Toxicity of Bisphenol A and its Replacements in the Mice Leydig Cells In Vitro

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Summary

The aim of the study was to examine the potential impacts of bisphenol A (BPA) and its analogues BPB, BPF, and BPS on mice TM3 Leydig cells, with respect to basal cell viability parameters such as metabolic activity, cell membrane integrity, and lysosomal activity after 48-h exposure. In addition, monitoring of potential bisphenol´s actions included evaluation of ROS production and gap junctional intercellular communication (GJIC) complemented by determination of testosterone secretion. Obtained results revealed significant inhibition in mitochondrial activity started at 10 μg/ml of bisphenols after 48-h exposure. Cell membrane integrity was significantly decreased at 5 μg/ml of BPA and BPF and 10, 25, and 50 μg/ml of BPA and BPS. The lysosomal activity was significantly affected at 10, 25, and 50 μg/ml of applied bisphenols. A significant overproduction of ROS was recorded mainly at 5 and 10 μg/ml of tested compounds. In addition, significant inhibition of GJIC was observed at 5 μg/ml of BPB followed by a progressive decline at higher applied doses. In the case of testosterone production, a significant decline was confirmed at 10, 25 and 50 μg/ml.

Key words

Viability • Reactive oxygen species • Gap junctional intercellular communication • Testosterone

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Introduction

With the development of industries and technology, xenobiotics have become a significant potential concern to animals and humans. Growing evidence shows that environmental contaminants can pose negative risks to animal and human health and reproduction [1,2]. Bisphenol A (BPA; 2,2-bis(4-hydroxyphenyl)propane) is one of the most widely produced chemicals in the world and is found in numerous consumer items such as food containers, paper products, thermal receipts, water pipes, and toys, medical equipment, personal protection equipment, sports equipment, and electronics. BPA is ubiquitous in the environment, and individuals are exposed to this chemical through both dietary and nondietary sources [3]. BPA exposure in humans is difficult to determine, owing to its short biological half-life and rapid excretion. Furthermore, several studies have identified complications with external contamination during sampling and/or analytical processes [4,5]. Thus, biomonitoring studies have determined widespread and everyday exposure to BPA, with urine BPA detected in more than 90 % of the general European and US populations at low concentrations [4,7]. The European Chemicals Agency recently classified BPA as a reproductive toxicant and a substance of very high concern. As a result of extensive human exposure and related health risks, regulations on the manufacturing and use of BPA have been implemented in North America, Australia, Canada, India, and the European Union [8,9].

The public's concern over BPA, as well as related legislation, prompted the development and production of alternative chemicals to replace BPA in a wide range of applications. Several chemicals with structural similarities to BPA are already utilized in the production of polycarbonate plastics and epoxy resins. These substances are known as bisphenol analogues as they have a similar structure with two hydroxyphenyl functionalities. For industrial uses, a total of 16 bisphenol analogues have been documented [7,10]. BPF (4,4′-methylenediphenol) and BPS (4-hydroxyphenyl sulfone) are two of the most common BPA alternatives used in the production of polycarbonate plastics and epoxy resins. BPB (2,2-Bis(4-hydroxyphenyl)butane) is a common component in the manufacture of phenolic and polycarbonate resins [11]. Bisphenol analogues have been shown to cause endocrine disruption, cytotoxicity, genotoxicity, reproductive toxicity, dioxin-like effects, and neurotoxicity confirmed by animal studies [10,12,13].

Male fertility decrease is a highly disputed issue in the current population. Especially, BPA and other environmental contaminants, have been proposed as causes of the decline in sperm counts, sperm concentrations, sex-hormone depletion, and subfertility initiation [10,7]. In addition, various studies produced contradictory results, which may be related to variances in exposure protocols, treatment duration, administered dosage, route, and exposure window [14,15]. BPA disrupts steroid signalling, affects developmental processes, and damages tissue by inducing oxidative stress. BPA also directly targets Leydig cells, the major effects of BPA on Leydig cell function include disruption of the hormonal micro-environment in the testis and up-regulation or downregulation of essential steroidogenic enzymes, resulting in changes of steroid hormone synthesis [16]. BPA analogues have been extensively studied and the results indicate that BPF, BPS, and BPB have endocrine-disrupting potencies comparable to or greater than BPA [15]. Overleaf, the mechanism of action and potential pathways that could affect molecular and cellular changes are not exactly identified [17,18].

The capacity of a cell to maintain a balance in chemical reduction and oxidation (redox) is critical to all aspects of cellular development, growth, and survival. Endogenously generated oxygen radicals can also cause damage to lipids in cellular membranes, proteins, and nucleic acids, however, the mechanisms through which bisphenols causes oxidative stress are not fully characterized [19-21]. Cell-cell communication is critical for tissue homeostasis as it allows for accurate signalling in response to both external and internal stimuli. These essential communication systems, such as gap junction intercellular communication (GJIC), are required for cells to stay dormant or to undertake proliferation, differentiation, or apoptosis. At the same time, GJIC plays a significant role in sex-steroid hormone secretion, and any inhibition could affect steroidogenesis, redox status, and the malignant growth of cancer cells [22]. Overleaf, there have been conducted a limited number of studies, that could clarify the potential of BPA analogues to inhibit GJIC and affect sex-steroid hormone secretion [16,23,24,25].

The objective of our study was to assess the current threats of bisphenol A and its analogues B, S, and F on the male reproductive function *in vitro.* Concretely, we evaluated basic cellular aberrations in mice TM3 Leydig cell line such as metabolic activity, cell membrane integrity, and lysosomal activity after bisphenol exposure. In addition, the production of intercellular ROS, and GJIC activity was accompanied by analyses of testosterone secretion after 48-h exposure.

Material and Methods

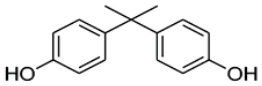
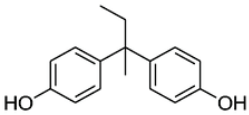
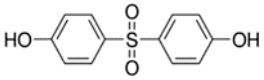
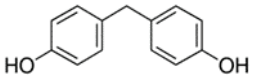
Chemicals

Bisphenol A (BPA; purity: >99 %), bisphenol B (BPB; purity: >98 %), bisphenol S (BPS; purity: >98 %), and bisphenol F (BPF; purity: >98 %) were purchased from Sigma-Aldrich (St. Louis, USA) and dissolved in 96 % of ethanol (EtOH; p.a. CentralChem, Bratislava Slovak Republic) as a stock solution. The maximum solvent concentration was set at 0.5 % (v/v). Table 1 displays the test compounds used in our study.

TM3 Leydig cell culture

The mice TM3 Leydig cells were purchased from the American Type Culture Collection (ATCC #CRL-1714TM; Manassas, VA, USA). Cells were cultured in Dulbecco´s Modified Eagle´s Medium/Nutrient Mixture (Ham´s) F12 with HEPEs and phenol red for cell culturing /without phenol red for experiments (DMEM/F12; Sigma-Aldrich, St. Louis, USA) supplemented with 5 % horse serum (HS; Gibco-Life Technologies, New Zealand), 2.5 % heat-inactivated fetal bovine serum (FBS; BiochromAG, Berlin, Germany), 2.5 mM L-glutamine (Sigma-Aldrich, St. Louis,

Table 1. Overview of the test chemicals used in *in vitro* study, including their structural formulas and CAS numbers.

Tested compounds	**Abbreviation**	**Formula**	**2-D Structure**	**CAS No.**
Bisphenol A	BPA	$C_{15}H_{16}O_2$		# 80-05-7
Bisphenol B	BPB	$C_{16}H_{18}O_2$		# 77-40-7
Bisphenol S	BPS	$C_{12}H_{10}O_4S$		# 80-09-1
Bisphenol F	BPF	$C_{13}H_{12}O_2$		# 620-92-8

USA) and 1 % penicillin/streptomycin (Sigma-Aldrich, St. Louis, USA) at 37 °C in 5 % CO_2 and 95 % saturated atmospheric humidity. TM3 cells were passaged after reaching 90 % confluence and sub-cultured in 1:50 ration in 75 cm^2 flasks (TPP, Trasadingen, Switzerland). Cells from passage no. 7 up to passage no. 22 were used for these experiments. Cells were routinely screened for contamination by bacteria, fungi, and mycoplasma, i.e. regular assessment of cellular morphology, microbial contamination testing, and mycoplasma detection using PlasmoTestTM (InvivoGen, San Diego, CA). TM3 cells were seeded in gelatine (Sigma-Aldrich, St. Louis, USA) pre-coated (0.1 % w/v) culturing 96 or 6-well plates (TPP, Trasadingen, Switzerland) 24-h before the exposure. Cells without any treatment served as a control group (Ctrl). The vehicle concentrations did not exceed 0.5 % (v/v) of EtOH. Vehicle-treated cells served as negative control (NC) in each experiment. The range of applied bisphenols concentration was chosen based on our previous pilot range-finding study [26]. Additionally, all obtained data were expressed as a percentage of the control (non-treated) group and experimental (treated) cells. At least, three separate repeats were carried out in each group.

Cell viability assay

The Leydig cell viability was determined using the MTT assay (3-4,5-dimetyltiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, St. Louis, USA) according to [27], considered as a standard colorimetric method which measures the reduction of a yellow water-soluble tetrazolium salt to insoluble blue formazan particles *via* mitochondrial succinate dehydrogenase enzyme activity of living cells. In brief, Leydig cells were pre-cultured at the density of 4×10^3 cells/well in 96-well plates. After adhesion, the cell culture medium was removed, and the fresh medium containing experimental doses (1-50 µg/ml) of BPA, BPB, BPS, and BPF was applied for 48-h. After exposure, TM3 cells were washed with Dulbeccos´s phosphate-buffered saline (DPBS; Sigma-Aldrich, St. Louis, USA) and incubated with DMEM/F12 containing MTT tetrazolium salt (final concentration: 1 mg/ml) for 3 h in a CO_2 incubator (37 °C; 5 % CO_2 and 95 % atmospheric humidity). Then, the MTT solution was removed, and the formed formazan crystals were dissolved with isopropanol (IPA; p.a. CentralChem, Bratislava Slovak Republic) during 20 min. Finally, a microplate reader Multiscan FC (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used to measure the absorbance of each well at 570 nm against 620 nm wavelengths.

Cell membrane integrity

5-carboxyfluorescein diacetate, acetoxymethyl ester (CFDA-AM; Thermo Fisher Scientific, Waltham, Massachusetts, USA) is a nontoxic esterase substrate that could be metabolized by nonspecific esterase in living cells from a membrane-permeable and nonfluorescent molecules to polar, fluorescent carboxyfluorescein. This conversion indicates alterations in cell membrane integrity [28]. Briefly, TM cells were seeded at the density of 4×10^3 cells/well in 96-well plates 24-h before exposure. Pre-cultured cells were treated with corresponding experimental doses (1-50 µg/ml) of BPA, BPB, BPS, and BPF for 48-h. After this time, the medium was removed, Leydig cells were washed by DPBS and subsequently incubated with fresh DMEM/F12 containing CFDA-AM (final concentration: 4 µM). After 30-min incubation in a CO_2 incubator (37 °C; 5 % CO_2

and 95 % atmospheric humidity), fluorescence (excitation/emission: 485/530 nm wavelength) was measured using a microplate reader GloMax®-Multi+ (Promega Corporation, Madison, USA) group.

Lysosomal activity

The neutral red uptake (NRU; Thermo Fisher Scientific, Waltham, Massachusetts, USA) assay has been used to evaluate the ability of viable cells to incorporate the supravital dye neutral red into cell membranes and concentrate in the lysosomes, where it binds to anionic and phosphate groups of the lysosomal matrix [29]. TM3 cell line was seeded and pre-cultured (4×10^3 cells/well) in 96-well plates for 24-h. Subsequently, mice Leydig cells were incubated with experimental concentrations starting from 1 to 50 µg/ml of BPA, BPB, BPS, and BPF for 48-h. After this time, Leydig cells were incubated with neutral red (final concentration: 0.005 % w/v) dissolved in DMEM/F12 for 2 h in a CO_2 incubator. The cells were then washed with DPBS, and accumulated dye was extracted with lysis buffer containing 1 % (v/v) acetic acid and 50 % (v/v) EtOH for 20 min. The absorbance was measured at 525/690 nm wavelength by the microplate reader GloMax®-Multi+.

Superoxide radical production

Nitroblue-tetrazolium (NBT; Sigma-Aldrich, St. Louis, USA) assay is a colorimetric method conducted by assessing blue formazan deposits, formed by the reduction of the membrane-permeable and yellow-colored nitroblue-tetrazolium chloride (2,2´bis(4-nitro-phenyl)-5,5´-diphenyl-3,3´-dimethoxy-4,4´-diphenylene) diterazolium chloride; Sigma-Aldrich, St. Louis, USA) by the superoxide radicals [30]. Mice TM3 cells were pre-cultured at the density of 4×10^3 cells/well in 96-well plates for 24-h. Afterward, cell culture media was replaced by DMEM/F12 supplemented with corresponding doses (1-50 µg/ml) of BPA, BPB, BPS, and BPF for 48-h. Subsequently, the NBT salt (final concentrations: 1 mg/ml) was dissolved in DMEM/F12 containing 1.5 % DMSO and added to the Leydig cells. After a 3 h incubation (37 °C; 5 % CO_2 and 95 % atmospheric humidity) formed blue deposits were solubilized by 2 M potassium hydroxide (KOH; p.a. CentralChem, Bratislava, Slovak Republic) dissolved in DMSO. The optical density was measured at a wavelength of 620 nm against 570 nm as a reference by using a microplate reader Multiscan FC.

Gap junctional intercellular communication assay

The scalpel loading/dye transfer (SL/DT) assay relies on the introduction of small (MW<900), membrane nonpermeable dyes Lucifer yellow CH dilithium salt (LY; Sigma-Aldrich, St. Louis, USA) into living cells that are traced in their intercellular movement through gap junctions. LY is negatively charged, and it has a high fluorescence efficiency [31]. Leydig TM3 cells were seeded into 6-well plates at a density of 1.25×10^5 cells/well for 24-h. After adhesion, cells were exposed to the corresponding concentrations (1-50 µg/ml) of BPA, BPB, BPS, and BPF for 48-h. After respective treatment, a gap junction permeable tracer LY (final concentrations: 1 mg/ml in calcium- and magnesium-supplemented PBS (CaMg-PBS; pH 7.2) was added to the cells and introduced into them by three parallel cuts made by a scalpel. After 6 min of incubation in the dark, the cells were washed with CaMg-PBS and fixed with 4 % paraformaldehyde (PFA; Boster, Pleasanton, California, USA). Finally, the images were captured using the Leica Application Suite X (LAS X) software suitable for fluorescent microscope DMI 6000 B (Leica Microsystems, Wetzlar, Germany), and DCF 345FX camera. The distance at which the LY diffuses through the gap junction of TM3 cells was evaluated using ImageJ software [32].

Steroid hormone secretion

The production of testosterone was determined by an enzyme-linked immunosorbent (ELISA) assay. ELISA method is an immunological reaction that combines the specific reactivity of antigens and antibodies with the efficient catalytic action of enzymes on substrates. TM3 cells were plated into 96-well plates at a density of 4×10^3 cells/well for 24-h. Pre-cultured cells were subsequently treated with experimental doses (1-50 µg/ml) of BPA, BPB, BPS, and BPF for 48-h and incubated in a CO_2 incubator. After this time period, the cell culture media was aspirated from each well, centrifuged (3000 rpm; 10 min; 4 °C), and supernatants were stored in Eppendorf tubes at -80 °C until steroid determination. The procedure for testosterone analyses was carried out according to the manufacturer´s instructions in ELISA kits (Dialab, Wiener Nudorf, Austria). The optical density was measured at 450 nm wavelength by a microplate reader Multiscan FC. The lowest detection limit of the ELISA kit was ≤0.10 ng/ml. The intra- and inter-assay coefficients of variability for testosterone were estimated as ≤7.0 % (intra-variability) and ≤8.3 % (inter-variability).

Statistical analyses

All statistical analyses were performed by GraphPad Prism 6.01 (GraphPad Software Incorporated, San Diego California, USA). Descriptive characteristics (minimum, maximum, mean, and standard error of the mean, etc.) were evaluated at first. One-way analysis of variance (ANOVA) and Dunnett´s multiple comparison tests were used to examine differences among bisphenol treatments and the control groups. The geometric mean of the values from at least three (n=3) independently repeated experiments was calculated and reported with a 95 % confidence interval. The results were expressed as the mean ± standard error of the mean (SEM).

Results

Effect of bisphenols on TM3 cell viability

As shown in Figure 1, the metabolic activity of exposed Leydig cells has not been significantly affected by lower doses (up to 5 µg/ml of bisphenols). However, the significant ($p<0.01$) cytotoxic effects have been confirmed at 10 µg/ml of BPB (Fig. 1A) (75.70±3.50 %), BPA (Fig. 1B) (79.80±6.55 %), and BPF (Fig. 1C) (81.40±3.94 %) compared to the control group. The same concentration of BPS (Fig. 1D) has shown less significance ($p<0.05$) but still a cytotoxic effect (85.36±4.29) compared with a control group. The remaining bisphenol doses (25 and 50 µg/ml) have had enormous toxicity after 48-h cultivation *in vitro*. The highest dose of BPB and BPA inhibit metabolic activity by almost 90 % (±1.87) and 95 % (±0.93), respectively. Similarly, BPF and BPS at the highest concentration reduced significantly (30.70±0.58 % or 57.86±1.23 %) cell viability significantly ($p<0.0001$) compared to the control group.

Effect of bisphenols on TM3 cell membrane integrity

As seen in Figure 2 significant ($p<0.05$) inhibition has been recorded at 5 µg/ml of BPB (Fig. 2A) (86.75±5.38 %) and BPF (88.30±1.89 %) (Fig. 2C) compared to the control group. In general, treatment with higher concentrations of bisphenols (10, 25, and 50 µg/ml) radically inhibited this parameter. Concretely, the level of BPB´s inhibition fluctuated between 86.75±5.38 % at 10 µg/ml and 7.24±1.00 % at 50 µg/ml, while the BPA (Fig. 2B) reduced this parameter to 78.43±1.19 % or 7.43±0.79 % respectively. In the case of BPF a significant inhibition has been confirmed at 5 µg/ml ($p<0.05$), 10 µg/ml ($p<0.001$), 25 and 50 µg/ml ($p<0.0001$), while BPS (Fig. 2B) significantly ($p<0.05$, $p<0.0001$) inhibited the presented parameter at 10 µg/ml (88.53±1.23 %), 25 µg/ml (68.55±1.49 %) and 50 µg/ml (52.39±5.69 %).

Effect of bisphenols on TM3 cell lysosomal integrity

As presented in Figure 3, the level of lysosomal activity was reduced to 59.84±2.03 % (10 µg/ml), 36.86±2.65 % (25 µg/ml), and 17.46±2.19 % (50 µg/ml) after BPB (Fig. 3A) exposure. Similarly, BPA (Fig. 3B) inhibited this parameter at almost 66.21±3.39 % (10 µg/ml), 42.11±5.68 % (25 µg/ml) and 20.42±2.01 % (50 µg/ml). Overleaf, the same doses of BPF (Fig. 3C) initiated a significant ($p<0.001$; $p<0.0001$) reduction in lysosomal integrity which fluctuated between 70.81±7.03 % at 10 µg/ml to 36.06±5.33 % at 50 µg/ml. In the case of BPS treatment (Fig. 3D), 10 µg/ml affected this parameter moderately, but significantly ($p<0.05$; 83.99±3.15 %). Significant ($p<0.0001$) changes, with a direct decrease in lysosomal integrity, were observed at 25 µg/ml (62.12±7.21 %) and 50 µg/ml (51.24±3.14 %).

Assessment of superoxide generation

Analyses of intercellular generation of superoxide radical presented in Figure 4 shows gradual increase at lower concentrations of BPB (Fig.4A) and BPA (Fig. 4B) with significant ($p<0.05$; $p<0.01$; $p<0.001$) impact at 5 µg/ml (123.20±7.30 %; 121.20±2.51 %) and 10 µg/ml (132.90±5.24 %; 122.20±4.61 %). However, 25 µg/ml and 50 µg/ml in both significantly ($p<0.01$; $p<0.001$; $p<0.0001$) decreased superoxide release compared to the control group. In case of BPF (Fig. 4C), 10 µg/ml (126.80±8.38 %) increased superoxide production significantly ($p<0.01$), while the highest concentration (50 µg/ml) decreased this production significantly ($p<0.001$) to 66.26±3.32 %. A moderate, but significant ($p<0.05$) growth in superoxide radical release was confirmed at 5 µg/ml (120.90±1.99 %) and 10 µg/ml (125.10±4.72 %) of BPS (Fig. 4D), without progressive inhibition at the highest concentrations. All treated groups were compared with the control groups.

Assessment of intercellular communication

The changes in GJIC after 48-h exposure to experimental bisphenols are shown in Figure 5. A significant ($p<0.001$) inhibition has been started at 5 µg/ml (74.24±4.24 %) of BPB (Fig. 5A), followed by progressive disruption of connexin channels communication at 10 µg/ml (59.30±1.63 %), 25 µg/ml (24.91±0.58 %) and 50 µg/ml (5.54±1.57 %) compared to

the control group. BPA reelevated (Fig. 5B) the negative impact and initiate a significant ($p<0.001$) decline of GJIC activity at the highest doses (10, 25, and 50 µg/ml) at almost 34 %, 67 %, and 90 %. A similar situation was recorded after BPF (Fig. 5C) and BPS (Fig. 5D) treatment. In case of 10 µg/ml of both a significant ($p<0.001$; $p<0.0001$) reduction (68.14±2.20 % or 78.96±3.76 %) was observed. The remaining doses (25 and 50 µg/ml) also reduce GJIC (55.62±3.00 %; 25.72±1.89 %) after BPF treatment, followed by the same tendency in BPS (57.40±2.16 % or 49.26±1.31 %) compared to the control group. The representative images of GJIC inhibition are displayed in Figure 5E.

Assessment of testosterone secretion

Data presented in Table 2 demonstrated the concentration-dependent effects of bisphenols. Lower experimental concentrations of BPB and BPA (up to 5 µg/ml) enhanced testosterone production, with significant ($p<0.05$) impact at 2.5 µg/ml (119.10±3.13 % and 118.10±3.73 %) compared to the control group (100.00±4.81 % and 4.75 %). After treatment with 10, 25, and 50 µg/ml significant ($p<0.0001$) decline in testosterone secretion was recorded. This inhibition was at almost 90 % to 80 %. Overleaf, lower concentrations (up to 5 µg/ml) of BPF and BPS induce moderate and non-significant changes in this parameter. In the case of 10 µg/ml (77.50±4.54 %) of BPF, a significant ($p<0.05$) decline was observed. Increasing concentrations in both progressively affected secretion of testosterone *in vitro*. The highest doses of 25 µg/ml (55.35±4.19 %) and 50 µg/ml (35.20±2.75 %) of BPF significantly ($p<0.0001$) decreased this parameter. The same concentrations of BPS caused significant ($p<0.001$; $p<0.0001$) decline to 62.41±2.88 % at 25 µg/ml and 41.75±6.74 at 50 µg/ml compared to the control group (100.00±3.56 %).

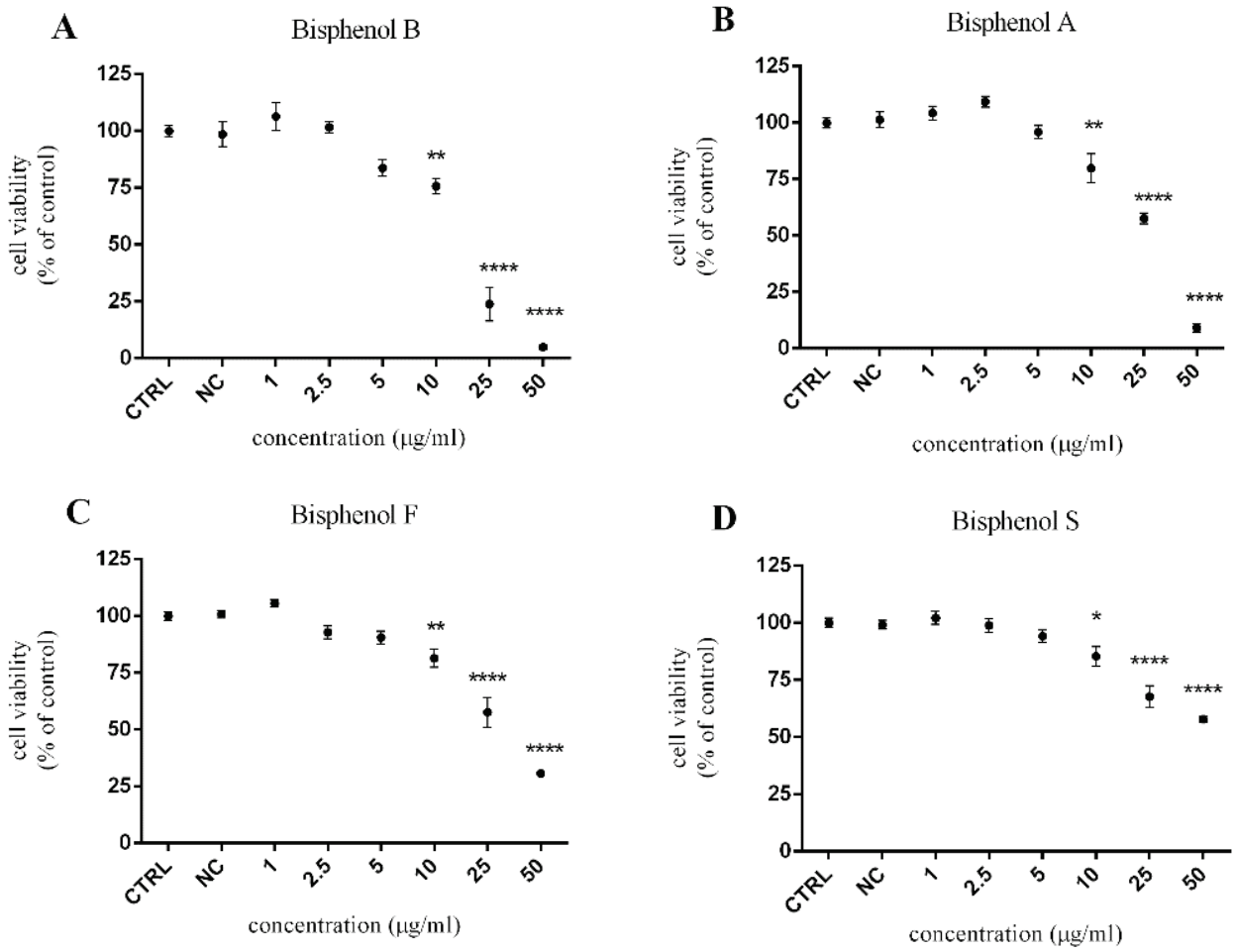


Fig. 1. Characterization of bisphenols-induced cytotoxicity after 48 h exposure in TM3 mice Leydig cells *in vitro*. CTRL – control group, NC – negative control. Results of cell viability were expressed by percentages of living cells over control (non-treated) cells. Data are presented as means (±SEM) from three independent experiments performed in triplicates. Levels of significance were established at **** ($p<0.0001$); ** ($p<0.01$), and * ($p<0.05$). Statistical differences between the values of control and treated groups are indicated by an asterisk (*).

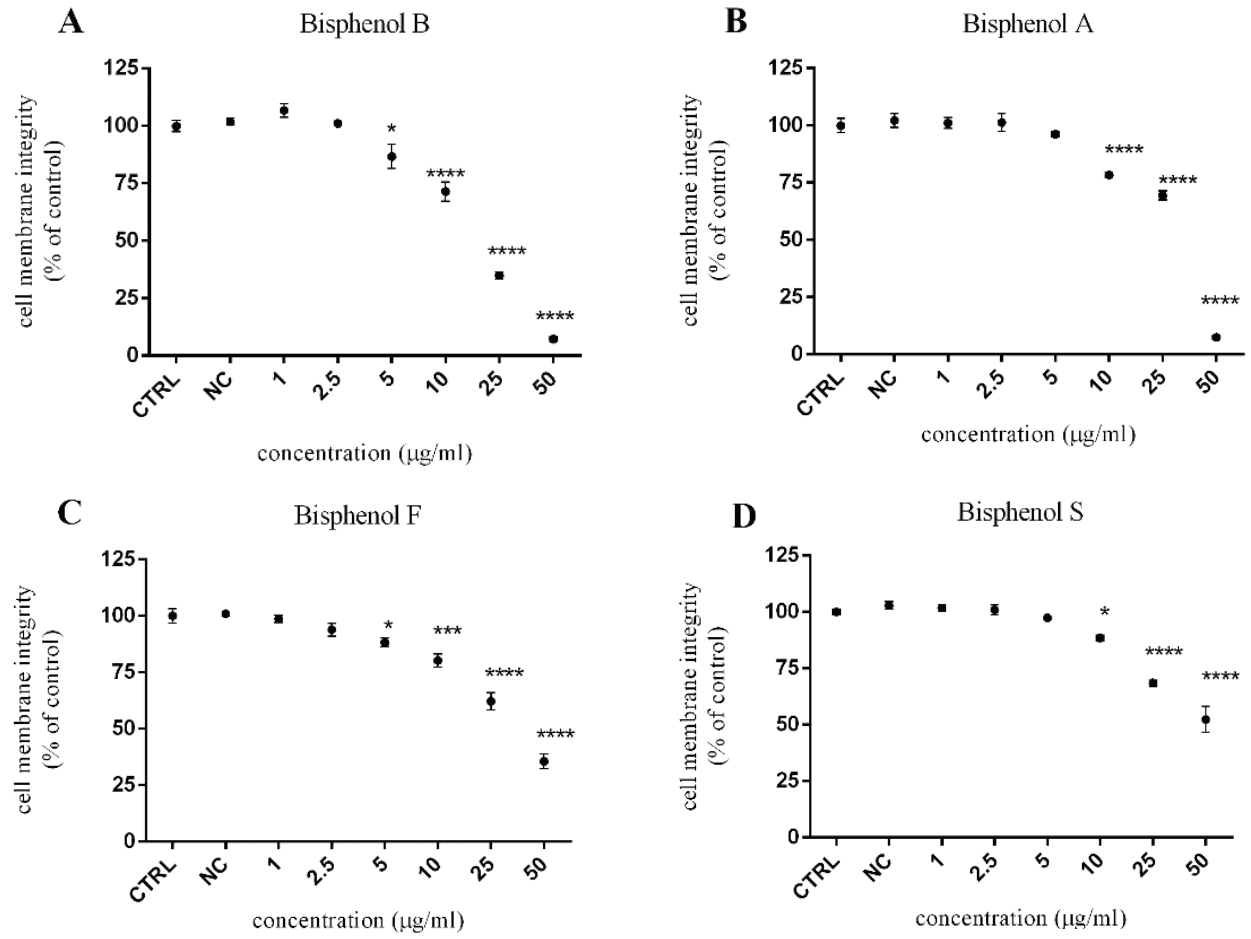


Fig. 2. The effects of bisphenols on TM3 cell membrane integrity after 48 h exposure *in vitro*. CTRL – control group, NC – negative control. The data are presented as means (± SEM) optical density percent of the control (untreated) and bisphenol´s treated groups. The data were collected from three independent experiments performed in triplicates. Levels of significance were established at **** ($p<0.0001$); *** ($p<0.001$), and * ($p<0.05$). Statistical differences between the values of control and treated groups are indicated by an asterisk (*).

Discussion

Our previous study revealed that short-term exposure (24-h) of mice Leydig cells to experimental concentrations of bisphenols could significantly affect steroidogenesis and inhibited mitochondrial functions *in vitro* [26]. To confirm the hypothesis about another potential pathway of bisphenol´s action, the prolonged time of cultivation (48-h) associated with changes in mitochondrial activity, cell membrane integrity, lysosomal activity, gap junctional intercellular communication, reactive oxygen species production, and testosterone secretion were evaluated.

Our data suggest that higher concentrations (10, 25, and 50 μg/ml) of all experimental bisphenols – BPB, BPA, BPF, and BPS significantly decreased the viability of TM3 Leydig cells after 48-h cultivation *in vitro*. Similar effects were described by Li *et al.* [33]. The results confirmed significant inhibition ($p<0.05$; $p<0.001$) at ≈5 μg/ml, ≈10 μg/ml and ≈20 μg/ml of BPA. In line with our results, the authors confirmed the biphasic effect, when the lower applied doses can slightly stimulate cell viability, while the highest concentrations could initiate a significant cytotoxic effect resulting in gradual apoptosis. The cytotoxicity of BPF and BPS was determined in mice Leydig cells after 48-h exposure by Rajkumar *et al.* [34] *in vitro*. Authors confirmed decreased in cell viability at ≈9.5 μg/ml of BPF and at ≈7.5 μg/ml of BPS. Together with this study, our result corresponds with Lan *et al.* [35] who reported that BPS doses higher than 30 μM (≈7.5 μg/ml) could decrease mitochondrial metabolism and induce cytotoxicity in tumor mice MA-10 Leydig cells. Similarly, doses greater than 100 μM were required for BPA (≈23 μg/ml) or BPF (20 μg/ml) cytotoxicity. In contrast, our study showed higher sensitivity of TM3 cells to BPF and BPA exposure started at 10 μg/ml concentrations of both. Zhang *et al.* [36] evaluated the

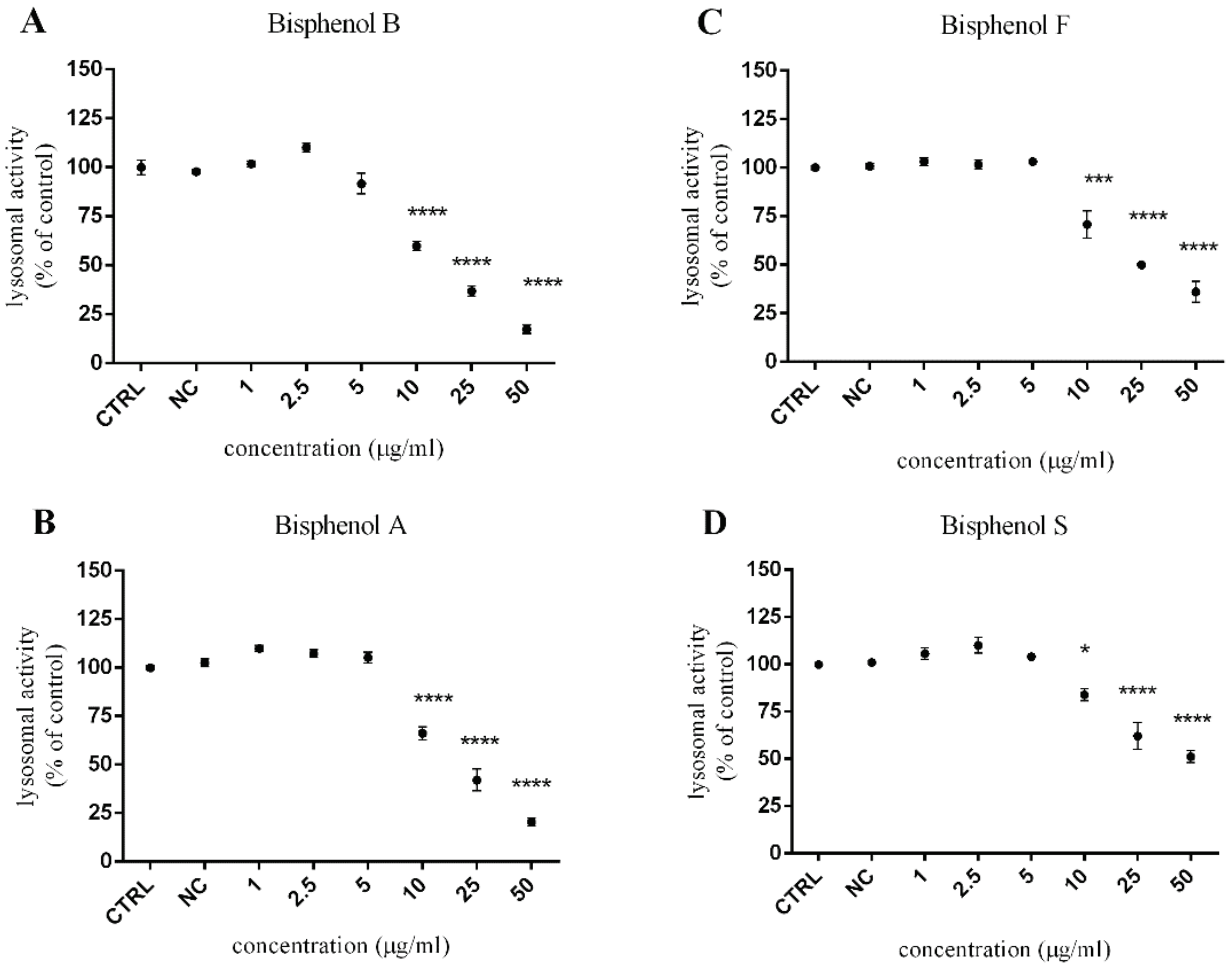


Fig. 3. The effects of bisphenols on lysosomal activity in TM3 Leydig cells after 48 h exposure *in vitro*. CTRL – control group, NC – negative control. The data are presented as means (± SEM) optical density percent of the control (untreated) and bisphenol´s treated groups. The data were collected from three independent experiments performed in triplicates. Levels of significance were established at **** ($p<0.0001$); *** ($p<0.001$), and * ($p<0.05$). Statistical differences between the values of control and treated groups are indicated by an asterisk (*).

cytotoxicity induced by BPS in mice Leydig cells *in vitro* after 48-h exposure. Obtained results suggest that applied doses below 25 µg/ml had no significant impact on the presented parameter. However, a significant decline in mitochondrial activity was recorded in the higher concentrations of BPS (from 25 to 200 µg/ml). Goncalves *et al.* [37] revealed the decline in the kinetics of cell growth and irregular shapes of exposed TM3 cells at 250 µg/ml after 48-h exposure. The testicular co-culture model represented by mice spermatogonia cell line C18, Sertoli TM4, and Leydig TM3 cells were treated with various concentrations of BPA and BPS (from 5 to 100 µM) for 24 and 48-h. Gained results showed, that 100 µM (≈23 µg/ml of BPA, and 25 µg/ml of BPS) of bisphenols noticeably decreased cell density, disrupt the cytoskeleton, and induced the elevation of DNA damage marker – γH2AX [38]. Significant changes in Leydig cell morphology and mitochondrial permeability affected by the BPA and it´s analogues action was evaluated by Rajkumar *et al.* [34]. Exposure to BPA (2.5-25 µg/ml) during 48-h significantly increased lysosome numbers and increased mitochondrial permeability. In summary, our study revealed greater sensitivity and earlier onset of decline in mitochondrial activity of TM3 Leydig cells treated with bisphenol A and its analogues compared to the discussed studies above which used TM3 or another cellular model.

If we want to understand the cytotoxic effect of bisphenols in detail, it is necessary to analyze additional changes in cellular parameters *in vitro*. The cytotoxic effects of bisphenols could be also evaluated using the combination of two indicator dyes: CFDA-AM and NR. Results of our study confirmed that membrane integrity of TM3 cells was significantly affected by 10, 25, and 50 µg/ml of BPB, BPA, BPF, and BPS with initial damages recorded at 5 µg/ml of BPB and BPF. Similarly,

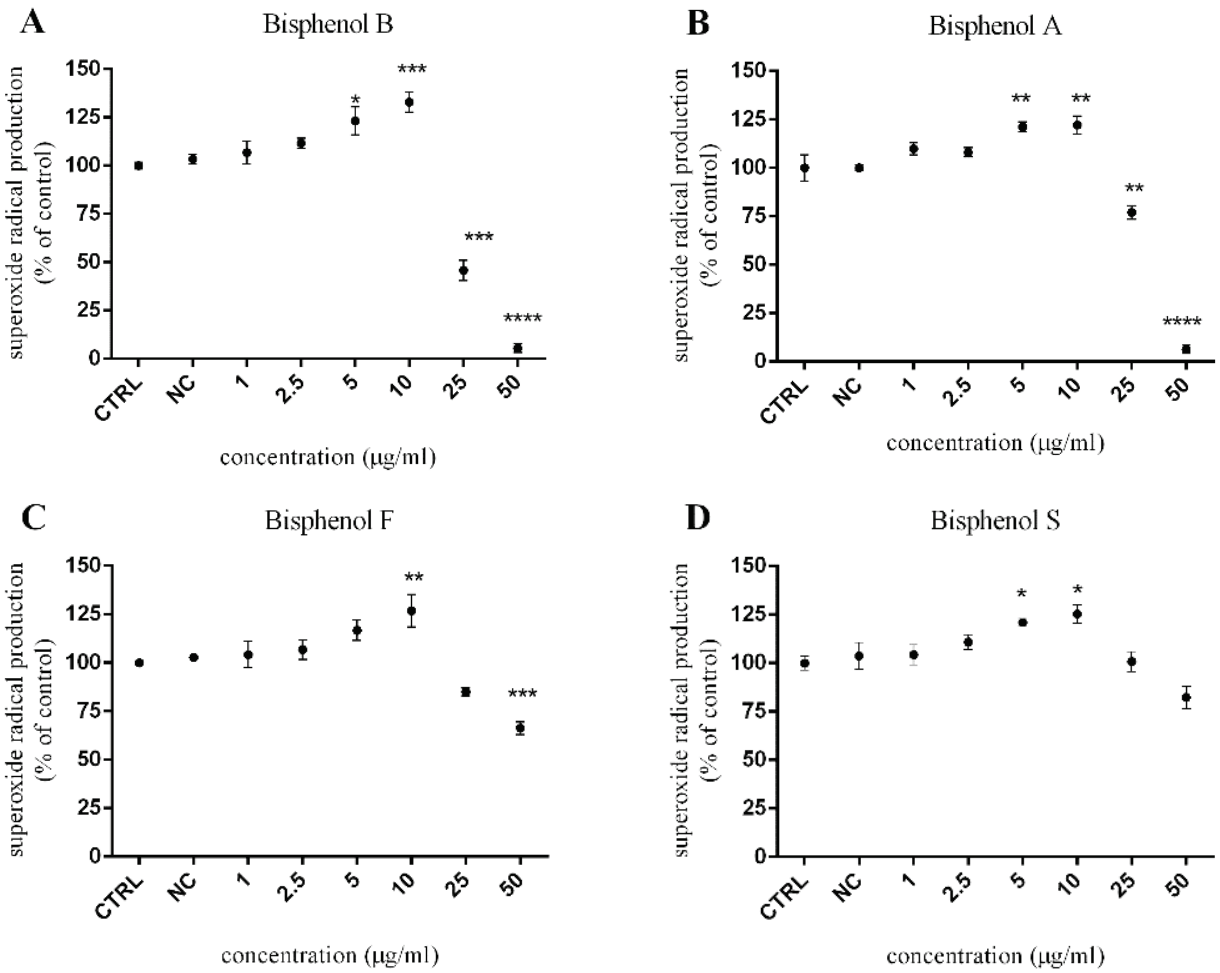


Fig. 4. The effects of bisphenols on intracellular production of superoxide radicals in TM3 Leydig cells after 48 h exposure *in vitro*. CTRL – control group, NC – negative control. The data are presented as means (± SEM) optical density percent of the control (untreated) and bisphenol´s treated groups. The data were collected from three independent experiments performed in triplicates. Levels of significance were established at **** ($p<0.0001$); *** ($p<0.001$) ** ($p<0.01$, and * ($p<0.05$). Statistical differences between the values of control and treated groups are indicated by an asterisk (*).

the lysosomal integrity of exposed cells was significantly reduced at 10, 25, and 50 µg/ml of BPB, BPA, BPF, and BPS.

Yawer *et al.* [16] confirmed that the highest dose (≈11.37 µg/ml to 45.5 µg/ml) of BPA decreased cell membrane integrity and lysosomal integrity after 24-h exposure. Yawer *et al.* [16] confirmed that the highest dose (≈11.37 µg/ml to 45.5 µg/ml) of BPA decreased cell membrane integrity and lysosomal integrity after 24-h exposure.

The same negative effect of bisphenols on lysosomal integrity determined by NR uptake was confirmed by Yin *et al.* [38]. The TM3 Leydig and Sertoli TM4 cells were treated by 2.5 to 400 µM of BPA and BPS and results revealed significant decreased of lysosomal integrity at concentrations of 200 µM (≈45.5 µg/ml BPA; 50 µg/ml BPS) and 400 µM (≈91 µg/ml BPA; 100 µg/ml BPS) for 24-h, and at 100 µM for 48 and 72-h, respectively. As we mentioned before, disruption of cell membrane integrity caused by higher experimental concentrations may immediately alter mitochondrial membrane potential. Goncalves *et al.* [37] also declare that the highest applied dose of BPA (≈22.75 µg/ml) initiates a significant decrease of membrane potential in TM3 Leydig cells after 48-h exposure. Taking it together, we may declare that TM3 mice Leydig cells are significantly vulnerable to BPB exposure, while BPS and BPF are less toxic compared to BPA action. At the same time, regarding the previously discussed studies is essential to highlight that even a lower dose of bisphenols could negatively affect other cellular parameters and processes, which could have a potential detrimental effect on male reproductive functions.

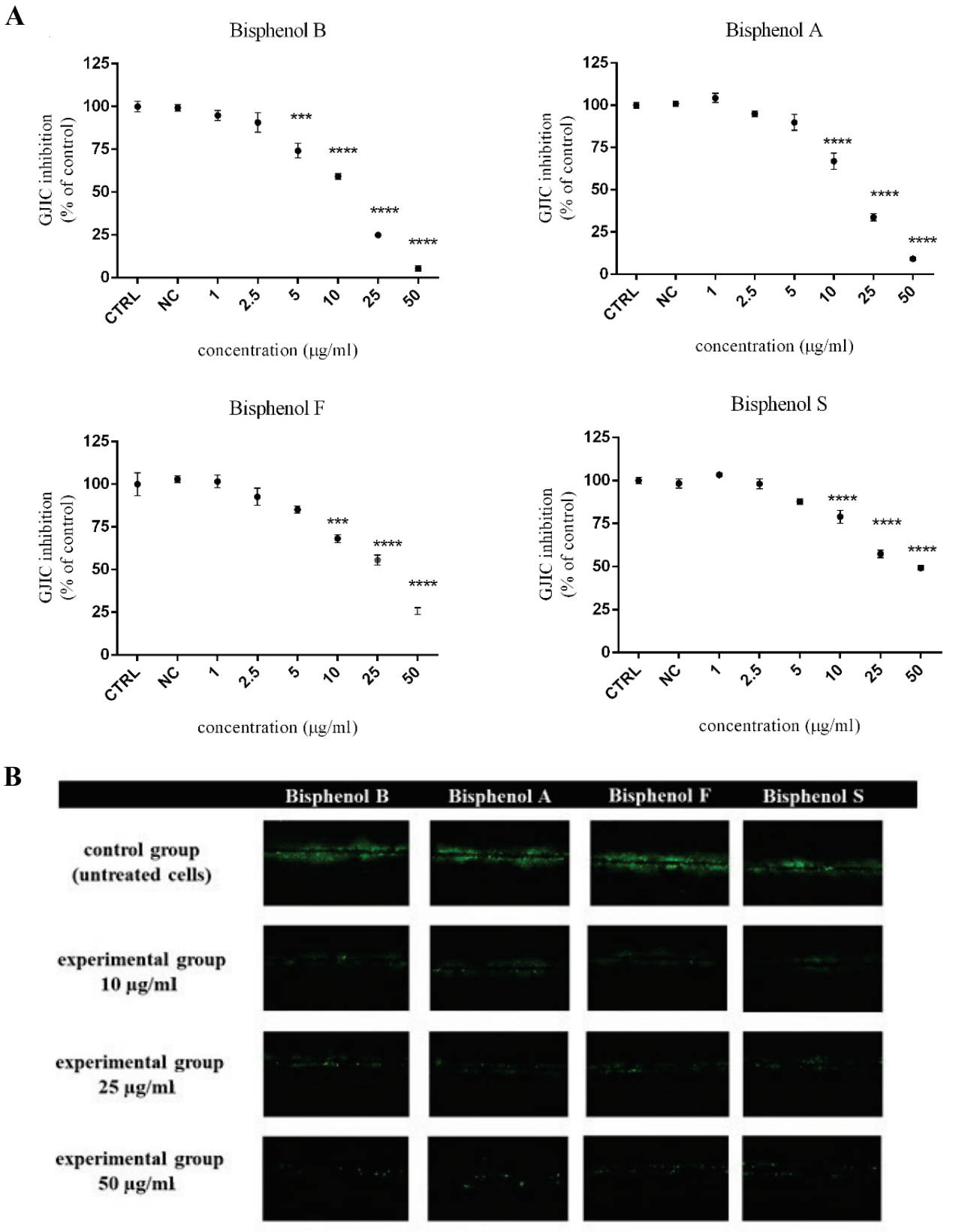


Fig. 5. The effects of bisphenols on GJIC in TM3 Leydig cells after 48 h exposure *in vitro*. (**A**) GJIC-inhibitory activity of bisphenols. (**B**) The representative images after 48 h exposures to BPB, BPA, BPF, and BPS were performed by SL/DT technique. The LY dye spreading into the TM3 Leydig cells is related to the GJIC extent. CTRL – control group, NC – negative control. The data are presented as means (± SEM) optical density percent of the control (untreated) and bisphenol´s treated groups. The data were collected from three independent experiments performed in triplicates. Levels of significance were established at **** ($p<0.0001$) and *** ($p<0.001$). Statistical differences between the values of control and treated groups are indicated by an asterisk (*).

Table 2. Testosterone production after 48 h exposure to bisphenols in TM3 mice Leydig cells *in vitro*.

Concentrations (μg/ml)	**BPB**	**BP**	**BPF**	**BPS**
CTRL	100.0±4.8 %	100.0±4.7 %	100.0±1.0 %	100.0±3.5 %
NC	104.8±1.7 %	102.1±3.8 %	104.1±5.4 %	99.2±1.8 %
1	112.8±6.6 %	106.3±3.7 %	112.2±2.9 %	105.3±7.3 %
2.5	119.1±3.1 %*	118.1±3.7 %*	96.9±4.7 %	111.7±4.1 %
5	105.8±3.9 %	108.3±1.3 %	86.3±6.7 %	110.6±8.6 %
10	56.5±2.8 %****	65.5±4.1 %****	77.5±4.5 %*	87.0±3.1 %
25	27.4±4.6 %****	43.3±3.3 %****	55.3±4.1 %****	62.4±2.8 %***
50	10.5±0.3 %****	21.9±4.0 %****	35.2±2.7 %****	41.7±6.7 %****

CTRL – control group, NC – negative control. The data are presented as means (± SEM) optical density percent of the control (untreated) and bisphenol´s treated groups. The data were collected from three independent experiments performed in triplicates. Levels of significance were established at **** ($p<0.0001$); *** ($p<0.001$), and * ($p<0.05$). Statistical differences between the values of control and treated groups are indicated by an asterisk (*).

Physiological levels of ROS are important for normal steroidogenesis and spermatogenesis maintenance. Overleaf, uncontrolled ROS production induced by bisphenols could progressively affect molecular pathways and cell cycle in exposed male germ cells [19,39]. Our result revealed, that 5 and 10 μg/ml of bisphenols could significantly increase the level of superoxide radical production, which indicated their pro-oxidant potential. The fact, that BPA could increase ROS production and induce oxidative stress was confirmed by previous studies [40-42]. However, the ROS-inducing potential of currently used BPA analogues such as BPB, BPF, or BPS is more debatable, and conclusions are diverse. Rajkumar *et al.* [34] evaluated the potential of BPA to induce uncontrolled growth of ROS in mice Leydig cells *in vitro*. Experimental cells were treated in various doses starting from 0.001 to 50 μM for 48-h. The published results suggest that the highest dose of BPA may significantly increase the level of ROS and induce oxidative stress. Compared to our study, gained results revealed that even lower doses than 50 μM of BPA (and 10 μg/ml) have the potential to increase superoxide radical production. Ullah *et al.* [43] performed *in vitro* evaluation of BPB and BPS (1, 10, and 100 ng/ml) in adult male Sprague-Dawley rats' testicular tissue after 2-h exposure. A significant increase in ROS was recorded in the 10 ng/ml (0.001 μg/ml) of BPB, and 100 ng/ml (0.1 μg/ml) of BPS compared to the control group. At the same time, significant accumulation of malondialdehyde (MDA) was confirmed only at the highest concentration of BPS. Zhang *et al.* [44] evaluate whether BPS could increase intercellular ROS generation in mice TM3 Leydig cells *in vitro* after 48-h exposure. The level of ROS was significantly increased at all applied doses of BPS (starting from 25 to ≈100 μg/ml). In addition, BPS exposure causes an increased formation of lipid peroxidation product – MDA. This study showed the same negative effect, and the presented data are in line with our results. In addition, the dose-dependent elevation of ROS after 48-h exposure has been observed. Ullah *et al.* [45] cultured testicular tissues of adult Sprague Dawley male rats together with different concentrations of BPF (1, 10, 25, 50, and 100 ng/ml) *in vitro* after 2-h exposure. The results revealed a significant dose-dependent increase in ROS levels, concretely at 25 ng/ml (0.025 μg/ml), 50 ng/ml (0.050 μg/ml), and 100 ng/ml (0.1 μg/ml). Consistent with our results low applied doses of BPF did not induce substantial changes in superoxide radical generation compared to the control group. In summary, unregulated ROS production induced by bisphenols may be a trigger for oxidative stress in mice Leydig cells. Considering to gained results and discussed studies we can conclude that our experimental analogues of BPA are definitely able to increase superoxide radical production and it seems that this ability of bisphenols could be involved in other intercellular changes resulting in the suppression of reproductive capacity of males.

It is well known, that Leydig cells are responsible for steroidogenesis and the production of sufficient levels of testosterone in males. However, bisphenols exposure could decrease the expression of several key steroidogenic enzymes (CYP17A1, HSD17B3, etc.) and impair testosterone secretion [33]. In

this point of view, the intercellular activity of GJIC plays an essential role confirmed by recent studies [24,25]. Results of our *in vitro* study revealed a significant reduction of GJIC activity at 5 μg/ml of BPB and 10, 25, or 50 μg/ml doses of BPA, BPF, and BPS. Inhibition of GJIC in mice Leydig cells has been determined previously by Iwase *et al.* [46]. TM3 cells were exposed to 5, 10, and 20 μM of diethylstilbesterol for 24 and 72-h, and Lucifer yellow was used for GJIC quantification. The results showed that prolonged time of exposure significantly decreased GJIC activity at 10 μM (≈3 μg/ml) and 20 μM (≈6 μg/ml). Goldenberg *et al.* [47] proclaim that increased testosterone secretion was accompanied by GJIC changes in mice TM3 cells. Results of the previous study [16] revealed that significant inhibition in GJIC activity was observed at ≈5 μg/ml and ≈10 μg/ml of BPA after 30 min and 24-h exposure. In addition, increasing experimental concentrations (≈22.5 and ≈45.5 μg/ml) cause significant damage to connexin channels at each time point with undetectable activity at 200 μM (≈45.5 μg/ml) after 24-h of BPA treatment. The same authors evaluated the potential to dysregulate GJIC by BPA in mice TM4 Sertoli cells *in vitro*. The gained results confirmed that applied concentrations of BPA (from ≈11.37 to 22.75 μg/ml) could rapidly affect presented parameters after 24-h exposure [48].

The current studies provide limited information about the potential of bisphenols to decrease testosterone production accompanied by changes in GJIC activity. However, different cellular models confirmed the importance of GJIC in steroid hormone production, oocyte maturation, protein kinase pathway modulation as well as in endothelial angiogenesis [49-51]. Gingrich *et al.* [24] evaluated the effects of BPA, BPS, and BPF (1, 100, and 1000 ng/ml) on GJIC in a time- and dose-dependent manner in ovine pre-luteinized theca cells. The results showed moderate inhibition after 1 μg/ml of BPA with no effect on GJIC after BPF exposure for 24-h. Surprisingly, BPS exposure increased the activity of the observed parameter starting at exposure to 0.01 μg/ml after the same time of cultivation. Alterations in GJIC activity were determined by Sabry *et al.* [52] recently. Bovine oocytes were exposed to 0.05 mg/ml BPA and BPS for 24-h. Results did not confirm any significant changes in Cx43 mRNA expression and no changes in GJIC regulation. Taking together, in the context of the male reproductive functions our gained results emphasize the significant importance of GJIC. It seems that cell communication mediated through Cx43 channels plays an essential role in steroid hormone production, and this issue is not still clearly solute. The results of our study confirmed an alteration in GJIC activity induced by experimental doses of bisphenols, which could lead to changes in steroid hormone production.

In line with the design of our study and all presented parameters described above, several prior studies have shown that testosterone secretion in different Leydig cell lines is significantly attenuated after bisphenols exposure [26,35,37]. The results of our *in vitro* study confirmed the same tendency, when higher concentrations starting at 10 μg/ml of BPB, BPA, BPS and BPF may cause a significant inhibition of this parameter after 48-h cultivation. The capacity of mice TM3 Leydig cells to produce testosterone after 48-h exposure to BPA was evaluated previously [25]. The results suggest that already lower experimental concentrations starting at 2.5 μg/ml) of BPA followed by ≈5 μg/ml, ≈10 μg/ml, and ≈20 μg/ml may significantly ($p<0.05$) inhibit testosterone secretion. Goncalves *et al.* [37] confirmed the same tendency with significant decline in testosterone secretion compared to the untreated cells. The ability of BPA to suppress testosterone secretion in tumor MA-10 cells was confirmed previously [35], when significant changes were recorded at 1 nM of BPA. Gao *et al.* [53] monitored the effects of BPS and BPF on steroid hormone secretion and total cholesterol level in mice TM3 Leydig cells during 24-h exposure. The ELISA methods confirmed a significant decrease in testosterone production at ≈50 μg/ml) of BPS and ≈20 μg/ml of BPF. Together with the inhibition of testosterone release, the total cholesterol level was significantly reduced at the corresponding concentration evaluated by total cholesterol assay kits. The potential of bisphenols to modulate testosterone secretion *in vitro* has been evaluated previously by Roelofs *et al.* [54]. Mice MA-10 tumor Leydig cells were cultured with different concentrations of BPF and BPS (from 0.01 to 100 μM) for 48-h. In both, a slight decrease in testosterone production was recorded up to 30 μM (≈7.5 μg/ml of BPS and 6 μg/ml of BPF) while at 100 μM (20 μg/ml) of BPF a significant increase was recorded. If we summarize obtained information about the effect of bisphenols on testosterone release, the negative correlations are sufficiently examined. We may declare that higher experimental concentrations together with prolonged time of cultivation have an unequivocally

negative impact on steroidogenesis, but biphasic properties of lower doses are not still clearly clarified. Current data about the interaction of male reproduction and bisphenol A analogues are varied and conclusions depend on many factors. Definitely, there are still other molecular pathways, which could be taken into account for a better understanding of this issue such as the activity of steroidogenic enzymes, the receptor-binding affinity of bisphenols as well as modifications of proteins included in many molecular pathways.

Conclusions

Results of our *in vitro* study suggest that exposure to bisphenols could impaired cell viability together with cell membrane integrity, decrease lysosomal activity, induced ROS production, inhibited GJIC as well as decreased testosterone production. Based on this fact, we may conclude that Leydig cells may serve as a potential target for bisphenols-induced testicular toxicity, and increased ROS generation together with altered GJIC activity may be the main factors contributing to the testosterone decline. Therefore, an increased incidence of male reproductive pathologies could be potentially associated with growing bisphenol exposure in daily life.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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