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Interfering effects of bisphenol A on *in vitro* growth of preantral follicles and maturation of oocyes

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ABSTRACT

Aims: In order to investigate the effects and mechanism of Bisphenol A (BPA) on the growth of preantral follicles and the maturation of oocytes *in vitro*, preantral follicles were harvested from mouse ovaries and *in vitro* cultured for 11 days with different concentrations of BPA (0, 4.5 and 45 μM) for calculating the percentages of antral follicles, denuded oocytes, degenerative oocytes and the maturation rate of oocytes, besides measuring the diameter of follicles and the thickness of cumulus cell layers.

Methods: The contents of estradiol (E_2) in the culture media on Day 4, 8 and 10 were detected by ELISA. The estrogen receptor (ER) expression, spindle morphology and chromosome distribution in oocytes on Day 10 and 11 were observed by immunofluorescence. Western blotting was used to detect the expressions of growth differentiation factor 9 (GDF-9), bone morphogenetic protein-15 (BMP-15), phosphorylated extracellular signal-regulated kinase 1 (p-Erk1) and phosphorylated Ca_2^+/calmodulin-dependent protein kinase II (p-CaMKII) in the oocytes.

Results: Compared with control, BPA (45 μM) significantly reduced percentages of antral follicles (9.25% vs. 91.17%, $P < 0.05$) and the maturation rate of oocytes (7.61% vs. 79.83%, $P < 0.05$), but increased the percentages of denuded oocytes (30.29% vs. 3.36%, $P < 0.05$) and degenerative oocytes (45.70% vs. 2.45%, $P < 0.05$). The diameter of follicles and the thickness of the cumulus cell layers were decreased significantly ($P < 0.05$). Moreover, BPA (45 μM) significantly decreased E_2 contents in the culture medium on Day 8 and 10 ($P < 0.05$) and the expressions of ER, GDF-9 and BMP-15 in oocytes ($P < 0.05$). Furthermore, BPA (4.5 and 45 μM) treatment resulted in the abnormal spindle morphology and chromosome distribution, and the decreased expressions of p-Erk1 and p-CaMKII in the MII oocytes.

Conclusion: Together, these results clearly demonstrated BPA retarded the preantral follicle growth *in vitro* through interfering with the synthesis and secretion of E_2 and reducing the expressions of ER, GDF-9 and BMP-15, and led to the abnormal meioses of oocytes through reducing p-Erk1 and p-CaMKII expressions in the preantral follicles, which will help us to further unsderstand the mechanism of BPA exposure retarding *in vitro* growth of preantral follicles and maturation of oocyes.

1. Introduction

Bisphenol A (BPA) is one of the most widely used industrial compounds in the world [1], which can be detected in water bottles, medical equipment and food packages in the daily life, and releases when these products are heated or aged [2]. Canada is the first country recognizing BPA as a toxic substance in 2010, and then European Union, Canada and the United States have banned BPA in bottles for baby feeding. BPA is a xenoestrogen with a similar structure of estradiol and diethylstilbestrol, and acts as an endocrine interference with a role like estrogen or antagonist of androgen [3, 4]. The exposure of BPA may disturb the development and function of the reproductive system, which is detected in the urine of > 90% of participants in the National Health and Nutrition Examination Survey, USA and has also been detected in various human fluids including follicular fluids [5]. Clinical studies on the infertile women suggest high concentrations of BPA in

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the urine are associated with a decrease of primordial follicle numbers [6]. As a result, the effects of BPA on the female reproductive system attract increasing attention.

The development and maturation of follicles is a complex hormone-regulated process in the female reproductive system, including oocytes, granulosa cells and membrane cells co-participation and interaction [7, 8]. Present studies have already shown that maternal exposure to BPA during gestation can cause adverse development in their offspring [9], including early puberty onset, changes in weight gain, early vaginal opening, and ovarian morphological abnormalities in the offspring [10]. Several studies investigated the effect of BPA on proliferation of granulosa cells [11–13], since the proliferation and differentiation of granulosa cells are key factors on the growth of follicles, and the hormones secreted by granulosa cells are essential substances for the development and maturation of follicles [14]. Jackye et al. found BPA inhibited the proliferation of granulosa cells and induced follicular atresia [15]. The normal proliferation of granulosa cells depends on growth differentiation factor 9 (GDF-9) and bone morphogenetic protein-15 (BMP-15) secreted by the oocyte [16, 17]. Thus, one important question is whether and how BPA influences the secretion of growth factors secreted by oocytes, which ultimately results in the inhibition of granulosa cell proliferation.

In this study, we used the *in vitro* culture system to study the interfering effects of BPA on the growth of preantral follicles, the proliferation of granulosa cells and the maturation of oocytes. Importantly, the underlying mechanism for this interfering effect was further investigated by examining the expression changes of essential growth factors for granulosa cell proliferation including GDF-9 and BMP-15, and the key kinases for the formation of normal oocyte spindle including extracellular signal-regulated kinase 1 (Erk1) and Ca_2^+/calmodulin-dependent protein kinase II (CaMKII).

2. Materials and methods

2.1. Animals

14-day-old female Kunming mice were provided by experimental animal center of Jilin University. The animals were provided free access to a standard laboratory mouse diet and sterile water. This study was conducted with approval from the Ethics Committee of Jilin Medical University.

2.2. In vitro culture of preantral follicles

Female Kunming mice were sacrificed by cervical dislocation, and the bilateral ovaries were rapidly removed into L-15 medium with 10% FBS (Gibco BRL, Gaithersburg, MD). Follicles were obtained mechanically after the tissues around the ovaries were removed. The preantral follicles were selected according to the Pedersen and Peter's grading criteria for grade 4 or 5a follicles [18]. Namely, the preantral follicles consisted of a round zona pellucida-wrapped oocyte in the middle of the follicle, 2–4 layers of granulosa cells, intact basement membrane and several theca cells attached to the basement membrane. The selected preantral follicles were cultured in α-MEM medium (Gibco BRL, Gaithersburg, MD) supplemented with 5% FBS, 1% ITS (Insulin-Transferrin-Selenium, Gibco BRL, Gaithersburg, MD) and 0.1 IU/mL r-FSH (Merck Serono, Darmstadt, Germany) with different concentrations of BPA (0, 4.5 and 45 μM, Sigma Chemical Co., St. Louis, MO), and incubated overnight at 37 °C in an atmosphere of 5% CO_2 in air. The next day, the follicles of 110–160 μm in diameter were selected and cultured for another 10 days. Preantral follicles cultured with 0.1% DMSO were used as negative controls. The medium was semi-quantitatively changed every other day and the growth of the follicles was recorded at the same time. At the end of the culture period, follicles were cultured in α-MEM medium containing 2.5 U/mL hCG (Merck Serono, Darmstadt, Germany) for another 16 h. The cultured oocytes would expel the first polar body and became mature. The oocyte maturation was observed under stereoscopic microscope. The software of Image Pro-Plus 6.0 was used to measure the diameter of the follicle and the thickness of the granulosa cell layers.

2.3. Immunofluorescence staining

MII oocytes were fixed in 4% paraformaldehyde for 0.5 h and washed with 0.01 M PBS (pH 7.0–7.2) for three times. The Oocytes were then permeabilized in PBS containing 0.1% Triton-X100 for 15 min and blocked with PBS containing 1% BSA and 0.01% Triton-X100 for 1 h, subsequently incubated for 2 h at 37 °C with estrogen receptor (ER) antibody (1:200, bs-0174R, Bioss Antibodies, Beijing, China) or α-tubulin antibody (1:200, bs-0159R, Bioss Antibodies, Beijing, China). Following three times (5 min/time) washing with 0.01 M PBS (pH 7.0–7.2), goat anti-rabbit FITC-conjugated secondary antibodies (1:200, Bioss Antibodies, Beijing, China) were added for 1 h incubation. After washing, oocytes were incubated with Hochest33342 (5 μg/mL) for 10 min at room temperature, and then moved to slides and observed under fluorescence microscopy (IX73, Olympus, Japan).

2.4. ELISA detection of E_2 in culture media

Media from follicle culture on Day 4, 8, and 10 were collected and subjected to ELISA for measurement of estradiol (E_2) levels. The measurement was carried out according to the manufacturer's instructions (ELISA kit, fd5327yt). All samples were run in duplicates and all intra- and inter-assay coefficients of variability were < 10%.

2.5. Western blotting

A total of 80 follicles from each group were collected at Day 11 of *in vitro* culture. Total proteins were extracted from follicles with the use of RIPA lysis buffer (1% PMSF) and separated by SDS-PAGE. Proteins were transferred onto PVDF membrane. After blocking with 5% non-fat milk for 1 h, the membrane was incubated with GDF-9 (1:200, bs-4720R) or BMP-15 (1:400, bs-66I2R) antibody at 4 °C overnight. After washing, the membrane was incubated with goat anti-rabbit HRP conjugated secondary antibody (1:5000) at room temperature for 2 h, and then developed by enhanced chemiluminescence method and scanned using the ChemiDOC XRS + imaging systems (Bio-Rad Laboratories, Hercules, CA, USA). The images were analyzed using Image J software (http://rsb.info.nih.gov/ij/). β-actin was used as an internal control for protein loading. The relative expression levels of GDF-9 and BMP-15 were calculated based on the density of β-actin.

For analysis of p-Erk1 and p-CaMKII expressions in oocytes, 33 of MII oocytes were collected after the ovulation. Total proteins were extracted with SDS buffer (2% SDS, 0.125 M Tris-HCl, 5% β-mercaptoethanol, 10% glycerin and 0.01%bromophenol blue) and separated by SDS-PAGE. Following transfer and blocking with 5% BSA for 1 h, the membrane was incubated with p-Erk1 (1:200, bs-1645R) or p-CaMKII (1:200, bs-1647R) antibody at 4 °C overnight. After washing, the membrane was incubated with goat anti-rabbit HRP-conjugated secondary antibody (1:5000) at room temperature for 2 h. Film development and densitometry analysis were performed as described above.

2.6. Statistical analysis

Data analysis was carried out using the SPSS 13.0 (IBM Corp, Chicago, IL, USA) and expressed as mean ± SD. Differences between groups were evaluated for significance using one-way ANOVA followed by Post Hoc test. $P < 0.05$ was considered a level of significance.