Comparative Study of Bisphenol A and Its Selected Analogues on the Induction of Mitochondrial Mass Loss and Apoptosis in Human Granulosa Cells

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Abstract: It has been confirmed that the occurrence of a variety of chronic diseases including female infertility is closely related to bisphenol A (BPA) exposure. BPA is now being gradually replaced by its analogues. Although the adverse health effects of BPA have been extensively studied, little is known about the female reproductive toxicity of its analogues, particularly BPS, BPF and BPAF. The present study evaluated the effects of BPA and its analogues BPS, BPF and BPAF on mitochondrial mass and potential to induce apoptosis in KGN cells. We observed that BPA and its analogues, especially BPA and BPAF exposure can significantly reduce mitochondrial mass. This reduction in mitochondrial mass was confirmed by flow cytometry after labelling the NAO probe. The significant induction effect of BPA and its analogues on KGN cell apoptosis was detected using flow cytometry. Compared with BPA and BPAF, the interference effect of BPS and BPF on KGN cells was smaller, although there were significant differences between the high-concentration treatment groups and the control group. Our study revealed the possible mechanisms of BPA and its analogues on granulosa cell damage and female sterility. In addition, it also suggests that the safety of BPA analogues in female reproduction needs to be reconsidered.

Keywords: Bisphenol A, Ovarian Follicle Dysfunction, Bisphenol A Analogues, Mitochondrial Dysfunction, Apoptosis, Granulosa Cells

1. Introduction

Bisphenol A (BPA) is a key component to produce various consumer products such as household electronics, medical and dental equipment, beverage cans, kitchen appliances, water and food containers and paper products [1-3]. It has now been confirmed that the occurrence of a variety of chronic diseases including female infertility is closely related to BPA exposure [4]. For these reasons, the use of BPA is being limited and gradually replaced with other bisphenols.

Many chemicals similar in structure to BPA are gradually being used to manufacture polycarbonate plastics and epoxies [1, 5]. Bisphenol S (BPS) is the most commonly used replacement monomer in BPA-free products. BPS is commonly used as an ordinary plastic, a washing and curing agent in cleaning products, and a coating and thermal paper developer [6, 7]. Bisphenol F (BPF) is also used to produce materials that require increased thickness and durability, such as pipe linings, adhesive plastics, and water pipes. Furthermore, BPF epoxy resins are used in various consumer products such as paints, varnishes, dental sealants, mouth restorations, tissue substitutes, and food-packaging coatings [8]. Bisphenol AF (BPAF) is a crosslinker for fluorooelastomers, electronics, and fibers. It is also a high-performance monomer for polymides, polyamides, polyesters, polycarbonate copolymers, and other specialty polymers [9].
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 10-nonyl acridine orange (NAO) and JC-1 were purchased from US Everbright Inc (Suzhou, China). Acridine orange (AO) were purchased from KeyGEN Biotechnology (Nanjing, China). Caspase-3 assay kit and Annexin V-APC apoptosis kit were purchased from US Everbright Inc (Suzhou, China).

2.2. Cell cultures and Treatment

Human granulosa cell line KGN 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. The concentrations of BPA and its analogues applied in this study were determined based on the results of our previous experiments [14]. The volume of DMSO as solvent was the same in the control group as in the different concentrations of the treatment groups.

2.3. Detection of Mitochondrial Mass

Cells cultured in 12-well plates were washed once with PBS after 24 h of BPA and its analogues exposure. The cells were digested with trypsin and centrifuged at 300 g for 5 min. The cell pellets were resuspended in serum-free medium containing 100 nM NAO and incubated at 37°C for 30 min in the dark. After the incubation step, the cells were centrifuged and resuspended in a serum-free medium. The fluorescence intensity of NAO was detected by flow cytometry (ACEA Biosciences, USA).

2.4. Apoptosis Detection

Cells were treated with bisphenols for 24 h. The cell culture medium was aspirated and washed twice with pre-cooled PBS. A total of 5 µL of Annexin V binding solution containing 1×10⁴ cells and gently mixed. After an incubation step at room temperature for 15 min in the dark, the samples were analyzed using a flow cytometer. In addition, cells were harvested, pelleted, and resuspended in DMEM/F12 containing 10µg/ml AO dye. DNA fragmentation induced by bisphenols was detected by flow cytometry (ACEA Biosciences, USA).

2.5. Caspase-3 Activity Detection

Cells were exposed to BPA and its analogues for 24 h. The cells were then digested with trypsin and resuspended in cell culture medium to a cell density of 10⁶ cells/mL. A cell suspension volume of 0.2 mL was transferred into a flow cytometer tube, and 5 µL of 0.2 mM substrate was added and immediately mixed to a substrate concentration of 5 µM. After incubating the cells at room temperature for 30 min, caspase-3 activity in the cells was measured by flow cytometry (ACEA Biosciences, USA).

2.6. Mitochondrial Membrane Potential Detection

Cells were treated with bisphenols for 24 h. The cells were digested with trypsin and centrifuged at 400 g at room temperature for 5 min. After removing the supernatant, cells were resuspended with 0.5 mL of JC-1 working solution and incubated at 37°C for 15 min. The cells were washed twice with PBS, and then resuspend with 0.5 mL 1× assay buffer. Changes in mitochondrial membrane potential were detected by a flow cytometer (ACEA Biosciences, USA).

2.7. 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 significant.

3. Results

3.1. BPA and Its Analogues Exhibit Different Potentials for Reducing Mitochondrial Mass

Previous studies have found that mitochondrial damage is one of the mechanisms by which BPA induce cell dysfunction. We used NAO to detect mitochondrial mass changes after exposure to different concentrations of BPA and its analogues for 24 h. We found that BPA and BPAF significantly reduced mitochondrial mass at 100 µM, respectively. However, the significant decrease in mitochondrial mass by exposure to BPS and BPF was not observed (Figure 1A and B). Laser confocal microscopy showed that the intracellular mitochondrial mass was significantly reduced after exposure to high concentrations of BPA and BPAF (Figure 1C).
Figure 1. BPA and its analogues exhibit different potentials for reducing mitochondrial mass. A and B. Flow cytometry was used to measure changes in mitochondrial mass after exposure to bisphenols. C. KGN cells were exposed to BPA and its analogues, and laser confocal microscopy was used to detect changes in mitochondrial mass (100×). The data are expressed as mean ± SD (n=3). Different superscript letters indicate statistically significant differences between the treatment groups (P < 0.05).

3.2. BPA and Its Analogues Induces KGN Cell Apoptosis

We assessed apoptosis in granulosa cells after treatment with BPA and its analogues using the flow cytometry. We observed that BPA, BPS and BPF significantly induced apoptosis at 100 µM, respectively. However, BPAF significantly induces apoptosis at 10 and 100 µM.

Figure 2. KGN cell apoptosis is induced by BPA and its analogues. KGN cells were exposed to bisphenols for 24 h, and then KGN cell apoptosis was measured using flow cytometry. The data are expressed as mean ± SD (n=3). Different superscript letters indicate statistically significant differences between the treatment groups (P < 0.05).
3.3. BPA and Its Analogues Decreases Mitochondrial Membrane Potential and Caspase-3 Activity of KGN Cells

A decrease in mitochondrial membrane potential is a landmark event in apoptosis. We used the JC-1 probe to detect changes in mitochondrial membrane potential. As shown in the Figure 3A, BPA, BPS, BPF and BPAF significantly reduced the aggregates levels of JC-1 at high concentrations. In contrast, the levels of monomers increased significantly after exposure to bisphenols. In addition, a significant increase in DNA fragmentation (Figure 3B) and caspase activity (Figure 3C) in KGN cells were also observed using flow cytometry after treatment with bisphenols at 100 µM.

![Figure 3](image)

**Figure 3.** Exposure to BPA and its analogues decreases mitochondrial membrane potential and caspase activity of KGN cells. After KGN cells were exposed to 100 µM of BPA and its analogues, flow cytometry was used to detect changes in mitochondrial membrane potential (A), DNA fragmentation (B) and caspase activity (C). 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

BPA is an endocrine disruptor widely distributed in the environment, causing reproductive dysfunction, especially by interfering with ovarian follicular development [15-18]. Although BPA is gradually being replaced by its analogues, these substitutes’ toxicity in reproduction has not been fully elucidated. Here, we assessed the induction of mitochondrial mass loss and apoptosis in KGN cells cultured in vitro by BPA and its analogues.

In our study, NAO probe was used to assess mitochondrial mass changes after bisphenols treatment. We found that BPA significantly reduced the mitochondrial mass at high concentrations. We also found that BPAF elicited a more significant reduction in mitochondrial mass compared to BPA, BPS, and BPF. Our previous report showed that under the same exposure concentrations, BPAF is more cytotoxic to granulosa cells than BPA, PBS and BPF [14].

Controlling mitochondrial mass is the basis for cell survival and function. The maintenance of mitochondrial mass plays an important regulatory role in ovarian follicle development [19-21]. Combined with our previous research results, we speculate that a reduction in mitochondrial mass is one of the mechanisms by which exposure to BPA and its analogues, especially BPAF, induces female infertility.

Apoptosis in granulosa cells is not only the main cause of follicular atresia but is also considered a mechanism of ovarian dysfunction, such as ovarian cysts, polycystic ovary syndrome and premature ovarian failure [22, 23]. Our results indicate that high concentrations of BPA can induce granulosa cell apoptosis. This result is consistent with other studies in non-ovarian cell line [24-27].

In addition, we also found significant differences in the potential of BPA analogues on apoptosis. In particular, BPAF
has a significant induction effect on the apoptosis of granulosa cells compared with BPA. We revealed the underlying mechanism of ovarian dysfunction induced by bisphenols. By comparing the viability and toxicity of BPA and its analogues to granulosa cells, we found that BPAF exhibited strong cytotoxicity to germ cells compared to other bisphenols tested at lower concentrations. These results suggest that the safety of alternative bisphenols, especially BPAF, needs to be reconsidered.

5. Conclusion

In conclusion, in the present study we found that BPA and its analogues, particularly BPA and BPAF, caused a significant decrease in mitochondrial mass and induce apoptosis in KGN cells. To our knowledge, this is the first report on BPA and its analogues inducing a decrease in mitochondrial mass in granulosa cells. Our study suggests that the female reproductive toxicity of BPA analogues, particularly BPAF, should be taken into account in future studies.

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