Bisphenol A and Its Analogues Exhibit Different Cytotoxic and Mitochondrial Dysfunction Potential in Human Granulosa Cells

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Abstract: Bisphenol A (BPA) is an environmental endocrine disruptor and has been strongly associated with the development of numerous diseases, including ovarian follicle development disorders. BPA is being replaced by structurally similar chemicals, such as bisphenol S (BPS), bisphenol F (BPF) and bisphenol AF (BPAF). However, the toxicity of these analogues in female reproduction is unclear. Here, we investigated the induction of cytotoxicity and mitochondrial dysfunction in the human granulosa cell line KGN by BPA and its selected analogues. We found that BPA and its analogues, especially BPAF, significantly reduced cell viability and caused cytotoxicity. Furthermore, we observed that BPA and BPAF significantly reduced mitochondrial function, including decreasing ATP generation, promoting ROS production and increasing intracellular Ca²⁺ levels. An oxidative-antioxidant imbalance was also detected after exposure to these chemicals. In contrast, the total antioxidant capacity was significantly reduced. To our knowledge, this is the first report on the evaluation of the potential of BPA and its analogues to induce cytotoxicity and mitochondrial dysfunction in ovarian granulosa cells. Our study revealed the possible mechanism of BPA and its analogues inducing granulosa cell damage and suggested that mitochondrial dysfunction may play an important regulatory role in bisphenol-induced follicular development disorders.

Keywords: Bisphenol A, Cytotoxicity, Mitochondrial Dysfunction, Granulosa Cells, Bisphenol A Analogues

1. Introduction

Bisphenols are widely spread in the environment and social surroundings. In particular, bisphenol A (BPA) extensively used in the manufacture of epoxy resins, phenolic resins and polycarbonate plastics [1]. Under harsh conditions, including high temperature, and acidity/alkalinity, BPA is released into the environment. Humans are exposed to this chemical through dietary and non-dietary routes such as skin contact and furniture dust inhalation [1].

Previous reports indicate that BPA is present in various human body fluids [2, 3]. Also, elevated levels of BPA are closely related to various diseases and health conditions [4-9]. Therefore, BPA is now being replaced by its analogues. Bisphenol S (BPS), bisphenol F (BPF) and bisphenol AF (BPAF) are the main alternatives to BPA in industrial applications and the production of daily necessities [10, 11].

Different research groups have investigated the interference effect of BPA on the development of ovarian follicles [12-14]. Although BPA analogues’ metabolic and endocrine-disrupting properties have not been determined, they began to appear as biological contaminants. The presence of BPA analogues has been detected in human urine and serum [15, 16]. Due to the structural similarity of these
alternatives to BPA, there is an urgent need to investigate their potentially harmful effects.

Studies including ours have shown that mitochondrial dysfunction is the basic mechanism of BPA-induced toxicity [17-21]. However, little is known about the effects of BPA analogues on mitochondrial damage in the female reproductive system. The present study’s main objective was to investigate the effects of BPA and its analogues on mitochondrial respiratory chain damage and oxidative stress system imbalance in granulosa cells.

2. Materials and Methods

2.1. Reagents

BPA, BPS, BPF and BPAF (all of 99-99.5% purity) were purchased from Sigma-Aldrich (Darmstadt, Germany). The fluorescent probes for reactive oxygen species (ROS) and Fluo-3-AM ester were purchased from US Everbright Biotechnology (Suzhou, China). MTT were purchased from KeyGEN Biotechnology (Nanjing, China). Lactate dehydrogenase (LDH) cytotoxicity assay kit and total antioxidant capacity (T-AOC) assay kit were purchased from Jiancheng Biotechnology (Nanjing, China). Adenosine 5'-triphosphate (ATP) assay kit was purchased from Beyotime Biotechnology (Shanghai, China).

2.2. Cell Cultures

KGN cells (a human granulosa-like tumor cell line) were provided by the Laboratory of Inflammation and Allergy of Southwest Medical University. The cells were cultured in DMEM/F12 culture medium (Hyclone, USA) with 100 IU/mL penicillin/streptomycin (NCM Biotech, China), 10% FBS (Gibco, USA) and incubated at 37°C under 5% CO₂ conditions.

2.3. Mitochondrial Function and Cell Cytotoxicity Assays

Mitochondrial function was measured using an MTT assay kit. KGN cells were seeded in 96-well plates at a density of 1×10⁵ cells/well. The cells were cultured for 48 h and treated with different concentrations of bisphenols for 24 h. Then, 10 µL of MTT solution was added to each well, followed by incubation under the same conditions for 4 h. Formazan lysate of 100 µL was added to each well, followed by incubation under the same conditions. The absorbance was measured at 570 nm after formazan was completely dissolved. In addition, the cytotoxicity induced by BPA and its analogues was detected according to the instructions of the LDH assay kit.

2.4. Detection of Cell Viability

Cells were cultured in a 12-well cell culture plate. After 24 h of exposure to bisphenols, the culture medium was aspirated, and the cells were washed once with PBS. Calcein AM detection working solution (Beyotime Biotechnology, China) of 500 µL was added to each well and incubated at 37°C for 30 min in the dark. Cell viability was observed using a fluorescence microscope (Olympus, Japan).

2.5. Detection of ATP Generation

Cells were plated onto 6-well plates and lysed with ATP lysates after bisphenols treatment for 24 h. The supernatant was collected for subsequent measurement after being centrifuged at 12,000 g for 5 min at 4°C. ATP test working solution of 100 µL was added to the test well and incubated at room temperature for 5 min. Then, 20 µL of the sample was added to the well and quickly mixed with a micropipette. The RLU value was measured with a multi-function microplate reader (Molecular Devices, USA) for chemiluminescence detection.

2.6. Intracellular Calcium Detection

Cells were cultured in a 12-well cell culture plate. After 24 h of exposure to bisphenols, the medium was removed, and the cells were washed three times with PBS. Fluo-3, AM working solution was added to the cell culture plate and incubated at 37°C for 30 min. After the Fluo-3, AM working solution was removed, the cells were washed three times with PBS. Cells were resuspended in PBS to make a solution of 1×10⁵ cells/mL and incubated at 37°C for 10 min to ensure complete de-esterification of AM bodies. Flow cytometry (ACEA Biosciences, USA) was used to detect changes in intracellular calcium levels.

2.7. Intracellular ROS Detection

Cells cultured in a 12-well cell culture plate were trypsinized after 24 h of bisphenols exposure. Intracellular ROS level was determined using the DCFH-DA fluorescent probe according to the product manual and the fluorescence intensity were detected by a flow cytometer (ACEA Biosciences, USA).

2.8. Detection of Total Antioxidant Capacity

Cells were treated with bisphenols for 24 h. The cells were scraped off and placed in 200 µL of pre-cooled PBS solution. Ultrasound was used to sufficiently disrupt the cells, and the lysate was centrifuged at 12,000 g at 4°C for 5 min. The supernatant was collected for subsequent analysis. To each well of the 96-well plate, 180 µL of FRAP working solution was added. A total of 5 µL of the sample was added to the sample test wells and mixed gently. After 5 min incubation at 37°C, the absorbance was measured at 593 nm using a microplate reader (Molecular Devices, USA).

2.9. Statistical Analysis

Data are presented as mean ± standard deviation (Mean ± SD) from at least three independent experiments. Comparisons between groups were performed using one-way ANOVA. The significance of the differences between the control group and each treated group was determined using a Tukey’s test. A value of P<0.05 was considered statistically
3. Results

3.1. BPA and Its Analogues Reduce Mitochondrial Function and Cell Viability of KGN Cells

We used MTT to measure the mitochondrial function and cell viability in KGN cells after treatment with different concentrations (0, 0.1, 1, 10 and 100 µM) of BPA, BPS, BPF and BPAF for different times (24 and 48 h). As shown in Figure 1A, BPA and its analogues reduce mitochondrial function and cell viability in a dose-dependent and time-dependent manner. As an indicator of cytotoxicity, LDH activity was detected to increase significantly after exposure to BPA and BPAF at 10 and 100 µM, BPS and BPF at 100 µM, respectively (Figure 1B). Furthermore, after labeling with Calcein AM probe, a decrease in KGN cells’ viability was also observed after exposure to bisphenols at 100 µM (Figure 1C).

3.2. Exposure to BPA and Its Analogues BPS, BPF and BPAF Reduced ATP Production

We examined intracellular ATP levels after treatment with bisphenols for 24 h. Intracellular ATP levels decreased significantly after treatment with BPA at concentrations of 10 and 100µM. Significant reductions in intracellular ATP levels were detected in KGN cells exposed to BPAF at concentrations of 1, 10 and 100 µM. In contrast, BPS and BPF had a weaker effect on the reduction of intracellular ATP, with significant differences only at 100 µM exposure (Figure 2A).

3.3. Exposure to BPA and Its Analogues Increased Ca²⁺ Levels of KGN Cells

Mitochondria are one of the organelles that store Ca²⁺ in cells. An abnormal release of Ca²⁺ from mitochondria increases the concentration of free Ca²⁺ in the cells, leading to cell death or dysfunction. Here, the Fluo-3 AM fluorescent probe was used to detect changes in Ca²⁺ levels in KGN cells after exposure to different concentrations of BPA and its analogues. A dose-dependent increase in intracellular Ca²⁺ levels was detected after exposure of KGN cells to BPA and its analogues (Figure 2B).

3.4. Exposure to BPA and Its Analogues Induces Oxidative Stress in KGN Cells

We used flow cytometry to examined the changes in intracellular ROS levels after exposure to BPA and its analogues. As shown in the Figure 3A, BPA and BPAF significantly increased ROS levels at 1, 10 and 100 µM, respectively. Studies on BPS and BPF have shown that the levels of ROS have also increased significantly after high-concentration treatment (10 and 100 µM). Furthermore, we also detected that BPA, BPS, BPF and BPAF reduce the levels of intracellular T-AOC in a concentration-dependent manner (Figure 3B).
Figure 2. Exposure to BPA and its analogues reduced ATP production and increased Ca\(^{2+}\) levels of KGN cells. A. KGN cells were exposed to BPA and its analogues for 24 h, and then changes in intracellular ATP levels were measured according to the instructions. B. Flow cytometry was used to measure changes in intracellular Ca\(^{2+}\) in KGN cells after exposure to different concentrations of BPA and its analogues. The data are expressed as mean ± SD (n=3). Different superscript letters indicate statistically significant differences between the treatment groups (P < 0.05).

Figure 3. Exposure to BPA and its analogues induces oxidative stress in KGN cells. A. Flow cytometry was used to measure the changes of intracellular ROS levels in KGN cells after exposure to BPA and its analogues for 24 h. B. T-AOC levels were measured after exposure to bisphenols. The data are expressed as mean ± SD (n=3). Different superscript letters indicate statistically significant differences between the treatment groups (P < 0.05).
4. Discussion

In this study, we evaluated the effects of BPA and its analogues on the reduction of mitochondrial function, including the induction of reduced ATP production and increased intracellular ROS and Ca\(^{2+}\) levels as well as the induction of oxidative stress in KGN cells. The maintenance of mitochondrial function is a prerequisite for cell survival and biological function. ATP generation, Ca\(^{2+}\) storage and ROS production are the main functions of mitochondria [22, 23]. ATP play essential roles in female reproduction. The ATP levels in follicular fluid are related to follicle size and gonadotropin antagonism. Also, abnormal ATP production is closely related to ovarian dysfunction [24-26].

We found that high concentrations of BPA and its analogues, especially BPAF, significantly reduce ATP generation in granulosa cells. These results agree with previous studies on reducing ATP production in cell lines caused by exposure to BPA and its analogues [27]. Although in vitro studies, including ours, have shown that bisphenols have a reduced effect on the intracellular ATP production, there are no reports on whether they are involved in the development of follicular dysgenesis by reducing ATP production, which requires further investigation.

Oxidative stress is caused by an imbalance between the generation and removal of oxygen free radicals. We observed that BPA induces significantly increased intracellular ROS in granulosa cells. In contrast, we also observed a decrease in granulosa cells’ total antioxidant capacity induced by BPA.

Oxidative stress is the main mechanism causing follicular developmental disorders, particularly polycystic ovary syndrome, one of the major diseases inducing female infertility [28, 29]. Our results and previous studies suggest that oxidative stress induced by BPA may play a regulatory role in the development of follicular developmental disorders. We also observed that high BPS, BPF and BPAF induced oxidative stress in granulosa cells. In particular, BPAF showed a strong potential for inducing oxidative stress compared to other BPA analogues in cells. In addition, the granulosa cells’ antioxidant capacity showed a downward trend after exposure to high levels of bisphenols. Bisphenols represents an environmental problem due to its high volumes produced. They have been detected in everyday items, including indoor dust, surface water, sediment and sewage [30-33]. Although the BPA analogues are widely used in everyday products production, their toxic effects on female reproduction are still unknown and require further evaluation.

5. Conclusion

In summary, we demonstrated that BPA and its analogues, particularly BPAF, significantly induced mitochondrial dysfunction, including reduced ATP production, induced oxidative stress generation and increased intracellular Ca\(^{2+}\) levels. In particular, BPA and BPAF have more significant effects on cell damage. Our study revealed the possible mechanism of BPA and its analogues inducing granulosa cell damage. In addition, it also suggests that it is necessary to evaluate the biological effects and potential risk of alternatives to BPA.

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Bisphenol A and Its Analogues Exhibit Different Cytotoxic and Mitochondrial Dysfunction Potential in Human Granulosa Cells

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