

BPA interferes with granulosa cell development and oocyte meiosis in mouse preantral follicles

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Impact Statement

We investigated the effects of bisphenol A (BPA) on granulosa cell development and meiosis of oocytes using *in vitro* culture system of mouse preantral follicles. The results showed that BPA promoted the development of granulosa cells in the late stage of follicular development; increased the expressions of proliferating cell nuclear antigen (PCNA) and estrogen receptor α (ER α) on granulosa cells and the secretion of estrogen and progesterone but reduced the expansion of cumulus cells around cell-oocyte complexes (COCs), Cx37 expression, and the gap junction communication between cumulus cells and oocytes; promoted the recovery of oocyte meiosis; and led to the developmental arrest of oocytes after germinal vesicle breakdown (GVBD), which may be related to the abnormal expression of cyclin B1. This study more systematically clarified the adverse effects of BPA as an environmental estrogen-like chemical on the development of granulosa cells and oocytes.

Abstract

Bisphenol A (BPA) is an established environmental endocrine disruptor and can interfere with the development of female germ cells. However, the underlying mechanisms are still unclear. We investigated the effects of BPA on granulosa cell development and meiosis of oocytes using *in vitro* culture system of mouse preantral follicles. Preantral follicles from D14 mouse ovary were treated with 10 μ g/mL BPA *in vitro* for 11 days. The adherent area of follicles was measured. On D11, cumulus cell expansion was observed. The meiosis recovery rate was calculated. Western blot detected P53, proliferating cell nuclear antigen (PCNA), estrogen receptor α (ER α), and cyclin B1. ELISA measured estrogen and progesterone levels. Immunofluorescence detected Cx37 on oocyte membrane. Gap junction communication was assessed. We found that BPA significantly promoted the expressions of PCNA and ER α in granulosa cells and the secretion of estrogen and progesterone by granulosa cells on D10 and significantly increased the attachment area of the follicles on D8 and D10. However, it reduced the expansion of cumulus cells, Cx37 expression, and the gap junction communication between cumulus cells and oocytes on D11. BPA promoted the recovery of oocytes from meiosis, interrupted the expression of cyclin B1 protein in arrested germinal vesicle breakdown (GVBD) oocytes, and reduced the *in vitro* maturation rate of oocytes. These GVBD oocytes were live without apoptosis or death. Conclusively, BPA disturbs the development of granulosa cells and the meiosis progression of oocytes by decreasing gap junction communication between oocytes and the granulosa cells as well as regulating cyclin B1 expression in GVBD oocytes.

Keywords: Bisphenol A, granulosa cell, oocyte, preantral follicle, meiosis

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Introduction

Bisphenol A (BPA) is one of the most widely used industrial compounds in the world. It is contained in mineral water bottles, medical equipment, and food packaging. BPA can be released from food packaging to food or beverages. Different concentrations of BPA have been found in human urine, blood, follicular fluid, and amniotic fluid.^{1–4} The structure of BPA is similar to estradiol and diethylstilbestrol. It is an estrogen-like endocrine disruptor and can act as estrogen and antagonize androgens in the body.^{5,6} Clinical study has found that the high concentration of BPA in the urine

of infertile women is clearly related to the decrease in the number of primordial follicles.⁷ BPA can also interfere with the synthesis and secretion of hormones, and cause embryonic development failure.^{8–10} Therefore, the effect of BPA on female germ cells has received more and more attention.

The effects of BPA on ovarian development have been investigated *in vivo*. Because normal ovarian follicle development and sex hormone secretion are necessary for female fertility,¹¹ these *in vivo* studies have focused on the effects of BPA on ovarian follicle number and sex hormone secretion.^{12–14} Rivera *et al.*¹² found that intraperitoneal injection of 50 μ g/kg bw/d of BPA in lambs 30 days after

birth significantly reduced the reserve of primordial follicles and increased the number of multiocyte and atretic follicles. Rodríguez *et al.*¹³ reported that intraperitoneal injection of 20 mg/kg bw/d of BPA significantly reduced the number of primordial follicles in eight-day-old rats. After exposure of neonatal mice and adult rats to BPA, the serum estrogen levels were significantly increased. Patel *et al.*¹⁴ revealed that different concentrations of BPA had different effects on ovarian follicle numbers and sex hormone levels at different ovarian developmental stages. However, the mechanisms underlying the effects of BPA on ovarian/follicle development need to be further clarified by *in vitro* experiments.

BPA can also affect the development and function of granulosa cells. For example, Pogrmic-Majkic *et al.*¹⁵ found that BPA significantly increased the expression of *STAR* mRNA and increased the synthesis and secretion of progesterone in human granulosa cells. Lee *et al.*¹⁶ found that BPA reduced the secretion of 17 β -estrogen by rat granulosa cells. However, in the *in vitro* culture system of pig cumulus cell-oocyte complexes (COCs), Song *et al.*¹⁷ reported that BPA significantly increased the secretion of 17 β -estrogen by granulosa cells and promoted the proliferation of granulosa cells. Huang *et al.*¹⁸ found that BPA induced KGN (a human granulosa-like tumor cell line) cell apoptosis by activating the reactive oxygen species/Ca²⁺-apoptosis signal-regulating kinase-c-Jun N-terminal protein kinase (ROS/Ca²⁺-ASK-JNK) pathway. Xu *et al.*¹⁹ reported that BPA induced apoptosis and G2-to-M arrest of murine ovarian granulosa cells. Different effects of BPA on the development and function of granulosa cells have been found in different species, even in the same species, using different experimental model systems.

BPA can reduce the expression of connexin 43 (Cx43) on human granulosa cells through the estrogen receptor (ER) and mitogen-activated protein kinase (MAPK) pathway, thereby reducing the gap junction between granulosa cells.²⁰ During the culture of mouse COCs, it is found that BPA did not affect the expression of gap junction protein Cx37.²¹ However, during rat COCs culture, BPA changed the expression of gap junction genes (*Gja1* and *Gja4*) and transfer rate.²² Thus, the effects of BPA on the gap junction between granulosa cells or between cumulus cells and oocytes and on the development of oocytes are still inconclusive.

At present, studies on the mechanism of BPA's adverse effects have been carried out in the *in vitro* cultured granulosa cells, oocytes, or COCs^{15–18,20–22} However, since the development of oocytes in follicle is inseparable from granulosa cells, the results obtained in these previous studies may have a certain degree of one-sidedness. Granulosa cells and oocytes form a functional unit in the follicle. They communicate with each other through gap junctions. Oocytes can affect the development of granulosa cells, and granulosa cells can also affect the development of oocytes. *In vivo*, BPA affects the development of granulosa cells through follicles but does not directly affect granulosa cells and oocytes. Therefore, in order to better simulate the developmental environment of oocytes and granulosa cells and to more accurately explore the effect of BPA on the development of female germ cells, we used preantral follicles in this study

and cultured them *in vitro* for 11 days. The dose of BPA was determined at 10 μ g/mL according to a previous study.²³ The effects of 10 μ g/mL BPA on the development of granulosa cells around oocytes, the expansion of cumulus cells, the secretion of estrogen and progesterone, gap junction communication, and oocyte development were evaluated. Our findings may provide stronger evidence for understanding the effects and mechanisms of BPA on granulosa cell development and oocyte meiosis.

Materials and methods

Experimental animals

Female mice of Kunming strain ($n=90$; 14-day old) were purchased from Yisi Experimental Animal Technology Co., Ltd. (Changchun, China). They were housed in a temperature-controlled room ($21 \pm 2^\circ\text{C}$) with a light cycle of 12h light/12h dark and free access to food and water. The study was carried out in compliance with the Animal Research: Reporting in Vivo Experiments guidelines. All animal experimental procedures were approved by the Ethics Committee of Jilin Medical University.

In vitro culture and treatment of mouse preantral follicles

Kunming female mice were sacrificed by cervical dislocation, and their bilateral ovaries were quickly isolated and incubated with L-15 working solution (containing 10% fetal bovine serum [FBS]) (11415114, Gibco, Carlsbad, CA, USA). Follicles were mechanically separated under a stereoscope.²³ According to the previously described grading standard,²⁴ grade 4 or 5a follicles were selected, that is, preantral follicles containing 2–4 layers of granulosa cells. The selected preantral follicles were cultured in minimum essential medium-alpha (α -MEM) culture medium (12571063, Gibco, Carlsbad, CA, USA), which contained 5% FBS (10091155, Gibco), 1% insulin-transferrin-selenium (ITS) (41400045, Gibco, Carlsbad, CA, USA), and 0.1 IU/mL recombinant follicle stimulating hormone (r-FSH) (Gonal-F, Merk Serono, Darmstadt, Germany), for 11 days at 37°C and 5% CO₂, in the presence of 10 μ g/mL of BPA (239658, Sigma, St. Louis, MO, USA) (BPA group), or 0.1% dimethyl sulfoxide (DMSO) (D2650, Sigma, St. Louis, MO, USA) (DMSO Group). The preantral follicles without treatment served as normal control. The dose of BPA has been confirmed to have germ cell toxicity.²³ The medium was changed semi-quantitatively every other day.

On the 10th day, 10 COCs were collected from follicles of each group. The remaining follicles were continuously cultured in the α -MEM culture medium containing 2.5 U/mL human chorionic gonadotropin (hCG) (Livzon Pharmaceutical Factory, Zhuhai, China). After 14–16h (on the 11th day), COCs were obtained from mature follicles, and the extent of cumulus cell expansion was compared with the COCs obtained from D10. Then, the cumulus cells around the oocytes were removed by hyaluronidase. The follicles at various developmental stages, and the meiosis and maturity of the oocytes were observed and recorded under Olympus IX-83. The diameter of oocytes was measured,

and the attachment area of the follicles on the petri dish was analyzed by Image J software. The development rate of oocytes in each stage was calculated as the ratio of the number of oocytes at this stage to the total number of living oocytes at all stages.

Mitochondrial membrane potential assay

MII oocytes from control group, germinal vesicle breakdown (GVBD) oocytes from BPA group, and dead oocytes treated with 100 μM H_2O_2 for 30 min were washed three times with phosphate-buffered saline (PBS), and then incubated with a culture medium containing 0.5 $\mu\text{mol/L}$ JC-1 (Invitrogen, Grand Island, NY, USA) at 37°C and 5% CO_2 in an incubator for 30 min, as previously described.²⁵ When the mitochondrial membrane potential is normal, JC-1 could enter the mitochondria and gather in the mitochondrial matrix to form J-aggregates, producing red fluorescence; if there is cell apoptosis with decreased or lost mitochondrial membrane potential, there would be J-monomers in the cytoplasm, exhibiting green fluorescence. The oocytes were observed under the Olympus IX-83 fluorescence microscope. The membrane potential of mitochondria was evaluated by the ratio of red fluorescence intensity to green fluorescence intensity.

Oocyte viability assessment

MII oocytes from control group, GVBD oocytes from BPA group, and dead oocytes treated with 100 μM H_2O_2 for 30 min were incubated with 10 $\mu\text{g/mL}$ propidium iodide (PI) and 2 μM Calcein acetoxymethyl ester (Calcein AM) (C2012, Beyotime, Shanghai, China) at 37°C for 15 min, as described previously.²⁶ After washing three times with PBS, the fluorescence of the oocytes was observed using the Olympus IX-73 fluorescence microscope. Oocytes with green fluorescence in the cytoplasm were considered alive, and those with red fluorescence in the nucleus were considered dead.

Western blotting analysis

In each group, granulosa cells were obtained from 20 follicles on the 4th or 10th day of *in vitro* culture, and the protein was extracted with radio immunoprecipitation assay buffer (RIPA) (containing 1% phenylmethanesulfonyl fluoride [PMSF]) (P0013B and P1006, Beyotime, Shanghai, China). Meanwhile, 60 GVBD oocytes were obtained from the COCs discharged from D11 of each group and subjected to lysis with protein lysis buffer (Laemmli sample buffer + β -mercaptoethanol + protease inhibitor) at room temperature. The proteins were transferred to the polyvinylidene fluoride (PVDF) membrane. After blocking with 5% skimmed milk for 1 h, the membrane was probed with anti-PCNA (proliferating cell nuclear antigen) (ab92552, Abcam, Cambridge, UK), anti-P53 (ab26, Abcam, Cambridge, UK), anti-ER α (ab32063, Abcam, Cambridge, UK), anti-cyclin B1 (ab72, Abcam, Cambridge, UK), and anti- β -actin (ab8226, Abcam, Cambridge, UK) primary antibodies overnight at 4°C. After washing the membrane with phosphate buffered saline with Tween 20 (PBST), the membrane was incubated in goat anti-rabbit

horseradish peroxidase (HRP)-labeled secondary antibody (31210, Thermo Scientific Pierce, Waltham, MA, USA) for 2 h at room temperature, and then subjected to enhanced chemiluminescence color development.²³ The ChemiDOC XRS+ imaging system (Bio-Rad Laboratories, Hercules, CA, USA) was used to scan the film. Image J software was used to analyze the relative expression levels of PCNA, P53, ER α , and cyclin B1.

ELISA assay

The follicle culture medium on the 10th day was collected for analyzing estrogen and progesterone levels, which were measured with ELISA kits (BPE20376 and BPE20381, Shanghai Lengtong Bioscience Co., Ltd., Shanghai, China) according to the instructions. Briefly, the samples and detection reagents were added to a 96-well plate and incubated at 37°C for 30 min. After washing, the chromogenic solution was added and incubated at 37°C for 10 min in the dark.²³ The absorbance value OD450 was measured with a SpectraMax Absorbance Reader (Molecular Devices, Sunnyvale, CA, USA).

Immunofluorescence

After the oocytes were fixed in 4% paraformaldehyde for 0.5 h, they were permeabilized in PBS containing 1% Triton-X100 for 0.5 h. After washing with PBS twice, blocking with 1% BSA for 1 h was performed. Then, the oocytes were incubated with rabbit anti-Cx37 antibody (bs-4067R, Bioss, Beijing, China) at 37°C for 1 h. After washing again, the oocytes were incubated with goat anti-rabbit fluorescein isothiocyanate (FITC)-labeled secondary antibody at 37°C for 1 h. Then, the nucleus was stained with 5 $\mu\text{g/mL}$ Hoechst 33342 at room temperature for 10 min.²⁶ Finally, the oocytes were mounted on the glass slides and observed under a laser confocal microscope (FV3000, Olympus, Tokyo, Japan). Image J software analyzed the average fluorescence intensity of Cx37 protein in oocytes, and 10 oocytes of each group were analyzed.

Communication analysis of gap junction

We analyzed the gap junction communication of cumulus cells and oocytes using the method described by Sasseville *et al.*,²⁷ with slight modifications. In detail, the COCs were incubated with Calcein AM-PBS (C2012, Beyotime, Shanghai, China) for 15 min to transfer Calcein AM from cumulus cells to oocytes through gap junctions.²⁶ Then, the cumulus cells around the oocytes were digested and removed with 0.1% hyaluronidase. The oocytes were observed under Olympus IX-83. Image J software analyzed the average fluorescence intensity of Calcein AM in oocytes. Ten COCs in each group were analyzed, and the experiment was repeated three times.

Statistical analysis

All data are expressed as mean \pm standard deviation and analyzed by SPSS 17.0 statistical software. One-way analysis of variance (ANOVA) followed by least significant difference (LSD) post doc was used for multigroup comparison. $P < 0.05$ suggested significant difference.

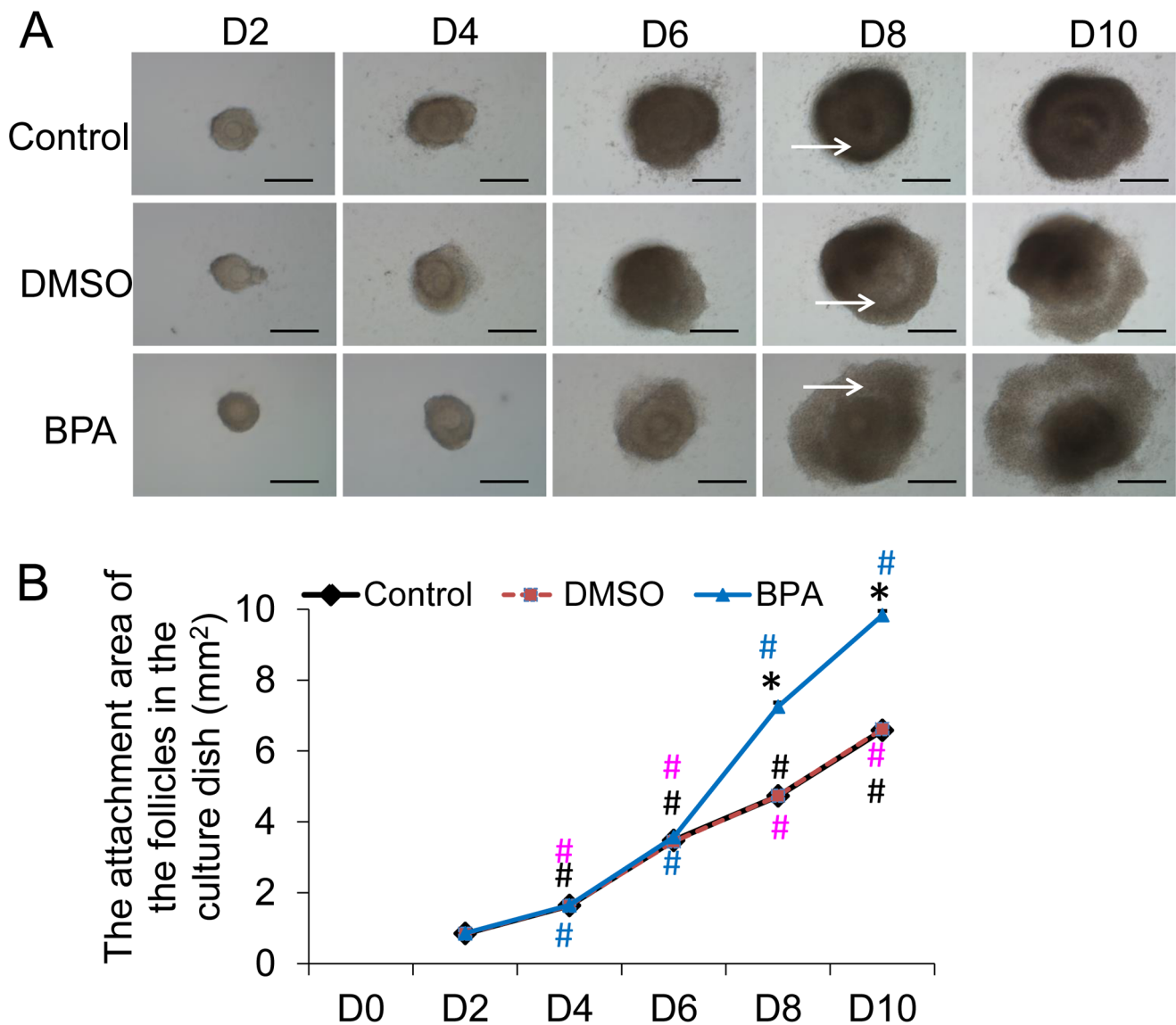


Figure 1. The *in vitro* development of follicles at each stage and the adherence area on the bottom of the petri dish. (A) *In vitro* development of follicles at each stage (scale bar=40 μ m). The white arrow indicates the follicular cavity. (B) The adherent area of follicles in each stage at the bottom of the petri dish (mm²) ($N=10$). Compared with control, * $P<0.05$. Within control group, D4 versus D2, D6 versus D8, and D10 versus D8, # $P<0.05$ (black). Within DMSO group, D4 versus D2, D6 versus D8, and D10 versus D8, # $P<0.05$ (pink). Within BPA group, D4 versus D2, D6 versus D8, and D10 versus D8, # $P<0.05$ (blue).

Results

BPA promotes the development of granulosa cells in the late stage of follicular development

Preantral follicles with a diameter of 110–130 μ m were isolated from the ovary of 14-day-old mouse and cultured *in vitro* for 11 days. The developmental status of the follicles was observed on Day 2 (D2), Day 4 (D4), Day 6 (D6), Day 8 (D8), and Day 10 (D10) (Figure 1(A)). On D2, the outer cells of the follicles began to grow outward. With the development of follicular granulosa cells, the volume of the follicle gradually increased and the follicular cavity with follicular fluid appeared in the follicle on D8. The attachment area of the follicles in the petri dish was measured (Figure 1(B)). The results showed that within each group, the attachment area of

follicles on D4, D6, D8, and D10 increased significantly with prolonged culture time ($P<0.05$, Figure 1(B)). Compared with the normal group, 0.1% DMSO had no significant effect on the attachment area of the follicles at each time point ($P>0.05$, Figure 1(B)). The attachment area of the D2, D4, and D6 follicles in the 10 μ g/mL BPA group was not significantly different than that of the normal follicles ($P>0.05$). However, the attachment area of D8 and D10 follicles was significantly larger than that of the normal group and the DMSO group ($P<0.05$). Thus, 10 μ g/mL BPA significantly promoted the development of granulosa cells starting from D8.

The granulosa cells of D4 and D10 follicles were collected, and Western blotting analysis of P53 and PCNA was performed (Figure 2(A)). Compared with the control group and the DMSO group, 10 μ g/mL of BPA had no significant effect

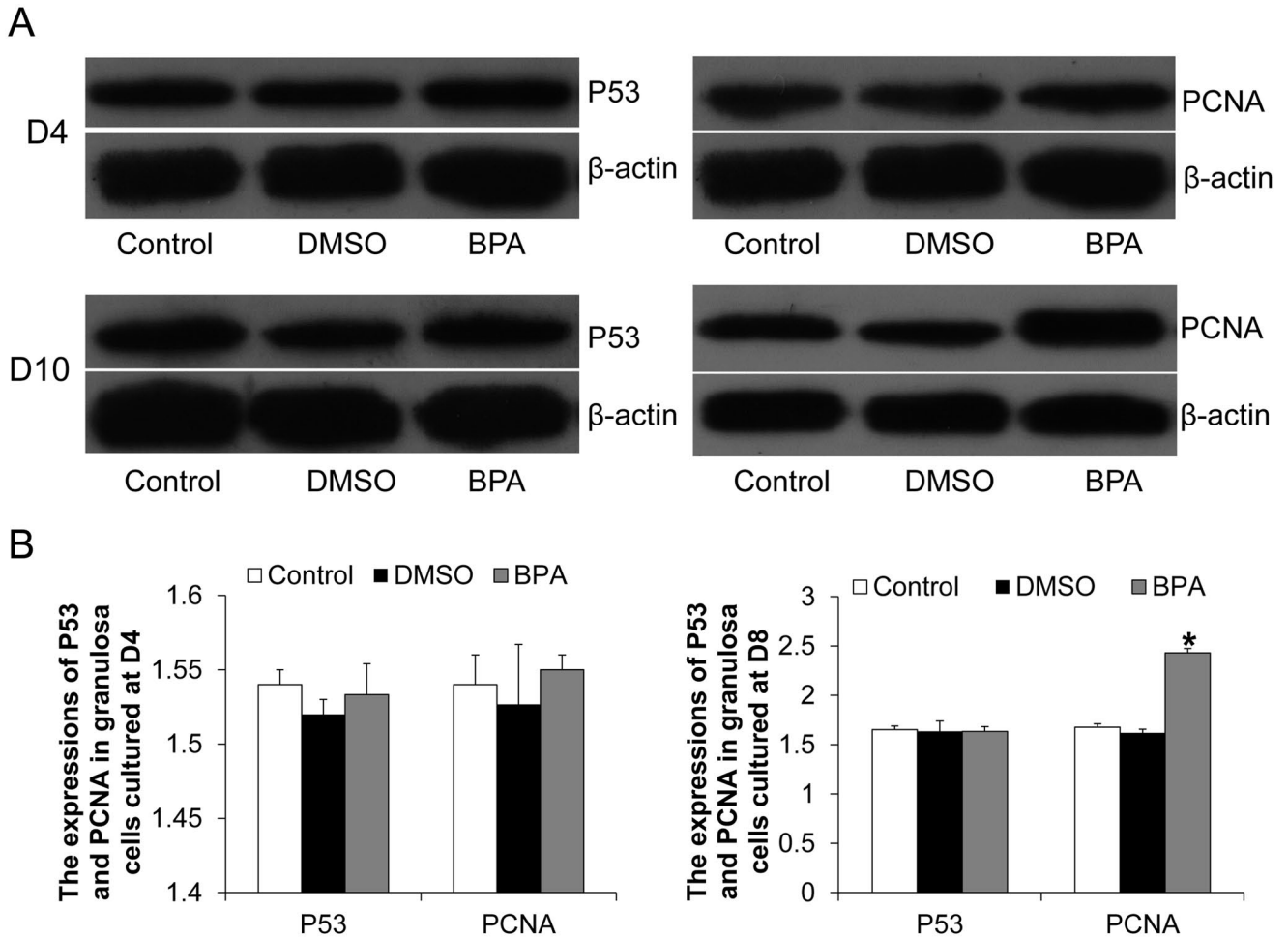


Figure 2. The expression of P53 and PCNA in granulosa cells. (A) Western blotting detected the expression of P53 and PCNA in D4 and D10 follicular granulosa cells. Representative results were shown. (B) Image J software analyzed the relative expression levels of P53 and PCNA. Compared with control, * $P < 0.05$.

Table 1. Estrogen and progesterone secreted by follicles on day 10.

Group	No. of mice	The number of replicates	Estrogen (ng/L)	Coefficient of variability (%)	Progesterone (ng/mL)	Coefficient of variability (%)
Control	9	3	21.65 ± 2.73	12.61	0.64 ± 0.09	14.06
DMSO	9	3	21.88 ± 2.82	12.89	0.65 ± 0.09	13.85
10 µg/mL BPA	9	3	42.60 ± 2.85*	6.69	0.86 ± 0.07*	8.14

DMSO: dimethyl sulfoxide; BPA: bisphenol A. Compared with control group, * $P < 0.05$.

on P53 expression in D4 and D10 granulosa cells ($P > 0.05$, Figure 2(B)) and on PCNA expression in D4 granulosa cells ($P > 0.05$, Figure 2(B)). However, BPA significantly increased PCNA expression in D10 granulosa cells ($P < 0.05$, Figure 2(B)), suggesting that it may promote proliferation of granulosa cells.

BPA improves the secretion of estrogen and progesterone and the expression of ERα by granulosa cells

The levels of estrogen and progesterone secreted by D10 follicles were measured by ELISA. The results showed that

compared with the control group and the DMSO group, the levels of estrogen and progesterone in the culture medium of the 10 µg/mL BPA group were significantly increased ($P < 0.05$, Table 1), indicating the promotive effect of the 10 µg/mL BPA on secretion of estrogen and progesterone by granulosa cells.

Western blotting analysis revealed that compared with the control group and the DMSO group, the expression of ERα in granulosa cells in the BPA group was significantly increased (Figure 3(A) and (B)). This demonstrates that BPA could stimulate the expression of ERα in granulosa cells in late follicular development.

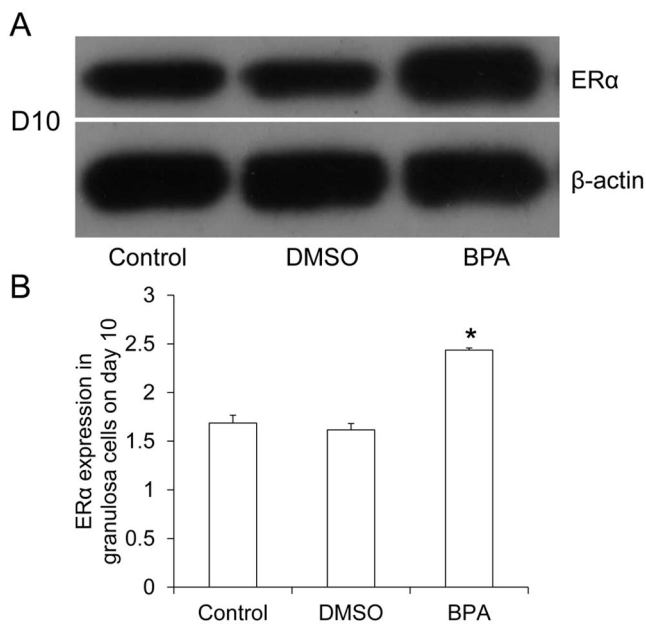


Figure 3. Expression of ER α in D10 follicular granulosa cells. (A) Western blotting analyzed ER α expression in D10 follicular granulosa cells. Representative results were shown. (B) Image J software analyzed the relative expression level of ER α . Compared with control, * $P < 0.05$.

BPA reduces the expansion of cumulus cells

The COCs were collected from antral follicles cultured on D10 and D11 for comparing cumulus cell expansion. As shown in Figure 4, the cumulus cells of COCs on D10 were poorly expanded, while the COCs on D11 had expanded cumulus cells under the action of hCG. The percentage of well and poorly expanded cumulus cells by COCs of D11 were calculated (Table 2). It was found that in control and DMSO groups, the percentage of COCs with poorly expanded cumulus cells was significantly lower than that with well-expanded cumulus cells ($P < 0.05$, Table 2). However, there was no significant difference in BPA group. For comparison among different groups, the percentage of COCs with well-expanded cumulus cells in the BPA group was significantly lower than that of the control group and the DMSO group, but the percentage of COCs with poorly expanded cumulus cells was significantly higher in the BPA group ($P < 0.05$, Table 2). Therefore, BPA could reduce the expansion of cumulus cells.

BPA promotes the recovery of oocytes meiosis but inhibits the development of GVBD oocytes

The COCs were collected from antral follicles on D11 of culture. After the cumulus cells were removed, the meiosis recovery and maturation of the oocytes were evaluated. Oocytes with germinal vesicle (GV) were GV-stage oocytes (Figure 5(A)); oocytes with GV disappeared but not discharged from the first polar body were GVBD oocytes (Figure 5(B)); and oocytes with the first polar body were mature oocytes, also called MII oocytes (Figure 5(C)). Both GVBD oocytes and MII-stage oocytes were oocytes with recovered meiosis. GVBD oocytes were oocytes with developmental arrest after meiosis recovery. The development rate of mouse oocytes in each stage was assessed. As shown

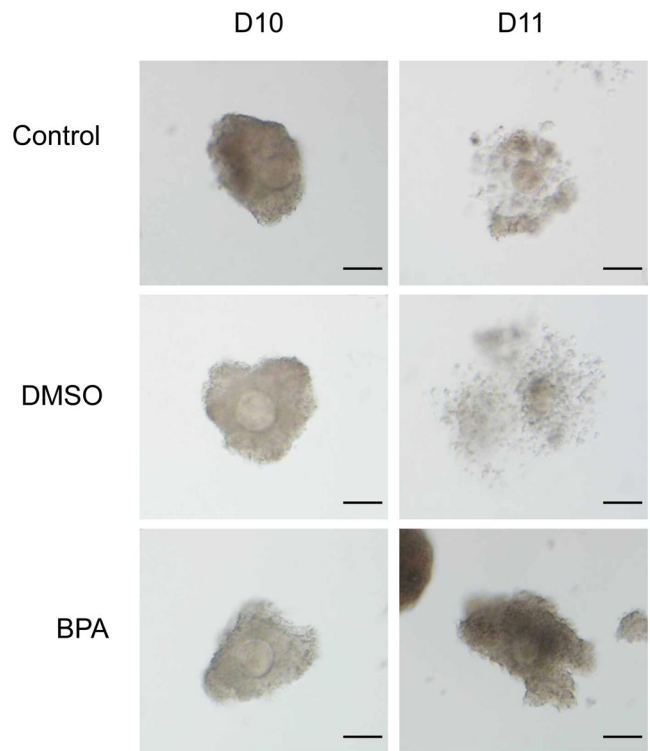


Figure 4. COCs on D10 and D11 of each group cultured *in vitro*. Scale bar=80 μm .

Table 2. Ratio of COCs with well-expanded and poorly expanded cumulus cells on D11.

Group	The total number of COCs	The total number of COCs with well-expanded cumulus cells (%)	The total number of COCs with poorly expanded cumulus cells (%)
Control	42	39 (92.76 \pm 1.04)	3 (7.24 \pm 1.04) [#]
DMSO	50	46 (92.11 \pm 2.83)	4 (7.89 \pm 2.83) [#]
10 $\mu\text{g}/\text{mL}$ BPA	40	21 (52.60 \pm 6.12) [*]	19 (47.40 \pm 6.12) [*]

COCs: cumulus cell-oocyte complexes; DMSO: dimethyl sulfoxide; BPA: bisphenol A.

Compared with control group, * $P < 0.05$. Compared within group (total number of COCs with well-expanded cumulus cells versus total number of COCs with poorly expanded cumulus cells), [#] $P < 0.05$.

in Table 2, in the control group, DMSO group, and BPA group, the proportion of oocytes with recovered meiosis was significantly higher than that of MII-stage oocytes, but the proportion of GV-stage oocytes was significantly lower than that of MII-stage oocytes ($P < 0.05$). The proportion of MII-stage oocytes in the control group and DMSO group was significantly higher than that of GVBD oocytes, while the proportion of MII-stage oocytes in BPA group was significantly lower than that of GVBD oocytes ($P < 0.05$). This suggests that BPA causes developmental arrest after meiotic recovery of oocytes. Compared with the control group and the DMSO group, 10 $\mu\text{g}/\text{mL}$ of BPA significantly reduced the percentages of GV-stage oocytes and mature oocytes ($P < 0.05$, Table 3). However, it significantly increased the percentage of GVBD oocytes and the recovery rate of meiosis ($P < 0.05$, Table 3). These data reveal that BPA could promote the recovery of oocytes from meiosis but inhibit the maturation of oocytes.



Figure 5. Mouse oocytes at different development stages. (A) GV-stage oocytes. There were obvious vesicles in the middle of the oocytes (scale bar=20 μm). (B) Oocytes after GVBD. The vesicles disappeared, but the oocytes did not discharge the first polar body (scale bar=20 μm). (C) MII-stage oocytes. The oocytes had the first polar body and were also called mature oocytes (scale bar=20 μm).

Table 3. *In vitro* development rate of mouse oocytes.

Group	The total number of live oocytes	The number of GV-stage oocytes (%)	The number of GVBD-stage oocytes (%)	The number of MII-stage oocytes (%)	The number of meiosis recovery oocytes (%)
Control	216	24 (10.59 ± 1.44) [#]	17 (8.31 ± 1.48) [#]	175 (81.03 ± 2.93)	192 (89.41 ± 1.44) [#]
DMSO	215	25 (11.44 ± 1.55) [#]	20 (9.48 ± 1.63) [#]	170 (79.08 ± 2.66)	190 (88.56 ± 1.55) [#]
10 μg/mL BPA	247	18 (7.31 ± 1.24) ^{#*}	122 (49.23 ± 3.81) ^{#*}	107 (43.46 ± 2.86) [*]	229 (92.69 ± 1.24) ^{#*}

GV: germinal vesicle; GVBD: germinal vesicle breakdown; DMSO: dimethyl sulfoxide; BPA: bisphenol A. Compared with control group, ^{*} $P < 0.05$. Compared with the number of MII-stage oocytes within each group, [#] $P < 0.05$.

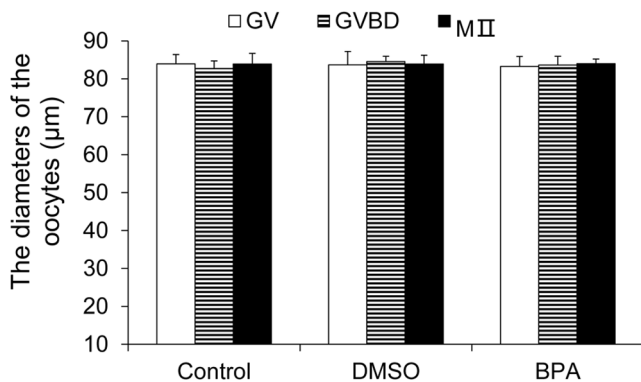


Figure 6. The diameter of mouse oocytes at different development stages of the BPA group (μm).

BPA does not change the size of oocytes, nor does it induce apoptosis and death of GVBD oocytes

At 14–16 h after hCG injection, the diameters of GV-stage oocytes, GVBD-stage oocytes, and MII-stage oocytes in each group were measured. There was no significant difference in the diameter of different development stages in each group and among the three groups (control group, DMSO group, and BPA group) ($P > 0.05$, Figure 6). Thus, BPA did not significantly affect the size of oocytes.

The staining with Calcein AM and PI was used to determine whether the oocytes are alive or dead. As shown in Figure 7, the green fluorescence of the cytoplasm of dead oocytes was very weak, and the nucleus showed red fluorescence. The cytoplasm of GVBD oocytes after treatment by BPA and MII oocytes in control group showed strong

green fluorescence, while there was no red fluorescence in the nucleus, suggesting that these oocytes were alive. Thus, BPA did not cause the death of GVBD oocytes.

The JC-1 was used to detect the mitochondrial membrane potential of GVBD oocytes in BPA group and MII-stage oocytes in control group. As shown in Figure 8(A), GVBD oocytes in BPA group and MII-stage oocytes in control group showed strong red fluorescence while weak green fluorescence (Figure 8(A)). Statistically, there was no significant difference in the ratio of red fluorescence to green fluorescence between GVBD oocytes in BPA group and MII-stage oocytes in control group ($P > 0.05$, Figure 8(B)), indicating that there was no abnormality in the mitochondria membrane potential of GVBD oocytes in BPA group. Thus, BPA did not induce the apoptosis of GVBD oocytes.

BPA reduces the gap junction communication between cumulus cells and oocytes and the expression of Cx37 on the oocytes

To determine the degree of gap junction communication between cumulus cells and oocytes, the collected COCs were incubated with Calcein AM for 15 min, and the fluorescence intensity of Calcein AM was detected (Figure 9(A)). The green fluorescence intensity in the oocytes of the BPA group was significantly reduced than the control group and the DMSO group (Figure 9(B)). Thus, 10 μg/mL BPA significantly reduced the ability of gap junction communication between cumulus cells and oocytes.

The collected oocytes were subjected to immunofluorescence staining of Cx37 protein (Figure 10(A)). Cx37 protein is the main structural protein of the gap junction between

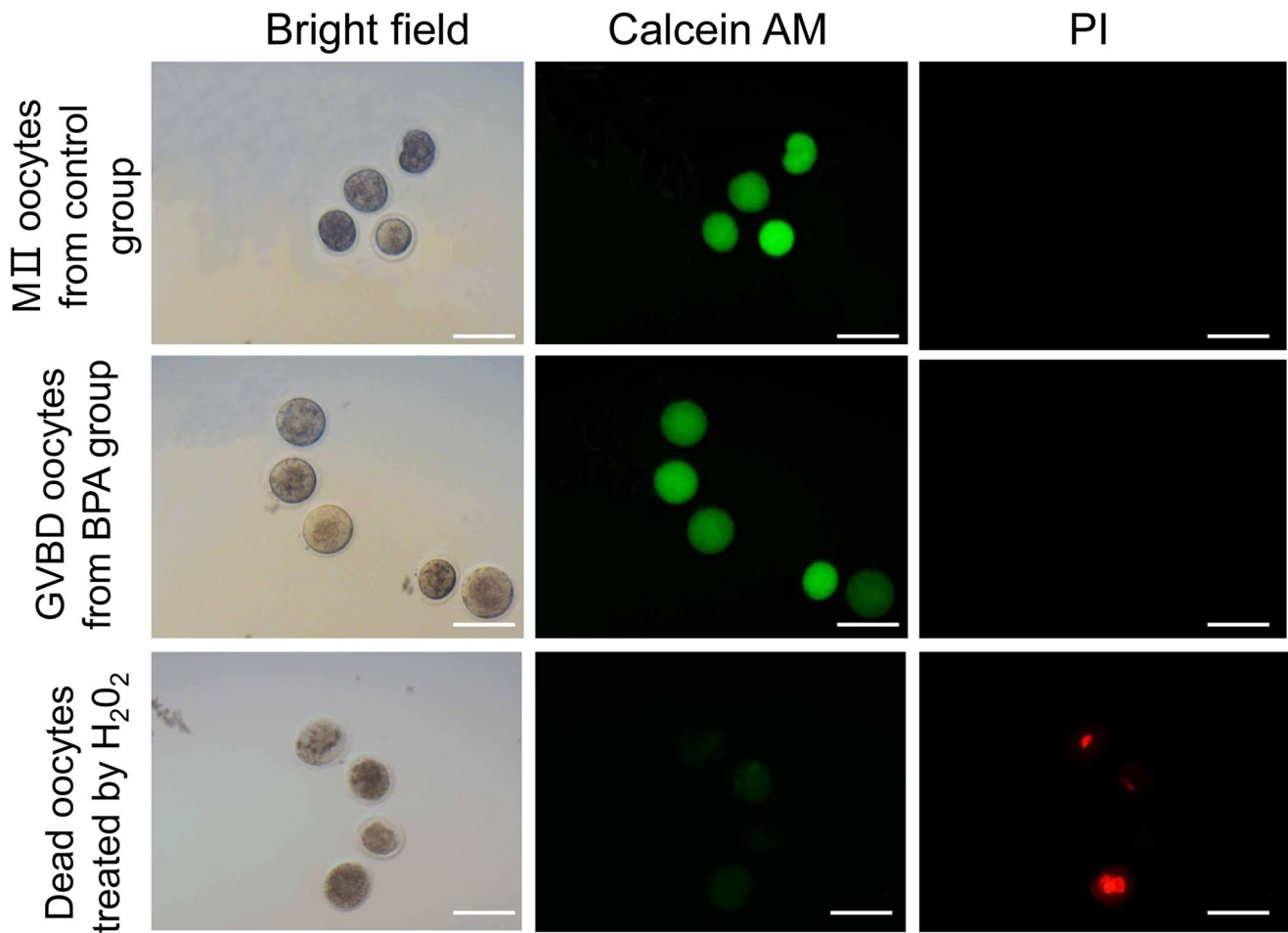


Figure 7. The live and dead oocytes in BPA group. Calcein AM and PI were used to stain oocytes. Oocytes with green fluorescence in the cytoplasm were considered alive, and those with red fluorescence in the nucleus were considered dead. Scale bar = 100 μm

cumulus cells and oocytes. We observed that it was expressed on the oocyte membrane (Figure 10(A)). Compared with the control group and the DMSO group, the average fluorescence intensity of Cx37 on the oocyte membrane of the BPA group was significantly reduced (Figure 10(B)). This may be the main reason for the decrease of gap junction communication between cumulus cells and oocytes.

BPA increases the expression of cyclin B1 protein in arrested GVBD oocytes

The arrested GVBD oocytes were collected 14–16 h after hCG injection and subjected to Western blotting analysis of cyclin B1. The representative Western blotting results were shown in Figure 11(A). Statistically, compared with the control group and the DMSO group, there was significantly higher expression of cyclin B1 in the arrested GVBD oocytes in the BPA group (Figure 11(B)). The abnormal increase of cyclin B1 may affect the procession of GVBD to maturation of the oocytes.

Discussion

The adverse effects of BPA are caused by its chronic accumulation.²⁸ At present, most studies on the adverse effects

of BPA on female reproductive cells were conducted with separated granulosa cells, oocytes, or COCs of the ovary.²⁹ The study system cannot better simulate the process of human exposure to BPA. Therefore, in this study, preantral follicles were isolated and cultured *in vitro* for 11 days in the presence of BPA. We found that BPA significantly promoted the development of granulosa cells in the late stage of follicular development, increased the secretion of estrogen and progesterone and the expression of ER α by granulosa cells, and, promoted oocyte meiosis recovery. However, BPA reduced the expansion rate of COCs cumulus cells and gap junction communication, induced the developmental arrest of the oocytes after GVBD, and reduced the maturation rate of the oocytes. GVBD oocytes treated by BPA were live oocytes with normal diameters and normal mitochondrial membrane potential. In addition, the developmental arrest of oocytes after GVBD caused by BPA may be related to the reduction of the gap junction communication and the abnormal expression of cyclin B1 in oocytes after GVBD.

Granulosa cells, as important constituents of follicles, can secrete estrogen and progesterone to participate in the maintenance of follicular development, oocyte maturation, female reproductive cycle, fertilization, and so on.³⁰ As an environmental estrogen-like chemical, BPA can disrupt the

secretion of estrogen and progesterone.^{17,18} Consistently, our results showed that BPA (10 µg/mL) significantly increased the secretion of estrogen and progesterone by granulosa cells. Our previous research also found that BPA increased the expression of *CYP19A1* and *HSD17B* by granulosa cells,²³ which are important enzymes that regulate estrogen synthesis. Castro *et al.*³¹ also found that BPA increased the secretion of estrogen by increasing the expression of aromatase in the rat ovary. Pogrmic-Majkic *et al.*¹⁵ reported that BPA promoted the synthesis of progesterone by human granulosa cells through the peroxisome proliferator-activated receptor γ (PPAR γ)-dependent epidermal growth factor receptor (EGFR) and ERK1/2 pathway, but it reduced the expression of *CYP19A1* mRNA and the synthesis of estrogen. Qi *et al.*³² believed that BPA might reduce the synthesis of progesterone by human granulosa cells by up-regulating the expression of ABAC1. BPA may affect the synthesis and secretion of estrogen and progesterone by granulosa cells through different signaling pathways, and this effect may be related to its concentration, species, and experimental model.

Estrogen can inhibit the apoptosis and promote the growth and differentiation of granulosa cells.¹⁷ As an environmental estrogen-like chemical, 10 µM BPA significantly promoted the proliferation of granulosa cells and attenuated their apoptosis by improving estrogen synthesis and estrogen-mediated signaling pathways.¹⁷ Similarly, our study found that BPA significantly increased the expression of PCNA in granulosa cells and promoted the development of granulosa cells in the late stage of follicular development. Studies have found that BPA interferes with cell development and function mainly through regulating the expression of ER and its downstream signaling pathways.^{33–37} Bruno *et al.*³⁴ found that BPA exposure activated ER α and ER β in the spleen at 24 h after infection and phosphorylated ER α and ER β during myocarditis. BPA and its alternatives act as ER α agonists to activate downstream ER signaling³⁵ and induce tumor-like outgrowths in female transgenic mice.³⁶ However, the suppressive effects of BPA on the expression levels of sex steroid receptors (ER α and ER β) in the rat uterus were observed.³⁷ In this study, we found that BPA promoted the expression of ER α in granulosa cells on day 11 of the culture. BPA may have different effects on ER expression and function due to the differences in cell sensitivity to BPA. Studies^{18,38,39} have found that BPA can bind to G protein-coupled estrogen receptor (GPER) in addition to ER α and ER β . In the human granulosa cell line KGN, BPA could induce granulosa cell apoptosis by binding to GPER and activating the ROS/Ca²⁺-ASK1-JNK pathway.¹⁸ In the mouse spermatocyte cell line GC-2, BPA could bind to a membrane ER GPR30, up-regulating *c-Fos* gene expression and down-regulating *Cyclin D1* gene expression, thereby inhibiting growth and inducing apoptosis of spermatocyte.³⁹ BPA not only affects the expression of ERs, but also has different effects on the growth of different types of cells through different pathways by binding to different ERs.^{18,38,39} Furthermore, studies are needed to investigate the regulation of BPA on different types of ERs and their downstream signaling pathways in granulosa cells.

In the follicles, the cumulus cells can nourish the oocytes via the gap junctions and can regulate the arrest or recovery of the oocyte meiosis. BPA can reduce the expansion of

cumulus cells and affect the maturation quality of oocytes;²¹ meanwhile, it can reduce the gap communication between cumulus cells and oocytes, resulting in decreased cyclic adenosine monophosphate (cAMP) and/or cyclic guanosine monophosphate (cGMP) in oocytes, leading to premature meiosis and GVBD.²¹ In this study, we found that BPA reduced the expansion of mouse COCs cumulus cells and the expression of gap junction protein Cx37, and reduced the transmission of Calcein AM between cumulus cells and oocytes through gap junctions. In addition, we found that BPA significantly increased the percentage of GVBD oocytes and the recovery rate of oocytes from meiosis, but reduced the rate of *in vitro* maturation of oocytes. Lin *et al.*²⁰ also found that BPA reduced the expression of Cx43 on granulosa cells through ER and MAPK pathways. Campen *et al.*²² found that BPA accelerated the recovery of oocyte meiosis by changing the expression of gap junction proteins and the communication ability of gap junctions. Jiao *et al.*⁴⁰ showed that BHPF (9,9-bis(3,4-dihydroxyphenyl)fluorene), a substitute for BPA, had similar adverse effects as BPA, which inhibited the expansion of cumulus cells, reduced the excretion rate of the first polar body, and suppressed oocyte maturation. The down regulation of gap junction intercellular communication by single nucleotide polymorphisms (SNPs) in cardiomyocytes is mediated through down regulation of Cx43 and upregulation of P-Cx43. SNPs-induced apoptosis through the disrupted functional gap junction was correlated with abnormal expressions of the proteins involved in the mitochondrial pathway-related apoptosis such as Bcl-2/Bax, cytochrome C, Caspase-9, and Caspase-3.⁴¹ In the present study, BPA decreased Cx37 expression and reduced Gap junction intercellular communication between oocytes and cumulus cells. However, it was found that GVBD oocytes in BPA group had the same diameter and mitochondrial membrane potential as MII oocytes in control group. GVBD oocytes after 14–16 h of hCG were arrested without apoptosis and death. BPA caused oocyte developmental arrest at the GVBD stage and significantly reduced the oocyte maturation rate. However, the mitochondrial membrane potential of the developmentally arrested oocytes was normal. Thus, the decrease of gap junction intercellular communication by BPA is likely to disturb the normal progression of the oocyte cell cycle.

The occurrence of GVBD is regulated by maturation promoting factor (MPF), composed of cyclin B1 and cyclin-dependent kinase 1, which regulates the procession of oocytes to resume meiosis.^{42,43} When the MPF reaches its peak, the oocyte develops GVBD or progresses to the MI stage.⁴⁴ Soon after the occurrence of GVBD, cyclin B degradation will occur, MPF will be inactivated, and the oocyte will progress from M phase to late phase.⁴⁵ The abnormal degradation and accumulation of cyclin B1 can cause defective anaphase-promoting complex/cyclosome activation and the arrest of oocytes in anaphase.⁴³ During the maturation of oocytes, cumulus cells will transmit cyclic nucleotides to oocytes through gap junction, thus affecting the expression of cyclin B1 and MPF activity in oocytes and altering the procession of oocyte meiosis.⁴⁶ Based on these findings and our results that BPA decreased Cx37 expression, reduced Gap junction intercellular communication between oocytes and cumulus cells and

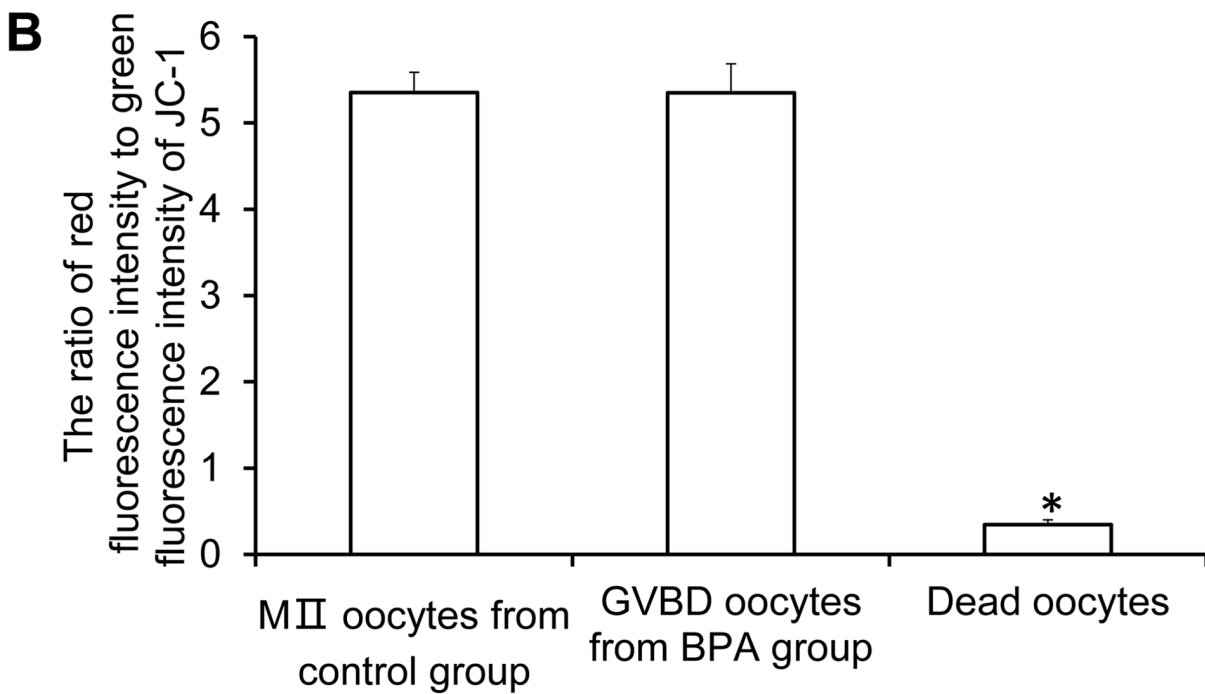
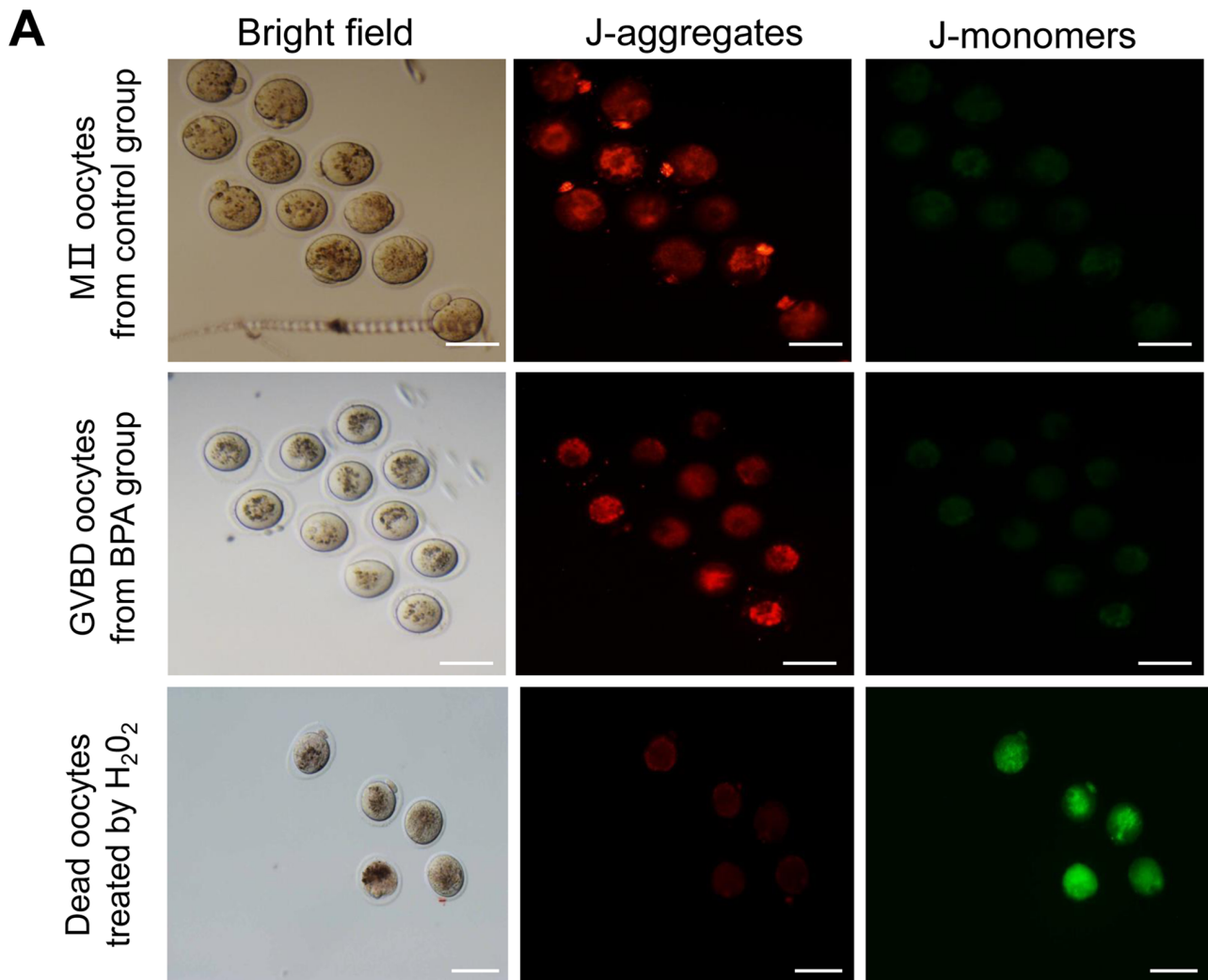


Figure 8. Mitochondrial membrane potential of oocytes after GVBD and MII-stage oocytes in the BPA group. (A) The representative images of JC-1 staining. J-aggregates produce red fluorescence while J-monomers exhibit green fluorescence. Scale bar=80 μ m. (B) Ratio of red fluorescence intensity to green fluorescence intensity of JC-1. Compared with GVBD and MII, * $P < 0.05$.

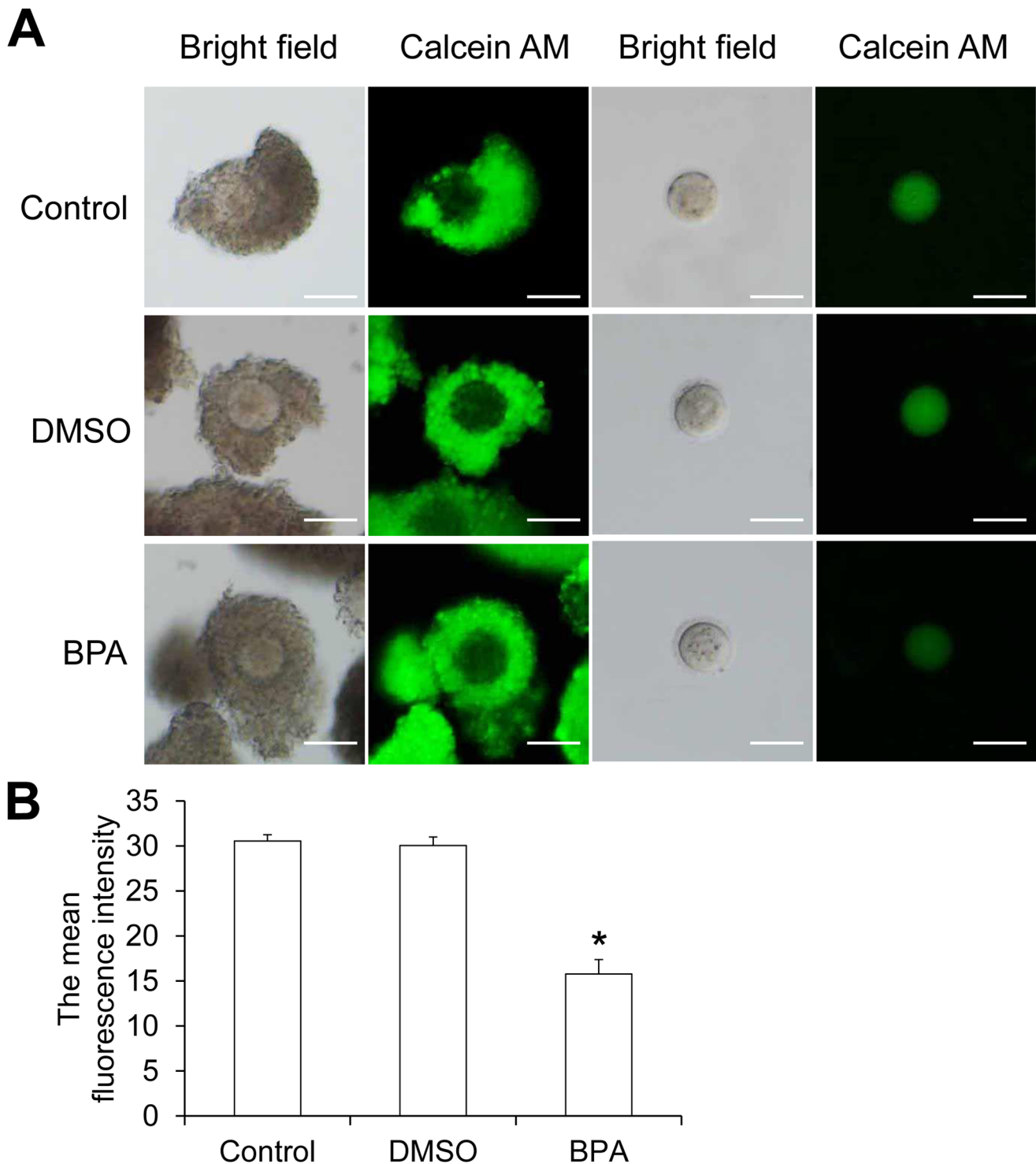


Figure 9. Gap junction communication between cumulus cells and oocytes assessed by Calcein AM. (A) COCs were incubated with Calcein AM for 10 min or 20 min. After removing the cumulus cells, the green fluorescence of Calcein AM transferred to oocytes through gap junctions was detected. Scale bar = 80 μ m. (B) The average fluorescence intensity of Calcein AM in oocytes measured using Image J image analysis ($N=10$). Compared with control, $*P < 0.05$.

cause the developmental arrest of oocytes after GVBD, we speculate that BPA might also affect the expression of cyclin B1. To verify this, we detected the expression of cyclin B1 in the oocytes that were developmentally arrested after GVBD and were unable to discharge the first polar body. The results showed that the expression of cyclin B1 in the arrested GVBD

oocytes of the BPA group was significantly higher than that of the control group. The developmental arrest of oocytes after GVBD caused by BPA may be related to the abnormal expression of cyclin B1. Oocyte development arrest at the GVBD stage will lead to follicular atresia and the follicles cannot mature, rupture, or ovulate, resulting in female infertility.^{47,48}

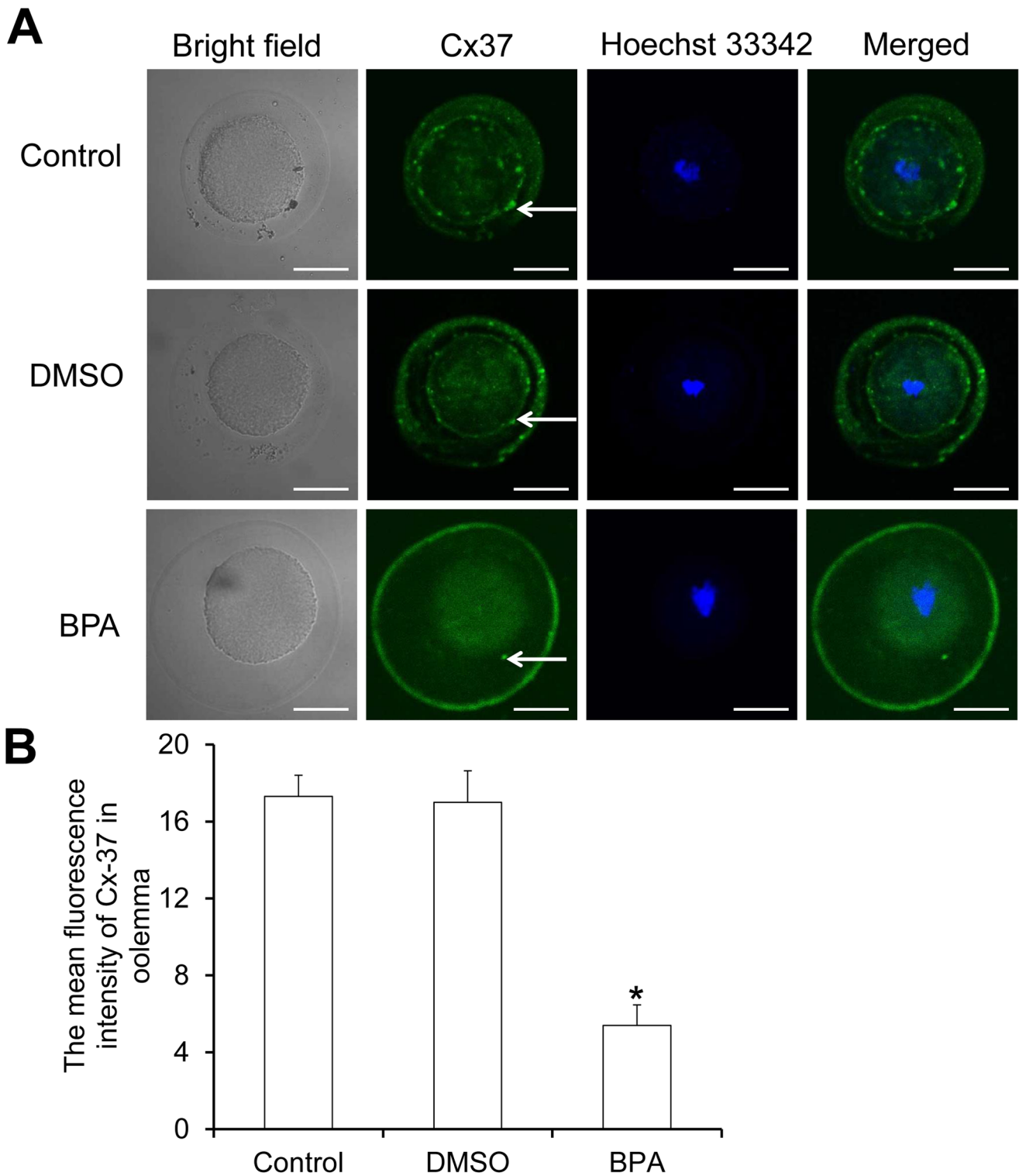


Figure 10. The expression of Cx37 on the oocytes of each group. (A) Immunofluorescence labeling of Cx37. Cx37 on the oocyte membrane shows green fluorescence, and the oocyte nucleus shows blue fluorescence. Scale bar=40 μ m. (B) The average fluorescence intensity of Cx37 detected with Image J software ($N=10$). Compared with control, * $P < 0.05$.

In summary, the *in vitro* culture system of preantral follicles in this study could maintain the connection between granulosa cells and oocytes, and more closely simulate the *in vivo* development of oocytes and granulosa cells. Our findings demonstrate that BPA promoted the development

of granulosa cells in the late stage of follicular development; increased the expressions of PCNA and ER α on granulosa cells and the secretion of estrogen and progesterone but reduced the expansion of cumulus cells around COCs, Cx37 expression, and the gap junction communication

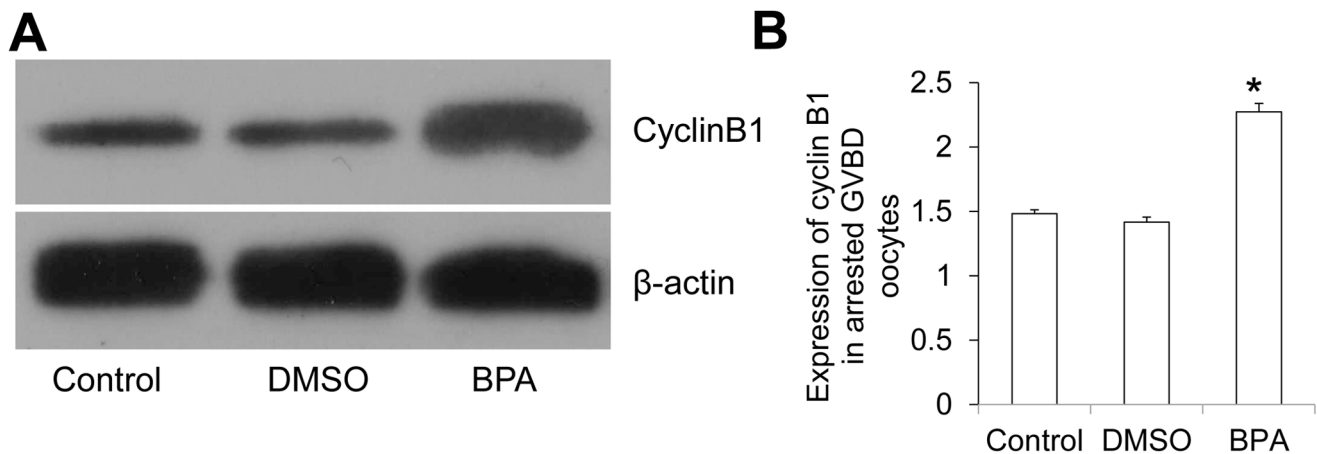


Figure 11. Expression of cyclin B1 in GVBD oocytes. (A) The expression of cyclin B1 in GVBD oocytes was measured with Western blotting. (B) Image J software assessed the relative expression level of cyclin B1. Compared with control, * $P < 0.05$.

between cumulus cells and oocytes; promoted the recovery of oocyte meiosis; and led to the developmental arrest of oocytes after GVBD, which may be related to the abnormal expression of cyclin B1. This study more systematically clarified the adverse effects of BPA as an environmental estrogen-like chemical on the development of granulosa cells and oocytes. These adverse effects caused by BPA will lead to follicular atresia, affect the production of mature oocytes, and cause female infertility. These findings suggest that we should speed up the development of BPA substitutes that have no adverse effects on reproductive capacity, thus avoiding the female reproduction damage caused by BPA.

AUTHORS' CONTRIBUTIONS

XP designed the study. YL, SL, FG, ZP, JZ, SL, and DL collected and analyzed the data. YL wrote the paper. XP collected the funds and revised the paper. All authors have read and approved the submitted manuscript.

DECLARATION OF CONFLICTING INTERESTS

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