Long-term exposure to very low doses of bisphenol S affects female reproduction

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Abstract

Bisphenols belong to the endocrine disruptors, affecting reproduction even in extremely low doses. Bisphenol S (BPS) has become widely used as a substitute for the earlier-used bisphenol A; however, its harmlessness is questionable. The aim of this study was to evaluate the effect of BPS on folliculogenesis and oocyte quality after *in vivo* exposure to low doses of BPS. Four-week-old ICR females ($n=16$ in each experimental group) were exposed to vehicle control (VC), BPS1 (0.001 ng BPS.g/bw/day), BPS2 (0.1 ng.g/bw/day), BPS3 (10 ng.g/bw/day) and BPS4 (100 ng.g/bw/day) for 4 weeks. Ovaries were subjected to stereology and nano liquid chromatography-mass spectrometry (LC/MS). Simultaneously, metaphase II oocytes were obtained after pregnant mare serum gonadotrophin and human chorionic gonadotrophin administration, followed by immunostaining. In particular, mating and two-cell embryo flushing were performed. We observed that BPS decreases the amount of ovarian follicles and BPS2 (0.1 ng.g/bw/day) affects the volume of antral follicles. Accordingly, ovarian proteome is affected after BPS2 treatment. While BPS2 dosing results mainly in cytoskeletal damage in matured oocytes, the effects of BPS3 and BPS4 seem to be due instead to epigenetic alterations in oocytes. Arguably, these changes lead to observed affection of *in vivo* fertilization rate after BPS3 and BPS4 treatment. BPS significantly affects female reproduction astoundingly in extremely low doses. These findings underline the necessity to assess the risk of ongoing BPS exposure for public health.

Reproduction (2018) **156** 47–57

Introduction

Bisphenol S (BPS) is an industrially produced compound found in many plastic items and, accordingly, a widespread environmental contaminant (Glausiusz 2014). BPS is found in water, house dust and/or food (Ike *et al.* 2006, Viñas *et al.* 2010, Liao *et al.* 2012a, Zhao *et al.* 2014, Li *et al.* 2015), entering the human body through ingestion, inhalation and dermal absorption (Liao *et al.* 2012b). Indeed, BPS has been detected in human blood and urine (Cobellis *et al.* 2009, Liao *et al.* 2012a, Yang *et al.* 2015). BPS is capable of disrupting the hormonal balance of the humans and influencing several physiological functions, similar to many other endocrine disruptors (EDs) (Colborn *et al.* 1993, Rochester & Bolden 2015). These chemical compounds mimic hormonal action in subtoxic doses and induce

hormonal imbalance, leading to inadequate regulation of folliculo- and oogenesis (Mok-Lin *et al.* 2010, Vandenberg *et al.* 2012, Rivera *et al.* 2015). Recently, BPS has been used as a 'safe substitution' of mass-produced bisphenol A (BPA), previously determined to be a compound with ED features (Le Fol *et al.* 2017, Gingrich *et al.* 2018).

The negative effects of BPA on both male (Rahman *et al.* 2015, 2018) and female reproduction (Eichenlaub-Ritter *et al.* 2008, Rivera *et al.* 2015), on human reproductive health (Peretz *et al.* 2014, Mannelli *et al.* 2015), as well as on assisted reproductive technologies (ARTs) (Mok-Lin *et al.* 2010), have been described. Based on these alarming findings, the tolerable daily intake (TDI) for BPA (EFSA 2014, available from www.efsa.europa.eu) has been

established at 50 ng.g/bw/day, without manifestation of toxic effects on live organisms. Recently, many plastic goods have a low BPA content, and some of them are labeled as BPA-free. However, BPA is gradually substituted by alternative compounds, most often structure analogues of BPA, including other bisphenols. Substantial evidence points to BPS as being the most used BPA alternative (Liao *et al.* 2012c, Barrett 2013, Eladak *et al.* 2015). An increasing number of studies (Rochester & Bolden 2015, Sartain & Hunt 2016, Žalmanová *et al.* 2017) underscore the possible deleterious substitutions known in other cases (Coggon 2002, Howard 2014).

A limited number of studies have focused on the biological mechanism of BPS and have indicated a BPA-like effect of BPS, with the ability of the artificial simulation of hormonal action, including interaction with estrogen receptors (Hashimoto *et al.* 2001, Chen *et al.* 2002, Kitamura *et al.* 2005, Kuruto-Niwa *et al.* 2005, Delfosse *et al.* 2012, Grignard *et al.* 2012, Rosenmai *et al.* 2014). According to those studies, genotoxicity, acute poisoning and/or inadequate estrogen activity associated with BPS were observed (Liao *et al.* 2012a). Moreover, in zebrafish offspring of exposed parents, there was also evidence of a BPS-induced decrease in the weight of gonads, disrupted production of steroidogenesis and increasing incidence of developmental malformations (Ji *et al.* 2013, Naderi *et al.* 2014). Regarding mouse sperm, a reduced quantity of spermatozoa and decreased sperm motility were observed after BPS *in vivo* treatment (Shi *et al.* 2017). The first evidence of the negative effect of BPS on mammalian oogenesis was recently published (Žalmanová *et al.* 2017), and damage to the meiotic spindle in mature oocytes treated *in vitro* has been described. In addition, BPS induces changes in the epigenetic pattern in somatic cells (Verbanck *et al.* 2017). A similar effect in gametes is possible.

To the best of our knowledge, there is a lack of study simulating temporal exposure of humans to BPS. Hence, this study addresses the impact of long-term *in vivo* dosing of mice with BPS in low concentrations, simulating real conditions of human exposure. In addition, experiments are designed with respect to the impact of BPS-exposed ART-subjected women. Our study simulates the exposure of the first and subsequent ovarian cycles until the reproductive peak is reached (~25 years in women, corresponding to 8-week-old mice), through hormonal ovarian stimulation followed by ART.

Materials and methods

Chemicals

All chemicals were purchased from Sigma-Aldrich if not otherwise stated.

Animals and ethical statements

All animal procedures were conducted in accordance with Act No. 246/1992 Coll., on the Protection of Animals against Cruelty, under the supervision of the Animal Welfare Advisory Committee at the Ministry of Education, Youth and Sports of the Czech Republic, approval ID MSMT-11925/2016-3. Four-week-old ICR mice were purchased from Velaz Ltd. (Prague, Czech Republic), housed in intact polysulfonate cages and maintained in a facility with a 12h light:12h darkness photoperiod, a temperature of $21 \pm 1^\circ\text{C}$ and a relative humidity of 60%. A phyto-estrogen-free diet 1814P (Altromin, AnimaLab, Poznan, Poland) and ultrapure water (in glass bottles, changed twice per week) were provided *ad libitum*.

Animal dosing and sample collection

Immediately after admission to the Animal Research and Care Facility, mice were randomly assigned to the experimental groups and allowed to acclimate for 1 week. BPS was then administered to the mice, and they underwent vaginal opening, for following 4 weeks through drinking water. The exposure consisted of five BPS dose treatments: 0, 0.004, 0.375, 37.5 and 375 ng/ml, that is vehicle control (VC) and BPS1–BPS4 groups, respectively. With respect to recorded body weight and known water intake (Bachmanov *et al.* 2002), the following doses were used: 0, 0.001, 0.1, 10 and 100 ng.g/bw/day (see ‘Animal dosing’ in the [Supplementary methods](#) for additional details; see section on [supplementary data](#) given at the end of this article). Appropriately comprehensive range of doses aimed to the endocrine-disrupting effect of BPS. Selected doses are in accordance with real oral exposure of human (Oh *et al.* 2018) and included the EFSA and NIH limits for the intake of BPA. The chosen window of exposure corresponds to at least three waves of cyclic follicle recruitment, beginning at the time of initial recruitment and continuing until attainment of the reproductive peak (McGee & Hsueh 2000, Moore-Ambriz *et al.* 2015).

Mice were euthanized by cervical dislocation and ovaries were collected. Body weight and weight of ovaries were recorded and ovaries were fixed as described in the following section. In addition to ovaries, blood was collected by intracardiac blood puncture. Serum samples were prepared by centrifugation (10,000g, 10 min, 4°C) and stored at -80°C until usage.

Ovarian histology

Ovaries free of oviduct and bursa were fixed in 2% paraformaldehyde for at least 48h at room temperature. Histological analysis was performed as previously described (Hernandez-Ochoa *et al.* 2010) in accordance with the experimental design (Fig. 1). Seven left ovaries per each experimental group were used for histological and stereological analysis. Samples were dehydrated, embedded in paraffin blocks, cut into 10 µm-thick histological sections and mounted with five sections per slide. The histological cutting plane of each sample was randomized using an orientator (Mattfeldt *et al.* 1990). Every fifth slide was stained

Figure 1 Experimental design. The scheme of hormonally unstimulated ovary collection for histological and proteomic analysis after 4-week bisphenol S (BPS) treatment, followed by evaluation of gonadotropin responsiveness expressed by flushing of *in vivo*-matured oocytes on Day 3. The following essential methodological approaches, such as ovarian histology, liquid chromatography-mass spectrometry (LC/MS-MS) and immunocytochemistry (ICC), are shown (blue lines). The number of mice per group used in a particular experiment is indicated.

in hematoxylin-eosin. From each stained slide, two adjacent sections, in predetermined order, were recorded. The primary, preantral, antral and atretic follicles and their volumes were morphologically assessed, with respect to analysis of recruited disruption-prone follicles, using a number of stereological techniques. For more details see 'Ovarian histology' in the [Supplementary methods](#).

Proteomic analysis

Based on a pilot analysis covering all VC and BPS groups, ovaries of hormonally unstimulated mice ($n=3$) of VC and BPS2 groups were collected for the complete proteomic analysis. Nano liquid chromatography-mass spectrometry (LC/MS) was used for protein identification and quantification. For additional details, see 'Protein identification by nano LC/MS' in the [Supplementary methods](#).

Assessment of ovarian gonadotropin responsiveness

Following pregnant mare serum gonadotrophin (PMSG)/human chorionic gonadotrophin (hCG) treatment of females (treated as illustrated in [Fig. 1](#)), flushing of *in vivo*-matured oocytes was performed. The total number and percentage of vital matured oocytes were counted and these oocytes were used for immunocytochemistry.

In vivo fertilization assay

Following PMSG/hCG treatment of females as described earlier, an *in vivo* fertilization assay was performed according to [Zudova et al. \(2004\)](#). Embryos were flushed from the oviduct and the fertilization rate was calculated as the percentage of cleaved embryos of the total recovered embryos/oocytes. For more details, see [Fig. 1](#) (Experimental design).

Immunocytochemistry (ICC)

Oocytes were fixed and permeabilized using two different methods according to visualized factors (for more details, see 'Immunocytochemistry' in the [Supplementary methods](#)). After blocking in 1% BSA in PBS with Tween 20, incubation with specific antibodies followed: anti- α -tubulin (Sigma-Aldrich),

pericentrin (PCNT; Abcam), anti H3K27me2 (H3K27me2; Abcam) and anti-5'-methyl cytosine (5meC; Abcam). Thereafter, samples were washed and incubated with a cocktail of anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 647. Concurrent with washing, phalloidin was applied for β -actin visualization. The signal intensity (i.e. integrated density) was measured using ImageJ software (NIH).

Statistical analysis

The data were processed with Statistica Cz 12 (StatSoft, Inc., Tulsa, OK, USA). For overall comparison of the study groups, Kruskal-Wallis ANOVA (for quantitative variables) or chi-square tests (for proportions) were used. In the case of a significant overall finding, differences between individual group pairs were assessed *post hoc*, using multiple comparisons of mean ranks, the Mann-Whitney *U* test with Bonferroni correction, or Fisher's exact test with Bonferroni correction. Where appropriate, correlations among variables were assessed using Spearman's method. The level of statistical significance was set at $\alpha=0.05$ and all reported *P* values and tests were calculated as two-tailed.

Results

Effect of BPS on ovarian follicles and histological quality

The goal of this experiment was to test the effect of BPS on the ovaries of hormonally unstimulated outbred mice. The BPS treatment covered the developmental time period, which includes initial follicle recruitment and subsequent cyclic ovarian recruitments, until the reproductive peak was reached, as evidenced by tracking of all recruited follicle stages (i.e. primary, preantral and antral follicles). The primary quantitative data, with the results of statistical and correlational analysis, are provided in the [Supplementary results](#) ([Supplementary Table 2](#)).

A significant reduction in the total volume of the ovary and relative ovary weight was found ([Fig. 2A and B](#)). This can largely be attributed to the reduction of the corpus luteum volume, as confirmed by the correlational

Figure 2 Ovarian histology after treatment with different doses of BPS. The weight of ovaries related to total body weight (A) and volume of ovaries (B), including ovarian cortex (C) and medulla (D), were recorded. Different superscripts indicate statistically significant differences ($P \leq 0.05$). Absolute numbers (E, F, G and H) and indicate volumes (E', F', G' and H') of primary, preantral, antral and atretic follicles are evidenced per one (left) ovary. Data are expressed as the mean $\pm$ SEM. Different superscripts indicate statistically significant differences ($P \leq 0.05$).

assessment (Supplementary Table 2 of Supplementary results), as well as to the decrease of both the number of antral follicles and their volume (Fig. 2G and G'). In addition to the cortex structures, medulla volumes were also reduced after BPS treatment (Fig. 2D).

No significant differences were found among the mean volumes of primary or preantral follicles in the administered BPS concentrations (Fig. 2E' and 2F'). In contrast, the mean volume of antral follicles was increased almost twofold in animals receiving BPS in low concentrations (Fig. 2G'). While the number of primary and preantral follicles followed a linear dose-dependent curve (Fig. 2E and F), the volume of the antral and atretic follicles shows a non-linear effect (Fig. 2G' and 2H'). Moreover, in the BPS3- and BPS4-treated animals, a visible antrum appears in follicles with fewer layers of granulosa cells (Supplementary Fig. 1). These findings were reflected by a significant decrease in plasma 17 β -estradiol after BPS3 and BPS4 treatment (Supplementary Fig. 2).

Proteomic profiling of the ovary after BPS treatment

This experiment was focused on possible protein targets of BPS, and an LC/MS-MS analysis was performed on hormonally unstimulated mouse ovaries. Based on proteomic analysis of BPS-wide exposure, BPS2 treatment was selected for additional detailed screening. There were 171 identified and quantified proteins, varying in densitometry analysis between VC and BPS2.

Apparent up-/or downregulated protein targets were identified, belonging to proteins with catalytic activity (e.g., superoxide dismutase (SOD)), transporter activity (e.g., aldo-keto reductase), translation regulator activity (elongation factors), structure molecule activity (e.g., tubulin α -1B chain, talin), receptor activity (laminin subunit), basal metabolism (subunit β of electron transfer flavoprotein) and binding proteins (galectin, Ca²⁺-binding Ser/Thr-protein phosphatase 2A). Proteins identified as affected by the BPS2 treatment are

Figure 3 Quantification analysis of ovarian proteins after BPS2 treatment. Proteins with significantly different levels ($P \leq 0.05$) in the BPS2 group, compared to vehicle control (VC), are shown and summarized. Nano LC/MS protein detection was performed, followed by analysis using ProteinPilot software and interpretation with the use of Panther 12.0 software. There are 15 protein hits, representing consistent upregulated (up) or downregulated (down) trends, at least in two individual samples per analyzed experimental group (i.e. VC and BPS2; $n=3$ each, from independent experiments). Proteins include binding proteins and proteins with different activities (transporter, translation regulator, structural, receptor and catalytic).

summarized in Fig. 3 (also see the separately provided Supplementary Tables 1 and 2 for complete output).

Effect of BPS on chromosome misalignment and spindle malformation in oocytes

The goal of this experiment was to evaluate the influence of BPS treatment on spindle formation and chromosome segregation in *in vivo*-matured and flushed oocytes. In addition to spindle α -tubulin and chromatin staining, subcellular immunolocalization of PCNT was performed.

An increased incidence of spindle malformation and abnormal chromosome alignment was observed in BPS-treated groups (Fig. 4A). After BPS2 and BPS3 treatments, there were increased numbers of abnormal oocytes, including those with spindle- and/or chromatin-derived oocyte abnormalities, (30.6 ± 5.9 and $34.3 \pm 3.2\%$, respectively, vs. $15.0 \pm 3.4\%$ in VC). These deviations seem to have been caused by spindle malformation, for which statistically significant differences were observed after BPS2 and BPS4 treatment (27.8 ± 10.7 and $33.7 \pm 5.5\%$, respectively, vs. $9.0 \pm 2.6\%$). To the contrary, there were no significant differences in chromosome misalignments among the groups. Data are summarized in Fig. 4B.

The observation of malformed spindle α -tubulin was supported by the results of the PCNT staining (see the representative pictures in Fig. 4C). Indeed, a PCNT signal was observed seemingly external to the spindle pole in the BPS1 group (dashed arrowhead in Fig. 4C), while oocyte spindle abnormalities in the BPS2 group were accompanied by a cap-shaped pole (empty arrowhead). Similarly, divided PCNT poles in the matured oocytes in metaphase II (MII) oocytes were

found in greatest abundance in BPS4-treated mice (white arrowhead).

Effect of BPS on oocyte yield, fertilization ability and epigenetic quality of oocytes

The goal of this experiment was to examine the physiological function of BPS-influenced ovaries of mice. PMSG-hCG hormonal stimulation was used and efficiency of *in vivo*-matured oocytes in MII stage was evaluated. Subsequently, genome-wide 5-methylated cytosine (5meC) and di-methylated histone H3 on lysine K27 (H3K27me2) were analyzed, as the DNA and histone markers of heterochromatin establishment and chromatin stability. In addition, an *in vivo* fertilization assay was performed as a marker of the fertilization ability of oocytes.

There was an increased number of MII oocytes flushed after hormonal stimulation following BPS1, BPS3 and BPS4 treatments; however, these differences were not statistically significant (Fig. 5A). The 5meC and H3K27me2 were immunolocalized in MII oocytes thereafter, and the signal intensity was related to the control oocytes (Fig. 5B). Although 5meC was not affected after BPS treatment compared with the VC group, differences among individual BPS treatment groups were observed (Fig. 5C). In contrast to 5meC, the methylation of H3K27 was affected after BPS3 and BPS4 treatment (Fig. 5D). In addition to oocyte epigenetic quality, the fertilization rate (fertilized oocyte/two-cell embryo ratio) was affected after BPS exposure. Decreasing fertilization rate in dose-dependent manner is intimated; however, only BPS3 showed statistically significant decline of 19.2%. On the contrary, BPS4 significantly increased the fertilization rate. The number of two-cell embryos

Figure 4 Incidence of spindle misalignment and abnormal chromosome segregation of *in vivo*-produced oocytes following bisphenol S (BPS) treatment. Abnormal oocytes, including abnormal spindle and/or chromatin in metaphase plate (A), were counted and quantified (B). Groups BPS1–BPS4 were tested against the vehicle control (VC) group for significant differences in the proportion of abnormal findings, that is abnormal oocytes in general, abnormal spindle and abnormal chromatin alignment. Fisher's exact test was used; one star indicates significance at the basic level ($P < 0.05$), two stars indicate significance after Bonferroni correction for quadruple testing ($P < 0.0125$) and non-significant results are not shown. Observed numbers of oocytes with normal and abnormal phenotype are stated inside the appropriate columns. The width of the columns is proportional to the number of oocytes analyzed in each group (number of flushed females = 3 per each experimental group). In addition to spindle α -tubulin, pericentrin (PCNT) on the poles of the spindle was tracked. Arrowheads indicate abnormal formation of PCNT on spindle poles (C). Scale bars represent 25 μ m.

flushed per mouse did not show significant differences (Figure 5E).

Discussion

Our study provides the first evidence of the effect of BPS on mammalian general ovarian morphology and physiology, including folliculogenesis, responsiveness to hormonal stimulation and the quality of *in vivo*-matured oocytes. Tested doses (0.001–100 ng.g/bw/day) with observed BPS effects are very low, comparable to established values for the TDI (50 ng.g/bw/day), no-observed-adverse-effect level (5 μ g.g/bw/day) or low-observed-adverse-effect level (50 μ g.g/bw/day) for BPA (Tyl 2009). Moreover, there was no possibility of strictly testing BPS with regard to these values, because they

have not yet been established. Tested very low doses of BPS represent real environmental exposure levels of people (Liao *et al.* 2012a,b, Rocha *et al.* 2015) and deserve rigorous attention.

We simulated the heterogeneity in the human population by using hormonally unstimulated mouse females. Although mice were in various stages of the estrus cycle, we observed a significant reduction in the total number of activated primary and preantral follicles after animals were exposed to various doses of BPS. The decrease in the number of follicles was accompanied by an increased volume of antral follicles, which could be a compensatory reaction, as the follicle population is regulated by follicular interactions, perhaps by a quorum sensing mechanism (Bristol-Gould *et al.* 2006). This compensation seems to have its limits, because

Figure 5 The effect of bisphenol S (BPS) on *in vivo* oocyte production, oocyte fertilization ability and epigenetic quality. Efficiency of *in vivo* flushing of matured oocytes (number of matured oocytes in metaphase II (MII) oocytes per one mouse) after BPS treatment, followed by gonadotropin stimulation (A). Immunostaining (B) and relative signal intensity (vehicle control (VC) = 1) of 5-methylcytosine (5meC) (C) and H3K27me2 (D) in *in vivo*-matured oocytes. 5meC and H3K27me2 data are expressed as means. The boxplot shows 25% and 75% quantiles, as well as 5% and 95% quantiles of observed it in the groups. One star indicates significance at the basic $P \leq 0.05$ level (C and D). Scale bar represents 25 μ m. Fertilization rate of ovulated oocytes was recorded after *in vivo* fertilization assay (E). Groups BPS1–BPS4 were tested against the VC group for significant differences in the proportion of fertilized oocytes (fertilization rate) using Fisher's exact test (one star indicates significance at the basic level $P < 0.05$, two stars indicate significance following Bonferroni correction for quadruple testing, $P < 0.0125$, non-significant results are not shown). Numbers of non-fertilized oocytes (no fertilization) and 2C embryos are stated inside the appropriate columns. The width of the columns is proportional to the number of flushed oocytes/2C embryos for the particular group. Numbers of flushed females are indicated in brackets. There is no difference in the number of flushed two-cell (2C) embryos per mouse.

at higher doses of BPS, we observed a decreased volume of antral follicles, which could be associated with suppression of their development (Altunkaynak *et al.* 2016). These findings are supported by hormone profiling and the observed decrease of β 17-estradiol in blood serum (Supplementary results). Although BPS is considered to be an analogue of BPA, the effects of the two bisphenols could differ in many aspects, as Moore-Ambriz *et al.* (2015) did not observe effects of BPA on histological features of ovaries after comparable dosing of mice. Contrary to our findings, which did not reveal an increase in follicle atresia after BPS treatment, Soleimani Mehranjani and Mansoori (2016) observed

an increased number of atretic follicles after treatment of mice with BPA.

Because the effect of BPS on ovaries and folliculogenesis was observed even at a low dose (0.1 ng.g/bw/day), the proteome of hormonally unstimulated ovaries after BPS2 treatment followed. The decreasing level of SOD suggests a limited ability to be resistant to oxidative stress, similar to the previously described suppression of SOD expression and activity after treatment of bisphenols (Maćczak *et al.* 2017, Rahman *et al.* 2017). This assumption is further supported by our observation of the affected expression of proteins associated with basal metabolism

(aldo-keto reductase, hydroxymethylglutaryl-CoA synthase and phosphoglycerate kinase) and protein-glutamine γ -glutamyltransferase, pro-apoptotic and inflammatory markers. In addition to cellular stress, based on BPS-wide proteomic screening, the factors belonging to steroidogenesis, such as 7-dehydrocholesterol reductase, apolipoprotein and lipid transfer protein, were altered. In particular, 7-dehydrocholesterol, involved in steroid metabolism (Shackleton 2012), was significantly decreased after BPS1, BPS2 and BPS3 treatment. This effect can be considered as being a feature of endocrine disruption, with respect to the significant decrease of circulating 17β -estradiol, mentioned earlier. This assumption is in accordance with BPA-disrupted steroidogenesis and estradiol biosynthesis (Peretz *et al.* 2011), as well as the observation of BPA-comparable estrogenic activity of BPS (Grignard *et al.* 2012). In addition, BPS treatment decreased the amount of protein-arginine N-methyltransferase 1, and post-translational modifications of various proteins, including factors essential for fertilization and early embryonic development (Panamarova *et al.* 2016), are considerably affected. This observation confirms the likelihood of BPS-mediated disruption of epigenetic modulation. In addition to steroidogenesis and epigenetics, cytoskeletal and associated factors (tubulin α -1B chain, talin-1, Ser/Thr-protein phosphatase 2A) are affected and considerably affiliated with other findings on cytoskeletal structures, accordingly with previous studies on BPA (Rahman *et al.* 2016).

Although the follicle populations were mostly reduced after BPS treatment and proteomics were significantly affected, the responsiveness on gonadotropin stimulation expressed by the number of flushed *in vivo*-matured oocytes was unchanged. Similar to oocyte number, the number of flushed two-cell embryos did not show a significant change in mice treated with BPS. This could indicate that superovulatory treatment still permits recruitment of a satisfactory number of follicles from a reduced follicular population. However, in humans, there is a reduced response to ovarian stimulation in women with reduced population of antral follicles in ovaries (Hendriks *et al.* 2005). The quality of flushed embryos was not determined in our study. Because we did not observe fragmented embryos, it does not appear that apoptosis poses a problem in these very early stages of embryonic development (Kamjoo *et al.* 2002, Tarín *et al.* 2002). On the other hand, we can suggest impaired quality of embryos with later manifestations as a result of detrimental BPS effect on oocytes (Krisher 2004, Sirard *et al.* 2006). Further experiments are needed to follow the effects of BPS exposure on embryo quality.

An epigenetic effect of EDs is one of their well-defined molecular mechanisms. Accordingly, we analyzed DNA and histone methylation, tracking 5mC and dimethylation of lysine K27 on histone H3

(H3K27me2), respectively. Changes in signal intensity of H3K27me2 were observed after BPS3 and BPS4 treatment, demonstrating an epigenetic mode of BPS action. Our observations are in accordance with previous studies describing BPA-affected methylation of core histones H3 and H4 in oocytes (Trapphoff *et al.* 2013, Wang *et al.* 2016). This suggests that BPS-modulated epigenetically derived gene imprinting occurs, as previously demonstrated for BPA (Trapphoff *et al.* 2013). In general, although the quantity of flushed oocytes and embryos are not affected, the quality of the cells can be influenced with numerous consequences in subsequent processes, such as fertilization, embryonic development and/or gametogenesis of offspring where transgenerational inheritance can occur (Dolinoy *et al.* 2007, Ziv-Gal *et al.* 2015). This assumption is supported by the aforementioned BPS3/BPS4-shifted fertilization rate.

Observed aberrant spindles of BPS-derived *in vivo*-matured oocytes are in accordance with the previously described negative effect of BPA on the meiotic spindle of mammalian oocytes (Hunt *et al.* 2003, Can *et al.* 2005, Viñas *et al.* 2010, Wang *et al.* 2016, Zhang *et al.* 2017). So far, the BPS destructive effect on oocyte spindles has been described *in vitro* (Žalmanová *et al.* 2017). In addition to spindle α -tubulin, PCNT was tracked and its disruption was detected. In parallel with our observation, abnormal BPA-induced localization of PCNT has been described (Eichenlaub-Ritter *et al.* 2008). However, the molecular mechanism remains unknown and, based on differences in the BPS-dose-dependent pattern of PCNT localization, the BPS effect seems to be dose-specific. In accordance with a previous study (Jiao *et al.* 2017), it is likely that there are BPS-targeted proteins belonging to the cytoskeleton and to up-stream factors leading to cytoskeleton formation. In addition to the effects on cytoskeletal proteins, abnormalities in chromosome segregation were observed and the mechanism of chromosome alignment is presumably affected, similarly to previous BPA studies (Hunt *et al.* 2003). Nevertheless, further experiments focused on the assessment of chromosome alignment are needed.

As a group, higher BPS concentrations (BPS3 and BPS4, i.e. 10 and 100 ng.g/bw/day) seem to be targeting steroidogenesis. In addition to this hormonal effect, BPS3/4-modulated epigenetics is clear, and the changes in hormonal signalization, epigenetic changes and fertilization capability are clearly connected. However, the ambivalence of the BPS effect on fertilization capability notes a difference in the molecular mechanism, even between BPS3 and BPS4. In contrast to BPS3 and BPS4, the dose of BPS2 (0.1 ng.g/bw/day) led to cytoskeletal damage with clear manifestations. The further impact of different forms of BPS-damaged oocytes on embryonic development remains to be elucidated.

Conclusions

In conclusion, this study is the first thorough evaluation of female reproduction following *in vivo* BPS treatment. In comparison with BPS-substituted BPA, BPS shows comparable biological effects even at much lower doses. Moreover, BPS is chemically more stable, making it worse in terms of biodegradability than BPA, and it shows higher levels of dermal penetration than BPA (Ike *et al.* 2006, Danzl *et al.* 2009, Liao *et al.* 2012a). Therefore, our conclusions indicate the necessity to estimate BPS intake in human populations and to assess the risk of ongoing BPS exposure to public health.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-18-0092>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was supported by the Charles University Research Fund (Progres Q39) and the National Sustainability Programme I (NPU I) Nr. LO1503 provided by the Ministry of Education, Youth and Sports of the Czech Republic, European Human Biomonitoring Initiative HBM4EU provided by H2020; T Z, K H and J P were supported by the National Agency of Agriculture Sciences (NAZV QJ1510138) and the Czech Ministry of Agriculture (MZeRO 0714).

Acknowledgements

We would like to thank Markéta Šlajerová for her assistance with experiments and Prof. Sarah Leupen for manuscript editing.

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Received 21 February 2018

First decision 9 April 2018

Revised manuscript received 17 April 2018

Accepted 10 May 2018