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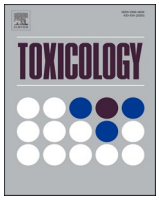

Differential susceptibility of Leydig and Sertoli cells to bisphenol A

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ABSTRACT

Bisphenol A (BPA) is an endocrine-disrupting chemical that is increasingly becoming a vital factor in public health due to its ubiquity and toxicity. BPA is associated with male infertility via the disrupted function of Leydig and Sertoli cells. Despite extensive research, the current understanding of the specific pathological concentrations and the mechanisms following BPA exposure still remain questionable. Therefore, we investigated the susceptibilities and underlying mechanisms in Leydig and Sertoli cells following treatment with various BPA doses (0.0001–100 μM in a 10-fold serial dilution). Our results showed that the lowest BPA levels (10^{-4} μM) decreased mitochondrial membrane potential and ATP levels. In contrast, ROS levels were increased at high BPA levels regardless of exposure time (24 or 48 h) in both cell types. Mitochondrial-mediated apoptosis was identified along with increased ROS levels and abnormal mitochondrial dynamics, but both cell types showed different susceptibility to BPA toxicity. Subsequently, BPA had detrimental impacts on the mRNA expression levels of steroidogenic enzymes and testosterone synthesis in Leydig cells and reduced anchoring junction proteins in Sertoli cells. Consequently, our results demonstrated that both cells were affected via estrogen receptor alpha. However, protein kinase A was oppositely expressed following BPA exposure in each cell type. Therefore, it is plausible to suggest that each cell has distinct sensitivities and mechanisms in response to BPA.

1. Introduction

The dysfunction of human reproduction has been increasing in recent years, leading to growing global social concerns. Worldwide, approximately 48.5 million couples are affected by infertility, and male factors solely or complexly cause 50 % of infertility (Kasonga et al., 2021; Santiago et al., 2021). Male infertility is a complicated disease caused by several factors, such as the environment and diet (Vandenberg et al., 2012b). Several epidemiological, experimental, and clinical studies have demonstrated the detrimental effects of endocrine-disrupting chemicals (EDCs) on male reproductive disorders (Balabanic et al., 2011; Futran Fuhrman et al., 2015; Manikkam et al., 2013). Bisphenol A (BPA) is the most commonly used EDC in plastic products, epoxy resins, food/drinking coating materials, thermal paper, and water pipes (Fenichel et al., 2013; Tomza-Marciniak et al., 2018). Numerous studies have detected BPA in human serum and urine (Inoue et al., 2000; Kim et al., 2003; Lopez-Cervantes and Paseiro-Losada, 2003; Teeguarden et al., 2011; Vandenberg et al., 2012a; Vandenberg et al., 2007). The measured concentrations in serum were found to range from 0.2 to 20 ng/mL.(Vandenberg et al., 2007) Owing to its ubiquity, BPA is found in over 90 % of urine samples collected from the U.S. population (Lopez-Cervantes and Paseiro-Losada, 2003; Tan and Mustafa, 2003). Therefore, human exposure to this compound raises concerns because of its ability to act as an endocrine disruptor. Many studies have demonstrated its potential to interrupt the endocrine system owing to its hormone-like properties, thus affecting human diseases such as cancer, obesity, diabetes, and male fertility (Calafat et al., 2005; Erler and Novak, 2010; Peretz et al., 2014; Tsai, 2006). BPA mainly affects the male reproductive system by disrupting spermatogenesis, steroidogenesis, and sperm function (Liao and Kannan, 2011).

Spermatogenesis is a differentiation process that produces spermatozoa in the testes with the support of two major cell types, Leydig and Sertoli. Spermatogenesis is primarily regulated by testosterone, a crucial hormone for male reproduction that is synthesized in the mitochondria of Leydig cells. Mitochondria are crucial organelles for cell ATP synthesis via oxidative phosphorylation, which occurs in the respiratory chain complex. Midzak et al. (Midzak et al., 2011) demonstrated that steroidogenesis is closely associated with mitochondrial ATP synthesis in Leydig cells. Therefore, the aberrant function of Leydig cell mitochondria impairs the overall male reproductive system, resulting in male

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infertility (Yang et al., 2015).

Sertoli cells comprise another primary cell type for every stage of spermatogenesis. They firmly adhere to germ cells located in the lining of the seminiferous tubules and support proper circumstances for their development. Sertoli cells produce lactate and pyruvate, both necessary energy sources for germ cells (Grootegoed et al., 1984; Jutte et al., 1981; Jutte et al., 1982; Jutte et al., 1983). In addition, the blood-testis barrier (BTB) provides unique microenvironments in the apical section of the seminiferous epithelium, which consists of germ and Sertoli cells. It confers cell polarity and provides immune privileges to the testis. The BTB includes ectoplasmic specializations, tight junctions, desmosomes, and gap junctions, which play vital roles in spermatogenesis by regulating the intracellular communication and dynamics between Sertoli cells (Lee et al., 2003). However, the weak immunological barrier for germ cell autoantigens resides in the spermatogonia and spermatocytes detected outside the BTB. Therefore, these cellular events are coordinated by environmental alterations or intrinsic or extrinsic biological factors, thereby modulating spermatogenesis.(Gao et al., 2015; Weider et al., 2011).

A previous study demonstrated that testosterone production was affected following treatment with 10^{-8} M BPA in humans, while 10^{-5} M decreased testosterone production in rats (Ben Maamar et al., 2015; N'Tumba-Byn et al., 2012). Another research team found that different concentrations of BPA affect testosterone production in different breeds of mice (Horan et al. 2018). In addition, it was recently discovered that BPA alters proteins in the BTB associated with male reproduction, such as gametogenesis, steroidogenesis, and sperm functions (Fiorini et al., 2004; Salian et al., 2009; Sobarzo et al., 2006). Based on these studies, different species and breeds may be affected differently after treatment with various BPA doses (Horan et al., 2018). Although ample evidence shows that BPA exerts harmful effects on the male reproductive system by disrupting the functions of Leydig and Sertoli cells, determining the exact dose or critical developmental period at which pathological outcomes occur remains challenging (Acerini and Hughes, 2006). Therefore, the aim of our research was to expand current knowledge regarding the effects of BPA on male fertility by assessing the BPA toxicity in relation to pathological outcomes. Herein, we demonstrated that each cell type has different susceptibilities and mechanisms of response to BPA. These observations may provide insight for discovering the fundamental mechanisms of BPA-induced male infertility.

2. Materials and methods

2.1. TM3 and TM4 cell culture and BPA concentrations

The TM3 and TM4 mouse Leydig and Sertoli cell lines (CRL-1714™ and 1715™) were sourced from the American Type Culture Collection and cultured in a 1:1 mixture of Gibco Ham's F12 medium and Dulbecco's modified Eagle's medium. The medium was supplemented with 5 % (v/v) horse serum and 2.5 % (v/v) fetal bovine serum by the manufacturer's instruction (both from Invitrogen, Thermo Fisher Scientific, Inc.). To avoid contamination, streptomycin (100 μg/mL) and penicillin (100 U/mL) were included in the growth medium. The culture was kept at a temperature of 37 °C in an atmosphere with 5 % CO_2 and humidity. Both cell lines were passaged every 2–3 days based on the cell confluency. Despite various countries establishing reference doses and safe limits for daily human exposure to BPA through regulatory agencies, substantial evidence indicates the toxic effects of lower doses of BPA on public health (Ben Maamar et al., 2015; Prins et al., 2019; Rahman et al., 2021). Therefore, we exposed the two cell lines to varying BPA concentrations to assess their sensitivities and toxicity levels. Pure dimethyl sulfoxide (DMSO; D2650, Sigma, St. Louis, MO, USA) was used to dissolve bisphenol A (BPA; 239658, Sigma, St. Louis, MO, USA). The BPA solutions were diluted in the medium to achieve final concentrations ranging from 0.0001 to 100 μM in a 10-fold serial dilution. In all preparations, the final concentration of DMSO in the medium was maintained at 0.1 % (v/v) to minimize any potential cytotoxic effects of the solvent. A vehicle control containing 0.1 % DMSO without BPA was included as a baseline reference. For the biochemical properties of Leydig and Sertoli cells, such as mitochondrial membrane potential (MMP), reactive oxygen species (ROS) levels, and ATP, BPA concentrations ranging from 0.0001–100 μM in a 10-fold serial dilution were employed and incubated at 37 °C under 5 % CO_2 conditions for 24 h and 48 h, with a 0.1 % DMSO vehicle control as the baseline reference. Simultaneously, concentrations of 0.0001, 0.01, 1, 10, and 100 μM BPA were used to analyze the testosterone levels and apoptotic cells. Apoptosis was first observed at 0.01 μM BPA, indicating the point at which cell damage became irreversible. Based on this, 0.01, 1, and 100 μM of BPA concentrations were used to identify protein expression levels.

2.2. Measurement of intracellular ATP

The intracellular ATP was evaluated using an ATP was assessed using an ATP Bioluminescence Assay Kit HS II (Roche Molecular Biochemicals, Mannheim, Germany; 11699709001). Cells were seeded at a density of 1×10^4 per well in 96-well plates. After the treatment, Microplate Multimode Reader (GloMax-Multi; Promega, Madison, WI, USA) was used to detect ATP bioluminescence intensity (RLU) of the control and BPA-treated groups.

2.3. Measurement of intracellular ROS

The intracellular ROS was assessed using a fluorescent dye DCFDA (Abcam; ab113851), in accordance with the manufacturer's instruction. Cells were seeded at a density of 1×10^4 per well in 96-well plates. After the treatment, fluorescence measurements were taken at 485 nm (excitation) and 535 nm (emission). The microplate fluorometer (Gemini Em; Molecular Devices, Sunnyvale, CA, USA) and SoftMax Pro 5 software (Molecular Devices) were used to detect and analyze the fluorescence of the control and BPA-treated groups.

2.4. Measurement of intracellular MMP

The intracellular MMP was assessed using a fluorescent dye JC-1 (Abcam; ab113850), in accordance with the manufacturer's instruction. Cells were seeded at a density of 1×10^4 per well in 96-well plates. After treatment, the fluorescence of J-aggregates and J-monomers were measured with excitation at 535 and 595 nm and emission at 485 and 535 nm, respectively. The microplate fluorometer (Gemini Em; Molecular Devices, Sunnyvale, CA, USA) and SoftMax Pro 5 software (Molecular Devices) were used to detect and analyze the fluorescence of the control and BPA-treated group. Data are expressed as the ratio of aggregate/monomer.

2.5. Hoechst 33342/PI double fluorescence staining

Apoptosis was measured using Hoechst 33342/PI double staining. Cells were seeded at a density of 2×10^4 per well in 24-well plates. Following treatment, cells were stained with Hoechst 33342 and PI dye per the manufacturer's instructions. Apoptotic cells were determined using a fluorescence microscope (Nikon).

2.6. Western blot analysis

Cells were lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, with 150 mM sodium chloride, pH 8.0, 1.0 % Igepal CA-630 (NP-40), 0.1 % sodium dodecyl sulfate, and 0.5 % sodium deoxycholate) including complete protease inhibitor and phosphatase (Roche Applied Science, Indianapolis, IN), and centrifuged at 17,000 ×g for 30 min at 4 °C. The lysates were boiled at 100 °C for 5 min. Samples were loaded onto 12 % of SDS polyacrylamide gels and resolved by

electrophoresis (Ryu et al., 2022). The separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes. Antibodies against Cas3, estrogen receptor (ER)-α, Atp5a, Bax, Bcl-2, ER-β (Santa Cruz), Opa1 (LSBio), cleaved Cas3, protein kinase A (PKA; Cell Signaling), Zo-1, Cdh1, Cx43, Mfn2, Cyc1, Ndufv2, Atp5f1, and β-actin (Abcam) were developed using enhanced chemiluminescence. Protein quantification of each band was conducted using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Data are expressed as the ratio of protein band intensity to β-actin intensity.

2.7. Addition of luteinizing hormone (LH) and measurement of testosterone levels

To evaluate testosterone levels in Leydig cells, LH was added, as previously reported (Riccetti et al., 2017). Leydig cells were seeded at a density of 5×10^4 per well in 24-well plates and incubated 2 days before the addition of LH (0, 0.01, 0.1, 1, and 10 ng/mL). Testosterone levels were measured after 6 h of incubation using a testosterone ELISA kit (Novus Biologicals), in accordance with the manufacturer's instructions. Briefly, the treated cells were fixed on strip wells. The antigen bound to antibodies was colorimetrically evaluated at 450 nm using the SoftMax Pro 5 software. Simultaneously, Leydig cells were treated with doses of BPA (0, 0.0001, 0.01, 1, 10, and 100 μM) under 10 ng/mL of LH to evaluate the levels of testosterone following BPA exposure. Then, testosterone levels were measured.

2.8. RNA extraction, cDNA synthesis, and quantitative real-time PCR (qRT-PCR)

RNA was extracted using a PureLink™ RNA Mini Kit (Invitrogen, Carlsbad, CA, USA) and TRIzol (Invitrogen), in accordance with the manufacturer's instructions. Nanodrop spectrophotometer (NanoDrop

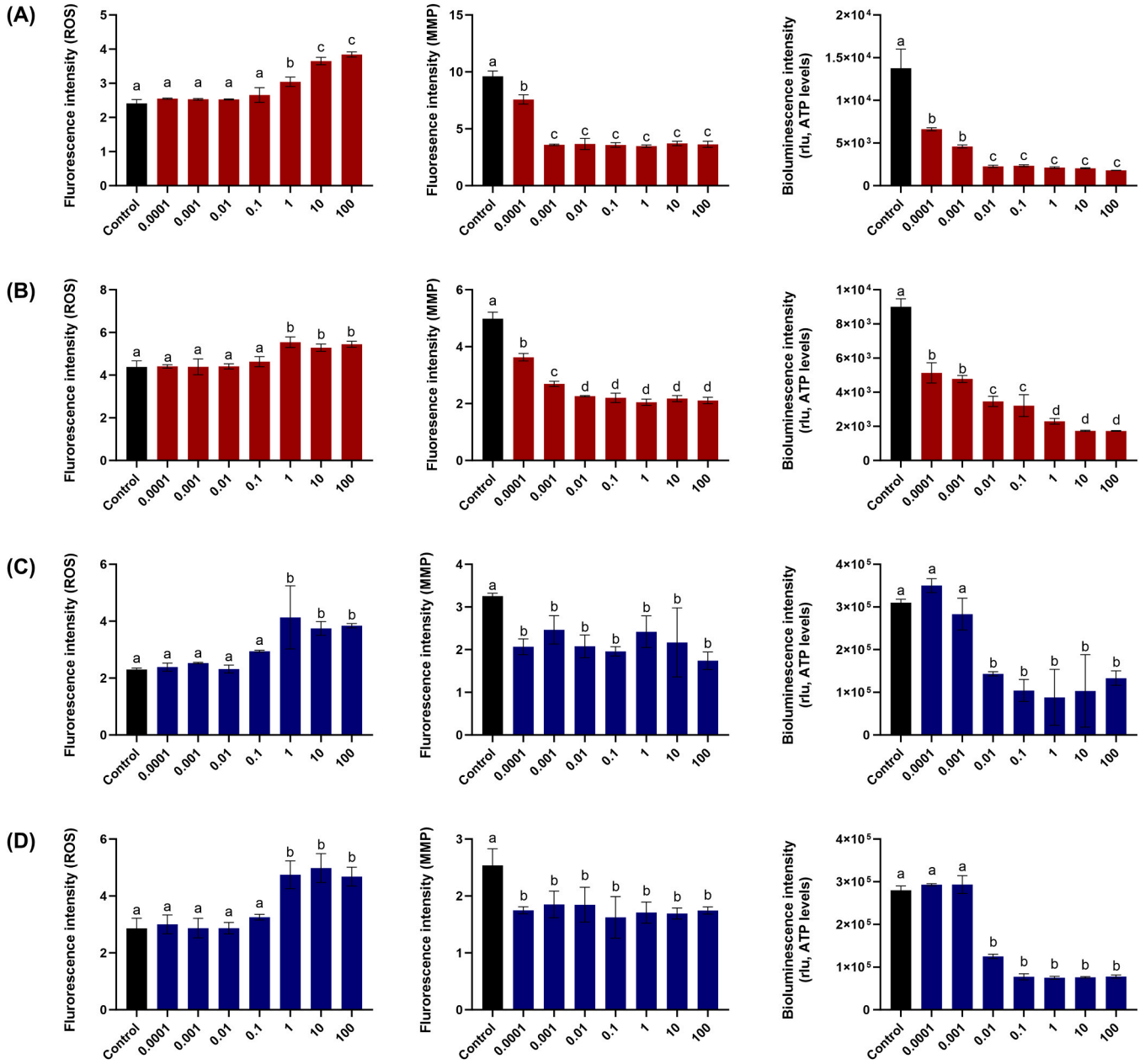

Fig. 1. Effects of bisphenol A (BPA) on intracellular reactive oxygen species (ROS), mitochondrial membrane potential (MMP), and ATP in Leydig (red bars) and Sertoli (blue bars) cells following 24-h and 48-h treatments **(A–D)**. Data represent the means of three replicates ± SD. Values with superscripts (^{a,b,c,d}) indicates significant differences between the control and BPA-treated groups, as determined by one-way ANOVA ($p < 0.05$) with post-hoc Tukey's test.

Technologies, Wilmington, USA) was used to evaluate the RNA concentration and 260/280 ratio. Then, cDNA synthesis was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), in accordance with the manufacturer's instructions. PrimerSelect software (DNASTAR, Madison, WI, USA) was used to design primers. Targeted cDNA was amplified by qRT-PCR and quantified using ABI PRISM 7500 (Applied Biosystems). Normalization was performed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene, and the $2^{-\Delta\Delta}$Ct method was used to analyze the relative abundance (Livak and Schmittgen, 2001).

2.9. Statistical analysis

Data analysis was performed using one-way analysis of variance (ANOVA) with Graphpad prism (version 9.0; CA, USA). Significant differences between groups were assessed by One-way ANOVA with post-hoc Tuckey's test. Differences between the control and BPA-treated groups were considered significant at p values less than 0.05. Data are presented as mean ± SD.

3. Results

3.1. BPA effects on MMP, ATP, and ROS levels in Leydig and Sertoli cells

We investigated the effects of BPA on MMP activity, intracellular ATP, and ROS levels in Leydig and Sertoli cells at 24 and 48 h. The lowest dose of BPA (0.0001 μM) significantly decreased MMP and intracellular ATP levels under both 24- and 48-h exposure in Leydig cells (Fig. 1, $p < 0.05$). On the other hand, MMP significantly decreased at the lowest concentration (0.0001 μM), whereas ATP levels significantly decreased at a relatively higher concentration (0.01 μM) in Sertoli cells (Fig. 1, $p < 0.05$). Although the lower dose of BPA significantly affected MMP and ATP levels, a comparatively higher dose of BPA (1 μM) increased the intracellular ROS levels in both Leydig and Sertoli cells (Fig. 1, $p < 0.05$). The biochemical parameters of Leydig and Sertoli cells exposed to BPA for different periods (24 and 48 h) showed no differences over time (Fig. 1). Therefore, all subsequent experiments were conducted following 24-h exposure to BPA.

3.2. BPA effects on apoptosis and mitochondrial dynamics in Leydig and Sertoli cells

We assessed the levels of apoptosis and proteins related to apoptotic mechanisms (caspase and Bcl pathways) in Leydig and Sertoli cells. Leydig cells exhibited increased apoptosis upon treatment with 0.01 μM BPA. In contrast, Sertoli cells showed a significant rise in apoptosis starting from a higher concentration of 1 μM BPA (Fig. 2A and B, $p < 0.05$ As apoptosis occurred at a minimum concentration of 0.01 μM, subsequent experiments with BPA concentrations of 0.01, 1, and 100 μM were conducted. Subsequently, we determined the Bax/Bcl-2 ratio and

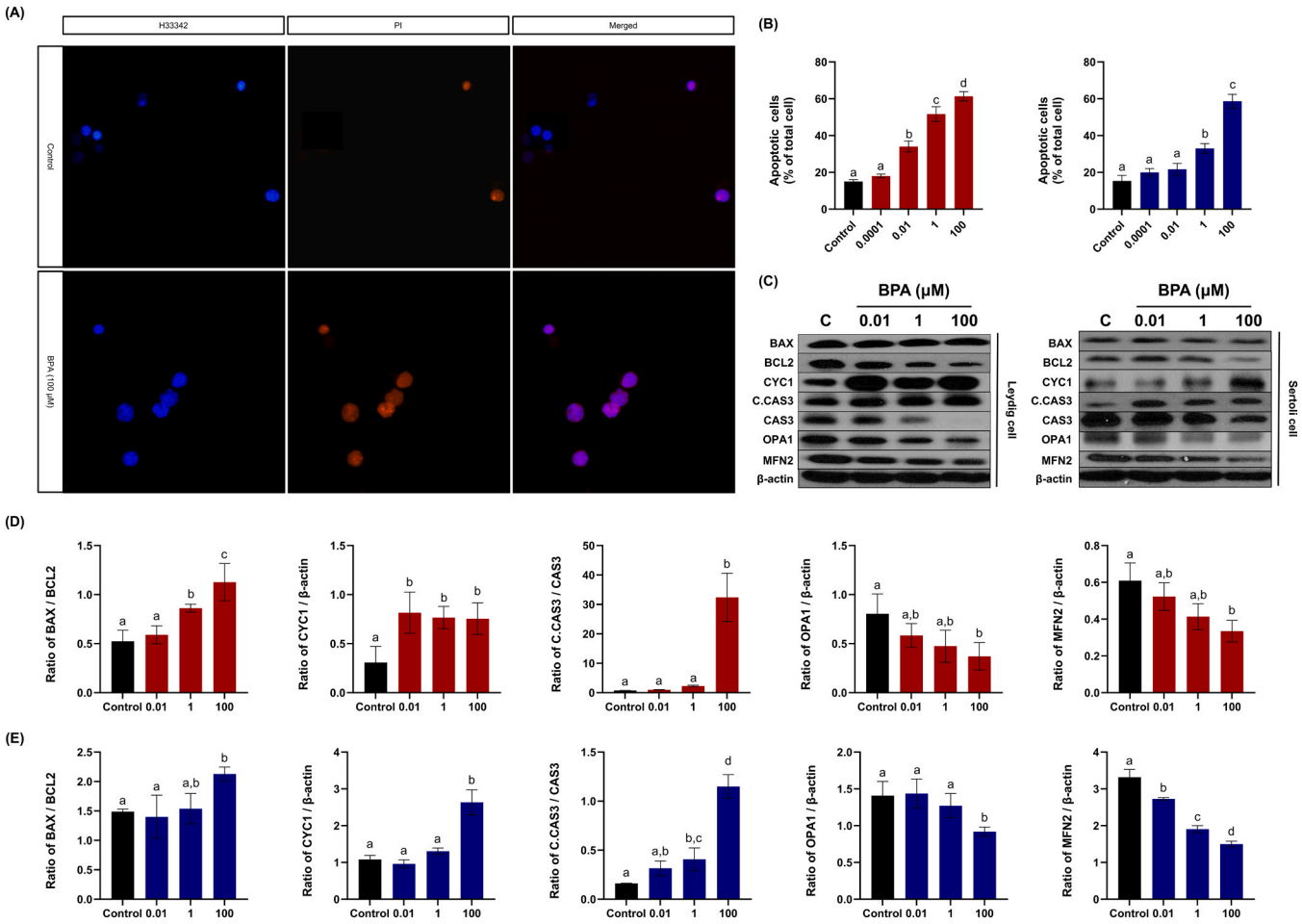

Fig. 2. Effects of bisphenol A (BPA) on apoptosis, apoptotic proteins, and mitochondria fusion protein in Leydig and Sertoli cells. **(A)** Representative images of Hoechst 33342/PI double fluorescence staining in control and 100 μM BPA-treated Leydig cells. **(B)** Changes in apoptotic cells (%) in the control and BPA groups in Leydig (red bars) and Sertoli (blue bars) cells. **(C)** Representative western blot images. Differences in protein expression levels between the control and BPA groups in **(D)** Leydig and **(E)** Sertoli cells. Data represent the means of three replicates ± SD. Values with superscripts (^{a,b,c,d}) indicates significant differences between the control and BPA-treated groups, as determined by one-way ANOVA ($p < 0.05$) with post-hoc Tukey's test.

Cyc1 expression, indicative of the Bcl-2 apoptosis pathway. In Leydig cells, the Bax/Bcl-2 ratio increased significantly, starting from 1 μM BPA (Fig. 2C and D, $p < 0.05$). In contrast, in Sertoli cells, Bax/Bcl-2 significantly increased at the highest dose of 100 μM BPA (Fig. 2C and E, $p < 0.05$). Next, we examined the proteins related to the caspase pathway, another apoptotic pathway. In Leydig cells, a significant increase in cleaved Cas3/Cas3 ratio was observed starting from the highest concentration of 100 μM BPA (Fig. 2C and D, $p < 0.05$). In Sertoli cells, a significant increase was observed at 1 μM BPA (Fig. 2C and E, $p < 0.05$). In addition, we measured mitochondrial fusion proteins to determine whether mitochondria-mediated apoptosis. Our results showed that both proteins decreased significantly under the highest concentration of BPA (100 μM) in the Leydig cell (Fig. 2C and D, $p < 0.05$). On the other hand, both OPA1 and MFN2 decreased significantly under 100 and 0.01 μM of BPA exposure, respectively (Fig. 2C and E, $p < 0.05$).

3.3. BPA effects on testosterone synthesis in Leydig cells

We measured testosterone levels to determine whether BPA treatment affected testosterone synthesis. Leydig cells were treated with LH to induce testosterone synthesis, which was successful under 10 ng/mL LH (Fig. 3A, $p < 0.05$). Therefore, we co-treated Leydig cells with various doses of BPA and 10 ng/mL LH to investigate the effect of BPA on testosterone synthesis. Testosterone levels decreased significantly following treatment with BPA (1, 10, and 100 μM) in Leydig cells (Fig. 3A, $p < 0.05$). Subsequently, qRT-PCR was performed to investigate the underlying mechanisms. Interestingly, most steroidogenic genes, except for HSD17B1, significantly increased in response to 1 μM of BPA (Fig. 3B, $p < 0.05$).

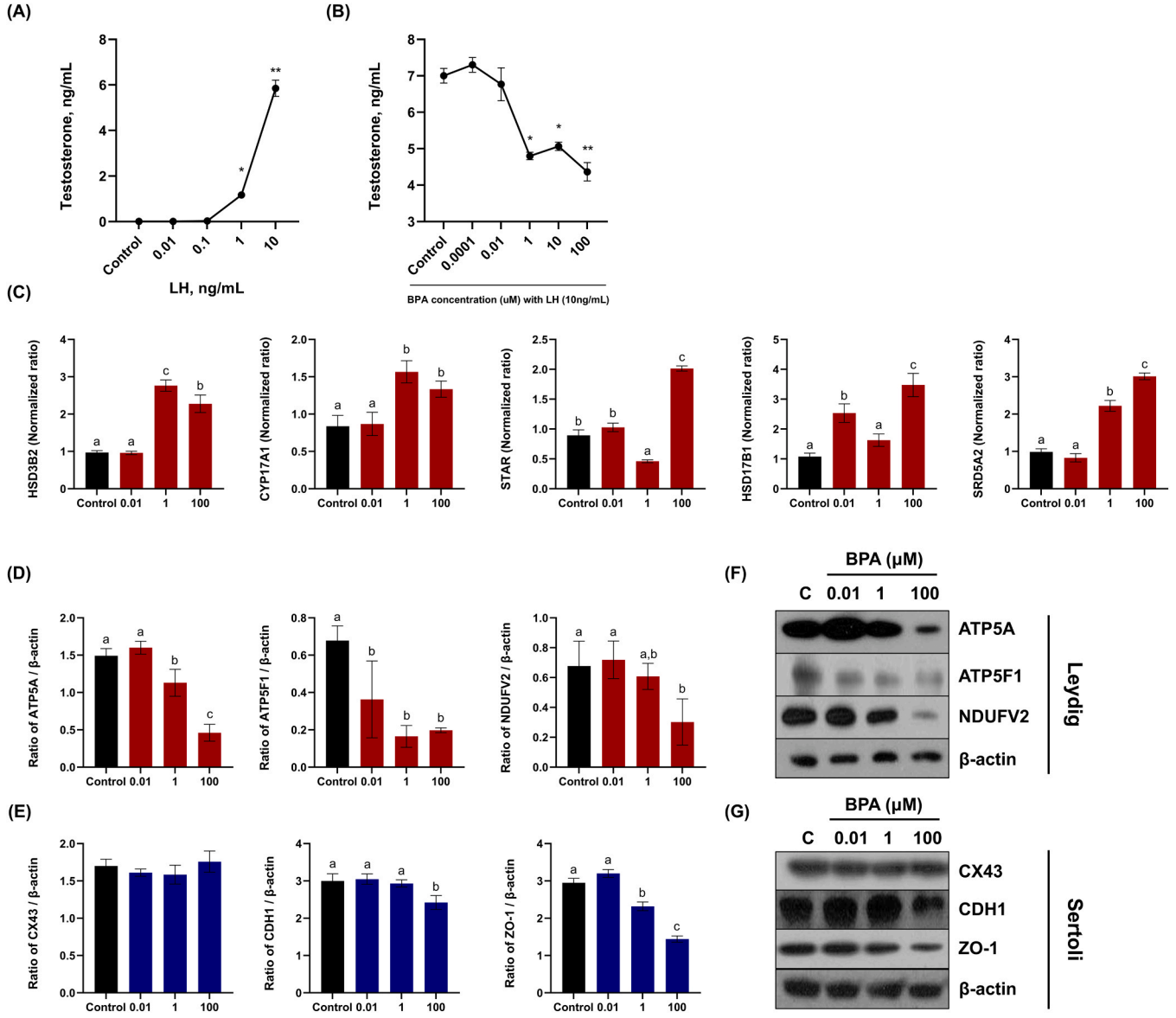

Fig. 3. Effects of bisphenol A (BPA) on testosterone synthesis in Leydig cells and blood-testis barrier (BTB) proteins in Sertoli cells. **(A)** Luteinizing hormone (LH) concentration dependence of testosterone production. **(B)** Differences in testosterone production under various BPA concentrations with 10 ng/mL of LH. **(C)** Differences in the mRNA levels of steroidogenic enzymes between the control and BPA groups in Leydig cells. Differences in protein expression levels between the control and BPA groups in **(D)** Leydig (red bars) and **(E)** Sertoli cells (blue bars). Representative western blot images in **(F)** Leydig and **(G)** Sertoli cells. Data represent the means of three replicates ± SD. Values with superscripts (^{a,b,c}) indicates significant differences between the control and BPA-treated groups, as determined by one-way ANOVA ($p < 0.05$) with post-hoc Tukey's test.

3.4. BPA effects on electron transport chain proteins in Leydig cell

We assessed the expression levels of electron transport chain proteins, including NUDFV2, ATP5A, and ATP5F1, to evaluate the effects of BPA on mitochondrial regulation in Leydig cells. All three proteins significantly decreased following treatment with different concentrations of BPA (Fig. 3D and F, $p < 0.05$).

3.5. BPA effects on BTB proteins in Sertoli cells

We assessed the expression levels of BTB proteins, including CX43, CDH1, and ZO-1, to evaluate the effects of BPA on various junctions in Sertoli cells. Our data revealed that BPA exposure did not affect the expression levels of CX43. In contrast, ZO-1 and CDH1 were significantly reduced in Sertoli cells following BPA exposure (Fig. 3E and G, $p < 0.05$).

3.6. BPA effects on signaling proteins in Leydig and Sertoli cells

We evaluated ER-α and -β expression levels in control and BPA-treated Leydig and Sertoli cells. Our results showed that ER-α decreased in Leydig and Sertoli cells when treated with 1 and 100 μM BPA, respectively (Fig. 4A, B, and C, $p < 0.05$). However, ER-β did not show any significant difference. While the 35 kDa PKA substrate increased under BPA in Sertoli cells, the 37, 45, and 50 kDa PKA substrates decreased significantly under 100 μM BPA in Leydig cells (Fig. 4A, B, and C, $p < 0.05$).

4. Discussion

Previously, we reported that BPA causes testicular damage and reduces the levels of ATP, along with aberrant levels of MMP, ROS, and apoptosis, leading to decreased sperm motility, motion kinematics, testosterone levels, and male fertility in mice (Ryu et al., 2022). Although the abnormal function of spermatozoa results from testicular damage, the underlying mechanism and effects of BPA on particular testicular cell types, Leydig and Sertoli cells, remain unclear. Several studies have demonstrated that oral gavage of BPA induces ROS generation in the testes, suggesting that it might also be related to ATP synthesis, MMP, testosterone synthesis, and apoptosis, which results in abnormal spermatogenesis, sperm motility and function, and male fertility (D'Cruz et al., 2012; El-Beshbishy et al., 2013; Li et al., 2009; Srivastava and Gupta, 2018; Wang et al., 2016). Our study also demonstrated that BPA promoted excessive ROS production, reduced ATP and MMP, and increased apoptosis in both Leydig and Sertoli cells. We found that BPA could cause excessive ROS generation following over 1 μM of BPA. In addition, ATP levels significantly decreased under 0.0001 and 0.01 μM in Leydig and Sertoli cells, respectively, in line with

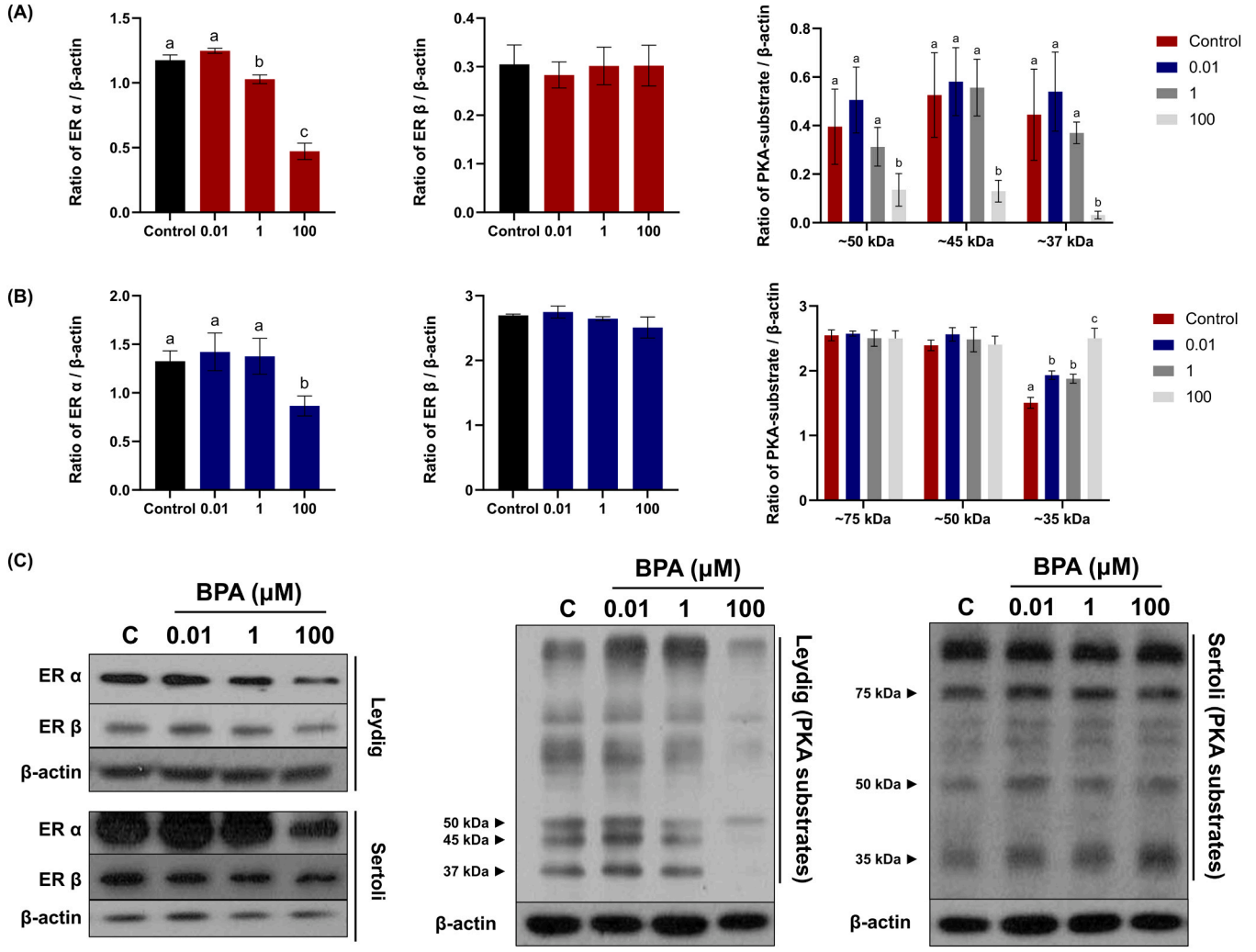

Fig. 4. Effects of bisphenol A (BPA) on signaling proteins in Leydig (red bars) and Sertoli (blue bars) cells. Difference in estrogen receptor and PKA substrate levels between the control and BPA groups in **(A)** Leydig and **(B)** Sertoli cells. **(C)** Representative western blot images. Values with superscripts (^{a,b,c}) indicates significant differences between the control and BPA-treated groups, as determined by one-way ANOVA ($p < 0.05$) with post-hoc Tukey's test.

our previous studies indicating that BPA can increase ROS levels and decrease ATP levels in mice spermatozoa (Rahman et al., 2020; Rahman et al., 2021). The mitochondrial respiratory chain is crucial in producing ROS and ATP, and excessive ROS generation induced by BPA can cause mitochondrial malfunctions (Lemarie and Grimm, 2011; Pun et al., 2014). Consistent with previous findings, MMP decreased significantly in response to the lowest concentration of BPA (0.0001 μM). In contrast to our findings that a low concentration of BPA (0.0001 μM) significantly reduced MMP and ATP levels, other studies demonstrated that a high concentration of BPA (100 μM) significantly decreased the intracellular levels of ATP and MMP in spermatozoa (Chen and Chan, 2009; Ryu et al., 2023). In addition, Agarwal et al. reported that MMP significantly decreased following BPA treatment (100 μM) in Leydig cells (Agarwal et al., 2020). Therefore, apoptosis was evaluated to examine the direct effects of BPA on these cells. Our results indicate that although very low concentrations of BPA affected ROS, ATP, and MMP, these effects are not toxic enough to induce apoptosis. However, we observed differences in the susceptibility to BPA toxicity between Leydig and Sertoli cells, as Leydig cells were affected at lower concentrations. Mitochondrial dysfunction is involved in apoptosis and disease pathology. Bcl-2 family members control protein release and activate various caspases in mitochondria. BPA affects Leydig cells via ROS-induced damage and apoptosis via apoptotic signaling in the mitochondria (Popgeorgiev et al., 2018). Therefore, to understand the pathways through which apoptosis occurs and the differences between the two cell types, we measured the expression of Bax, Bcl-2, Cyc1, Cas3, and cleaved Cas3. Additionally, we evaluated the expression levels of the mitochondrial fusion enzymes MFN2 and OPA1 to explore the underlying mechanisms of action of BPA in testicular mitochondria. We found that the Bax/Bcl-2 ratio, which determines the apoptotic potential of cells (Perlman et al., 1999), is significantly increased in response to higher BPA concentrations (1 μM), inconsistent with ROS results in Leydig cells. In addition, the cleaved Cas3/Cas3 ratio was significantly altered after BPA exposure. We found that low concentrations of BPA (1 μM) in Leydig cells induced early apoptosis via the Bax/Bcl-2 pathway, while the highest concentration (100 μM) directly triggered apoptosis through cleaved Cas3. In contrast, in Sertoli cells, the Bax/Bcl-2 ratio and Cyc1 increased only at the highest concentration (100 μM), and direct apoptosis was induced by cleaved Cas3 starting from lower concentrations (1 μM) of BPA. Several studies have reported that OPA1 and MFN2 are important proteins involved in mitochondrial fusion, which controls mitochondrial homeostasis and respiratory cells (Agarwal et al., 2020; Chen and Chan, 2009). Our results showed that OPA1 and MFN2 levels reduced following BPA exposure in both Leydig and Sertoli cells. Based on these results, very low concentrations of BPA affected ROS, ATP, and MMP levels in both cell types, but these levels were not sufficiently altered to induce apoptosis. However, exposure to BPA concentrations of 0.01 μM or higher appeared to decrease mitochondrial fusion, impacting ATP, ROS, and MMP levels and ultimately leading to apoptosis in both Leydig and Sertoli cells. Nonetheless, the two cell types exhibit different susceptibilities to BPA toxicity.

Various proteins and enzymes synthesize testosterone in the mitochondria of Leydig cells (Dufau, 1988; Fluck et al., 2003; Shi et al., 2007). Several studies have shown that BPA exposure alters testosterone levels in Leydig cells (Kobayashi et al., 2012; LaRocca et al., 2011; Salian et al., 2009). Nevertheless, there has been limited research on how BPA exposure alters the expression levels of critical proteins involved in steroidogenesis. Therefore, we evaluated testosterone levels and the mRNA levels of steroidogenic enzymes to determine the effects of BPA on testosterone synthesis. High doses of BPA (1 and 100 μM) decreased testosterone levels. In addition, the mRNA levels of steroidogenic enzymes tended to increase following exposure to high concentrations of BPA. This likely suggests that the decreased testosterone synthesis due to BPA led to increased expression of these genes as part of cellular homeostasis efforts to restore it. However, further experiments are required to confirm these findings. Furthermore, we evaluated the effects of BPA exposure on ATP synthesis, which is critical for steroidogenesis in the mitochondria of Leydig cells. Numerous studies have shown that exposure to BPA significantly reduces both intracellular ATP levels and the activity of the mitochondrial respiratory chain complex (Wang et al., 2019; Xiao et al., 2019). BPA also affects ATP synthesis and MMP, resulting in abnormal sperm motility and function (Chattopadhyay et al., 2010; Chitra et al., 2003). Therefore, we evaluated the expression levels of proteins related to the mitochondrial respiratory chain complex I (NDUFV2) and mitochondrial ATP synthase (ATP5A and ATP5F1) to explore the underlying mechanisms of BPA in the testicular mitochondria of Leydig cells. Consistent with earlier findings (Wang et al., 2019), BPA exposure significantly decreased the expression of NDUFV2, ATPF1, and ATP5A. In particular, ATP5F1 expression significantly decreased after exposure to a low dose of BPA (0.01 μM). Our results suggest that BPA affects ATP synthesis-related proteins, which may cause aberrant steroidogenesis and spermatogenesis. Therefore, our findings suggest that BPA exposure decreases mitochondrial fusion and MMP, which impairs mitochondrial function and leads to decreased respiratory chain complex proteins, and ATP synthase. This impairment may decrease mitochondrial ATP production and testosterone levels.

In mammalian testes, the BTB is an important immune barrier that protects spermatogenesis from harmful substances by connecting Sertoli cells with tight junctions, basal ectoplasmic specializations, desmosomes, and gap junctions (Li et al., 2010). However, several studies reported that EDCs, including BPA, can penetrate the BTB and lead to the disruption of spermatogenesis (Jin et al., 2013; Nielsen et al., 2000; Rosenfeld et al., 1998; Saunders et al., 1998; Strauss et al., 2009). Therefore, we evaluated the expression levels of various junction proteins (Cdh1, adherens junctions; Cx43, gap junctions; and Zo-1, tight junctions) to identify BPA toxicity in Sertoli cells. Our results showed that Zo-1 and Cdh1 were significantly decreased at 1 and 100 μM of BPA, respectively, while Cx43 remained unchanged. Based on our findings, adherens junctions might be disrupted following low BPA concentration, whereas tight junctions are disrupted at high BPA concentration in Sertoli cells. This disruption could potentially lead to abnormal opening and closing events in the BTB, resulting in premature spermiation and reduced sperm count (Adegoke et al., 2022).

Research has shown that BPA binds to ER-α and -β in Leydig and Sertoli cells (Akingbemi et al., 2003; Matthews et al., 2001). BPA binds to ERs and affects nuclear chromatin characteristics, causing cell differentiation, proliferation, and survival via abnormal transcription/translation (Pike et al., 1993; Wang et al., 2014). Several signaling cascades occur subsequently, such as the phosphorylation of PKA by BPA (Nguyen et al., 2019; Rosenfeld et al., 1998; Saunders et al., 1998). BPA affects the levels of ERs in Leydig and Sertoli cells, which are associated with hormone-mediated activity and, consequently, male fertility (Adegoke et al., 2023; Oliveira et al., 2004; Salian et al., 2009). Interestingly, we found that BPA significantly decreased the levels of ER-α expression in Leydig and Sertoli cells (1 and 100 μM, respectively), whereas ER-β expression remained unchanged. Previous studies showed that BPA has lower potency than estrogen for ES-α in human MCF7 cells (Leffers et al., 2001). An *in vivo* study revealed that the expression levels of ER-α increased significantly under different doses of BPA (Nanjappa et al., 2012). Based on these reports and our results, it is suggested that BPA acts by binding to different receptors at different doses in Leydig and Sertoli cells. Our results revealed that three different kDa weights of PKA substrates decreased significantly only under the highest concentration of BPA (100 μM) in Leydig cells. In contrast, only one PKA substrate (35 kDa) increased significantly following exposure to the lowest concentration of BPA. Therefore, these results indicate that both cell types are susceptible to BPA toxicity through different mechanisms. However, in addition to ERs, non-estrogen receptor-related pathways, such as p53, PPARγ, and JUN/FOS, which could also contribute to the pathological effects of BPA on male fertility, should be considered for their roles in the effects of BPA (Gao et al., 2020; Rosenfeld et al., 1998).

5. Conclusion

Although substantial evidence indicates that BPA adversely affects the male reproductive system by disrupting Leydig and Sertoli cell function, determining the specific dose or developmental time these pathological effects most likely occur remains challenging. Owing to the significant controversies surrounding the effects of BPA on male fertility, defining accurate toxicity levels for public health purposes and understanding the mechanisms and functional studies necessary to overcome BPA-induced male infertility pose considerable challenges. Therefore, our study established a broad range of BPA concentrations and systematically narrowed this range to determine the levels at which BPA toxicity may induce pathological results. Furthermore, we demonstrated that Leydig and Sertoli cells have different susceptibility and response mechanisms following BPA exposure. We postulate that this study may provide an opportunity to understand the fundamental mechanisms of BPA-induced fertility. In light of these results, we propose that minimizing BPA exposure, using antioxidants, and targeting estrogen receptor pathways may help reduce BPA-induced toxicity, particularly regarding male fertility. These strategies show promise in mitigating the harmful effects of BPA on reproductive health. However, since this study was conducted using an *in vitro* model, a relevant daily human dose should be determined and applied to *in vivo* models in future experiments.

CRediT authorship contribution statement

Pang Myung-Geol: Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Park Yoo-Jin:** Writing – review & editing, Data curation. **Rahman Md Saidur:** Writing – review & editing. **Pang Won-Ki:** Writing – review & editing, Investigation. **Ryu Do-Yeal:** Writing – original draft, Methodology, Investigation, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Myung-geol Pang reports was provided by National Research Foundation of Korea. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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