Assessment of the effective impact of bisphenols on mitochondrial activity and steroidogenesis in a dose-dependency in mice TM3 Leydig cells

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Running head: Bisphenols affect mitochondrial activity and steroidogenesis

Summary

The increasing worldwide production of bisphenols has been associated to several human diseases, such as chronic respiratory and kidney diseases, diabetes, breast cancer, prostate cancer, behavioral troubles and reproductive disorders in both sexes. The aim of the present *in vitro* study was to evaluate the potential impact bisphenols A, B, S and F on the cell viability and testosterone release in TM3 Leydig cell line. Mice Leydig cells were cultured in the presence of different concentrations of bisphenols (0.04-50 $\mu g.ml^{-1}$) during 24 h exposure. Quantification of the cell viability was assessed using the metabolic activity assay, while the

level of testosterone in cell culture media was determined by enzyme-linked immunosorbent assay. Within the panel of substances under investigations, the higher experimental concentrations (10; 25 and 50 $\mu g.ml^{-1}$) significantly (*P<0.001*) decreased Leydig cells viability, while the same doses of BPA and BPB also reduced testosterone production significantly (*P<0.001*). Taken together, the results of our study reported herein is a consistent whit the conclusion that higher experimental doses of bisphenols have a cytotoxic effect and could have a dose-dependent impact on testosterone production.

Key words: bisphenols, Leydig cells, viability, testosterone

Bisphenol A (BPA, 2,2-bis[4-hydroxyphenyl] propane) is one of the oldest and most studied synthetic substance known as an endocrine disruptor (ED). About 70 % of BPA production is used to produce polycarbonate plastics used in a variety of common products such as plastic packaging, cling film, epoxy resins, food cans and many others (Vandenberg *et al*. 2007). Many studies showed that BPA may definitely affect steroidogenic process through alterations in steroidogenic enzymes and transport proteins, including impairment of spermatogenesis followed by reduced semen quality parameters (Ye *et al*. 2011; Hulak *et al.* 2013; Vitku *et al.* 2015). Due to many negative effects, toxicity and widespread exposure, use of BPA has been banned in some consumer products such as reusable food or beverage containers, infant formula containers and baby bottles (Eladak *et al.* 2015). Nowadays, there are several analogues to BPA such as bisphenol S (BPS), bisphenol F (BPF) or bisphenol B (BPB). According to the previous studies, a direct inhibition of bisphenol alternatives on steroidogenesis or spermatogenesis, with irreversible changes in sperm morphology, Sertoli cells activity and hormonal imbalance is extensively discussed (Cao *et al.* 2012; Liao and Kannan, 2013). In addition, many authors confirmed neurotoxicity, genotoxicity, reproductive

toxicity and strong endocrine disruptive activity (Alves *et al.* 2013; Rosenmai *et al.* 2014). Nowadays, toxicological data are scarce and experimental studies evaluating the effects of bisphenol analogues are unclear. Under these endpoints, we have decided to urgently investigate the effects of BPA, BPB, BPS and BPF as well as their cellular toxicity and potential impact on steroidogenesis *in vitro.*

The TM3 Leydig cell line (ATCC #CRL-1714; Manassas, VA, USA) were cultured in Dulbecco´s Modified Eagle´s Medium/Nutrient Mixture (Ham´s) F12 with HEPEs and $NaHCO_3$ (DMEM/F12; Sigma Aldrich, St. Louis, USA) supplemented with 5 % horse serum (HS; Gibco-Life Technologies, New Zealand), 2.5 % fetal bovine serum (FBS; BiochromAG, Berlin, Germany), 2.5 mM L-glutamine (Sigma Aldrich, St. Louis, USA) and 1 % penicillin/streptomycin solution (Sigma Aldrich, St. Louis, USA). The Leydig cells density was adjusted to a final concentration of 4×10^3 cells/well and seeded in 96-well plate for 24 h. TM3 cells were maintained at 37 °C under a humidified atmosphere of 95 % air and 5 % CO_2. Afterwards, the medium was changed to include different concentrations of bisphenols A, B, S and F (Sigma Aldrich, St. Louis, USA), starting from 0.04 to 50 $\mu g.ml^{-1}$ and the cells remained cultured during the next 24 h. The applied concentrations range of bisphenols was selected according to the results of our pilot range-finding experiments. The viability of exposed cells was estimated using the MTT (3-4,5-dietyltiazol-2-yl)-2,5-diphenyltetratzolium bromide) assay, which measured the reduction of a yellow tetrazolium salt to blue formazan in viable cells (Mosmann, 1983). Formed formazan crystals were dissolved by isopropanol (p.a. CentralChem, Bratislava, Slovak Republic) and read by an ELISA reader (Multiscan FC, ThermoFisher Scientific, Vantaa, Finlad) at 570 nm against 620 nm wavelengths. Furthermore, the level of testosterone in cell culture media was determined by enzyme-linked immunosorbent assay using ELISA kits purchased from Dialab (Testosterone Cat. #K00234; Austria). The absorbance was measured at 450 nm by ELISA reader (Multiscan FC, ThermoFisher Scientific,

Vantaa, Finlad). The data were collected from four (n=4) independent experiments that were performed in triplicates and statistically analyzed using the GraphPad prism 5.0 (GraphPad Software Incorporated, San Diego California, USA). One-way analysis of variance (ANOVA) followed by Dunnett´s test was used for statistical evaluations. Results were presented as means (± SEM) of Leydig cell viability % and testosterone % of control (untreated) and treated groups. Differences were compared for statistical significance at $P<0.05$.

As presented in Figure 1. the metabolic activity was significantly ($P<0.05$; $P<0.001$) reduced at 10 (83.06±3.82%); 25 (85.63±3.36%) and 50 (52.79±6.05%) $\mu g.ml^{-1}$ of BPA. Significant ($P<0.001$) changes were also observed at the 25 (66.08±2.34%) and 50 (6.13±1.87%) $\mu g.ml^{-1}$ of BPB, while 10 (84.81±0.70% and 83.46±6.22%); 25 (79.47±2.97%; 81.82±2.00%;) and 50 (61.30±4.14% and 46.31±0.77%) $\mu g.ml^{-1}$ of BPS and BPF decreased Leydig cell viability significantly ($P<0.01$; $P<0.001$). The data suggest that the highest dose of BPB (50 µg.ml-1) is extremely cytotoxic and the suppression may be increased by other bisphenols with time. The cytotoxic effects of bisphenols are shown at the photomicrograph (Figure 2). BPA is one of the most well-studied endocrine disruptors (Kolatorova *et al.* 2017). The ability to affect the cell viability of Leydig TM3 line in dose- and time- dependent manner of BPA *in vitro* was evaluated by Goncalves *et al.* (2018). The data showed that at concentrations above 5 µM (10 – 500 µM) during 24 h exposure to BPA significantly inhibited mitochondrial activity when compared to the control group. Nonetheless, the Leydig cell viability did not decrease significantly after 48 h exposure to BPA at concentrations below 50 µM. In this case, higher experimental doses (100 - 500 µM) of BPA reduced mitochondrial activity significantly. Roelofs *et al.* (2015) determined the metabolic activity of MA-10 cells after 48 h BPS and BPF exposure. The results showed non-cytotoxic effect at 0.01-30 µM of BPS and 0.01-100 µM of BPF. The cytotoxic potential of BPS, BPB and BPF (10-300 µM) after 48 h incubation was investigated in the *in vitro* study by Russo *et al.* (2018). The biological

effect was evaluated using a well-established health and cancer cell lines (HeLa, MCF-7 and 3T3-L1). Moderate toxicity was observed for BPF and BPS on all cell lines, while BPB was clearly toxic only for 3T3-L1 (mouse embryonic fibroblast) and MCF-7 (human breast cancer) cells.

The results of our *in vitro* study indicate that experimental concentrations of BPA, BPB, BPS and BPF (0.04-50 $\mu g.ml^{-1}$) may affect the testosterone production in mice TM3 Leydig cells after 24 h exposure. A significant (*P<0.01; P<0.001*) reduction in hormone production was recorded at 10 (69.66±8.32% and 59.93±1.96%); 25 (30.88±2.91%; 22.94±4.41%) and 50 (20.54±3.51%; 9.60±2.46%) $\mu g.ml^{-1}$ of BPA and BPB compared to the controls. On the other hand, BPS and BPF slightly reduced testosterone synthesis at the same concentrations, but not significantly. We are convinced that the highest experimental dose of BPA and BPB have a strong inhibitory potential evoked by decreasing in steroidogenic enzymes activity such as 3β-HSD or 17 β-HSD. A summary of testosterone production after 24 h treatment by bisphenols A, B, S and F in TM3 Leydig cells is presented in Table 1. Similar tendency was observed in the previous study of Ok e*t al.* (2017). They showed significant inhibition of testosterone and progesterone production by 100 μM of BPA treatment in comparison to the control group after 24 h exposition in TM3 cell line *in vitro.* Goncalves *et al.* (2018) study showed that the concentrations 1, 10 and 100 μM of BPA are able to reduce testosterone production in TM3 Leydig cell line after 48 h incubation by approximately 22-39 % respectively, when compared to the non-treated cells. Testosterone production in MA-10 cells after 48 h *in vitro* exposure to experimental doses (0.01-100 μM) of BPF and BPS was evaluated by Roelofs *et al.* (2015). Exposure to both of selected bisphenols in concentrations ranges of 0.01-30 μM for BPS, and up to 100 μM for BPF showed that only BPF increased testosterone production, while BPS did not affect this production in exposed cells. Suppression of testosterone synthesis may be associated with inhibition of steroidogenic enzyme activity because there was a decrease in

steady state mRNA levels of the cytochrome P450 17α-hydroxylase/17,20 lyase, StAR and Hsb3b1, although statistical significance was not reached (Akingbemi *et al.*, 2004; Eladak *et al.*, 2015)

In conclusion, our results showed that higher experimental concentrations from 10 to 50 $\mu g.ml^{-1}$ of BPA, BPS and BPF as well as 25 or 50 $\mu g.ml^{-1}$ of BPB, may adversely affect the viability of TM3 Leydig cells after 24 h *in vitro* cultivation. The results also suggest that all experimental doses (0.04 – 50 $\mu g.ml^{-1}$) of bisphenols may affect testosterone release while the highest concentrations have inhibitive effects on steroid hormone production

Conflict of Interest

There is no conflict of interest.

Acknowledgments

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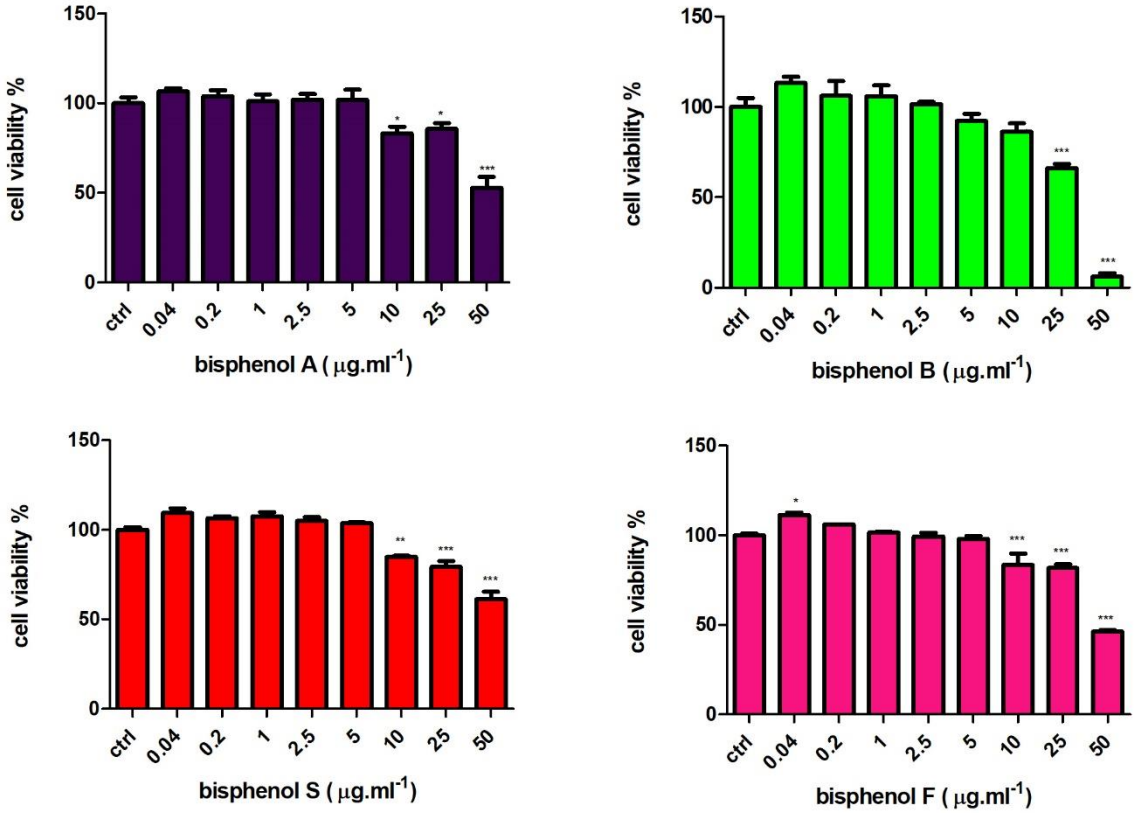
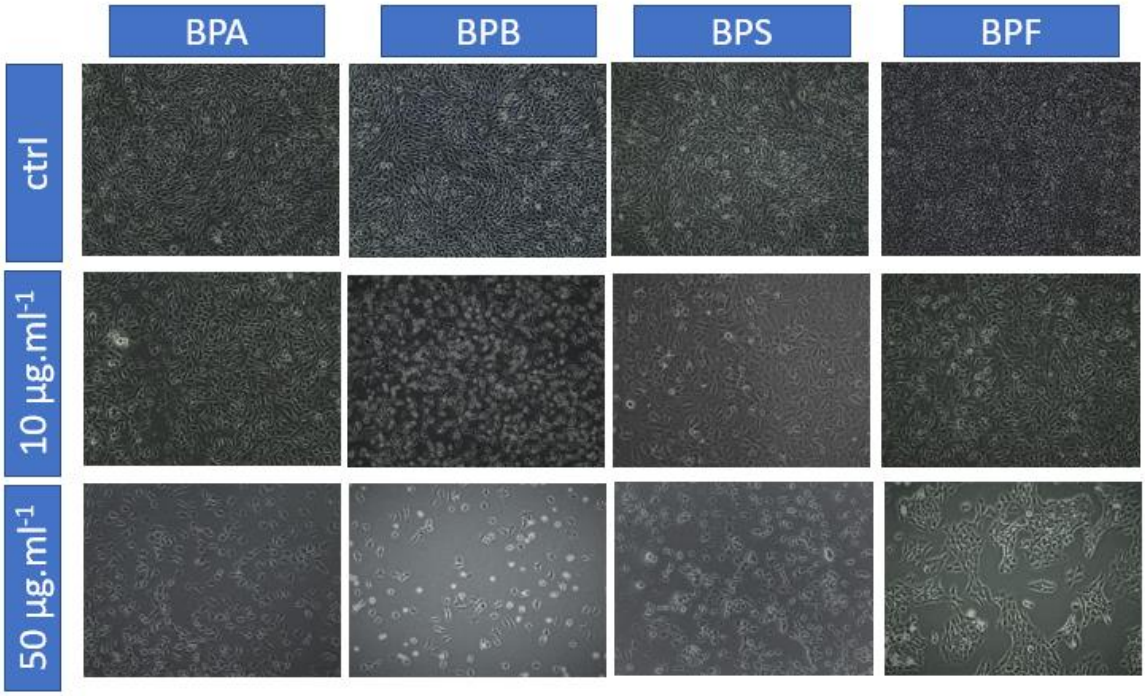
Fig. 1. The effects of bisphenols A, B, S and F on the TM3 cell viability after 24 h *in vitro* cultivation



Abbreviations: ctrl- control (non-treated) group. The numbers under each column (0.04-50 $\mu g.ml^{-1}$) represent experimental concentrations of bisphenols. Each bar represents the mean (±SEM) viability % of control and treated group. Data were obtained from four (n=4) independent experiments that were performed in triplicates. Level of significance was set at **(P<0.05), **(P<0.01)* and **** (P<0.001).* Statistical difference between the values of control and treated groups is indicated by an asterisk.

Fig. 2 A photomicrograph of TM3 cells after 24 h treatment with bisphenols A, B, S and F at 400x magnification



Abbreviations: ctrl- control (non-treated) group. The numbers 10 and 50 $\mu g.ml^{-1}$ represent experimental concentrations of bisphenols. BPA – bisphenol A, BPB – bisphenol B, BPS – bisphenol F, BPF – bisphenol F.

Table 1. A summary of testosterone (%) production after 24 h treatment by bisphenols A, B, S and F in TM3 Leydig cells

Test compound	Experimental doses of bisphenols (µg.ml^{-1})								
	ctrl	0.04	0.2	1	2.5	5	10	25	50
BPA	100.0±5.1%	113.7±7.3%	115.5±9.1%	119.4±2.4%	120.0±4.6%	116.0±3.7%	69.6±8.3%**	30.8±2.9%***	20.5±3.5%***
BPB	100.0±4.8%	110.3±1.6%	109.0±2.8%	111.3±5.0%	106.7±3.5%	108.7±3.2%	59.0±1.9%***	23.9±4.1%***	9.0±2.4%***
BPS	100.0±3.0%	108.6±5.8%	107.1±5.5%	105.0±3.8%	107.5±9.1%	115.1±1.4%	91.2±2.0%	93.1±1.1%	80.6±8.2%
BPF	100.0±4.6%	120.2±2.0%	115.7±5.0%	115.9±4.6%	102.0±8.5%	109.7±6.4%	99.9±7.6%	96.2±7.1%	86.2±2.1%

Abbreviations: ctrl – control group; BPA – bisphenol A, BPB – bisphenol B, BPS – bisphenol F, BPF – bisphenol F. Each number represent the mean (±SEM) testosterone % of control (untreated) and treated groups. Data were obtained from four (n=4) independent experiments that were performed in triplicates. Level of significance was set at ***(P<0.01)* and *** *(P<0.001).* Statistical difference between the values of control and treated groups is indicated by an asterisk.