day, although this is only an estimate using urine samples taken in the morning from human study subjects and could be higher [1, 4]. Although this is only speculation, bioaccumulation in fat is highly likely, making levels of BPA used in our study plausible for human exposure. This may be particularly important because studies indicate that some women undergoing in vitro fertilization treatment have high levels of BPA [47].

Overall, this study shows that exposure to BPA inhibits growth and induces atresia in antral follicles. Future studies should examine whether BPA affects follicle growth and atresia in humans.

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Article 63

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Article 63