Přílohy

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PROKEŠOVÁ, Šárka, Kamar GHAIBOUR, František LIŠKA, Pavel KLEIN, Tereza FENCLOVÁ, Miriama ŠTIAVNICKÁ, Petr HOŠEK, Tereza ŽALMANOVÁ, Kristýna HOŠKOVÁ, Hedvika ŘIMNÁČOVÁ, Jaroslav PETR, Milena KRÁLÍČKOVÁ a Jan NEVORAL. Acute low-dose bisphenol S exposure affects mouse oocyte quality. Reproductive Toxicology [online]. 2020, 93, 19-27 [cit. 2022-06-10]. ISSN 08906238. Dostupné z: doi:10.1016/j.reprotox.2019.12.005 **(IF= 3,143)**

Reproductive Toxicology 93 (2020) 19–27

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Acute low-dose bisphenol S exposure affects mouse oocyte quality

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ARTICLE INFO

Keywords:
Endocrine disruptors
Epigenetics
Histone modifications
DNA methylation
Meiotic spindle
Oocyte development

ABSTRACT

Bisphenol S (BPS) is widely used to replace the known endocrine disruptor BPA in various products. We evaluated the effect of acute *in vivo* BPS exposure on oocyte quality, simulating the oral route of exposure via oral gavage. Eight-week-old ICR female mice (N = 15 per experimental group) were exposed to vehicle or BPS1–BPS4 (0.001, 0.1, 10, and 100 ng BPS x g bw^{-1} day^{-1}, respectively) for seven days. Oocytes were isolated and matured *in vitro*. We observed that BPS exposure increased aberrant spindle formation in mature oocytes and induced DNA damage. Moreover, BPS3 significantly increased the chromatin repressive marks 5-methyl cytosine (5meC) and H3K27me2 in immature oocytes. In the BPS2 group, the increase in 5meC occurred during oocyte maturation. Transcriptome analysis revealed differential expression of early embryonic development transcripts in BPS2-exposed oocytes. These findings indicate that the biological effect of BPS is non-monotonic, affecting oocyte quality even at concentrations that are orders of magnitude below those measured in humans.

1. Introduction

Bisphenols are widely used in day-to-day consumer products including paper, cans, and baby bottles [1,2]. The most widely used bisphenol, bisphenol A (BPA), constitutes an endocrine disruptor with numerous deleterious effects on public health [3]. Further, very low (e.g., subtoxic) doses negatively affect health with a nonlinear effect [4,5]. These findings have led to BPA elimination based on tolerable daily intake (TDI) thresholds, including 25 ng x g body weight $(bw)^{-1}$ day^{-1} as determined by the United States Food and Drug Administration in 2014 [6] and more strictly as 4 ng x g bw^{-1} day^{-1} by the European Food Safety Administration in 2015 [7], following a complete ban on BPA in children's items and additional 'BPA-free' products by the latter agency in 2013 [8]. However, the resulting products are not truly bisphenol-free.

In particular, Bisphenol S (BPS) has become the most widely used replacement for BPA because it is more chemically stable and economical to use [9]. Although released into the environment in lower amounts than BPA [10], BPS is often detected in the environment, including in the air, water, food, and/or house dust [11]. Accordingly, increasing human BPS exposure has been confirmed [12]. Analogous to well-known BPA exposure routes, BPS enters the human body via ingestion, inhalation, or dermal contact [13]. Following entry into the body, BPS is rapidly metabolised and gradually excreted. However, despite such intensive BPS clearance, repeated acute human exposure is probable owing to the high stability and prevalence of BPS in the environment [14].

The body responds to endocrine disruptors in a nonlinear manner,

Abbreviations: 5meC, 5-methyl cytosine; BPA, bisphenol A; BPS, bisphenol S; bw, body weight; DAPI, 4′,6-diamidino-2-phenylindole; GV, germinal vesicle; GVBD, germinal vesicle breakdown; IBMX, isobutyl-methylxanthine; MII, metaphase II; NSN, non-surrounded nucleolus; PBS, phosphate buffered saline; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; SN, surrounded nucleolus; TDI, tolerable daily intake; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labelling

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https://doi.org/10.1016/j.reprotox.2019.12.005
Received 20 September 2019; Received in revised form 11 December 2019; Accepted 19 December 2019
Available online 24 December 2019
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with paradoxically stronger effects at lower doses [5]. Recent BPS exposure in the general population ranges from 0.01 to 10 ng/mL in several body fluid types including the blood, urine, breast milk, and follicular fluid [15–17]. The highest levels occur among individuals with occupational exposure [18], with an estimated daily intake of 0.06–1.7 ng × g bw^{-1} [11,19]. The biomonitoring data indicate a possible risk of such low doses for human health if the negative effects of BPS replicate those of BPA.

Notably, the reproductive system is a unique indicator of detrimental impact consequent to bisphenols and other hormonal disruptors in the environment owing to their stimulation of estrogenic signalling [20], alteration of post-translational modifications of functional proteins [21], and epigenetic shift in germ cells [22], which opens the possibility of transmitting the effect into subsequent generation(s) [23,24]. The female reproductive system is particularly extremely sensitive to environmental stress because the oocyte pool is not renewed. Furthermore, multiple features in this system may be adversely affected, thus impacting fertilisation and embryonic development [25]. Specifically, the two physiological checkpoints where oocyte meiosis is arrested represent critical exposure windows of oocyte susceptibility. Chromatin assembly initially occurs in germinal vesicles (GVs) in immature oocytes and is arrested at the meiotic prophase checkpoint [26]. Mouse prophase oocytes isolated from antral follicles in the first critical window may possess two chromatin configuration types. The first is the non-surrounded nucleolus (NSN) configuration, with more dispersed chromatin and high transcriptional activity characteristic of growing oocytes. The second is the transcriptionally inactive surrounded nucleolus (SN) configuration, which is attained upon oocyte growth and exhibits more condensed chromatin, with a significant fraction concentrated around the nucleolus [27]. Thus, fully grown oocytes isolated from antral follicles represent a non-homogenous population, in which oocytes have either NSN-type or SN-type chromatin conformation [28]. On such a background, epigenetic GV marks constitute a precise tool to evaluate oocyte health because oocyte chromatin uniqueness results from transcriptional silencing and many endocrine disruptors have an epigenetic mode of action [29,30]. Moreover, oocyte maturation, fertilisation, and embryonic development are fully dependent on the oocyte cytoplasmic mRNA pool and organelles. Contrary to the *status quo* description of immature oocytes, chromatin dynamics during oocyte maturation is essential. The chromatin changes necessary for successful fertilisation and embryonic development facilitate 1) restarting meiosis after the first checkpoint, manifested by GV breakdown (GVBD); 2) proper chromosome segregation and reduction to haploid order, leading to polar body extrusion; 3) chromosome alignment in the metaphase II (MII) plate; and 4) establishment of the second meiotic checkpoint and chromatin integrity as an outcome of DNA damage [31,32].

In mammalian oocytes, very low BPS doses cause various deleterious impacts in pig oocytes matured *in vitro* [33] and in mouse oocytes *in vivo* [34]. In these studies, BPS exposure causes meiotic spindle formation failure, improper chromosome alignment, and alters oocyte oestrogen receptor expression and distribution [33]. In our previous study, we demonstrated that long-term exposure to very low BPS doses causes decreased antral follicle size and number in female mice [34]. In addition to causing cytoskeletal changes in the oocyte spindle, BPS exposure results in inadequate genome-wide epigenetic changes [34]. However, the cytoskeletal and epigenetic disturbances in oocytes consequent to doses present in human populations remains unclear.

Therefore, the aim of this study was to assess both cytoskeletal and chromatin changes in oocytes following *in vivo* exposure, using appropriate markers. Moreover, mRNA analysis was performed to profile transcriptome-wide changes and predict the impact of BPS-affected oocytes on subsequent fate. Accordingly, we simulated acute oral exposure with wide-range subtoxic BPS doses *in vivo*, causing immature GV oocytes to arrest at the first meiotic checkpoint. To track the hormonally disruptive effects of BPS, we assessed the quality of immature oocytes, spindle assembly, chromatin integrity, and epigenetic modifications in mice without hormonal stimulation.

2. Materials and methods

2.1. Reagents

All chemicals were purchased from Sigma-Aldrich (USA) unless stated otherwise.

2.2. Animal use and housing

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. Six- to seven-week-old female ICR mice were purchased from Velaz Ltd. (Czech Republic), housed in intact polysulphonate cages, and maintained in a facility with a 12 h light/dark cycle, a temperature of 21 ± 1 °C, and a relative humidity of 60 %. A phyto-oestrogen-free diet (1814 P; Altromin, Germany) and ultrapure water (in glass bottles, changed twice per week) were provided *ad libitum*. Animals were allowed to acclimate for at least one week prior to initiation of treatment.

2.3. BPS exposure experiments

Animals (N = 75) were randomly separated into five experimental groups of 15 animals per group treated with one of four different BPS doses (0.001, 0.1, 10, and 100 ng BPS x g bw^{-1} day^{-1}, hereafter termed BPS1, BPS2, BPS3, and BPS4, respectively), and vehicle (see below). Experiments were performed in five independent replicates. BPS was dissolved in 50 μl 50 % glycerol containing 0.1 % dimethylsulphoxide and administered daily for seven days by oral gavage. After the exposure period, mice were euthanised by cervical dislocation and their ovaries were collected for further experiments.

2.4. Oocyte collection and in vitro maturation

Ovarian follicles were punctured using 27 gauge needles. Immature oocytes in the GV stage were collected and manipulated in M2 medium supplemented with 100 μM isobutyl-methylxanthine (IBMX), a specific endogenous phosphodiesterase inhibitor, to maintain intact GV oocytes [35]. Fully grown immature oocytes with intact GVs were placed in M16 culture medium with IBMX and allowed to recover their oocyte pool of proteins for at least 1 h at 37 °C and 5 % CO_2. Thereafter, oocytes were fixed in 4 % paraformaldehyde in phosphate buffered saline (PBS), supplemented with 0.1 % polyvinyl-alcohol, for 30 min at room temperature (22 °C), and stored at 4 °C until further usage. Alternatively, recovered GV oocytes were cultured in IBMX-free M16 culture medium for 16 h at 37 °C and 5 % CO_2 to obtain matured MII oocytes. Matured oocytes with extruded polar bodies were fixed and stored as described above.

2.5. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay

Fixed MII oocytes were permeabilised in 0.1 % Triton X-100 in PBS containing 0.05 % NaN_3 for 40 min. The oocytes were treated with fluorescein-conjugated dUTP and terminal deoxyribonucleotidyl transferase enzyme (In Situ Cell Death Detection Kit, cat. No. 11684795910, Roche, Germany) for 1 h in the dark at 37 °C. The positive control was prepared using a DNase I kit (AMP-D1, Sigma-Aldrich). Finally, the oocytes were mounted onto slides with Vectashield medium plus 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., USA). Signal intensity was measured using ImageJ software (National Institutes of

Health, USA).

2.6. Immunocytochemistry and image analysis

Fixed oocytes were permeabilised in PBS containing 0.04 % Triton X-100 and 0.3 % Tween-20 for 15 min. Oocyte heterochromatin marks were evaluated including 5′-methyl cytosine (5meC) and dimethylation of histone H3 on lysine K27 (H3K27me2). 5meC-H3K27me2 co-staining was performed using HCl and trypsin as previously described [36]. Then, oocytes were blocked in 1 % bovine serum albumin in PBS with Tween 20 for 15 min and incubated with anti-α-tubulin (1:200, Sigma-Aldrich) or a cocktail of anti-5meC (1:200, Sigma-Aldrich) and anti-H3K27me2 (1:200, Abcam, UK) antibodies. After washing, the oocytes were incubated with a cocktail of anti-mouse and anti-rabbit AlexaFluor 488 and 647 (1:200) antibodies, respectively. Phalloidin (1:200; Thermo Fisher Scientific, USA) was added to washes and used for β-actin visualisation. Stained oocytes were mounted onto slides in Vectashield medium with DAPI. Signal intensity was measured using ImageJ software. Chromatin configuration around the nucleolus (SN, NSN [37]) and extrusion of the polar body were also evaluated. Images were acquired using an Olympus IX83 spinning disc confocal microscope (Olympus, Germany) and VisiView software (Visitron Systems GmbH, Germany).

2.7. RNA isolation

For each sample, 50 oocytes were collected in TRIzol reagent (Invitrogen, USA) and homogenised using a TissueLyser LT (Qiagen, The Netherlands) for 5 min. Chloroform was used for phase separation, and the aqueous phase was mixed 1:1 with 70 % ethanol. RNA was purified using RNeasy MinElute spin columns (Qiagen) and quantified by quantitative reverse transcription-polymerase chain reaction (qRT-PCR; see section 2.8). Integrity was assessed using a 2100 Bioanalyzer and an RNA 6000 Pico Kit (Agilent, Germany).

2.8. qRT-PCR

Total RNA was reverse transcribed using SuperScript III (Thermo Fisher). Mouse phosphoglycerate kinase (*Pgk1*) cDNA was amplified using the following primers: Pgk1_c140F: 5′ GGTGTTGCCAAAATGTC GCT 3′ and Pgk1_c186R: 5′ AACGGACTTGGCTCCATTGT 3′; 186 bp amplicon size. The amplification reaction was performed in an Applied Biosystems 7900 H T thermal cycler using PowerUp SYBRGreen master mix (Thermo Fisher). *Pgk1* was selected from among three possible housekeeping genes based on stability and high oocyte expression. RNA samples were diluted to equal concentrations based on the relative *Pgk1* quantity (computed using the $2^{-\Delta C_t}$ method).

2.9. Microarrays

Transcriptome expression analysis was performed with four control samples and four BPS-exposed samples using Affymetrix Mouse Gene 2.1 ST Array Strips (USA). Each strip evaluated two controls and two exposed samples. The results were analysed using the Transcriptome Analysis Console (TAC, Affymetrix). The data discussed in this study have been deposited in the NCBI Gene Expression Omnibus [38,39] and are accessible through GEO Series accession number GSE140640 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE140640). We selected quantile normalisation and assessed BPS exposure as the main factor, controlling for scan date (i.e., the strip) as the confounding variable. Statistical differences were tested using two-way analysis of variance (ANOVA), with multiple comparison correction using the Benjamini-Hochberg (false discovery rate) method.

2.10. Statistical analysis

The data were processed using Statistica Cz 12 (StatSoft, Inc., USA). Kruskal–Wallis ANOVA was used for quantitative variables and chi-square tests were used for proportions. Significant differences between individual group pairs were assessed *post hoc* using multiple comparisons of mean ranks, Mann–Whitney U tests with Bonferroni correction, or Fisher's exact tests with Bonferroni correction. Where appropriate, correlations among variables were assessed using Spearman's method. Statistical significance was set at $\alpha = 0.05$, and all reported *P*-values and tests were two-tailed.

3. Results

3.1. BPS effects on oocyte quantity and meiotic ability

We first evaluated the effect of BPS on ovarian capacity and the maturation rate of GV oocytes isolated from BPS-exposed, hormonally-unstimulated female mice. SN and NSN statuses in GV oocytes and the GVBD/maturation rate in mature oocytes were analysed using DAPI-stained oocyte chromatin. We observed that the GV oocyte yield was not affected by BPS treatment (Fig. 1A). Similarly, the SN/NSN ratio did not differ between the control and BPS groups (Fig. 1B). Even the maturation rate and meiotic capability (Fig. 1D) showed no statistically significant differences, suggesting that BPS exposure at the tested levels in oocyte donors has no effect on the general oocyte quantity or quality.

3.2. Effects of BPS exposure on spindle formation in mature MII oocytes

We next assessed metaphase spindle formation of BPS-treated MII oocytes matured *in vitro*. Chromatin features established at the second matured oocyte arrest stage recapitulated both the molecular and cytoskeletal inheritance of immature GV oocytes exposed to BPS *in vivo*; i.e., no significant differences were detected. However, BPS-treated mature oocytes showed unconjugated tubules on the barrel and pole of the spindle and some double metaphase spindles occurred in the BPS2 group (Fig. 2A). Thus, BPS primarily induced spindle damage rather than chromatin misalignment (Fig. 2B–D). Notably, no consistent effect on spindle malformation was observed following 0.1 ng x g bw^{-1} day^{-1} (BPS2) treatment (Fig. 2D), suggesting that low-dose BPS exerts spindle-specific modes of action.

3.3. Effects of BPS exposure on DNA integrity in mature MII oocytes

DNA integrity of BPS-treated MII oocytes matured *in vitro* was also evaluated. TUNEL assays used to analyse DNA double-strand breaks revealed increased abnormal oocytes and elevated abnormal chromosome alignment and/or spindle malformation in all BPS-exposed groups, supporting the deleterious effect of BPS (Fig. 3A). Remarkably, no consistent effect on DNA integrity was detected following 10 ng x g bw^{-1} day^{-1} (BPS3) treatment (Fig. 3B), suggesting that low-dose BPS also exerts DNA-specific modes of action.

3.4. BPS alters H3K27 methylation in immature GV oocytes

Based on the cytoskeletal and chromatin changes in mature oocytes, we investigated epigenetic alterations to GV oocytes following BPS treatment. As genome-wide DNA methylation and histone H3 constitute markers of heterochromatin establishment and chromatin stability, 5meC and H3K27me2 were evaluated on a global level using immunocytochemistry (Fig. 4A). 5meC levels were significantly increased in the BPS3 ($P < 0.05$) and BPS4 ($P < 0.01$) groups compared to the BPS2 5meC level. However, no significant difference was observed compared to control oocytes (Fig. 4B). In comparison to the control, H3K27 dimethylation was increased in BPS3 oocytes (Fig. 4C).

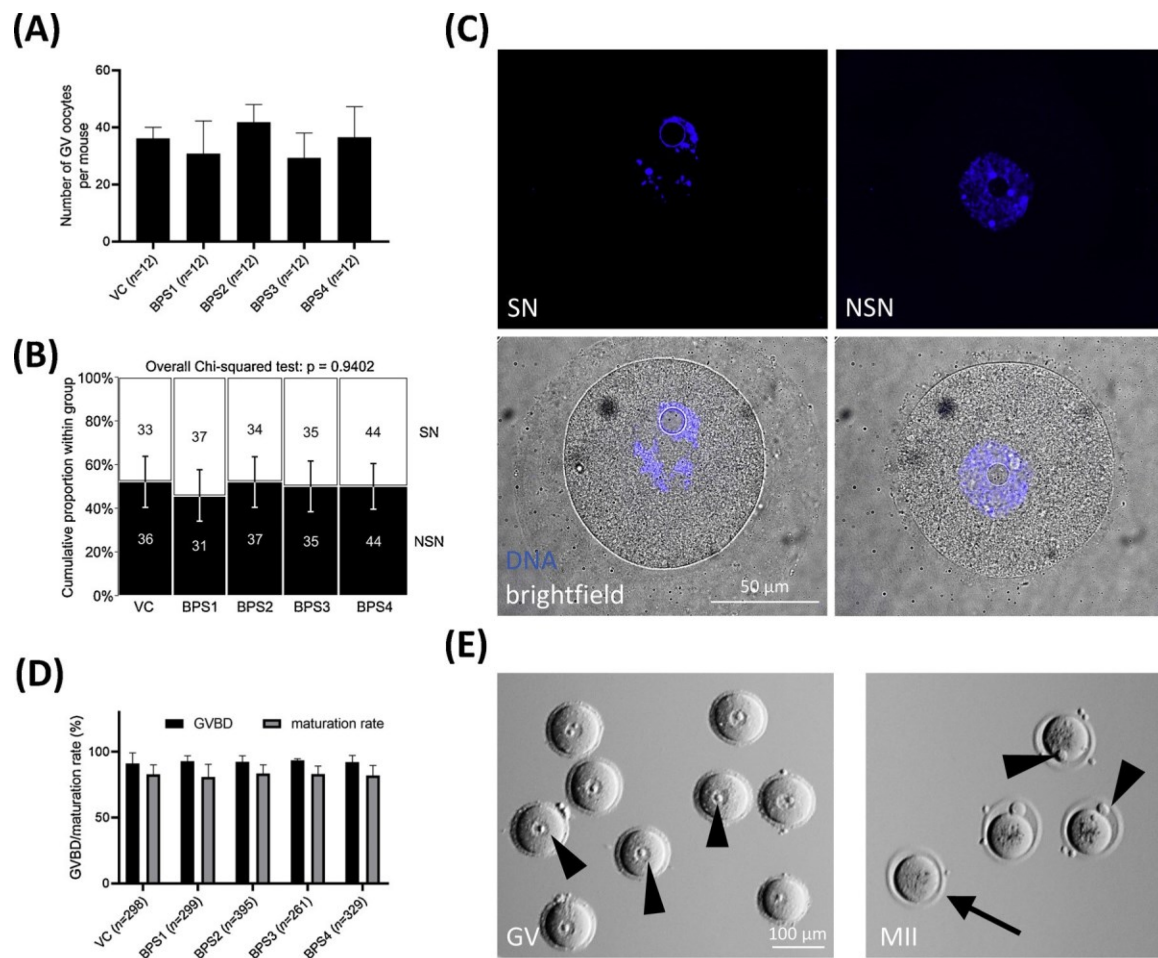

Fig. 1. Quantity and quality of germinal vesicle (GV) oocytes isolated following exposure of female mice to BPS. (A) Oocyte yield based on the number of oocytes isolated from one female. Data are expressed as median, minimum, and maximum lines. Numbers of females from four independent experiments are indicated in brackets. (B) Surrounded (SN) and non-surrounded nucleolus (NSN) proportions from six independent experiments. (C) Representative fluorescent pictures of SN and NSN oocytes in the GV stage. (D) GV breakdown (GVBD) and oocyte maturation rates (median and min–max), indicating GV oocyte capacity to reinitiate meiosis and achieve MII stage, respectively. The numbers of oocytes from five independent experiments are indicated in brackets. (E) Representative pictures of immature GV and matured MII oocytes in transmitted light. The arrow indicates the oocyte having undergone GBVD but not achieving the MII stage. Arrowheads indicate GV and extruded polar body, as markers of immature GV and matured MII oocytes, respectively.

3.5. *BPS modifies genome-wide DNA methylation in mature MII oocytes*

We next examined heterochromatin marks following *in vitro* oocyte maturation. Immunostaining of 5meC and H3K27me2 in metaphase chromosomes of matured oocytes (Fig. 5A) revealed significantly increased ($P < 0.05$) 5meC in the BPS2 group (Fig. 5B). However, H3K27me2 levels were not significantly different compared to those of controls (Fig. 5C). Notably, 5meC levels increased in BPS2 matured oocytes although 5meC of GV oocytes was intact.

3.6. *Microarray analysis of GV oocytes after in vivo BPS2 treatment*

As the altered 5meC levels in matured compared to GV oocytes suggested that upstream epigenetic factors may have been affected at the transcriptional level in immature BPS2 oocytes, we next evaluated the effect of BPS2 on gene expression in transcriptionally silenced GV oocytes. As the number of available samples was limited ($N = 4$ for both control and BPS2 groups), the results did not reach genome-wide significance after correcting for multiple comparisons. However, using arbitrary fold change cut-offs of > 1.5 and $P < 0.01$, 102 genes were up- (89) or downregulated (13) following BPS2 treatment (Fig. 6A and B). These results indicated that genes associated with cellular stress (in particular, *Cldn34b2*, *Gsdmc2*, and *Batf3*) were upregulated following BPS exposure. In contrast to our initial hypothesis, we observed alterations in factors related to embryonic development rather than epigenetic regulators (e.g., DNA methyl transferases and histone methyl transferases) in BPS-treated oocytes. In particular, *Ceacam10, Hist1h2af, Tma16*, and *Raptor* expression, which constitute markers of pre-implantation and embryonic development, were upregulated. High *Tma16* and *Batf3* transcript levels appeared to be indicative of BPS-mediated changes during early embryonic development.

4. Discussion

BPS is currently utilized in many common consumer products, including cases in which legal prohibitions against BPA allow products containing BPS to be labelled ‘BPA free’. Thus, considering that a recent biomonitoring study detected comparable instances of BPS-positivity to those for BPA [40,41] and BPS is more stable under heat and light than BPA [4], testing the biological effect of BPS is necessary to assess the risk to human health from BPS exposure [12].

For the present study, we chose an *in vivo* mouse model of acute BPS exposure. The tested doses were mostly lower than the established values for TDI (4 ng x g bw^{-1} day^{-1}) and much lower than the no-observed-adverse-effect level (NOAEL, 5 μg g bw^{-1} day^{-1}) and low-observed-adverse-effect level (LOAEL, 50 μg g bw^{-1} day^{-1}) for BPA

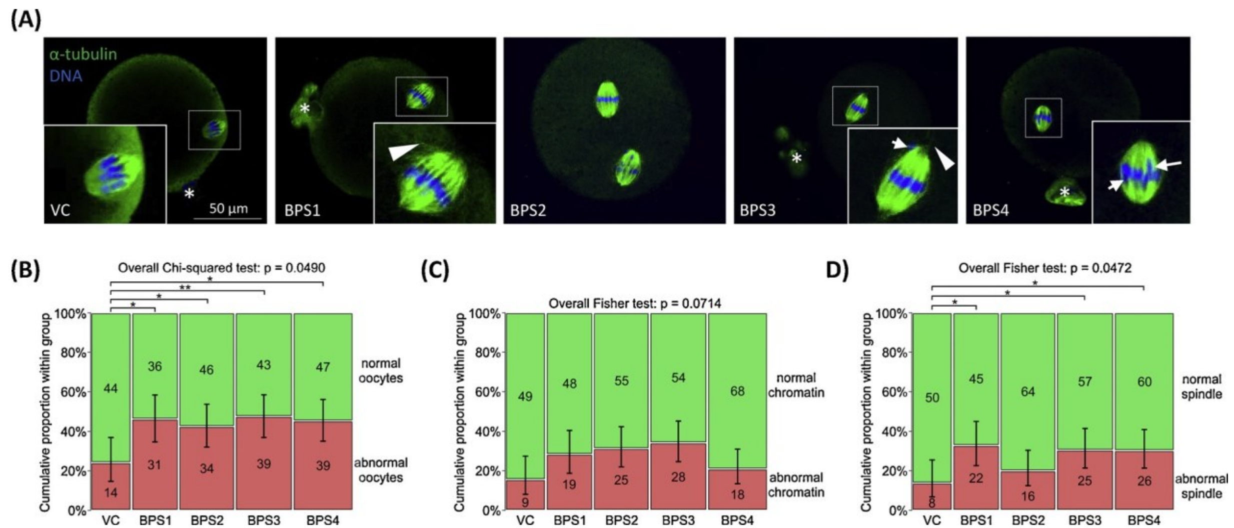

Fig. 2. Meiotic spindle and chromosomal alignment in BPS-exposed oocyte maturation. (A) Representative images of the chromatin and meiotic spindle of mature oocytes. Female mice were exposed to vehicle (VC, vehicle control) or different doses of BPS (BPS1–BPS4). Chromosome misalignment and spindle malformations were assessed based on DNA and α-tubulin staining, respectively. Arrows indicate individual chromatin aberrations, and arrowheads show unconjugated tubules on the spindle barrel and pole. (B–D) Chromatin and spindle malformations were identified in four independent experiments. The changes are expressed as the cumulative proportion of normal to abnormal oocytes, including oocytes with normal *vs*. abnormal chromatin and spindles. The values represent the number of oocytes in each experimental group. Error bars show 95 % confidence intervals for population proportions. Asterisks indicate statistical significance at $P < 0.05$ (*) and 0.01 (**) of pair-wise Fisher exact tests in a post-hoc role after overall significance testing as indicated.

[42]. However, a TDI for BPS has not yet been established, although very low doses of BPS have known risks [4]. Therefore, environmental BPS deserves rigorous attention, particularly in regard to human reproductive health.

We modelled population heterogeneity in the human population using a hormonally-unstimulated outbred mouse strain. To investigate the potential impact of BPS exposure on oocyte meiotic progression, we first evaluated oocyte number, the quality of immature oocytes (SN vs. NSN oocytes), re-initiation of meiotic maturation, and maturation rate. After noting no significant differences in these parameters, we focused on the oocyte quality in subsequent experiments and evaluated molecular markers of oocyte health.

First, the spindle apparatus was tested because it mediates chromosome alignment and segregation. The spindle also acts as the centre of physiologically asymmetric oocyte cytokinesis [35]. Defects in spindle assembly and abnormalities in chromosome alignment can result in meiotic progression failure and subsequently alter embryonic development consequent to fertilisation failure or aneuploidy [43,44]. Meiotic abnormalities in ova (e.g., unequal chromosome segregation) can cause changes in chromosome alignment and/or aneuploidy [45]. Moreover, presumed BPS-targeted cytoskeletal proteins and upstream factors are sensitive to BPS exposure [33]. In our experiments, abnormal oocyte levels were increased, including an increased incidence of abnormal chromosome alignment and/or spindle malformation, in all BPS-treated groups. Therefore, BPS causes spindle damage rather than chromatin misalignment. In particular, irregular spindle microtubule arrangement was the most frequent phenotype observed in our study, highlighting an oestrogen-like BPS effect similar to that reported in a previous study describing oestrogen-affected spindles [46].

Chromatin integrity was also assessed by quantifying double-strand DNA breaks. A deleterious effect of BPS on DNA integrity, indicated by increased TUNEL signal, was detected in BPS1, BPS2, and BPS4 groups.

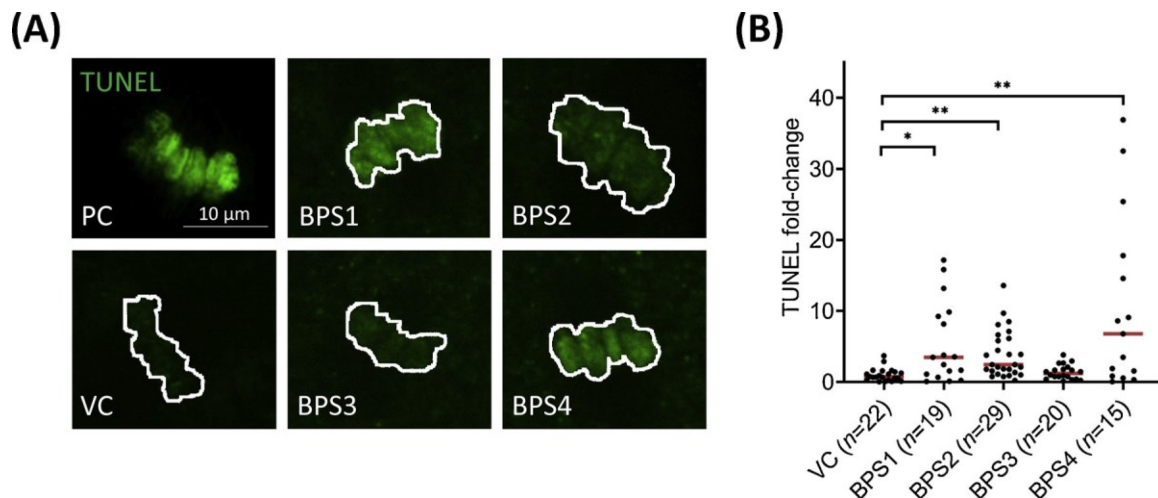

Fig. 3. DNA integrity in BPS-exposed oocyte maturation. (A) DNA integrity was evaluated using TUNEL assays. Pictures are representative of integrated density evaluated using ImageJ. Female mice were exposed to vehicle (VC, vehicle control) or different doses of BPS (BPS1–BPS4). PC: positive control. (B) Statistical differences were tested using a Kruskal-Wallis nonparametric test followed by Dunn's multiple comparison. Centre lines represent medians of individual values (*n*, the number of analysed oocytes, is noted in brackets for each experimental group). Asterisks indicate statistical significance at $P < 0.05$ (*) and 0.01 (**).

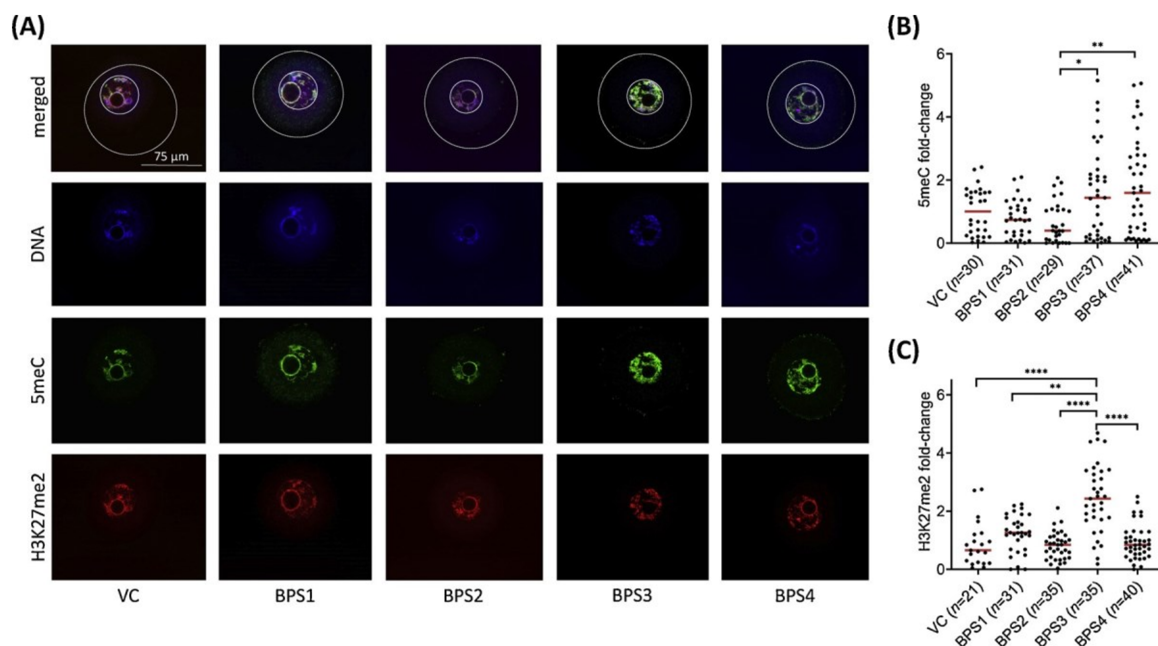

Fig. 4. Genome-wide epigenetic marks in immature germinal vesicle (GV) oocytes. (A) Representative images of 5meC (green) and H3K27me2 (red) chromatin-wide repressive marks in immature GV oocytes. Dashed and solid circles show the oocyte and GV border, respectively. (B, C) Integrated signal density for 5meC (B) and H3K27me2 (C) compared to that of the vehicle control group. Statistical differences were identified using the Kruskal–Wallis nonparametric test followed by Dunn's multiple comparison. Asterisks indicate statistical significance at $P < 0.05$ (*), 0.01 (**), or 0.0001 (****). Centre lines represent medians of individual values (*n*, the number of analysed oocytes, is noted in brackets for each experimental group).

These findings suggested that BPS functions as an endocrine disruptor, as observed in a recent endocrine disruptor study [47] and using a previously-established approach for DNA damage assessment [48]. This finding further underlines a non-monotonic curve in the response to BPS exposure, which was previously described as an endocrine disruption effect [5]. The effects of BPS as an endocrine disruptor support the findings in our reduced hormone stimulation donor mouse model.

In addition to cytoskeletal damage, endocrine disruptors also have a

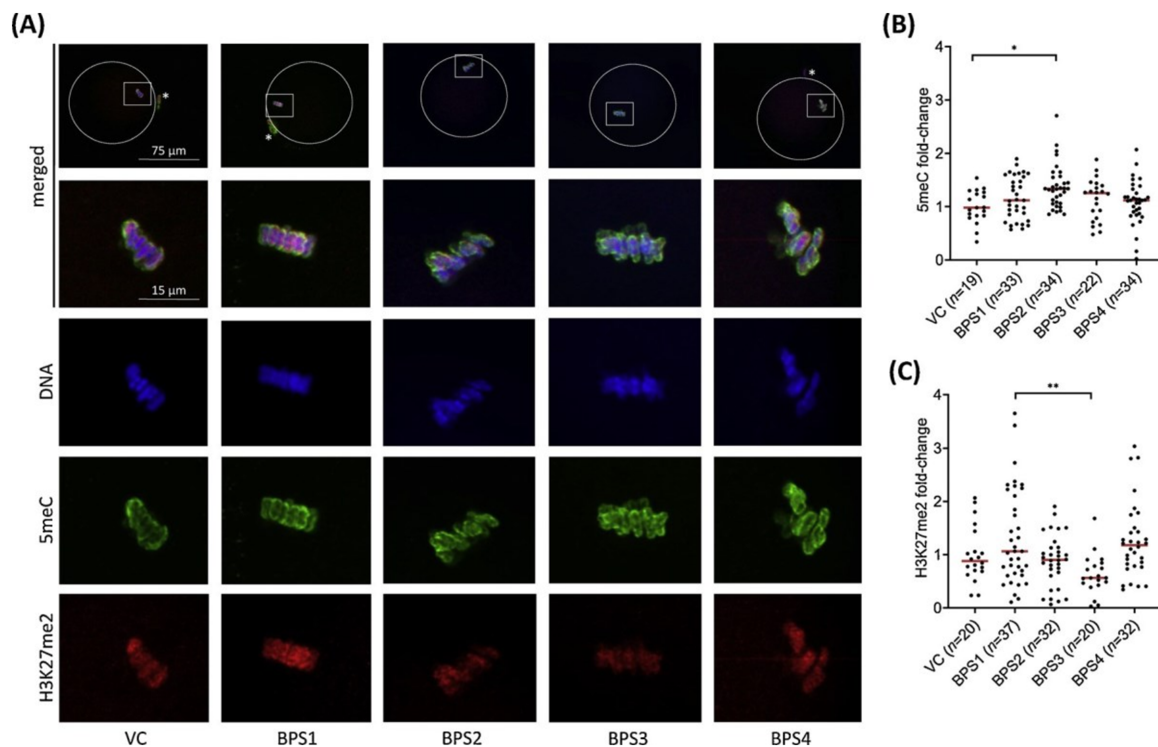

Fig. 5. Genome-wide epigenetic marks in mature MII oocytes. (A) Representative images of 5meC and H3K27me2 in mature MII oocytes. Dashed circle shows the oocyte border. The frame represents the emphasised area of the metaphase chromatin. The extruded polar body, which mark the mature oocyte, is indicated with an asterisk. (B, C) Integrated signal density of 5meC (B) and H3K27me2 (C) was compared to that of the vehicle control group. Statistical differences were tested using the Kruskal–Wallis nonparametric test, followed by Dunn's multiple comparison. Asterisks indicate statistical significance at $P < 0.05$ (*) or 0.001 (**). Centre lines represent medians of individual values (*n*, number of analysed oocytes, are noted in brackets for each experimental group).

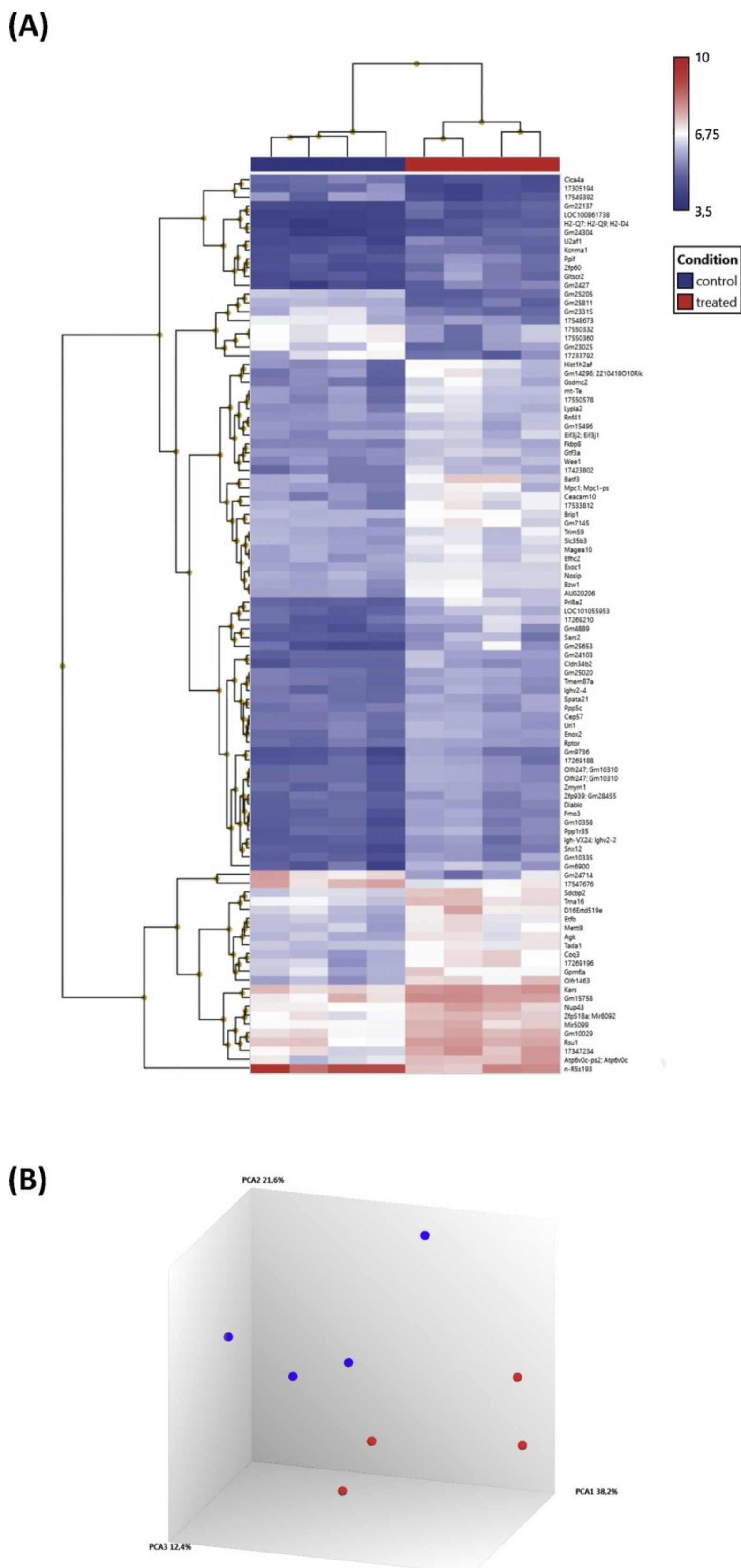

Fig. 6. Effect of BPS2 exposure on gene expression in mouse germinal vesicle (GV) oocytes. (A) mRNA expression heatmap in GV oocytes shows differences in gene expression between the vehicle control (control) and BPS2-treated oocytes. (B) Principal component analysis (PCA) and clustering of individual samples. Blue dots: control group, red dots: BPS2-treated group. Transcriptional profiling was performed using four pooled samples (equal to four independent experiments; 50 oocytes per sample from three animals) of the control and treated groups (i.e. n = 12 animals each).

well-defined epigenetic effect. We found that both genome-wide DNA and histone methylation (i.e., 5meC and H3K27me2), as genome-repressive marks, are vulnerable to disruption by BPS. In immature GV oocytes, H3K27 methylation was increased following BPS3 treatment. In contrast, 5meC levels increased in matured MII oocytes in the BPS2 group. Our observations thus support a previously-observed BPA effect on histone methylation in oocytes [49,50] and the BPS-induced H3K27me2 alterations [34]. However, the epigenetic effect varied with the mode of exposure (acute vs. chronic). The level of H3K27me2 increased following chronic exposure to BPS [34], whereas no effect was observed in present acute-exposure experiments. This discrepancy highlights the potential different targets of acute vs. chronic exposure, particularly preantral/antral follicles and earlier stages (i.e., primordial and/or primary follicles). Taken together, these findings suggest that BPS modulates epigenetically-driven gene expression, similar to BPA [51]. Furthermore, BPS-altered epigenomic programming can persist throughout the lifespan, thereby altering gene expression and possibly increasing disease susceptibility across generations [52].

Based on the findings that BPS2 oocytes also exhibit altered 5meC in matured MII oocytes, we considered GV ooplasmic factors that might underlie the epigenetic shift observed in MII oocytes. Moreover, the BPS2 dose of 0.1 ng BPS x g bw^{-1} day^{-1} is comparable to actual exposures [53] that exert detrimental effects on the cytoskeleton and DNA integrity. Because bisphenol alters epigenetic marks [54], we used microarray-based transcriptome analysis to profile the expression of genes related to genomic features and potential markers of oocyte quality of *in vivo*-exposed GV oocytes. We identified 102 genes as distinctly up- or downregulated following BPS treatment. In contrast to our hypothesis, rather than epigenetics-associated genes, the results suggested preferential biologically meaningful gene upregulation of preimplantation and embryonic development genes, such as *Raptor*, the central component of the mTOR complex 1, which is indispensable for oocytes and subsequent embryonic development [55]. Moreover, the two genes with the highest increased expression levels were *Tma16* and *Batf3*, which respectively encode a transcriptional machinery-associated protein and the basic leucine zipper transcription factor ATF-like 3, an RNA polymerase II proximal promoter sequence-specific DNA binding protein. Both genes regulate gene expression in early embryonic development. Therefore, fertilisation and early embryonic development appear to be highly sensitive to BPS, which may underlie the endocrine disruptor-induced reproductive failure.

In contrast, the transcriptome analysis results indicated that epigenetic factors (e.g., DNA methyl transferases, TET family dioxygenases) in the immature oocyte are likely affected by BPS in various ways other than through the targeting of transcription to exert an eventual epigenetic effect on matured oocyte chromatin. Such alternate BPS-sensitive mechanisms may include protein inactivation and/or inadequate post-translational modifications. Therefore, it is necessary to identify BPS molecular targets and interactions in gametes and early embryos, with the goal to protect human reproductive health. An obvious requirement also exists for further experiments focused on the fertilization and early embryonic development of BPS-affected oocytes, considering the qualitative changes between *in vitro*- and i*n vivo*-matured oocytes, possible counteractions of bisphenols and hormonal stimulation (when assisted reproductive technologies are applied), and the developmental success of embryos following natural or *in vitro* fertilization.

In conclusion, acute exposure to very low BPS doses affects oocyte quality, as evinced by changes in the genome-wide epigenetic code and the transcriptional profile in immature GV oocytes. Moreover, BPS exposure significantly impacted the quality of GV oocytes, particularly spindle formation, DNA integrity, and epigenetic modifications, during meiotic maturation. Our observations therefore indicate that BPS is not a suitable replacement for BPA, and BPS intake should be limited.

Declaration of Competing Interest

None.

Acknowledgements

We would like to thank our students for their help with the laboratory work, and Mr. Lukáš Farhád Mirchi (1st Faculty of Medicine, Charles University) for kind help with data deposition to Gene Expression Omnibus. This study was supported by the Czech Health Research Council (NV18-01-00544); the Charles University Research Fund (Progres Q39); the National Sustainability Programme I (NPU I) Nr. LO1503 provided by the Ministry of Education, Youth and Sports of the Czech Republic (MEYS CR); project No. SVV 02690 awarded by MEYS CR; and project No. CZ.02.1.01/0.0/0.0/16_019/0000787 “Fighting Infectious Diseases“, awarded by MEYS CR and financed by The European Regional Development Fund, European Human Biomonitoring Initiative HBM4EU provided by H2020. Š.P., T.Ž., K.H. and J.P. were supported by the Czech Ministry of Agriculture, institutional support MZE-RO 0718.

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ŘIMNÁČOVÁ, Hedvika, Miriam ŠTIAVNICKÁ, Jiří MORAVEC, Yaroslav KOLINKO, Olga GARCÍA-ALVAREZ, Peter R. MOUTON, Azalia Mariel CARRANZA TREJO, Tereza FENCLOVÁ, Nikol ERETOVÁ, Petr HOŠEK, Pavel KLEIN, Milena KRÁLÍČKOVÁ a Jan NEVORAL. Low doses of Bisphenol S affect post-translational modifications of sperm proteins in male mice. Reproductive Biology and Endocrinology [online]. 2020, 18(1) [cit. 2022-06-10]. ISSN 1477-7827. Dostupné z: doi:10.1186/s12958-020-00596-x **(IF= 5,017)**

Reproductive Biology and Endocrinology

RESEARCH Open Access

Low doses of Bisphenol S affect post-translational modifications of sperm proteins in male mice

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Abstract

Background: Bisphenol S (BPS) is increasingly used as a replacement for bisphenol A in the manufacture of products containing polycarbonates and epoxy resins. However, further studies of BPS exposure are needed for the assessment of health risks to humans. In this study we assessed the potential harmfulness of low-dose BPS on reproduction in male mice.

Methods: To simulate human exposure under experimental conditions, 8-week-old outbred ICR male mice received 8 weeks of drinking water containing a broad range of BPS doses [0.001, 1.0, or 100 μg/kg body weight (bw)/day, BPS1–3] or vehicle control. Mice were sacrificed and testicular tissue taken for histological analysis and protein identification by nano-liquid chromatography/mass spectrometry (MS) and sperm collected for immunodetection of acetylated lysine and phosphorylated tyrosine followed by protein characterisation using matrix-assisted laser desorption ionisation time-of-flight MS (MALDI-TOF MS).

Results: The results indicate that compared to vehicle, 100 μg/kg/day exposure (BPS3) leads to 1) significant histopathology in testicular tissue; and, 2) higher levels of the histone protein γH2AX, a reliable marker of DNA damage. There were fewer mature spermatozoa in the germ layer in the experimental group treated with 1 μg/kg bw (BPS2). Finally, western blot and MALDI-TOF MS studies showed significant alterations in the sperm acetylome and phosphorylome in mice treated with the lowest exposure (0.001 μg/kg/day; BPS1), although the dose is several times lower than what has been published so far.

Conclusions: In summary, this range of qualitative and quantitative findings in young male mice raise the possibility that very low doses of BPS may impair mammalian reproduction through epigenetic modifications of sperm proteins.

Keywords: Male reproduction, Endocrine disruptor, Low dose effect, Bisphenol S, Post-translational modification

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Introduction

Bisphenol A (BPA) is well-documented as an endocrine disruptor with detrimental effects on reproduction [1]; as a result of increasing scrutiny of BPA, there is a broad interest in substitution of alternative bisphenols for human consumption. The most common alternative bisphenol, Bisphenol S (BPS), includes a sulfone group (SO_2) in place of the dimethylmethylene group [C $(CH3)_2$] in BPA [2]. BPS has shown a range of deleterious effects following oral ingestion, inhalation or dermal absorption [3], with the most common route of intake for humans being exposure through contaminated water and food at relatively low doses [4]. To date, however, there have been only limited experimental studies of the possible harmfulness of low BPS doses.

Previous studies of BPS in male rats have reported a range of deleterious effects on hormonal balance, reduced germinal epithelium of seminiferous tubules and increased generation of reactive oxygen species [5, 6]. Recent studies have reported BPS induces epigenetic changes, including alterations in the histone code in oocytes, increased DNA methylation in mouse spermatocytes and changes to transcriptome and proteome of cells in testicular tissue and many other cells types [7–10]. Collectively, these findings suggest BPS may disrupt male reproductive functions through post-translational modifications (PTMs) of nucleic acids and proteins [1, 11, 12] and regulation of transcriptionally silenced spermatozoa [13]. In particular, lysine acetylation and tyrosine phosphorylation of sperm proteins regulate spermatogenesis and sperm capacitation [14–16]. Based on these studies, it is possible that low doses of BPS could modulate male reproduction through PTMs of protein and nucleic acid structure. BPS is classified as an endocrine disruptor and its dose-response is more likely to be nonmonotonic, hence, very-low doses may be more effective than high doses. Therefore, we have chosen wide range of much lower BPS doses than was published before [5, 6]. Using a wide range of low- and very-low doses BPS administered in drinking water for 8 weeks to young adult male mice, we want to determine the effect of BPS doses form the environment. Our findings provide one of the first indications that low doses of BPS regulate PTMs of spermatozoa and lead to possible negative effects on male reproduction.

Material and methods

All chemicals, including BPS (CAS: 80-09-1, cat. No. 103039) were purchased from Sigma-Aldrich (USA), unless stated otherwise.

Animals

All animal procedures were done in accordance with the Protection of Animals against Cruelty (Act No. 246/1992) under the supervision of the Animal Welfare Advisory Committee at the Ministry of Education, Youth, and Sports of the Czech Republic. Adult 7-week-old ICR male mice were purchased from Velaz Ltd. (Prague, Czech Republic), housed in standard cages in groups of 3 and maintained in a 12/12-h light/dark cycle at 21 ± 1 °C with a relative humidity of 60%. Bisphenol contamination was reduced using intact polysulfonate cages and glass drinking bottles. Mice were maintained on a phytoestrogen-free diet (1814P Altromin, Altromin Specialfutter GmbH & Co., Germany) with ultrapure water available ad libitum.

BPS dosage and sample collection

Mice were randomized into four experimental groups and allowed to adapt for 1 week. Vehicle control (0.1% ethanol; VC) and BPS for three treatment groups were administered through drinking water at final concentrations of 0, 0.0038, 3.8, and 380 μg/L, respectively, for 8 weeks (8–16 weeks of age). The following dosages were presumed [0, 0.001, 1, and 100 μg/kg body weight (bw)/day] with actual exposure estimated based on the knowledge of recorded body weight and water intake as previously reported [17]. A wide range of doses and the route of exposure have been chosen appropriate to the real human exposure; doses of experimental animals through the drinking water have been used with respect to the welfare of animals. Hereafter, experimental groups will be stated as BPS1, BPS2 and BPS3.

Nine mice per group were included in three individual independent experiments (n = 36). Animal weights were recorded at the end of the experiments mice euthanised by cervical dislocation. Blood samples were collected by cardiac puncture, and serum was stored at − 80 °C until hormonal assay performance. Left and right testes were collected, weighed, and processed for histology and proteomics, respectively.

Sperm isolation and assessment

From the mice described above, the cauda epididymidis was dissected in 0.5 mL Whitten's medium (Suppl. Table S1), and sperm were allowed to swim out for 30 min. Thereafter, sperm concentration and motility were evaluated using Makler chamber and light microscope (Olympus CKX 41; Germany) equipped with a 10× objective (CAchN NA 0.25). 10 μl of sperm suspension was pipette to the Makler chamber, thereafter spermatozoa were counted in 3 lines, each of 10 squares and divide by 3 to obtain average sperm concentration in million per milliliter. Simultaneously, each spermatozoon across the counted area was identified either as motile or immotile. Accordingly, the sperm motility was expressed as the ratio of motile to immotile spermatozoa. The analysis was performed blindly to avoid bias.

Hormonal profiling

Blood serum samples in three independent experiments (n = 5 mice per group) were assayed with Immunobeads Milliplex MAP kit (HPTP1MAG-66 K, MSHMAG-21 K; Merck Millipore, USA) for the following hormone levels: adrenocorticotropic hormone, follicle-stimulating hormone, growth hormone, luteinising hormone, thyroid-stimulating hormone, cortisol, progesterone, testosterone, triiodothyronine, and thyroxine.

Quantitative and qualitative analyses of testes

One testis from each animal (n = 9 per group) was fixed in Bouin solution, embedded in paraffin wax with random orientation, and sectioned completely into 10-μm-thick slides. The total testis volume, total germ epithelium volume, and interstitium volume were estimated according to the Cavalieri principle [18]. The fractions of spermatogenesis (pre-spermiation stages I–VI; middle spermiation stages VII–VIII; post-spermiation stages IX–XII) were found using the point grid approach [19, 20]. To determine the precision and accuracy of the stereological analysis, the coefficient of error was estimated (Suppl. Tab. S2) [18]. Qualitative analysis of seminiferous tubes was performed according to the methods described by the Society of Toxicologic Pathology [21, 22] to assess the following abnormalities: missing germ cell layers and germ cell depletion, retained spermatids (spermiation failure), multinucleate and apoptotic germ cells, and exfoliation of spermatogenic cells into the lumen. At least 100 seminiferous tubules were evaluated blind to treatment group for each testicular cross section. The quantitative assessment was performed on a Nikon Eclipse Ti-U microscope (Nikon, Japan) equipped with a motorised stage (Prior, UK) using a 10× objective (Plan Fluor, NA 0.3) and Stereologer 11 software (SRC, Biosciences Tampa, FL, USA) for histopathological evaluation was performed using a 40× objective (UPlanFl, NA 0.75).

Western blot

Testicular tissue and sperm were dissolved in lysis buffer (40 mM Tris base, 7 M urea, 2 M thiourea, 4% CHAPS, 120 mM dithiothreitol), enriched with Complete Mini Protease Inhibitor Cocktail (Roche, Switzerland), for 30 min on ice. Sperm samples of three individuals belonging to the same experimental group were pooled. Thereafter, samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis on 4–15% separating Mini-PROTEAN precast gels and blotted using a Trans-Blot Turbo Transfer System onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, France). The membranes were blocked in 1% bovine serum albumin in TBS with 0.5% Tween-20 for 60 min at room temperature and incubated overnight at 4 °C with primary antibodies diluted in blocking buffer. The following primary antibodies were used: anti-acetyl lysine antibodies (cat. no. ab80178; Abcam, UK), anti-phospho-tyrosine antibodies (cat. no. ab10321; Abcam), anti-acetylated α-tubulin antibodies, and anti-γH2AX antibodies. Mouse monoclonal anti-α-tubulin antibodies (cat. no. T6199; Sigma, St. Louis, MO, USA) and rabbit monoclonal anti-histone H3 antibodies (cat. no. D1H2; Cell Signaling Technology, Danvers, MA, USA) were used as the loading control for γH2AX and acetylated α-tubulin, respectively. Horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse or anti-rabbit IgG; dilution: 1:15,000; Invitrogen, Carlsbad, CA, USA) were applied for 60 min at 22 °C. Target proteins were visualised using ECL Select Western Blotting Detection Reagent (GE Healthcare Life Sciences, UK) and a ChemiDoc MP System (Bio-Rad). Alternatively, proteins were visualised using a colorimetric Opti-4CN substrate kit (Bio-Rad), followed by matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS) for peptide detection in the dissected bands.

Proteome profiling

Testis lysates from animals in the experimental groups were collected for complete proteomic analysis. Nano-liquid chromatography-MS (nano-LC-MS) was used for protein identification and quantification, as described previously [7]. The acetylome and phosphorylome were analysed separately.

Statistics

The data were processed with GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA). Based on Shapiro-Wilk normality distribution tests, analysis of variance (ANOVA) and Kruskal-Wallis tests were used for normally and non-normally distributed data. In cases of significant overall findings, differences between individual group pairs were assessed by Tukey's and Dunn's post-hoc tests, respectively. Results with P less than 0.05 were considered statistically significant. Normally and non-normally distributed data were expressed as means and medians, respectively.

Results

Hormonal profiles and sperm features of BPS-treated males

At the end of 8-week exposure to actual doses of BPS, the body and testes weights were recorded and relative testes weights (mg/g bw) were determined. There were no differences between the experimental groups and the vehicle control (Table 1). Hormonal assays showed no significant differences in plasma hormone levels between the BPS-treated and vehicle control groups (Suppl. Table S3). Moreover, the spermatozoa

Table 1 Characteristics of experimental animals

	VC	BPS1	BPS2	BPS3
Weight of mouse body (g)	41,82 ± 0,72[a,b]	42,44 ± 0,77[a,b]	45,24 ± 1,36[a]	41,31 ± 1[b]
Relative weight of testes (mg/g of bw)	12,81 ± 0,30	12,76 ± 0,15	10,87 ± 0,37	11,03 ± 0,17

Body and relative testis weights are shown as means ± SEM of animals included in the study ($n = 9$ per experimental group). One-way ANOVA was followed by Tukey's multiple comparison tests. Different letters in the same row indicate significant differences ($p < 0.05$). *VC* vehicle control, *BPS1–3* increasing doses of bisphenol S

count was not affected by BPS exposure (Fig. 1a), although treatment with 0.001 μg/kg bw BPS1 decreased the portion of motile spermatozoa (Fig. 1b).

Higher BPS exposure induced abnormal testicular histopathology

Histological assessment was performed to evaluate the impact of actual BPS doses on testicular tissues. Stereological analysis showed no differences between groups in terms of testis volume, germinal epithelium volume (Fig. 2a, b), interstitium volume, germ layer volume fraction, and interstitium volume fraction. To investigate the effects of BPS treatments on spermatogenesis, individual stages of the seminiferous epithelium were identified and no differences between experimental groups were found (Fig. 2c, c'). Histopathological analysis of testicular tissues from BPS-exposed male mice showed an increased incidence of abnormalities in mice treated with the highest BPS dose (BPS3; Fig. 2d). In addition to vacuolisation of germ layer cells and enlarged multi-nuclear germ cells, the atypical residual bodies demonstrated the effects of BPS3 on testicular tissues (Fig. 2d–g). There were fewer mature spermatozoa in the germ layer in the BPS2 experimental group (Fig. 2h). Representative images of individual histopathologies are shown (Fig. 2d'–h').

Proteomic analysis of testicular tissue

Based on the different modes of action of BPS at various doses, whole-proteome profiling of testicular tissues was performed. In total, 3044 proteins were detected. Unique protein expression in the control and BPS-treated groups is shown in the Venn diagram in Fig. 3a. However, after quantification of the levels of 1886 proteins, followed by subsequent principle component analysis (PCA), no distinct clusters of mice ($n = 24$) from individual groups were observed, thus indicating a lack of a consistent proteome pattern (Fig. 3b). In addition to total protein analysis, acetylated ($n = 15$) and phosphorylated ($n = 26$) peptides were quantified (Fig. 3c, d), and no significant differences were observed. Moreover, the deleterious effects of BPS3 were elucidated using antibodies against the phosphorylated form of H2AX (γH2AX) to label DNA double-stranded breaks; γH2AX is a representative PTM that can be used to identify DNA damage and cellular stress. Consistent with the increased incidence of abnormalities in seminiferous tubules in the BPS3 group, we observed an increase in the γH2AX signal as well (Fig. 3e).

Lower BPS exposure changed the post-translational quality of sperm proteins

In accordance with whole-proteome analysis of BPS-treated testes, analyses of the sperm acetylome and phosphorylome were performed using western blotting and MALDI-TOF MS. Because of the low protein amounts in sperm lysate extracts, sperm samples from three individuals belonging to the same experimental group were pooled, and three independent experiments

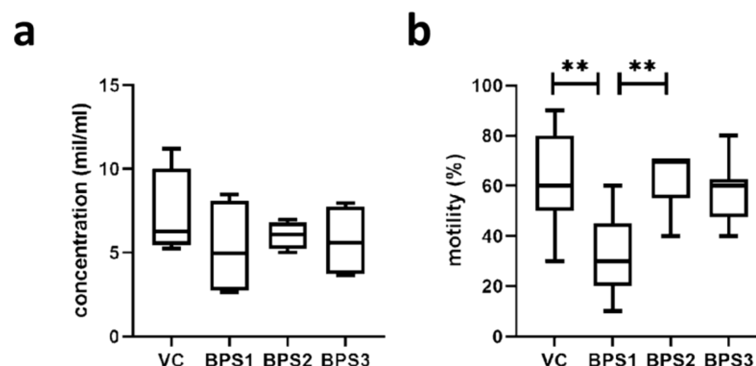

Fig. 1 Sperm features: (a) sperm concentration and (b) percentage of motile sperm. Data are shown as medians and 5–95% percentiles. Kruskal-Wallis tests followed by Dunn's multiple comparison tests were performed, and statistical significance is indicated (**$p < 0.01$). VC: vehicle control, BPS1–3: increasing doses of bisphenol S

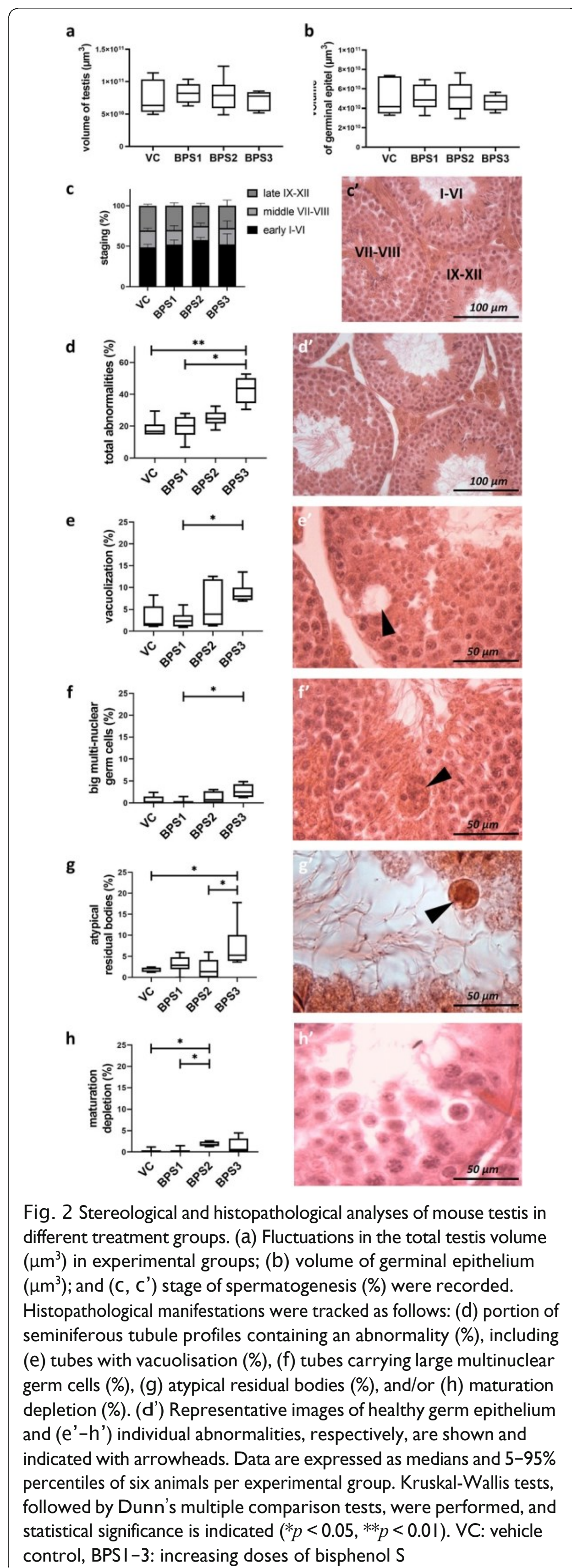

Fig. 2 Stereological and histopathological analyses of mouse testis in different treatment groups. (a) Fluctuations in the total testis volume (µm^3) in experimental groups; (b) volume of germinal epithelium (µm^3); and (c, c') stage of spermatogenesis (%) were recorded. Histopathological manifestations were tracked as follows: (d) portion of seminiferous tubule profiles containing an abnormality (%), including (e) tubes with vacuolisation (%), (f) tubes carrying large multinuclear germ cells (%), (g) atypical residual bodies (%), and/or (h) maturation depletion (%). (d') Representative images of healthy germ epithelium and (e'–h') individual abnormalities, respectively, are shown and indicated with arrowheads. Data are expressed as medians and 5–95% percentiles of six animals per experimental group. Kruskal-Wallis tests, followed by Dunn's multiple comparison tests, were performed, and statistical significance is indicated (*$p < 0.05$, **$p < 0.01$). VC: vehicle control, BPS1–3: increasing doses of bisphenol S

were performed. After loading equal amounts of protein per well, we found that the acetylation of proteins with molecular weights of approximately 37, 40, and 50 kDa were affected by treatment with BPS1 (Fig. 4a, b). Moreover, BPS1 also modified the phosphorylation of sperm proteins (37, 40, 85, and 100 kDa; Fig. 4c, d, d'). Candidate acetylated and phosphorylated proteins are summarised in Fig. 4 (e, f), and the results indicated the involvement of housekeeping proteins (ATP synthase subunit, hexokinase-1) and enzymes (DNA repair protein, E3 ubiquitin-protein ligase). In accordance with previous findings, demonstrating that BPS1 suppressed sperm motility, cytoskeletal factors (i.e., tubulin chains, actin; Fig. 4e) seems to be underwent to acetylation. Therefore, an antibody against acetylated α-tubulin (acTubulin) was used for a verification of tubulin as a candidate BPS target.

Next, we evaluated the densitometry of bands representing acetylated tubulin after treatment with BPS1 (Fig. 4g). Decreased tubulin acetylation was observed; however, the difference was not statistically significant, suggesting that other targets (such as ATP synthase and actin) may be related to sperm motility.

Discussion

Male reproduction involves sensitive machinery, which is required for spermatozoon development and can be affected by exposure to various environmental stimuli. Because mature spermatozoa have been transcriptionally silenced, changes in PTMs can regulate protein activity and modify other crucial biomolecules. Indeed, lysine acetylation and phosphorylation have been shown to be indispensable for the proper function of sperm [14, 15]. Our findings suggested that PTMs may be affected by pollutants from the environment. In our study, we simulated the exposure of adult males to BPS, a common endocrine disruptor, at very low doses (~ 0.001 and 1 µg/kg bw/day). Moreover, we chose ~ 100 µg/kg bw, which has been suggested to induce reproductive toxicity [3, 5]. The 8-week exposure covered the whole duration of spermatogenesis; therefore, we assumed that the sperm quality and testicular tissues would be affected at the tissue/cell and proteome levels. We also evaluated the effects of endocrine disruption on post-translational modifications of testicular/sperm proteins in accordance with our hypothesis of the "post-translational effect" of very low doses of these agents.

Recent studies have demonstrated that bisphenols alter steroid signalling pathways, having negative effects on male and female reproduction. Our observations did not reveal hormonal changes, even after higher BPS exposure, whereas comparable doses were found to be effective in rats [6]. However, earlier results showing that endocrine disruptors lead to hormonal imbalances

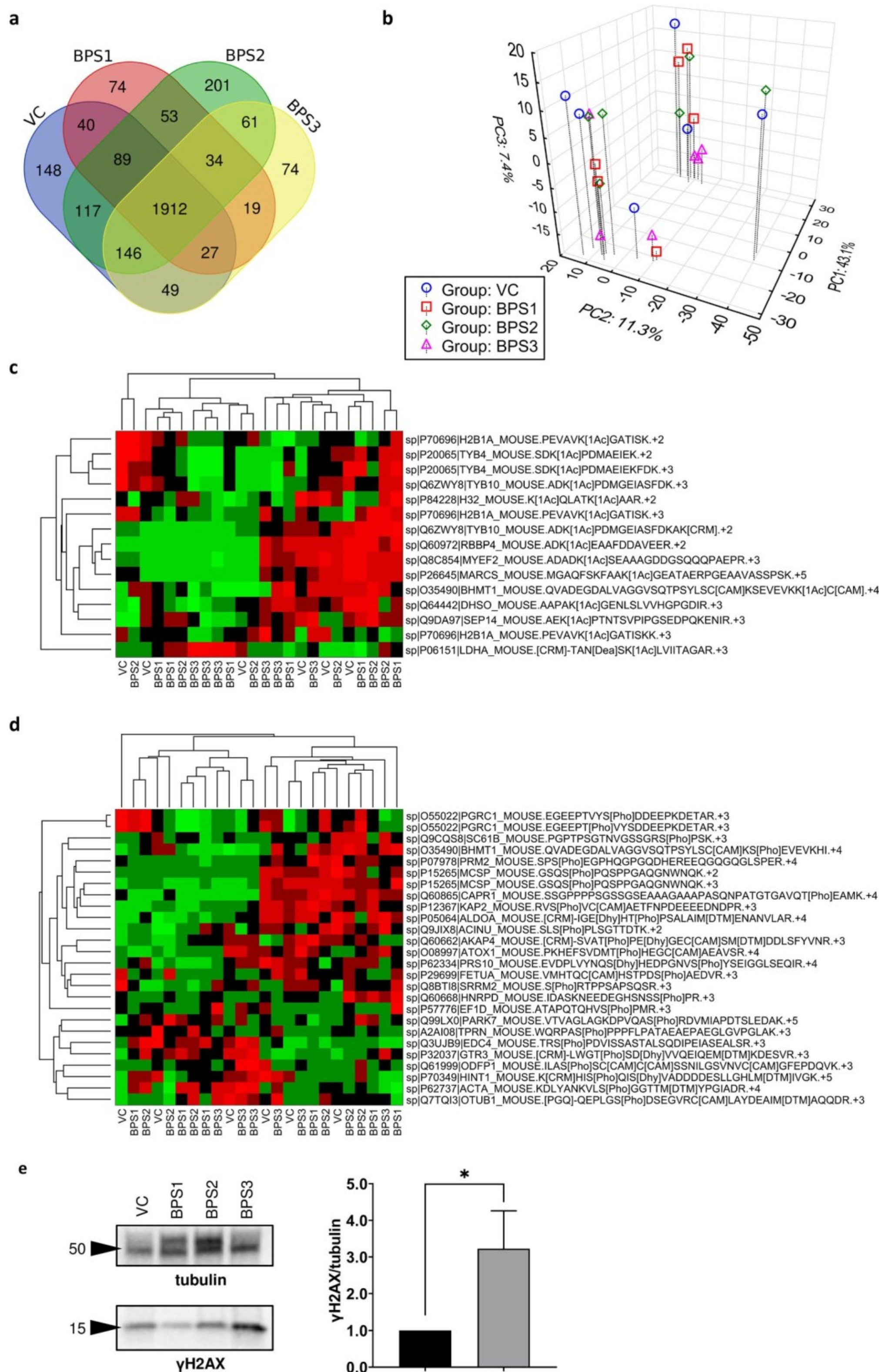

Fig. 3 (See legend on next page.)

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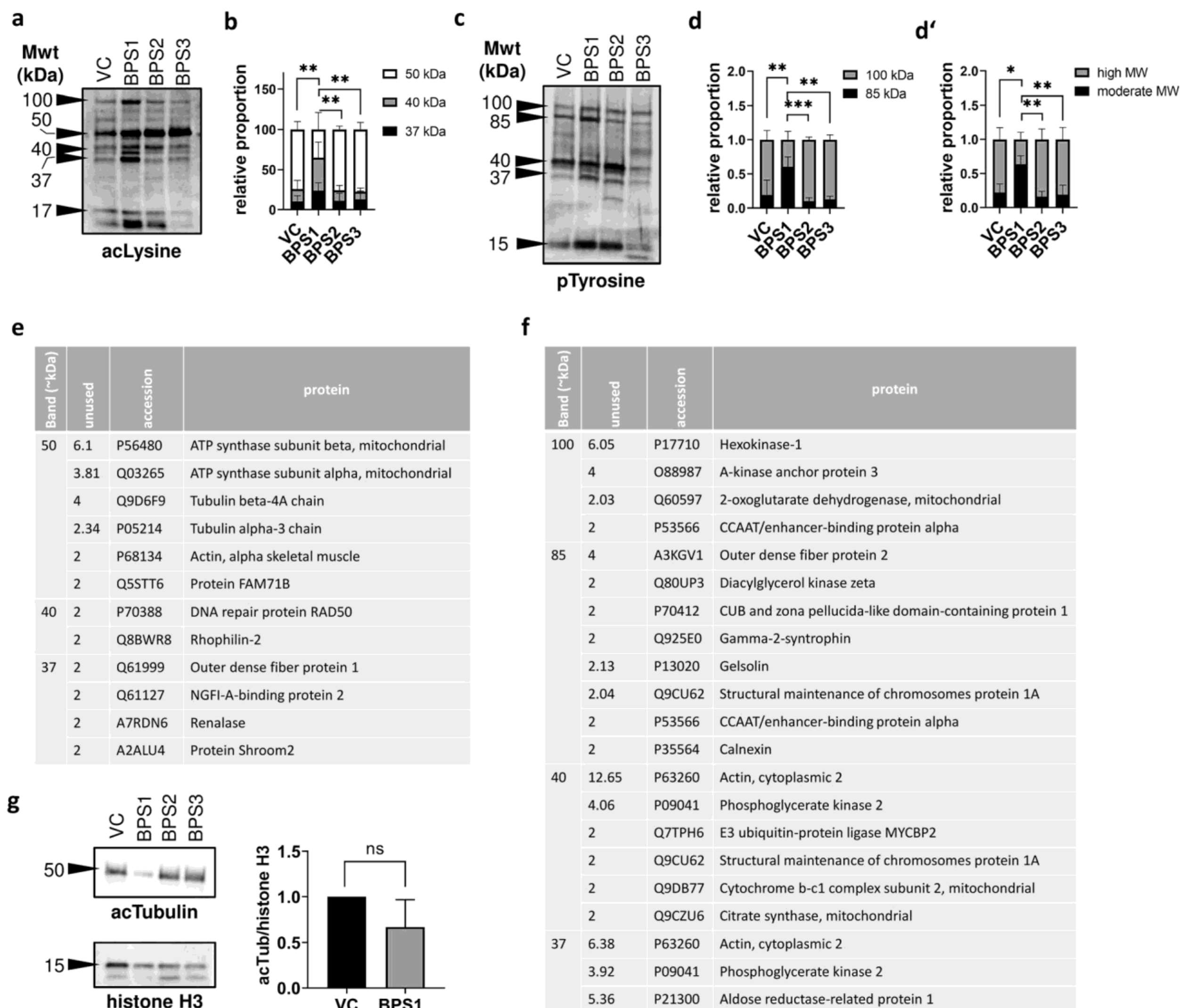
Fig. 3 Proteomic analysis of testicular tissues. (a) Venn diagram of total described testicular proteins in mice ($n = 4$) after various treatments in different experimental groups. (b) Projection of 24 experimental mice into the space of first three principal components according to PCA; percentages in the axis legends show the proportion of total variance explained by the particular component. (c) Overview of acetylated and (d) phosphorylated testicular proteins. (e) Analysis of γH2AX; band signals were normalized to α-tubulin and related to the vehicle control as the mean (range) of three independent experiments. Unpaired t tests were performed, and statistical significance is indicated (*$p < 0.05$). VC: vehicle control, BPS1–3: increasing doses of bisphenol S



e

Band (~kDa)	unused	accession	protein
50	6.1	P56480	ATP synthase subunit beta, mitochondrial
	3.81	Q03265	ATP synthase subunit alpha, mitochondrial
	4	Q9D6F9	Tubulin beta-4A chain
	2.34	P05214	Tubulin alpha-3 chain
	2	P68134	Actin, alpha skeletal muscle
	2	Q5STT6	Protein FAM71B
40	2	P70388	DNA repair protein RAD50
	2	Q8BWR8	Rhophilin-2
37	2	Q61999	Outer dense fiber protein 1
	2	Q61127	NGFI-A-binding protein 2
	2	A7RDN6	Renalase
	2	A2ALU4	Protein Shroom2

f

Band (~kDa)	unused	accession	protein
100	6.05	P17710	Hexokinase-1
	4	O88987	A-kinase anchor protein 3
	2.03	Q60597	2-oxoglutarate dehydrogenase, mitochondrial
	2	P53566	CCAAT/enhancer-binding protein alpha
85	4	A3KGV1	Outer dense fiber protein 2
	2	Q80UP3	Diacylglycerol kinase zeta
	2	P70412	CUB and zona pellucida-like domain-containing protein 1
	2	Q925E0	Gamma-2-syntrophin
	2.13	P13020	Gelsolin
	2.04	Q9CU62	Structural maintenance of chromosomes protein 1A
	2	P53566	CCAAT/enhancer-binding protein alpha
	2	P35564	Calnexin
40	12.65	P63260	Actin, cytoplasmic 2
	4.06	P09041	Phosphoglycerate kinase 2
	2	Q7TPH6	E3 ubiquitin-protein ligase MYCBP2
	2	Q9CU62	Structural maintenance of chromosomes protein 1A
	2	Q9DB77	Cytochrome b-c1 complex subunit 2, mitochondrial
	2	Q9CZU6	Citrate synthase, mitochondrial
37	6.38	P63260	Actin, cytoplasmic 2
	3.92	P09041	Phosphoglycerate kinase 2
	5.36	P21300	Aldose reductase-related protein 1

Fig. 4 Sperm acetylome and phosphorylome analyses. (a) Acetylated sperm proteins (acetylated lysine) with major bands. (b) Densitometric analysis of the ratio of candidate bands. (c) Phosphorylated sperm proteins (phosphorylated tyrosine) with major bands. (d) Densitometric analysis of the ratio of 100- to 85-kDa bands. (d') The ratio of 37–40-kDa (moderate) to 85–100-kDa (high) molecular weight bands. Differential counting was expressed as means (ranges) of three independent experiments. Differences were tested by two-way ANOVA, followed by Tukey's multiple comparison test, and asterisks indicate statistical significance *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. (e) Candidate acetylated and (f) phosphorylated proteins from individual bands were evaluated using MALDI-TOF MS-based peptide detection. Analysed sperm samples represent a pool of three animals per experimental group from three independent replicates. (g) Densitometric analysis of acetylated tubulin from BPS1-treated sperm. VC: vehicle control, BPS1–3: increasing doses of bisphenol S

should be revised because alternative mechanisms of hormone-derived actions have been noted. For example, oestrogen-like action results in carcinogenesis [23], and changes in the distributions of oestrogen receptors and androgen-converting enzyme aromatase have been reported [24]. Endocrine disruptors have also been shown to modulate downstream signalling of activated G protein-coupled oestrogen receptors [25]. It is difficult to identify bisphenol-affected mechanisms after systemic exposure; therefore, cellular and molecular markers are appropriate for assessment of the risk of bisphenol exposure. Based on our findings, we speculate that different doses of BPS may have different effects. For example, whereas extremely low doses (BPS1: ~ 0.001 μg BPS/kg bw) affected sperm motility, higher BPS doses (BPS3: ~ 100 μg/kg bw) showed significant effects on testicular tissues. Surprisingly, moderate doses of BPS (BPS2: equal to daily intake of approx. 0.1 μg/kg bw) did not show any effects on spermiogram recording and histological assessment. This finding was consistent with the phenomenon of nonlinear effects [26], with the lowest dose of BPS (BPS1) inducing motility failure rather compared with the other BPS doses. Therefore, proteome profiling was used to test a wide range of BPS doses and characterise the dose-dependent mode of action.

Because of the lack of effect of BPS on the whole proteome of testicular tissues, protein acetylation and phosphorylation were chosen for further analysis. Although no significant effects were observed in terms of acetylation and phosphorylation of the detected peptides, γH2AX, a mark of DNA damage, was increased in BPS3 testicular tissues, demonstrating the increased occurrence of abnormalities. In sperm lysates, protein acetylation and phosphorylation were detected using specific antibodies against acetylated lysine and phosphorylated tyrosine. The choice of PTMs was consistent with the earlier described biological role of both PTMs in sperm capacitation and fertilisation ability [14, 27]. Indeed, altered levels of acetylated and phosphorylated proteins were observed after exposure to very-low-dose BPS (BPS1). This finding was presumably associated with decreased motility, resulting in detection of candidate proteins. We can assume that differentially acetylated and/or phosphorylated may be responsible for motility failure, in accordance with the significance of PTMs in major proteins, including phospho-hexokinase-1 [28] and phospho-outer dense fibre protein-2 [29]. Decreased phospho-tyrosine signals at 100 kDa suggest a lack of hexokinase-1 activity, which is associated with male infertility [30]. Our findings supported the mechanism of action of BPA described previously through fertility-related proteins, including protein phosphorylation [31]. Our study suggested that in addition to phosphorylation, bisphenols altered other PTMs, particularly protein acetylation. However, western blot analysis using anti-acetylated tubulin did not show any decrease, as expected, and other protein targets for acetylation were considered, including ATP synthase and actin, both of which are involved in sperm motility [32, 33].

Conclusion

In conclusion, these studies are among the first to raise the possibility that low and very low doses of BPS may have a deleterious effect on sperm quality in mammals. Since human BPS exposure is much lower (0.004 μg/kg bw/day) than is commonly tested [34], our findings suggest that post-translational effects could play a role in idiopathic infertility. Furthermore, this work supports the view that substitution of BPS for BPA may be inadequate for elimination of the negative effects of these agents on public health. Further biomonitoring and testing of molecular targets of BPS could be relevant for accurate risk assessment and elimination of its potential negative impact on male fertility.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12958-020-00596-x.

Additional file 1: Table S1. Composition of Whitten's HEPES-buffered medium. Table S2. Coefficients of error (CE) for evaluated terms of performed stereological analysis (n = 9 per each group). VC: vehicle control, BPS1-3: increasing doses of bisphenol S. Table S3. Hormone profiling of males in different experimental groups. Values of adrenocorticotropic hormone (ACTH), follicle-stimulating hormone (FSH), growth hormone (GH), luteinising hormone (LH), thyroid-stimulating hormone (TSH), cortisol, progesterone, testosterone, triiodothyronine (T3), and thyroxine (T4) are expressed as medians ± SEM, n = 5 per experimental group. Kruskal-Wallis tests were followed by Dunn's multiple comparison tests. Different letters in the same row indicate significant differences (p < 0,05). VC: vehicle control, BPS1-3: increasing doses of bisphenol S.

Abbreviations

BPA: Bisphenol A; BPS: Bisphenol S; CHAPS: 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate; MALDI-TOF MS: Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry; MS: Mass spectrometry; nano-LC-MS: Nano-liquid chromatography mass spectrometry; PTMs: Post-translational modifications; TBS: Tris-buffered saline; VC: Vehicle control

Acknowledgements

We would like to thank Ms. Iveta Zimova for her kind help with the experimental work.

Ethics approval and consent to participate.

All animal procedures were done in accordance with the Protection of Animals against Cruelty (Act No. 246/1992) under the supervision of the Animal Welfare Advisory Committee at the Ministry of Education, Youth, and Sports of the Czech Republic.

Authors' contributions

Conceived project: JP, MK, JN. Animal experiment design: JN, OGA, PK. Execution of experiments: MŠ, NE, OGA, HŘ. Quantitative analyses of testes: YK, PRM, AMCT. Qualitative analyses of testes: MC. Proteome analysis: JM, JN, TF. Compiling the results: JN, MŠ, HŘ. Statistics: PH, JN, HŘ. Writing the manuscript and data interpretation: HŘ, MŠ, NJ, OGA. Proofreading: PRM. All authors read and approved the final manuscript.

Funding
This study was supported by the Czech Health Research Council (grant no. NV18-01-00544); the Charles University Research Fund (Progres Q39); the National Sustainability Programme I (NPU I) Nr. LO1503 provided by the Ministry of Education, Youth and Sports of the Czech Republic (MEYS CR); project no. SVV 02690 awarded by MEYS CR; and project no. CZ.02.1.01/0.0/0.0/16_019/0000787 "Fighting Infectious Diseases", awarded by MEYS CR and financed by The European Regional Development Fund, European Human Biomonitoring Initiative HBM4EU provided by H2020, and the United States Fulbright Commission (P001496 grant to P.R.M.).

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 27 January 2020 Accepted: 22 April 2020
Published online: 28 May 2020

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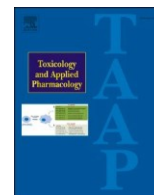
Toxicology and Applied Pharmacology 413 (2021) 115409

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/taap



Exposure to alternative bisphenols BPS and BPF through breast milk: Noxious heritage effect during nursing associated with idiopathic infertility

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ARTICLE INFO

Keywords:
Bisphenol
Oocyte maturation
Spindle formation
Epigenetics

ABSTRACT

There is increasing evidence that bisphenols BPS and BPF, which are analogues of BPA, have deleterious effects on reproduction even at extremely low doses. Indirect exposure *via* the maternal route (*i.e.* across the placenta and/or by breastfeeding) is underestimated, although it can be assumed to be a cause of idiopathic female infertility. Therefore, we hypothesised the deleterious effects of exposure to BPA analogues during breastfeeding on the ovarian and oocyte quality of offspring. A 15-day exposure period of pups was designed, whilst nursing dams ($N \geq 6$ per experimental group) were treated *via* drinking water with a low (0.2 ng/g body weight/day) or moderate (20 ng/g body weight/day) dose of bisphenol, mimicking real exposure in humans. Thereafter, female pups were bred to 60 days and oocytes were collected. Immature oocytes were used in the *in-vitro* maturation assay; alternatively, *in-vivo*-matured oocytes were isolated and used for parthenogenetic activation. Both *in-vitro*- and *in-vivo*-matured oocytes were subjected to immunostaining of spindle microtubules (α-tubulin) and demethylation of histone H3 on the lysine K27 (H3K27me2) residue. Although very low doses of both BPS and BPF did not affect the quality of ovarian histology, spindle formation and epigenetic signs were affected. Notably, *in-vitro*-matured oocytes were significantly sensitive to both doses of BPS and BPF. Although no significant differences in spindle-chromatin quality were identified in ovulated and *in-vivo*-matured oocytes, developmental competence was significantly damaged. Taken together, our mouse model provides evidence that bisphenol analogues represent a risk to human reproduction, possibly leading to idiopathic infertility in women.

1. Introduction

Human infertility has become a serious medical issue in developed countries, due to increased maternal age (Bukovsky, 2017), lifestyle practices (Leisegang and Dutta, 2020), and/or environmental threats (Di Renzo et al., 2015). However, many infertility cases have been classified as 'idiopathic', when the primary cause is unknown (Monteiro et al., 2020; Punab et al., 2016). In idiopathic infertility studies, the genetic background of patients is favoured (Mallepaly et al., 2017), and when environmental noxious stimuli are considered a cause of reproduction failure, direct exposure *via* different routes is assessed (Ndaw et al., 2018; Upson et al., 2014). At most, gestational exposure to environmental pollutants is considered an indirect burden (Fisher et al., 2020; Monteiro et al., 2020; Ziv-Gal et al., 2015). However, there is limited information regarding breast milk contamination with bisphenols (Tuzimski et al., 2020), as well as the possibly deleterious effects of nursing-mediated exposure in adulthood (Li et al., 2016).

Accordingly, we can consider the nursing period as crucial for testing environmental pollutants due to the following assumed reasons: i) breastfeeding is an exclusive food for infants; ii) pollutants are often liposoluble; iii) therefore, higher concentrations of these compounds may be consumed in fatty-rich breast milk; iv) elimination *via* the glucuronidation metabolic pathway in the infant kidney is not fully developed (Matalová et al., 2016) and, therefore, v) the exposure to low doses may act as a reproductive toxin, despite being harmless to the mother; and finally vi) despite the direct impact of chemical compounds, the metabolic effect of vertical exposure from mother to foetus is considered (summarised in Chemek and Nevoral, 2019).

A multitude of plastic compounds occurs in developed countries, threatening human reproduction. Bisphenols are a widely used plastic

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https://doi.org/10.1016/j.taap.2021.115409
Received 13 September 2020; Received in revised form 8 January 2021; Accepted 10 January 2021
Available online 18 January 2021
0041-008X/© 2021 Published by Elsevier Inc.

compound, with endocrine-disrupting properties that ubiquitously affect the endocrine system. Bisphenol A, the most widely used bisphenol compound, is well known for having a negative effect on human reproductive health (Brieno-Enriquez et al., 2012; Mínguez-Alarcón et al., 2015; Peretz et al., 2014) and therefore has gradually been eliminated from many commonly used products (Žalmanová et al., 2016). In particular, its use in baby bottles and toys has been prohibited. Consequently, obvious but regrettable substitutes have been found (Žalmanová et al., 2016): bisphenol S (BPS) and/or BPF that replaced BPA from many consumables, with deleterious BPA-like biological effects (Žalmanová et al., 2017).

While many studies have compared BPA *versus* alternative bisphenols, few studies describing BPA analogues on reproduction have been published in recent years (Desdoits-Lethimonier et al., 2017; Rehfeld et al., 2020; Warner and Flaws, 2018). However, little is known about the molecular mode of the action of bisphenols at relevant doses. Moreover, there is limited information available regarding exposure to BPA analogues, BPS and BPF. Current evidence indicates that gametes are more sensitive to low doses of BPS, whereas the oocyte cytoskeleton, epigenetic code, and/or protein post-translational modifications are affected in a subtle way, depending on the biological impact of bisphenol (Nevoral et al., 2018b; Prokešová et al., 2020; Řimnáčová et al., 2020). These observations appropriately supplement the findings of cohort studies and human biomonitoring, preceding the selection of experimentally used doses. In addition to the direct exposure of adults, gestational transplacental exposure has become a hot topic in human biomonitoring and toxicokinetic studies (Sharma et al., 2018). Similarly, the nursing period has recently come into the centre of interest and is being considered as an exposure window with significant impact on reproduction (Martini et al., 2020). There are several reasons to speculate about this exposure window as a cause of idiopathic infertility induced by environmental pollutants, concurrently being underestimated in the context of reproductive health.

In accordance with the aforementioned evidence, we hypothesised that neonatal exposure to bisphenol analogues leads to female reproductive failure in adulthood, which may otherwise be considered as 'idiopathic'. The aim of the study was to simulate the real-life exposure route to bisphenols being considered safe for reproductive health. Doses have been chosen based on the actual exposure of humankind (Rochester and Bolden, 2015). Moreover, they were used in animal experiments that induced non-fatal systemic responses in exposed animals (Nevoral et al., 2018b; Prokešová et al., 2020). In addition to standard molecular assessment of oocytes, developmental competence of them was evaluated, being considered as a key marker of female fertility (Sirard et al., 2006). Our study provides the first evidence of the biological effect of translactational transfer of alternative bisphenols on female reproduction, using the perinatal nursing window of exposure.

2. Material and methods

All chemicals were purchased from Sigma-Aldrich (MO, USA), unless stated otherwise.

2.1. Animals

Six- to seven-week-old female ICR mice ($n = 60$) were purchased from Velaz Ltd. (Czech Republic) and used as dams of F1 offspring subjected to experimental assessment. The animals were housed in intact polysulphona cages, maintained in 12 h-light/dark cycles at room temperature (21 ± 1 °C) and relative humidity (60%). A phyto-oestrogen-free diet (1814P; Altromin, Germany) and ultrapure water (in glass bottles, changed twice weekly) were provided *ad libitum*. All animal procedures were conducted in accordance with the Coll. Act on the Protection of Animals against Cruelty No. 246/1992 of the Czech Republic and under the supervision of the Animal Welfare Advisory Committee of the Ministry of Education, Youth, and Sports of the Czech Republic (approval number: MSMT-11925/2016–3).

2.2. Exposure and dosage

ICR dams were mated in the oestrus phase. Bisphenols were administered *via* drinking water (low and moderate BPS, 0.375 ng/mL and 37.5 ng/mL, respectively) during the nursing period of ICR dams between PND0 (*i.e.* day of delivery) and PND15. Vehicle control comprised treatment with 0.1% ethanol in sterile tap water. The exposure duration covered the sensitive exposure window and assured breast milk as an exclusive feeding route for offspring. The route of exposure to bisphenols was selected with respect to the welfare of females exposed during a highly sensitive period of nursing. Doses with known biological effects (Nevoral et al., 2018b) and appropriate to real-life human exposure (Rochester and Bolden, 2015) were chosen. After recording the drinking water consumption and body weight of nursing dams, the actual bisphenol exposure of dams was determined. Henceforward, corrected bisphenol intake was maintained at low and moderate BPS dosages.

2.3. Inspection of clinical signs of reproduction onset

The weight of the litter was inspected at delivery and defined as postnatal day (PND) 0 (PND0). Thereafter, weight was recorded at PND10 and PND21. Once pups were weaned at PND21, anogenital distance, a marker of androgenic activity, was measured as the distance from the superior edge of the external genitalia to the inferior edge of the anus, using a handheld calliper with precision to 0.1 mm. During post-weaning housing, the day of vaginal opening was recorded as the day of onset of puberty.

2.4. Histology and sample preparation

Young and adult ovaries were isolated on PND15 and PND60, when the exposure was terminated and the reproductive peak was achieved, respectively. Ovaries were fixed for 14 days in Bouin's solution, followed by storage in paraffin blocks. Blocks were processed into 10-μm-thick systematic serial sections with random orientations (100 ± 10 sections per animal) using a microtome (Leica RM2255). Every fifth section was mounted on to the slide, followed by staining with the standard protocol with haematoxylin-eosin. From 7 to 14 equidistant sections per ovary, depending on the ovary size, were used for the stereological analysis (Mouton, 2002).

2.5. Stereology and follicle count

Stereological analysis of ovaries was performed using Stereologer 11 software (SRC Biosciences, Tampa, Florida, USA) on a personal computer, attached to a Nikon Eclipse Ti−U microscope, equipped with high-speed XY stage and *Z*-axis motors (Prior, UK), camera (Promicra 3-3 cc), and a standard set of lenses (Plan Fluor). The total volume of the ovaries was estimated, using the point grid counting approach, based on the Cavaslieri principle (Gundersen et al., 1999). The quantification of follicles was performed using optical dissector techniques (Sterio, 1984) by counting visible cell nuclei. The follicles were divided into the following five groups, according to the morphological features described earlier (Nevoral et al., 2018b): primordial, primary, preantral, antral, and atretic follicles. The primordial follicle count was used to estimate the ovarian reserve of young ovaries (PND15) and was excluded from the analysis in adult ovaries (PND60).

2.6. Oocyte isolation

For immature oocytes in the GV stage, we used 8- to 10-week-old perinatally exposed females in the pro-oestrus or oestrus phase. Oocytes were isolated from ovarian tissue using a needle and a 27-G

syringe, and used subsequently for *in-vitro* maturation studies. Alternately, another female was administered PMSG and hCG 48 h later, followed by isolation of *in-vivo*-matured oocytes after 16 h. Ovulated cumulus-oocyte complexes were flushed from the oviduct and treated with 0.1% hyaluronidase for 5 min to remove cumulus cells. These oocytes were used for *in-vitro* fertilisation or immunocytochemistry.

2.7. In-vitro maturation assay

Immature oocytes in the GV stage were manipulated in M2 medium supplemented with 100 μM isobutyl-methylxanthine (IBMX), a specific endogenous phosphodiesterase inhibitor, to maintain intact GV oocytes. Fully grown immature oocytes with intact GVs were placed in M16 culture medium with IBMX and allowed to recover their oocyte pool of proteins for at least 1 h at 37 °C and 5% CO_2. Thereafter, oocytes were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), supplemented with 0.1% polyvinyl-alcohol, for 30 min at room temperature (22 °C), and stored at 4 °C until further use. Alternatively, recovered GV oocytes were cultured in IBMX-free M16 culture medium for 16 h at 37 °C and 5% CO_2 to obtain matured MII oocytes. Matured oocytes with extruded polar bodies were fixed and stored until further use at 4 °C.

2.8. Immunocytochemistry (ICC) and image analysis

In both *in-vitro*- and *in-vivo*-matured oocytes, α-tubulin and dimethylated histone H3 on lysine K27 (H3K27me2) were used for spindle visualisation and heterochromatin assessment, respectively. Firstly, 4% paraformaldehyde-fixed oocytes were permeabilised in PBS containing 0.04% Triton X-100 and 0.3% Tween-20 for 15 min. Then, oocytes were blocked in 1% bovine serum albumin in PBS with Tween 20 for 15 min and incubated with a cocktail of anti-α-tubulin (1:200, Sigma-Aldrich) and anti-H3K27me2 (1:200, Abcam, UK) primary antibodies. After washing, the oocytes were incubated with a cocktail of anti-mouse and anti-rabbit AlexaFluor 488 and 647 (1:200) antibodies, respectively. Phalloidin (1:200; Thermo Fisher Scientific, USA) was added to the washes and used for β-actin visualisation. Stained oocytes were mounted on to slides in Vectashield medium with DAPI. Signal intensity was measured using ImageJ software. Images were acquired using an Olympus IX83 spinning disc confocal microscope (Olympus, Germany) and VisiView software (Visitron Systems GmbH, Germany). Immunostained oocytes were subjected to measurement of the integrated density (expressing signal intensity) of H3K27me2, using ImageJ software (NIH, Bethesda, CA, USA). The integrated density values were related to control oocytes (VC = 1).

2.9. Parthenogenetic activation

Alternatively to ICC, *in-vivo*-matured oocytes of low and moderate BPS exposure groups were denuded from cumulus cells, as described above, and used for parthenogenetic activation. Oocytes were incubated in modified EmbryoMax KSOM Mouse Embryo (MR-121-D, Millipore) medium, supplemented with 0.1% bovine serum albumin (BSA), 2 mM EGTA, 10 mM $SrCl_2$, and 5 μg/mL cytochalasin B for 5 h at 37 °C and 5% CO_2. Embryos were cultured in KSOM with BSA for 24 h and 96 h to cleaved embryos and blastocyst stages, respectively.

2.10. Statistics

The data were analysed using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA). Based on Shapiro-Wilks normality distribution tests, differences were tested using an ordinary one-way ANOVA, followed by Tukey's multiple test. Alternatively, Kruskal-Wallis tests and Dunn's post-hoc tests were used for non-normally distributed data. Proportion data were analysed using Fisher's exact test with Bonferoni correction. P-values <0.05, 0.01, 0.001, and 0.0001 were considered statistically significant and indicated with asterisks (*), (**), (***), and (****), respectively. Normally and non-normally distributed data were expressed as means and medians, respectively.

3. Results

3.1. Exposure to very low doses of bisphenols does not induce androgenic and/or oestrogenic effects

We aimed to track essential non-invasive characteristics of individual litters, such as weight gain throughout the nursing period, followed by recording of anogenital distance at the day of weaning (defined as the 21st postnatal day, PND21) and the day of vaginal opening following exposure to low and moderate doses of BPS and BPF from delivery to PND15, *via* exposure to doses given to lactating dams. For comparison, two of the most commonly used alternative bisphenols, bisphenol S (BPS) and BPF, were chosen for the initial testing. Diethylstilbestrol (DES), a synthetic nonsteroidal form of oestrogen, was used as a positive control for the potential oestrogen-like effects of low doses of both bisphenols.

Based on the recording of water intake and body weight of nursing dams, the actual bisphenol exposure was calculated (Fig. 1A). There was no effect of perinatal bisphenol exposure on litter weight or weight gain during the nursing period (Fig. 1B). Neither anogenital distance nor vaginal opening, a marker of androgenic and oestrogenic activity, respectively, showed any differences compared to vehicle control (Fig. 1C).

3.2. Ovarian reserve estimation in bisphenol-exposed female pups and adult mice

Histological evaluation of ovarian tissue samples was performed at PND15 and PND60, corresponding to the end of bisphenol treatment and reproductive peak achievement, respectively. No effect of BPS was observed on the primordial follicle reserve in young ovaries (Fig. 2A and Supplementary Table S1). Treatment with low doses of DES did not show any differences. Thus the effect of DES exposure was not evaluated in adult ovaries. Similarly, exposure during lactation to either BPS or BPF did not affect the pool of primary, preantral, and antral follicles in adult ovaries (Fig. 2B and Supplementary Table S2). Even the number of atretic follicles in the low-concentration BPS group was not found as statistically significant ($P = 0.3911$). For complete histological data, see Supplemental Material (Supplementary Tables S1 and S2).

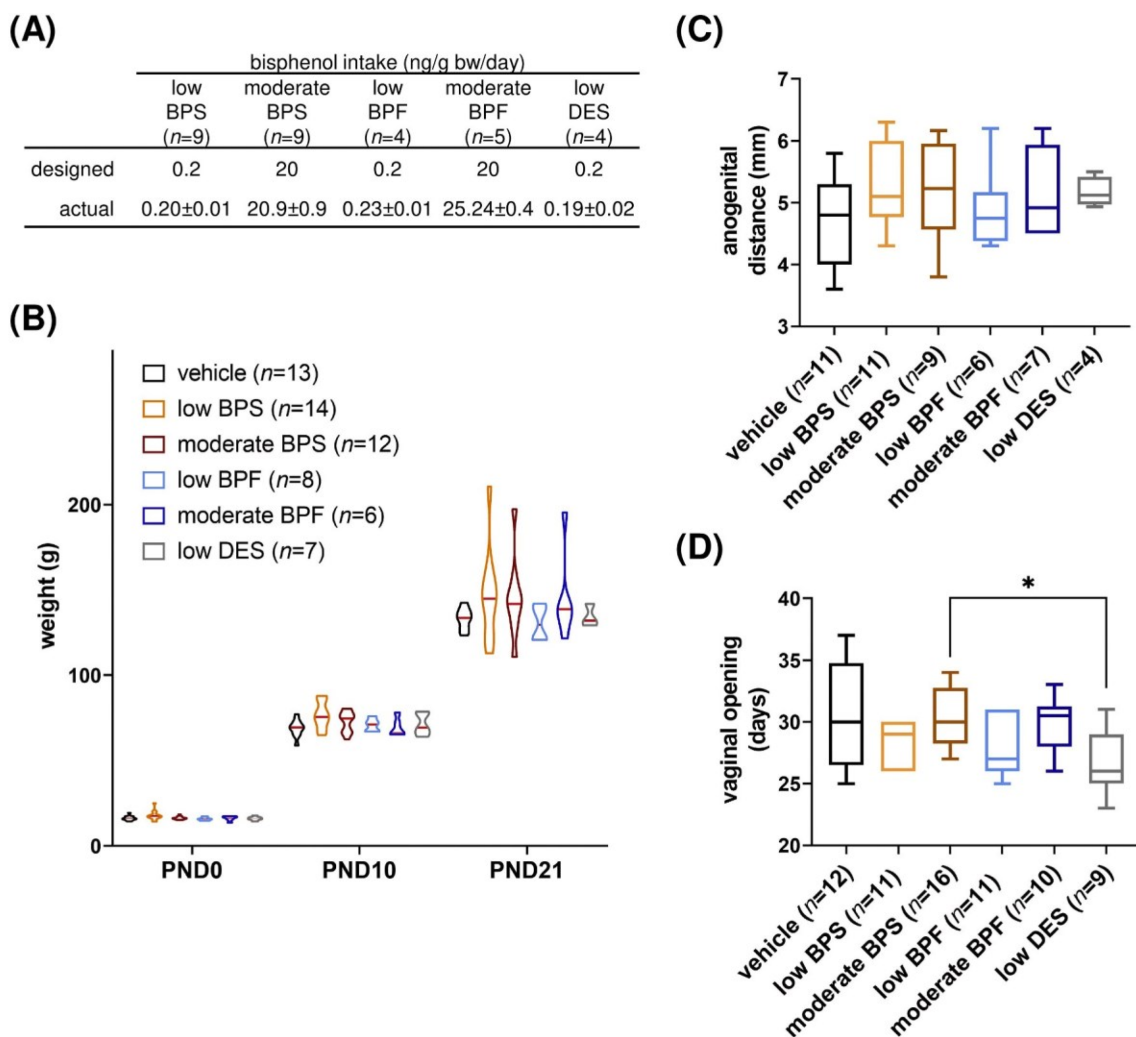
3.3. Assessment of the ovarian reserve of bisphenol-exposed female offspring

Following the findings of histological analyses, the physiological reserve of the ovaries was tested. Females in the proestrus/oestrus phase were used as donors of immature germinal vesicle (GV) oocytes, which are used for *in-vitro* maturation (IVM). Alternatively, hormonally stimulated females ovulated *in-vivo*-matured oocytes.

Based on the number of GV oocytes isolated per ovary, we did not observe any effect of nursing exposure to bisphenols (Fig. 3A). In addition, the ability of meiotic arrest breakdown (GVBD, germinal vesicle breakdown, GVBD) and the maturation stage (maturation rate) were not affected (Fig. 3B). Similarly, the number of flushed *in-vivo*-matured oocytes was not affected, and the number of atretic or fragmented oocytes, indicating oocyte quality, was not altered in bisphenol-exposed offspring (Fig. 3C).

3.4. Bisphenol-affected spindle assembly and histone code in oocytes matured in vitro

Based on the above findings, indicating bisphenol had no effect on oocyte yield or quality, we considered a more tenuous mechanism of



(A)

	bisphenol intake (ng/g bw/day)				
	low BPS (*n*=9)	moderate BPS (*n*=9)	low BPF (*n*=4)	moderate BPF (*n*=5)	low DES (*n*=4)
designed	0.2	20	0.2	20	0.2
actual	0.20±0.01	20.9±0.9	0.23±0.01	25.24±0.4	0.19±0.02

Fig. 1. Non-invasive features of litters and offspring. (A) Planned and effective exposure of dams to bisphenols (BPS, BPF) and diethylstilbestrol (DES), labelled as low and moderate. The number of dams is indicated in brackets. (B) Violin plots showing weight of litters, recorded at birth, *i.e.* postnatal days (PND) PND0, hence after PND10, and PND21. The number of recorded litters is indicated in the legend. The shape of violin plots represents the distribution of individual litters and line points median. (C) Anogenital distance (mm) of female offspring inspected at PND21. (D) The day of vaginal opening. (C,D) The number of inspected female offspring belonging to at least three independent litters is noted in brackets. Data are expressed as medians, and whiskers represent the range of minimum-maximal values. Statistical differences were tested using the Kruskal-Wallis nonparametric test, followed by Dunn's multiple comparisons test. The asterisk indicates statistical significance at $P < 0.05$ (*).

action for low doses of bisphenols at the intracellular level. Immunostaining of α-tubulin and demethylation of histone H3 on lysine K27 (H3K27me2) were chosen as markers of cytoskeleton and histone code-marked heterochromatin, respectively. We considered the native oestrus cycle and selected oocyte donors in the pro−/oestrus phase to avoid any potential interactions with administered gonadotropins.

In-vitro-matured oocytes showed a higher occurrence of spindle abnormalities, accompanied by chromosome misalignment (Fig. 4A). Moreover, persistent astral microtubules were observed in the ooplasm of several oocytes of the low BPS group, along with spindle malformation (Fig. 4B). Indeed, oocytes of female donors exposed to low BPS showed evidence of spindle malformation. Interestingly, only moderate BPF led to a significant increase of spindle misassembly in matured oocytes (Fig. 4C). In addition to abnormalities in ooplasmic microtubules in oocytes exposed to a low level of BPS, a statistically significant increased frequency of chromosomal misalignment was observed (Fig. 4D). A significant decrease in H3K27me2, a chromatin repressive marker, is observed in adult oocytes after a perinatal exposure to either a low dose of BPF or moderate doses of BPS or BPF (Fig. 4E).

3.5. *In-vivo maturation alleviates oocyte damage, but affects developmental competence*

To investigate the effect of nursing exposure on the quality of *in-vitro*-matured oocytes, the latter were subjected to cytoskeletal and histone code assessment of *in-vivo*-matured oocytes of hormonally-stimulated female donors, considered as these oocytes being more resilient to environmental stress (Fig. 5A). There is a significantly increased proportion of oocytes with spindle damage (α-tubulin) and chromosome misalignment in the low BPS group of females (Fig. 5B,C). No changes in histone heterochromatin marker methylation status (H3K27me2) were observed in all bisphenol groups (Fig. 5D).

Following the finding of spindle damage and chromosome misalignment in oocytes of low-BPS-nursing exposed females, *in-vivo*-matured oocytes were used to assess developmental competence. For this purpose, parthenogenetic activation was used, leading to blastocyst formation, while eliminating paternal contribution unlike *in-vitro* fertilisation. Only BPS-affected oocytes were assessed, due to the equality of BPS and BPF phenotypes observed on the oocyte spindle (Fig. 5B). Indeed, the parthenogenetic activation assay indicated a decrease in

Fig. 2. Histological assessment of young and adult ovaries. (A) Young haematoxylin-eosin-stained ovary on PND15, ovarian volume measurement, and primordial follicle counting. Arrows indicate the primordial follicles assessed. (B) Adult ovary at PND60 and the analysis of ovary volume and the amount of individual follicle stages: primary follicle reserve, preantral, antral, and atretic follicles, including representative pictures of individual stages, respectively. Arrows indicate the type of follicle assessed. The number of female offspring is indicated in brackets. Data had a parametric distribution and are expressed as mean and bars represent standard error of the mean (S.E.M.). Statistical differences were tested using an ordinary one-way ANOVA, followed by Tukey's multiple test.

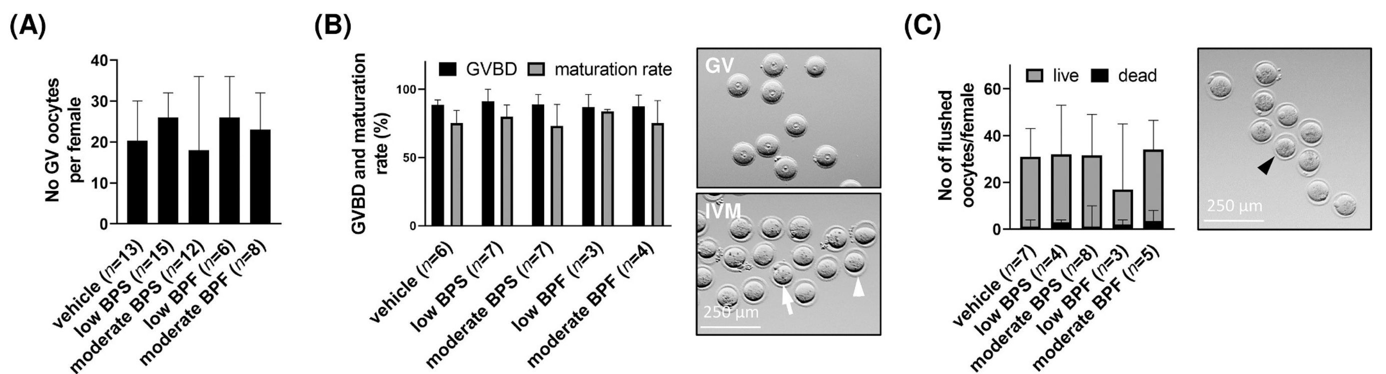


Fig. 3. Effect of perinatal exposure to BPS on female reproductive capacity in adulthood. (A) Follicle capacity expressed by the amount of isolated GV oocytes. The number of female descendent pups is indicated in brackets. (B) Oocyte maturation assessed by the ability to re-initiate meiotic division (GBVD, germinal vesicle breakdown), and to achieve a mature stage, defined as the maturation rate. The number of *in-vitro* maturation (IVM) assays is indicated in parentheses. Arrowhead points to GVBD-underwent oocyte; arrow indicates matured oocytes with extruded polar bodies. (C) The hormonal responsiveness of female descendant due to the counting of number of flushed *in-vivo*- matured and ovulated oocytes. The number of flushed females is indicated in brackets. Arrowhead points to the oocyte classified as dead. All data showed a parametric distribution and they are expressed as mean and bars representing standard error of the mean (S.E.M.). Statistical differences were tested using an ordinary one-way ANOVA, followed by Tukey's multiple test.

developmental competence of ovulated oocytes. The exposure to a low dose of BPS affected the activation and cleavage rate, as well as the blastocyst rate, in contrast to the moderate BPS group (Fig. 5D–E), indicating the association of embryonic development success with the cytoskeletal fitness of matured oocytes.

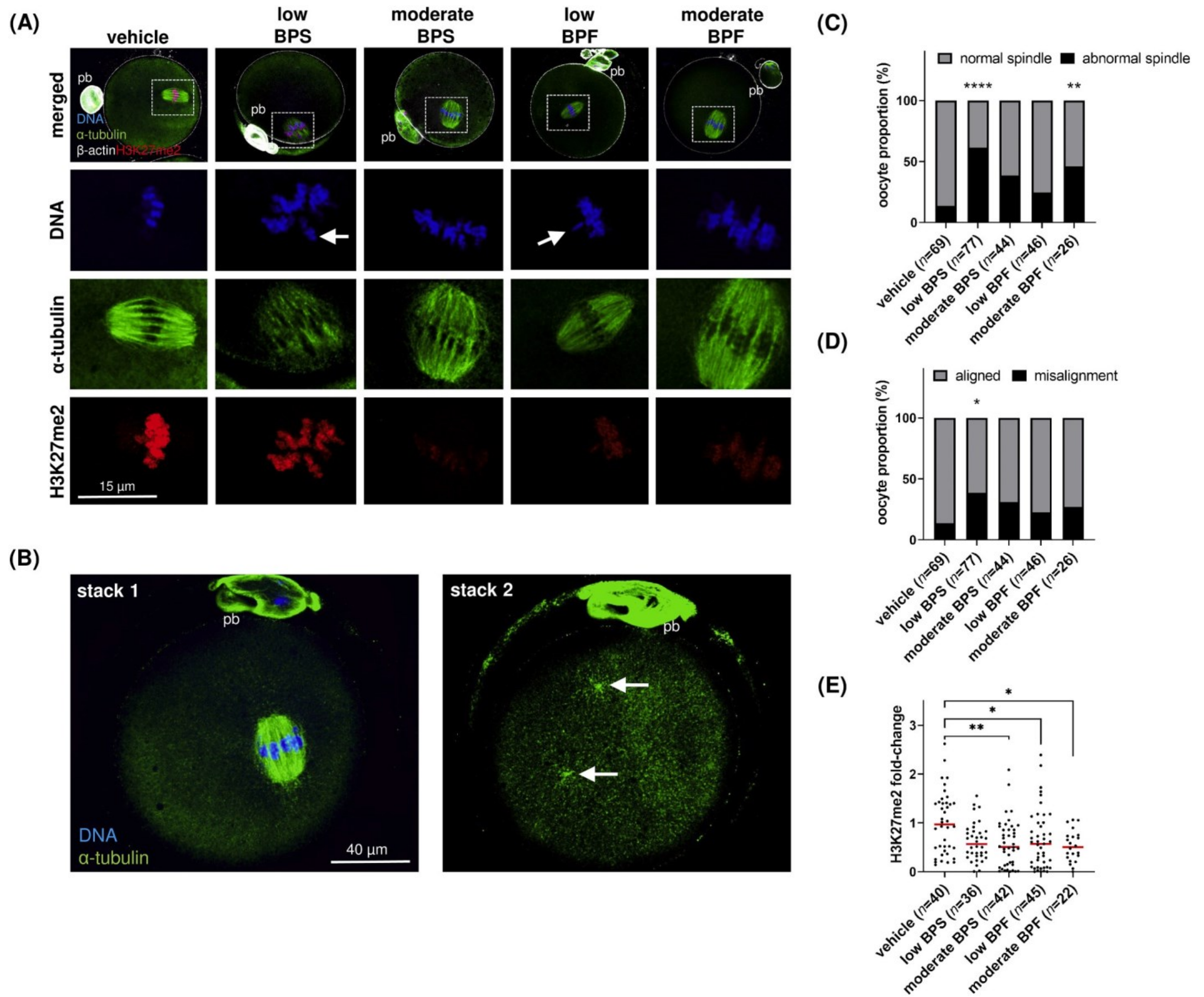


Fig. 4. Effect of nursing bisphenol exposure on cytoskeleton and epigenetic quality of *in-vitro*-matured oocytes. (A) Representative images of chromosome misalignment, cytoskeletal damage of the spindle, and chromosomal H3K27me2. The frame represents the emphasised area of the metaphase chromatin. Arrows indicate chromosome misalignment; pb: polar body. (B) Persisting astral microtubules in the ooplasm (indicated with arrows), accompanying spindle defects of low BPS oocytes. (C) Quantification of matured oocytes carrying the affected spindle, including astral microtubules noted above. (D) Frequency of chromosomal misalignment. (C,D) Data are expressed as cumulative proportion of oocytes, come from at least three unrelated female donors. The numbers of oocytes are indicated in brackets. Statistical differences between control and bisphenol-exposed groups were tested using Fisher's exact test with Bonferoni correction. Asterisks indicate statistical significance at $P < 0.05$ (*), 0.01 (**), and 0.0001 (****). (E) Quantification of H3K27me2 signal density in matured oocytes, related to unexposed control (mean of the vehicle = 1). Data show nonparametric distribution and lines indicating medians. The numbers of analysed oocytes are indicated in brackets. Statistical differences were tested using the Kruskal-Wallis nonparametric test, followed by Dunn's multiple comparisons test. Asterisks indicate statistical significance at $P <$ 0.05 (*), and 0.01 (**).

4. Discussion

Investigation into the biological effect of widely used BPA has produced much evidence supporting the deleterious effects of BPA on the endocrine system and on reproduction (Hunt et al., 2003; Moore-Ambriz et al., 2015; Pollock et al., 2014; Rahman et al., 2015; Wang et al., 2016; Ziv-Gal et al., 2015). This has led to a subsequent ban on its use in many commonly used products, such as in polycarbonate plastics and bottles, baby bottles, and toys. For this reason, the BPA analogues, BPS and BPF, have been introduced and used industrially in plastic compounds as a replacement for BPA (Sartain and Hunt, 2016). Recently, several studies have demonstrated the negative effect of these substitutes, and therefore, another 'regrettable substitution' has surfaced (Vandenberg et al., 2012). However, few studies have dealt with very low doses of bisphenols (Jan Nevoral et al., 2018b; Prokeˇsov´a et al., 2020; Zhang et al., 2020), which mimic actual intake levels *via* environmental exposure in developed countries (summarised by Wu et al., 2018), at most increased in several cases of occupational exposures (Ndaw et al., 2018; Russo et al., 2017; Thayer et al., 2016). In fact, indirect exposure to those low doses through the placenta and/or breast milk are not usually assessed, despite the fact that the exposure windows of *in-utero* development and nursing represent sensitive exposure windows (Dolinoy et al., 2007; Monteiro et al., 2020). Regardless of dosage, the effect of exposure during this exposure window on female reproduction is lacking, although some evidence about translactational exposure to BPA analogues has been published (LaPlante et al., 2017; Li et al., 2016).

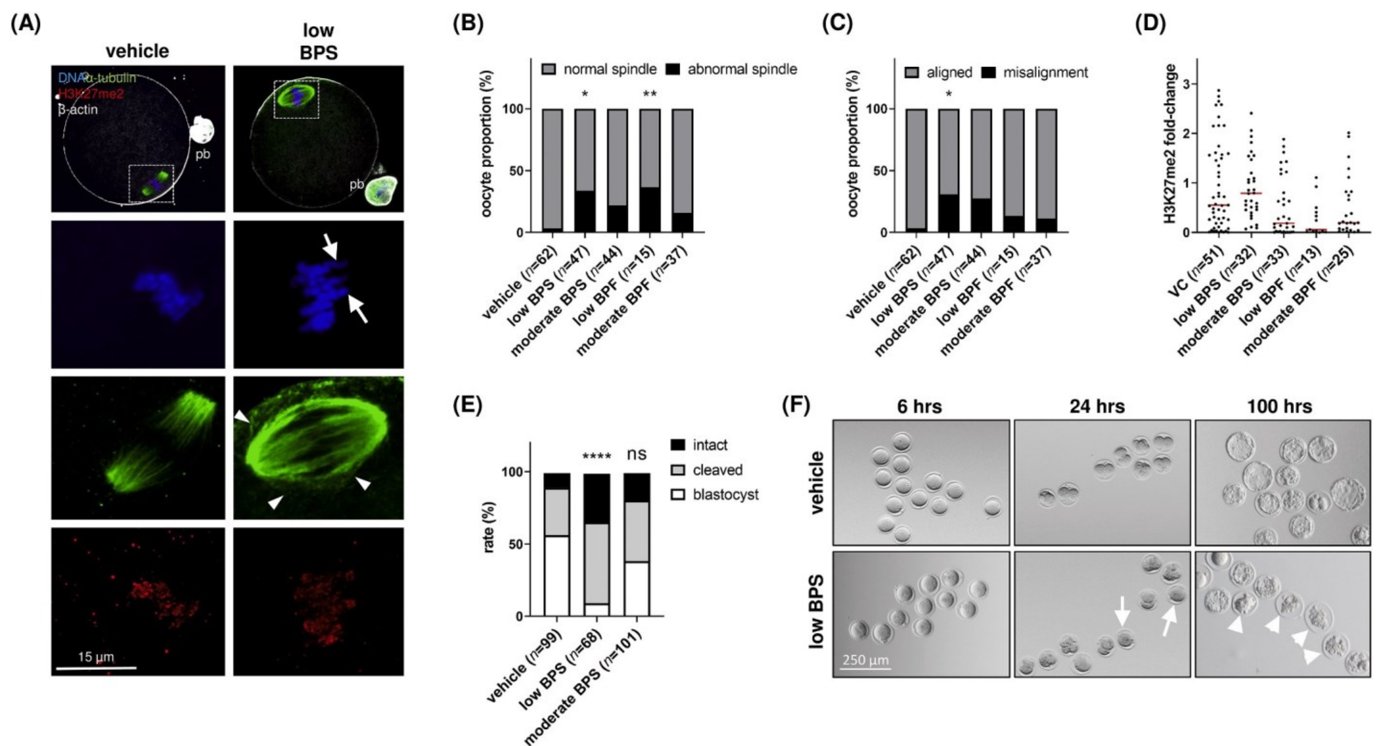


Fig. 5. Effect of nursing bisphenol exposure on the quality of *matured* and ovulated oocytes. (A) Visualisation of oocyte spindles (α-tubulin) and heterochromatin marker H3K27me2. The frame represents the emphasised area of the metaphase chromatin. Arrows indicate chromosome misalignment, arrowheads point to misadjacent tubules on the spindle; pb: polar body. (B) Quantification of matured oocytes carrying the affected spindle. (C) Frequency of chromosomal misalignment. (B,C) Data are expressed as cumulative proportion of oocytes, come from at least three unrelated female donors. The numbers of oocytes are indicated in brackets. Statistical differences between control and bisphenol-exposed groups were tested using Fisher's exact test with Bonferoni correction. Asterisks indicate statistical significance at $P < 0.05$ (*), and 0.01 (**). The numbers of analysed oocytes are indicated in brackets. (D) Quantification of H3K27me2 signal density in matured oocytes, related to unexposed control (mean of the vehicle = 1). Data show nonparametric distribution and lines indicate medians. Statistical differences were tested using the Kruskal-Wallis nonparametric test, followed by Dunn's multiple comparisons test. The number of analysed oocytes is indicated in brackets. (E) The parthenogenetically activated oocytes of BPS-exposed female offspring and the ability of cleavage and blastocyst achievement. Fisher's exact test with Bonferoni correction was used for statistical testing. Asterisks indicate statistical significance at $P < 0.0001$ (****); ns: no significance. The number of activated oocytes is indicated in brackets. (F) Illustrative pictures of activated oocytes of vehicle and low BPS groups, after 6 h, 24 h and 100 h of *in-vitro* embryo culture, leading to pronucleus formation, cleavage, and blastocyst achievement, respectively. Arrows and arrowheads indicate intact non-cleaved oocytes and lysed embryos, respectively.

Furthermore, existing knowledge has been derived mainly from BPA studies using toxic doses (Chen et al., 2020; Qiu et al., 2020), and no specific molecular markers of female fertility have been examined. In this study, we designed a mouse model of translactational exposure to female offspring, through which nursing mothers were exposed orally to bisphenols, to simulate the exposure of nursing mothers to real-life doses of alternative bisphenols, BPS and BPF. Moreover, cytoskeletal and epigenetic markers were analysed in offspring oocytes to uncover the subtle changes in molecular activity of very low doses, in accordance with knowledge of the perinatal period being highly sensitive due to DNA integrity maintenance (Stringer et al., 2020) and epigenetic changes leading to transgenerational inheritance (Pocar et al., 2017, 2012).

In our experiments, we did not observe any obesogenic, androgenic, and/or oestrogenic effects, based on litter weight recording, anogenital distance, and vaginal opening, respectively, in female pups of dams exposed to BPS or BPF. Conventional toxicological approaches cannot detect subtle changes in reproductive functions, leading to 'idiopathic infertility' when suckling offspring transition to adulthood. Therefore, we assumed that there could be impairments identifiable in follicles at different stages of development: i) in young ovaries at PND15, when most oocytes are transcriptionally active and physiologically not bearing meiotic competence and ii) in adult ovaries where follicle recruitment occurs and different stages of follicle development can be found (Sorensen and Wassarman, 1976). In this study, different doses of alternative bisphenols did not induce any significant changes in the number of follicles or in the number of atretic follicles. In accordance with the histological analyses, ovarian reserve was not affected, and the yield of immature and ovulated oocytes was similar for animals exposed to vehicle control and those exposed to both bisphenol doses. Nevertheless, oocyte quality could be affected, while ovarian assessment did not reveal any effects due to bisphenol at low doses.

Our findings showed that the oocyte cytoskeleton was impaired when females were exposed through breast milk to low doses of BPS and BPF, particularly with regard to the meiotic spindles. Moreover, persisting ooplasmic astral microtubules were observed in mature oocytes, seemingly reminiscent of the microtubule-organising centres physiologically occurring in immature oocytes (Verlhac et al., 1993), which persisted in oocytes treated with taxol, a microtubule stabilising agent (Mailhes et al., 1999). These aberrant particles may be considered as non-degraded pericentriolar material or as precursors of centrosomes in oogonia (Sathananthan et al., 2000; Simerly et al., 2018). In addition, spindle resemblance was similar to that observed following excessive polymerisation of tubulin, accompanied by widening of the spindle, and the presence of astral microtubules emanating from spindle poles and/or cytoplasmic foci, found in cryopreserved oocytes (Tamura et al., 2013). Nevertheless, this deviation must not be associated with aneuploidy (Forman et al., 2012). This phenotype is obviously an impact of the exposure on primary oocytes during the perinatal exposure window, while primordial follicles are being formed (Niu and Spradling, 2020).

In addition to spindle assessment, we assumed the epigenetic mode of action of bisphenols. Therefore, H3K27me2 was chosen as a multilateral marker of heterochromatin formation and stability, while its decrease is possibly due to induced apoptosis (Liu et al., 2017) and an obese-mouse oocyte phenotype (Hou et al., 2016). Indeed, *in-vitro*-matured oocytes showed a decrease in H3K27me2 in our experiments, indicating that chromosomal epigenetic assembly repressed heterochromatin formation. Epigenetic remodelling by endocrine disruptors is variable, with the effect of different molecules differing according to the chemical structure. In any case, the epigenetic changes in gametes can potentially lead to the modulation of epigenetic memory and/or shift of gene imprinting, resulting in transgenerational inheritance of these changes (Manikkam et al., 2013; Pocar et al., 2017). Essentially, incorrectly assembled spindles and impaired chromatin stability of *in-vitro*-matured oocytes indicate a defect in the chromosome-segregation machinery in immature oocytes, associated with specific alteration of transcription factors in the ovary of suckling females. Conversely, when the quality of *in-vivo*-matured oocytes was assessed following hormonal stimulation and ovulation of cumulus-oocyte complexes, no significant differences were found.

Our observations of spindle damage and epigenetic remodelling following bisphenol exposure in *in- vitro*- and *in-vivo*-matured oocytes are in accordance with our histological findings, highlighting the significance of an oocyte somatic cell-based environment. Whereas the follicle count was not affected, somatic cell interactions were not able to rescue oocyte quality (Li et al., 2008). This assumption is in accordance with the fact that granulosa cells are differentiated and established in the first days of perinatal life (Niu and Spradling, 2020). Although an *in-vivo* ovulation bottleneck can select oocytes with well-assembled spindles and metaphase plates (Hornak et al., 2012, 2011), we observed spindle damage even in *in-vivo*-ovulated oocytes. Although *in-vivo* oocyte maturation seems to be more robust than *in-vitro* systems, those oocytes can be affected even *in vivo* anyway, leading to the failure of embryonic development. Accordingly, we observed the declined developmental competence of BPS-affected *in-vivo*-matured oocytes using the parthenogenetic activation. Our findings are noticeably similar to earlier published work describing cytoskeletal and epigenetic damage of oocytes following gestational exposure to very low doses of bisphenol S (Zhang et al., 2020).

There are no doubts that further investigation is needed to characterise the targeted proteins. However, several aspects should be taken into consideration: i) developmental competence may be affected by the ability of oocytes to undergo meiotic disruption and maturation; ii) responsible factors are synthesised in young ovaries, seemingly creating a pool not significantly replenished past PND15, which is responsible for the cast-off infertility diagnosed as idiopathic; and iii) surprisingly, the exposure to lower doses of BPS exerts activity which is in contrast to moderate exposure levels of BPS, underlining the non-linear effect of BPS as an agonist of yet unknown targets, further supporting the role of perinatal-induced and bisphenol-driven idiopathic infertility.

5. Conclusions

Alternative bisphenols seem inappropriate and should not be considered as safe BPA substitutes. Both BPS and BPF exert distinct biological effects on oocytes during the perinatal exposure window when the ovarian pool meiotic competence of oocytes is established. Interestingly, individual bisphenols seem to differ in their molecular activity and result in different phenotypes in BPS- *vs.* BPF-exposed females. Further, each BPA substitute is characterised by a different 'effective dose', resulting in different threshold effects. Given the above evidence, bisphenol analogues represent compounds bearing a burden on human reproduction, and through the effects induced during an early exposure window in the breastfeeding infant, these may potentially result in idiopathic infertility in women.

Author contribution

JN designed the study, interpreted, and analysed the data for publication and manuscript preparation. JN, YK, TŽ, JP, and MK contributed to the design of the experiments and critical approval of the final article. JN, JH, YK, ŠP, TF, and LM performed experiments, acquired data, and performed statistical analysis. All co-authors contributed to the manuscript writing.

Funding sources

This study was supported by the Czech Health Research Council (NV18-01-00544), the Charles University Research Fund (Progress Q39), and the National Sustainability Programme I (NPU I) No. LO1503 was provided by the Ministry of Education, Youth and Sports of the Czech Republic (MEYS CR); project No. SVV 02690 was awarded by MEYS CR; and project No. CZ.02.1.01/0.0/0.0/16_019/0000787 'Fighting Infectious Diseases' was awarded by the MEYS CR and financed by the European Regional Development Fund, European Human Biomonitoring Initiative. HBM4EU was provided by H2020. S. P., T.Z., K.H., and J.P. were supported by the Czech Ministry of Agriculture (MZE-RO 0718).

Credit author statement

Jan Nevoral: designing of the study, funding acquisition, project administration, conceptualization, methodology, data curation, data analysis and interpretation for publication, manuscript writing (original draft, review, editing) and figures' preparation, formal analysis.

Jiřina Havránková: performing experiments, investigation, visualisation, data analysis (histology), contributing to the manuscript writing.

Yaroslav Kolinko: designing of the study, methodology, interpretation, software, validation, statistics.

Šárka Prokešová: performing of experiments (immunocytochemistry).

Tereza Fenclová: performing of experiments (image analysis).

Ladan Monsef: performed experiments (parthenogenetic activation).

Tereza Žalmanová: designing of the study, writing - review & editing.

Jaroslav Petr: funding acquisition, designing of the study, manuscript writing.

Milena Králíčková: funding acquisition, designing of the study, supervision.

Declaration of Competing Interest

No conflicts of interest are declared.

Acknowledgements

We would like to thank Lucie Marsalova and Marketa Slajerova for their technical support in the histological analysis, and Vaclav Rucka for his enthusiastic assistance with the animal facility.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.taap.2021.115409.

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frontiers in Physiology

ORIGINAL RESEARCH
published: 24 February 2022
doi: 10.3389/fphys.2022.725442

Nursing Exposure to Bisphenols as a Cause of Male Idiopathic Infertility

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Idiopathic infertility is a serious problem, which can be caused and explained by exposure to endocrine disruptors, such as bisphenols. In our study, we studied transactional exposure to bisphenol and its effects on newborn male mice throughout their reproductive life. Newborn male mice were exposed to bisphenol S and bisphenol F through maternal milk from post-natal day 0 to post-natal day 15 at concentrations of 0.1 ng.g/bw/day and 10 ng.g/bw/day, respectively. Although there were minimal differences between the control and experimental groups in testicular tissue quality and spermatozoa quality, we discovered an interesting influence on early embryonic development. Moderate doses of bisphenol negatively affected cleavage of the early embryo and subsequently, the blastocyst rate, as well as the number of blastomeres per blastocyst. In our study, we focused on correlations between particular stages from spermatogenesis to blastocyst development. We followed epigenetic changes such as dimethylation of histone H3 and phosphorylation of histone H2 from germ cells to blastocysts; we discovered the transfer of DNA double-strand breaks through the paternal pronucleus from spermatozoa to blastomeres in the blastocyst. We elucidated the impact of sperm DNA damage on early embryonic development, and our results indicate that idiopathic infertility in adulthood may have causes related to the perinatal period.

Keywords: bisphenol, DNA damage, idiopathic infertility, spermatogenesis, nursing exposure

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Physiology

Received: 15 June 2021
Accepted: 18 January 2022
Published: 24 February 2022

Citation:
Fenclová T, Rˇ imnácˇ ová H, Chemek M, Havránková J, Klein P, Králícˇ ková M and Nevoral J (2022) Nursing Exposure to Bisphenols as a Cause of Male Idiopathic Infertility. Front. Physiol. 13:725442. doi: 10.3389/fphys.2022.725442

INTRODUCTION

The impact of the environment on reproductive health may be an explanation for idiopathic infertility (Shi et al., 2017). Environmental contaminants with estrogenic and/or anti-androgenic activity include bisphenols (BPs) and endocrine disruptors (Glausiusz, 2014; Eladak et al., 2015; Rahman et al., 2015). Bisphenols are mostly present in manufactured plastics, paper, cans, and other products for daily use (Simoneau et al., 2011; Wong and Durrani, 2017). The most widely used bisphenol, bisphenol A (BPA), has various deleterious effects on human physiology and health,

Abbreviations: BPs, bisphenols; BPA, bisphenol A; BPS, bisphenol S; BPF, bisphenol F; DAPI, 4,6-diamidino-2-phenylindole; DFI, DNA fragmentation index (DFI); HDS, high DNA stainability; H3K4me2, dimethylation of lysine (K4) on histone H3; PBS, Phosphate buffered saline; PND, post-natal day; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; γH2AX, phosphorylation of histone H2.

including male (Salian et al., 2011; Rahman et al., 2015; Shi et al., 2019) and female (Rivera et al., 2015) reproduction and fertility (Vandenberg et al., 2007; Siracusa et al., 2018) as well as *in vitro* fertilization (IVF) (Mok-Lin et al., 2010). This effect has been observed even at very low (e.g., subtoxic) concentrations (Vandenberg et al., 2012; Eladak et al., 2015). BPA has been replaced in the manufacturing process with several analogs, including bisphenol S (BPS) and bisphenol F (BPF) (Eladak et al., 2015; Shi et al., 2019), which are more chemically stable than BPA but are worse in terms of biodegradability; they also show better dermal penetration (Ike et al., 2006; Danzl et al., 2009; Liao et al., 2012a,b). Bisphenol A analogs contaminate the environment (Chen et al., 2016), including water, air, house dust, and food (Ike et al., 2006; Viñas et al., 2010; Liao and Kannan, 2014; Wu et al., 2018). Therefore, the highest intake of bisphenols in humans occurs through the diet, primarily through the consumption of canned foods or drinking bottled water (Ehrlich et al., 2014; Eladak et al., 2015). However, we can consider more inconspicuous routes of exposure, such as gestational and/or perinatal exposure via the placenta and breast milk, respectively.

Prenatal exposure to bisphenols in male rats decreased sperm motility, counts, and quality (Salian et al., 2011). In addition, changes in testicular peripubertal development (Muñoz-de-Toro et al., 2005), testicular tissue, and testes gene expression have been observed in mice (Shi et al., 2018), and these changes were proven to be transferred to the next generation (Salian et al., 2011; Rahman et al., 2017). Moreover, bisphenols affect post-translational modifications in sperm, and dimethylation of lysine K4 on histone H3 (H3K4me2) has been established as an indicator of aberrant histone-protamine exchange, resulting in improper chromatin condensation (Štiavnická et al., 2020) and chromatin immaturity (Lambrot et al., 2019; Štiavnická et al., 2020). The epigenetic mode of action is similarly affected by bisphenol in oocytes (Žalmanová et al., 2017; Nevoral et al., 2018; Prokešová et al., 2020).

However, perinatal nursing exposure, as another indirect exposition route is currently underestimated, although the impact seems to be significant for the following reasons: (i) bisphenols are more concentrated in maternal milk; (ii) maternal milk (with bisphenols) is an exclusive food for newborns; and (iii) newborns do not have fully developed detoxification mechanisms, especially in the liver and kidneys (Matalová et al., 2016). In our experimental approach, dams were exposed to bisphenols via a common route of exposure during the breastfeeding period, leading to indirect exposure of their offspring (Chemek and Nevoral, 2019). The dosage of BPs is considered to be significant (EFSA Panel on Food Contact Materials, Enzymes, and Flavourings and Processing Aids [CEF], 2015).

In our experiment, we studied transactional bisphenol exposure and its effects on newborn male mice throughout their reproductive life. What is currently diagnosed as idiopathic infertility can, in fact, be the result of exposure to endocrine disruptors during the nursing period. Our study is unique owing to very low bisphenol doses in the early exposure window with consequences in adulthood, which is a model of idiopathic infertility. Our innovative approach revealed correlations between each stage of spermatogenesis, with overlap to early embryonic development, zygotes, and blastocysts.

MATERIALS AND METHODS

Chemicals

All basic chemicals were purchased from Sigma-Aldrich (Missouri, United States), and chemicals for sperm washing were purchased from Irvine Scientific (California, United States) unless otherwise noted. Antibodies against anti-H3K4me2 and anti-γH2AX and anti-mouse/rabbit IgG antibodies conjugated with fluorescein (Alexa Fluor 488/647) were purchased from Abcam (Cambridge, United Kingdom). Anti-tubulin antibody was used as an internal loading control and was purchased from Cell Signaling Technology (Massachusetts, United States). Anti-mouse and anti-rabbit antibodies conjugated with horseradish peroxidase were purchased from Sigma-Aldrich (Missouri, United States).

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 Committee of the Ministry of Education, Youth and Sports of the Czech Republic, approval ID MSMT-11925/2016-3.

Animals

Six- to seven-week-old female ICR mice were purchased from Velaz Ltd. (Czech Republic) and used as mothers of F1 offspring subjected to experimental assessment. Experimental design consisted of six groups; vehicle control, low diethylstilbestrol (DES), low BPS, moderate BPS, low BPF, and moderate BPF. We exposed nursing dams (at least $n = 6$ in each experimental group) of outbred ICR mice to low and moderate levels of BPS and BPF (0.2 and 20 ng/g body weight of dams per day, respectively) over the first 15 days of nursing. Within 18 months, we analyzed a total of 82 of their litters, when each group consisted of at least seven litters. We analyzed at least three male pups from each litter. All animals were housed in intact polysulfonate cages and maintained in a facility with a 12-h light/dark cycle, a temperature of 21 ± 1°C, and relative humidity of 60%. A phyto-estrogen-free diet (1814P; Altromin, Germany) and ultrapure water (in glass bottles, changed twice per week) were provided *ad libitum*.

Bisphenol Dosing

Dams were dosed with BPS or BPF through drinking water, 0.375 ng/mL and 37.5 ng/mL (hereafter denoted as low and moderate concentrations, respectively), and the other animals were exposed to the vehicle (0.1% ethanol). Dams were treated from delivery until the 15th post-natal day (PND) of the male offspring. Doses were chosen in accordance with known biological effect published previously (Nevoral et al., 2018; Prokešová et al., 2020) and to obtain an assumed exposure to 0.1 ng.g/body weight/day BPS and 10 ng.g/body weight/day BPF. The route of the exposure was used with respect to the welfare of nursing dams and real intake was precisely calculated within genuine water intake as 0.2

ng/g body weight/day and 20 ng/g body weight/day for BPS and BPF, respectively. The male offspring were weaned at PND 21 and housed individually in standard conditions until the 14th week of age, when reproductive maturity was achieved.

Embryo Flushing and *in vitro* Culture

Eight-week-old embryo donors were stimulated with pregnant mare serum gonadotropin and human chorionic gonadotropin 48 h later. Following overnight mating, females were euthanized by cervical dislocation, and one-cell zygotes were isolated from the oviduct in 0.1% hyaluronidase in M2 medium. After cumulus cell removal, zygotes were fixed in 4% paraformaldehyde for 30 min and stored at 4°C until further experimentation. Alternatively, zygotes were cultured for an additional 4 days *in vitro*, using the modified potassium simplex optimization medium covered with mineral oil. At the end of embryo culture, the blastocyst rate was recorded, and blastocysts were fixed as mentioned above and used for further experiments. The experimental males were euthanized by cervical dislocation. The cauda epididymis was isolated into human tubal fluid medium with 0.1% bovine serum albumin. Sperm were allowed to swim for 30 min and used for further experiments.

Sperm Characteristics and Assessment

Sperm concentration and motility were evaluated using a prewarmed Makler chamber and a light microscope (Olympus CKX 41; Germany) equipped with a 10 × objective lens (CAchN NA 0.25). Ten microliters of sperm suspension were placed into the Makler chamber. Thereafter, the average sperm concentration (million per milliliter) was counted in three lines, each of 10 squares, and divided by three. Simultaneously, each spermatozoon across the counted area was identified as either motile or immotile. Motility was expressed as the percentage of motile sperm from the total count. The analysis was performed in a single-blind manner by one person to avoid bias.

Sperm Chromatin Structure Assay

A sperm chromatin structure assay was performed according to a previously described protocol (Evenson and Jost, 2001). This technique is based on the vulnerability of sperm DNA to acid-induced denaturation *in situ* and subsequent metachromatic staining with acridine orange. The DNA fragmentation index (DFI) lrb% and high DNA stainability (HDS) lrb%, indicators of chromatin immaturity (*i.e.*, protamination completeness), were assessed. The samples were evaluated using a FACSVerse Flow Cytometer (BD Biosciences) controlled with BD FACSuite. For each sample, 5,000 events were recorded. Excitation of acridine orange was performed with a blue laser (488 nm); red fluorescence was detected with a 700/54 BP filter, and green fluorescence was detected with a 537/32 BP filter. Data were analyzed using WEASEL Ver. 3 (WEHI).

Electrophoresis and Western Blot

Electrophoresis and Western blotting were performed on testicular tissue and the sperm of experimental males. Testes samples were lysed in radioimmunoprecipitation assay buffer enriched with a complete mini protease inhibitor cocktail (Roche, Switzerland). Samples of sperm heads were prepared by sonication and lysis in radioimmunoprecipitation assay buffer supplemented with 100 mM DTT. Protein concentration was measured using the bicinchoninic acid method (Lovrien and Matulis, 2005; Olson and Markwell, 2016). Thereafter, the samples were mixed with Laemmli loading buffer supplemented with β-mercaptoethanol. For dodecyl sulfate polyacrylamide gel electrophoresis, 4–15% separating Mini-PROTEAN® TGX Stain-Free™ Precast Gels (Bio-Rad, France) were used; 30 μg of testicular proteins (60 μg) or sperm proteins were loaded into each gel chamber. For Western blotting, the Trans-Blot® Turbo™ Transfer System (Bio-Rad, France) was used. Polyvinylidene difluoride membranes (Bio-Rad, France) were blocked in 5% bovine serum albumin in TBS with 0.5% Tween-20 for 60 min at 21 ± 1°C and incubated overnight at 4°C with primary antibodies diluted in 1% bovine serum albumin in TBS with 0.5% Tween-20. Molecular sperm quality was evaluated using a rabbit polyclonal anti-H3K4me2 antibody (1:1,000; cat. no. ab7766; Abcam, United Kingdom) and a mouse monoclonal anti-γH2AX antibody (1:1,000; cat. no. ab26350; Abcam, United Kingdom). Rabbit polyclonal anti-α-tubulin antibody (1:1,000; cat. no. #2144; Cell Signaling, United States) was used as the housekeeping antibody. Horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse or anti-rabbit IgG; 1:15,000; Invitrogen, United States) were applied for 1 h at 21 ± 1°C. The targeted proteins were visualized using ECL Select Western blotting Detection Reagent (GE Healthcare Life Sciences, United Kingdom), and membranes were scanned on a ChemiDoc™ MP System (Bio-Rad, France). Images of membranes were processed using ImageLab 4.1 software (Bio-Rad, France). The same method was used to evaluate testicular tissue, which revealed changes in the context of spermatogenesis between experimental groups.

Immunofluorescence

For γH2AX and H3K4me2 immunostaining, 10 sections were dewaxed, rehydrated, and processed as described by Chemek et al. (2018) with some modifications. Antigen retrieval was performed by pressure-cooking slides for 10 min in 0.01 M citrate buffer (pH 6.0). Non-specific binding sites were blocked with a solution of 5% normal goat serum (NGS) and 0.1% TritonX-100 and 0.5% Tween 20 in phosphate-buffered saline (PBS-TT) for 60 min at 21 ± 1°C. Subsequently, the testis sections were incubated with a rabbit polyclonal anti-H3K4me2 antibody (1:100; cat. no. ab7766; Abcam, United Kingdom) or a mouse monoclonal anti-γH2AX antibody (1:200; cat. no. ab26350; Abcam, United Kingdom) before overnight incubation at 4°C. Non-specific binding of the secondary antibodies was tested by omitting specific primary antibodies and these slides were processed at comparable settings. After washing in PBS-TT solution containing 1% NGS, slides were incubated for 40 min with PNA lectin (Alexa Fluor 488, Abcam, United Kingdom) diluted 1:400 and with the appropriate secondary antibody (anti-mouse or anti-rabbit Alexa Fluor 647, Abcam, United Kingdom) diluted 1:500 in PBS-TT containing 1% NGS. The slides were mounted with Vectashield medium plus

4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., United States) for nuclear staining. Slides were then observed with a confocal microscope, and images were analyzed using ImageJ software (NIH, United States).

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling Assay

TUNEL assays were performed to analyze DNA double-strand breaks in blastocysts derived from zygotes flushed after exposed male mating. Fixed blastocysts were permeabilized in 0.1% Triton X-100 in PBS containing 0.05% NaN_3 for 40 min. The blastocysts were then treated with fluorescein-conjugated dUTP and terminal deoxyribonucleotidyl transferase enzyme (*In Situ* Cell Death Detection Kit, cat. no. 11684795910, Roche, Germany) for 1 h in the dark at 37°C. The positive control was prepared using a DNase I kit (AMP-D1, Sigma-Aldrich, United States). Finally, the blastocysts were mounted onto slides with Vectashield medium and DAPI (Vector Laboratories Inc., United States). Signal intensity was measured using ImageJ software (National Institutes of Health, United States).

Immunocytochemistry

Fixed zygotes were permeabilized in PBS containing 0.04% TritonX-100 and 0.3% Tween-20 for 15 min at 37°C. Zygote epigenetic marks were evaluated, including γH2AX and H3K4me2. Zygotes were then blocked in 1% bovine serum albumin in PBS with Tween-20 for 15 min and incubated with a cocktail of antibodies against mouse anti-γH2AX (1:200, cat. no. ab26350; Abcam, United Kingdom) and rabbit anti-H3K4me2 (1:200, cat. no. ab7766; Abcam, United Kingdom) antibodies for 1 h at 21 ± 1°C. Non-specific binding of the secondary antibodies was tested by omitting specific primary antibodies and these slides were processed at comparable settings. After washing, the oocytes were incubated with a cocktail of anti-mouse and anti-rabbit Alexa Fluor 488 and 647 (1:200) antibodies, respectively. Phalloidin (1:200; cat. no. #13054; Thermo Fisher Scientific, United States) was added to the wash and used for β-actin visualization. Stained zygotes were mounted onto slides in Vectashield medium with DAPI (Vector Laboratories Inc., Burlingame, CA, United States). Images were acquired using an Olympus IX83 spinning disc confocal microscope (Olympus, Germany) and VisiView software (Visitron Systems GmbH, Germany). The number of γH2AX loci in the developed paternal pronucleus was assessed. The integrated density of H3K4me2 in those pronuclei was measured using ImageJ (NIH, United States) software; the expanded pronucleus was considered as paternal, and the density was normalized to maternal density.

Statistical Analyses

The data were analyzed using GraphPad Prism 8.1.1 (GraphPad Software Inc., San Diego, CA, United States). Based on Shapiro-Wilk's normality distribution tests, differences were tested using an ordinary one-way analysis of variance, followed by Tukey's multiple test. Alternatively, Kruskal-Wallis and Dunn's *post hoc* tests were used for non-normally distributed data. *P*-values < 0.05, 0.01, 0.001, and 0.0001 were considered statistically significant and indicated with asterisks (*), (**), (***), and (****), respectively. Alternatively, daggers indicated differences from the positive control (low DES) (#, ##, ###, and ####, respectively). Normally and non-normally distributed data are expressed as means and medians, respectively.

RESULTS

Effect of Nursing Exposure to Bisphenols on Reproductive Signs and the Spermiogram

In our experiment, male offspring were exposed to BPS or BPF during the first 15 days of their lives through breast milk. Diethylstilbestrol was used as an estrogen positive control. The aim was to elucidate bisphenols' obesogenic and/or estrogen-like effect on morphological development of exposed males, due to body weight and anogenital distance, respectively. Moreover, the conventional sperm parameters and DNA integrity were assessed. There was no impact of exposure on the body weight of males at PND 21 or PND 90 (**Figure 1A**). However, low BPS exposure affects the anogenital distance, although the same dose of DES does not show any effect (**Figure 1B**). Sperm motility (**Figure 1C**) and sperm concentration (**Figure 1D**) were not significantly reduced in bisphenol-exposed groups; the DFI and sperm immaturity *via* HDS were also not affected (**Figure 1E**).

Nursing Exposure to Bisphenols Changes Histone Code in Germ Cells of Adult Testis

Because there was no observable effect of low doses of bisphenols as obesogenic, estrogen-like, and/or toxic compounds, we considered the epigenetic mode of bisphenol action toward early life-vulnerable germ cells. Therefore, we focused on well-established epigenetic markers of DNA damage—H3K4me2 and γH2AX. First, we evaluated the amount of H3K4me2 in seminiferous tubules at stages VII–VIII of spermatogenesis, as a parameter of DNA damage and integrity. These stages were recognized using FITC-conjugated peanut-agglutinin co-staining and corresponded to spermiation (**Figure 2A**). In the immunofluorescence assay, we observed a statistically significant increase in H3K4me2 levels in the testes of males exposed to moderate doses of BPS; however, there was no statistical difference between the control group and low BPS group (**Figure 2B**). We enhanced this finding with Western blotting and densitometry analysis, approved by the molecular weight of histone H3 (**Figure 2C**). In whole-testicular lysates, there was no statistical difference between the control and experimental groups (**Figure 2D**). In addition to H3K4me2, we analyzed the amount of γH2AX in seminiferous tubules at stages VII–VIII of spermatogenesis (**Figure 2E**). We analyzed the area of expression of the γH2AX and found no statistical difference between the control and experimental groups (**Figure 2F**). Neither Western blotting nor densitometry (**Figure 2G**) showed statistically

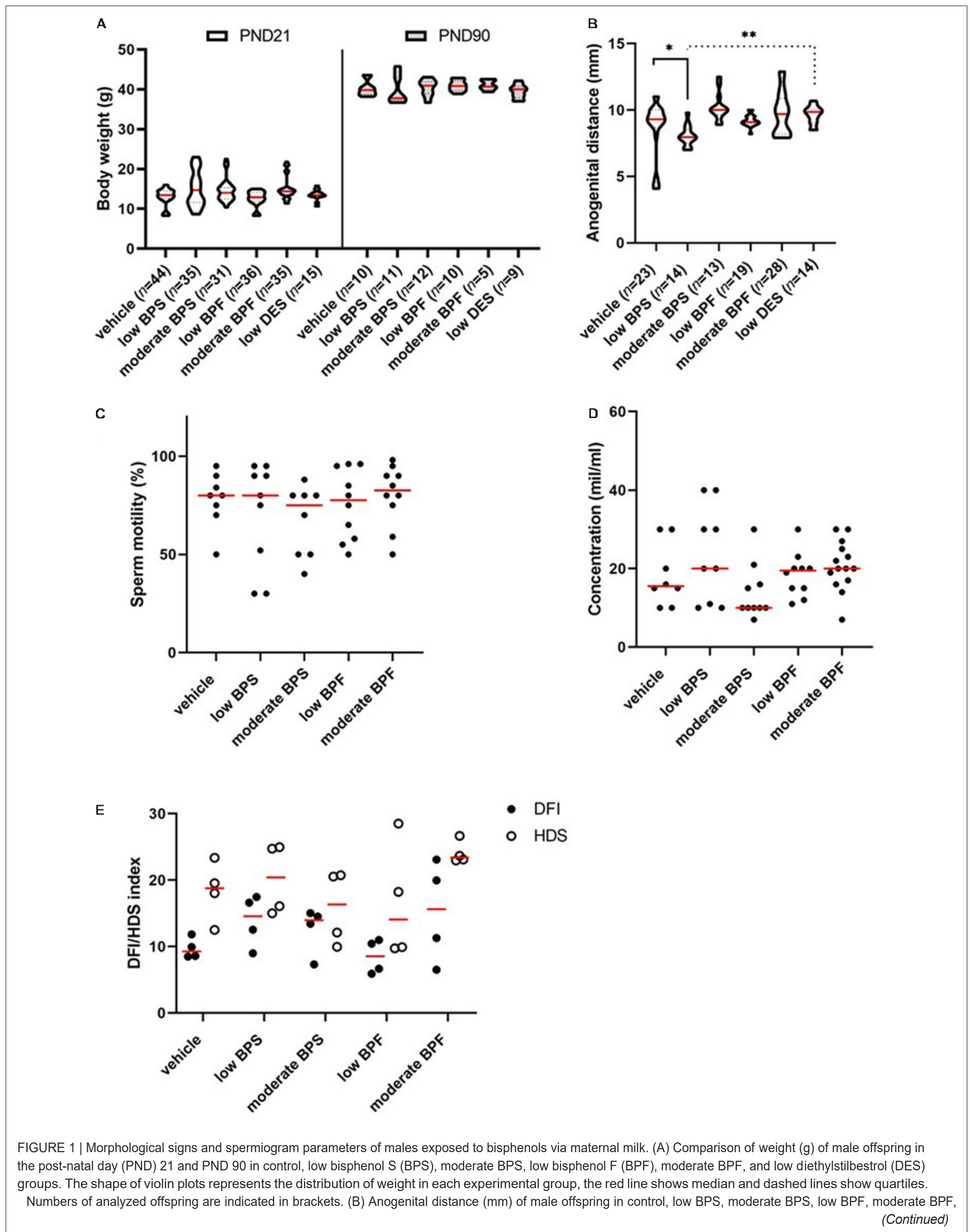


FIGURE 1 | Morphological signs and spermiogram parameters of males exposed to bisphenols via maternal milk. (A) Comparison of weight (g) of male offspring in the post-natal day (PND) 21 and PND 90 in control, low bisphenol S (BPS), moderate BPS, low bisphenol F (BPF), moderate BPF, and low diethylstilbestrol (DES) groups. The shape of violin plots represents the distribution of weight in each experimental group, the red line shows median and dashed lines show quartiles. Numbers of analyzed offspring are indicated in brackets. (B) Anogenital distance (mm) of male offspring in control, low BPS, moderate BPS, low BPF, moderate BPF,

(Continued)

FIGURE 1 | and low DES groups. The shape of violin plots represents the distribution of anogenital distance in each experimental group, the red line shows median and dashed lines show quartiles. Numbers of analyzed offspring are indicated in brackets. (C) Motility of spermatozoa (%) of male offspring in control, low BPS, moderate BPS, low BPF, and moderate BPF groups. Dots represent individual males; red lines show the median. (D) Concentration of spermatozoa (mil/ml) on male offspring in control, low BPS, moderate BPS, low BPF, and moderate BPF groups. Dots represent individual males; red lines show the median. (E) DNA fragmentation index (DFI) of male offspring in control, low BPS, moderate BPS, low BPF, and moderate BPF groups and high DNA stainability index (HDS) of male offspring in control, low BPS, moderate BPS, low BPF, and moderate BPF groups. Dots represent individual males; red lines show the median. Statistical differences were tested using the Kruskal-Wallis test, followed by Dunn's multiple comparisons test. Statistical differences are indicated from the vehicle ($*P < 0.05$, $**P < 0.01$). For DFI and HDS, statistical differences were tested using the two-way ANOVA followed by Sidak's multiple comparison test. BPF: bisphenol F; BPS: bisphenol S; PND: post-natal day; DES: diethylstilbestrol; DFI: DNA fragmentation index; HDS: high DNA stainability index.

significant differences between the control and experimental groups (**Figures 2G,H**).

Bisphenol Nursing Exposure Does Not Affect Histone Code in Spermatozoa of Adult Males

According to the results with testicular tissue, we determined the amount of DNA breaks due to the assessed epigenetic markers: the dimethylation of histone H3 (H3K4me2) and phosphorylation of histone H2 (γH2AX). Both markers were examined in spermatozoa by Western blotting, followed by quantification by densitometry (**Figure 3A**). There was no statistical difference between the control and experimental groups in the levels of H3K4me2 and γH2AX (**Figure 3B**). The specificity of antibody binding was elucidated by immunocytochemistry, and representative images of H3K4me2 and γH2AX localization in the sperm head are shown in **Figure 3C**.

Nursing Exposure to Bisphenols Impairs Sperm Contribution on Zygote Quality

According to the findings mentioned above, we assessed sperm chromatin quality after development to the paternal pronucleus, followed by an *in vivo* fertilization assay and zygote flushing. Zygotes were fixed, and H3K4me2 and γH2AX were co-stained using immunocytochemistry. Based on DNA staining of both pronuclei, progressive development was considered to be paternal. We analyzed the integrated density of H3K4me2 in the paternal pronuclei and observed decreased levels of H3K4me2 in the low DES group (**Figure 4A**), although no observable effect was observed in zygotes belonging to moderate-BPS-exposed males. In addition to H3K4me2, there was an increase in the number of γH2AX loci in all experimental groups compared to that in the vehicle control, indicating DNA damage in bisphenol-affected spermatozoa participating in fertilization. The exposure to low DES similarly induces increasing γH2AX (**Figure 4A**). Subcellular localization of both histone markers in the paternal pronucleus is shown in **Figure 4B**.

Bisphenol Nursing Exposure Affects Sperm Development Competence

Following the findings of a higher occurrence of DNA damage markers in zygotes after BPS/BPF exposure, we evaluated early embryonic development up to the blastocyst stage. Therefore, *in vivo* produced zygotes were flushed and cultured *in vitro* until the blastocyst stage, alternatively to the zygote immunostaining mentioned above. In addition to this evaluation, we assessed the quality of blastocysts achieved *in vitro*, based on blastomere cell counting and DNA damage evaluation.

First, we analyzed the pregnancy rate (**Figure 5A**) of females mated by bisphenol-exposed males, followed by the assessment of the fertilization rate as the ratio of zygotes to non-fertilized oocytes (**Figure 5B**). For both parameters, there was no statistical difference between the control and experimental groups. We then evaluated the quantitative indicators of embryonic development. For cleavage, there was a statistically significant decrease in the moderate BPS group by a median of 71.4% (**Figures 5C,D**). For the blastocyst rate, we did not record statistical differences between the control and moderate BPS groups when the blastocysts were related to cleaved embryos (**Figures 5E,F**). Although the amount of blastocysts dropped by 41.2% in the moderate BPS group (data not shown), we considered this to be a result of embryo cleavage. Qualitative indicators of embryonic development, such as the number of blastomeres per blastocyst and apoptotic index, were evaluated using DAPI staining and TUNEL assay. There was a statistical difference between the control group and both BPS groups in the number of blastomeres per blastocyst (**Figures 5G,H**). There was a statistical difference in the apoptotic index between the control and low DES groups (**Figure 5I**), while DES-derived blastocysts showed surprisingly increased blastomere counts. Obviously, zygotes derived from BPS-exposed males are affected in terms of further embryonic development. Similarly, the quality of blastocysts is affected in BPS-exposed males, although the incidence of DNA-damaged blastomeres does not increase. We also discovered an increase in γH2AX in blastomeres in the moderate BPS group by the TUNEL assay (**Figure 5J**).

Embryo Success Is Determined by Sperm Quality Achieved During Spermatogenesis

We used data from previous experiments to determine the statistical correlations between individual parameters. We used the Spearman correlation coefficient to evaluate dependencies, which we supplemented with confidence intervals. The expected relationship between the fertilization rate and blastocyst rate was confirmed based on the finding of a moderate positive correlation. Interestingly, we found a moderate negative correlation between H3K4me2 in testicular germ cells and cleavage of early embryos, as well as a high negative correlation between H3K4me2 and double-strand breaks in blastocysts.

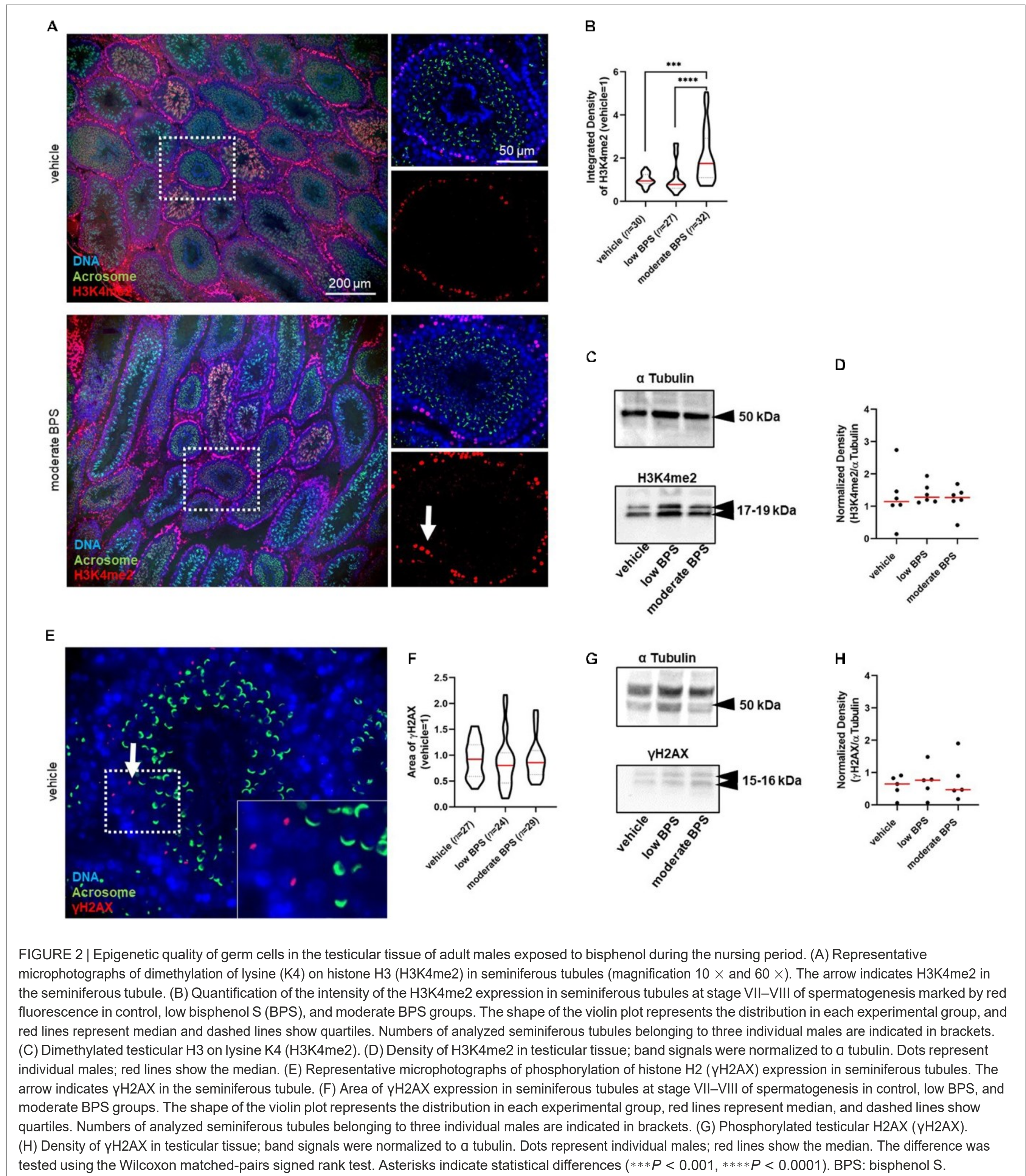


FIGURE 2 | Epigenetic quality of germ cells in the testicular tissue of adult males exposed to bisphenol during the nursing period. (A) Representative microphotographs of dimethylation of lysine (K4) on histone H3 (H3K4me2) in seminiferous tubules (magnification 10 × and 60 ×). The arrow indicates H3K4me2 in the seminiferous tubule. (B) Quantification of the intensity of the H3K4me2 expression in seminiferous tubules at stage VII–VIII of spermatogenesis marked by red fluorescence in control, low bisphenol S (BPS), and moderate BPS groups. The shape of the violin plot represents the distribution in each experimental group, and red lines represent median and dashed lines show quartiles. Numbers of analyzed seminiferous tubules belonging to three individual males are indicated in brackets. (C) Dimethylated testicular H3 on lysine K4 (H3K4me2). (D) Density of H3K4me2 in testicular tissue; band signals were normalized to α tubulin. Dots represent individual males; red lines show the median. (E) Representative microphotographs of phosphorylation of histone H2 (γH2AX) expression in seminiferous tubules. The arrow indicates γH2AX in the seminiferous tubule. (F) Area of γH2AX expression in seminiferous tubules at stage VII–VIII of spermatogenesis in control, low BPS, and moderate BPS groups. The shape of the violin plot represents the distribution in each experimental group, red lines represent median, and dashed lines show quartiles. Numbers of analyzed seminiferous tubules belonging to three individual males are indicated in brackets. (G) Phosphorylated testicular H2AX (γH2AX). (H) Density of γH2AX in testicular tissue; band signals were normalized to α tubulin. Dots represent individual males; red lines show the median. The difference was tested using the Wilcoxon matched-pairs signed rank test. Asterisks indicate statistical differences (*** $P < 0.001$, **** $P < 0.0001$). BPS: bisphenol S.

Although no difference in the phosphorylation of sperm histone H2AX (γH2AX) was observed between control and bisphenol-exposed adult males, there is a high positive correlation between γH2AX in spermatozoa and γH2AX loci in paternal pronuclei in zygotes. It is clear that testicular quality and sperm quality are associated with the success of early embryonic development and blastocyst quality, as well as the vulnerability of juvenile testis to environmental noxious influences. All

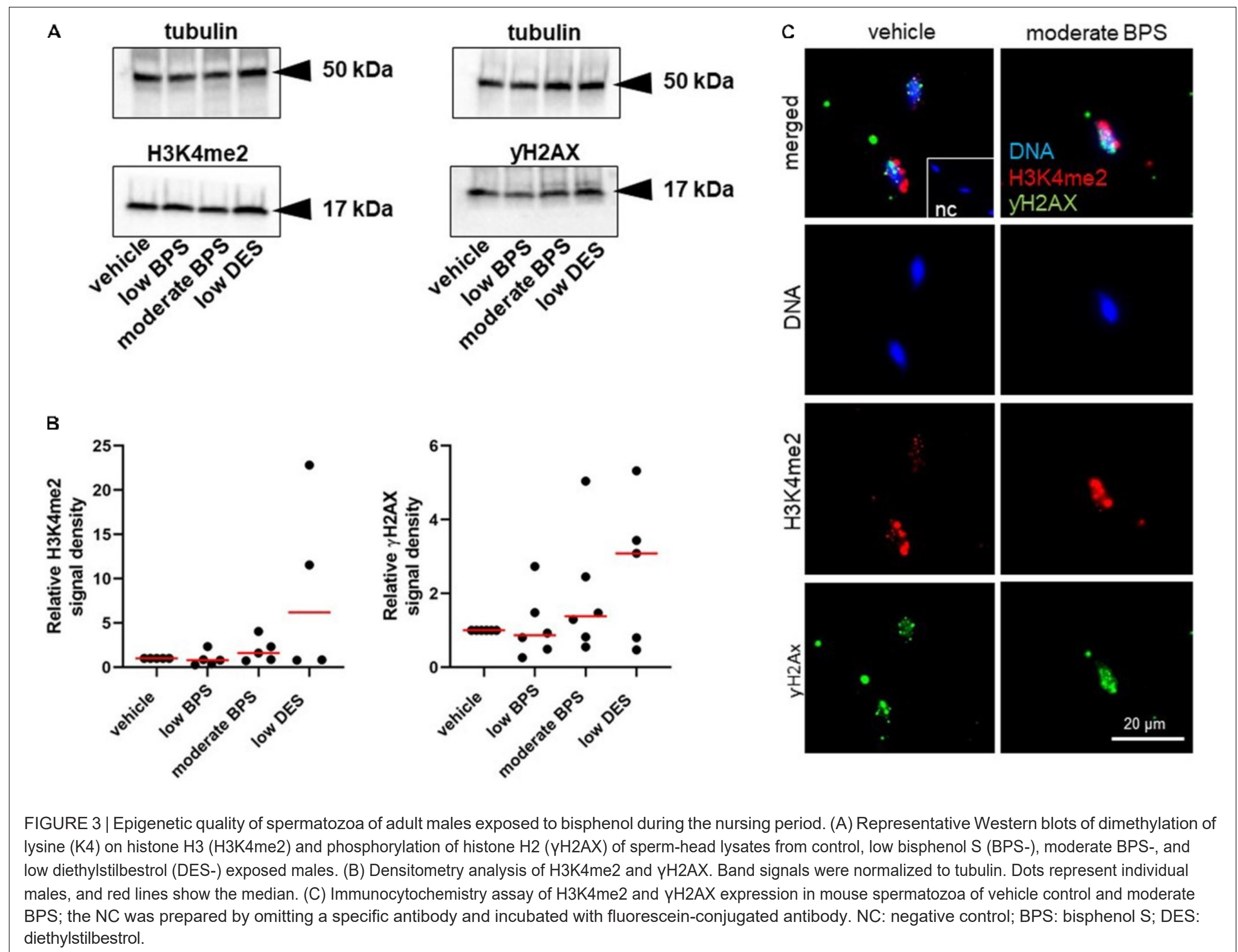


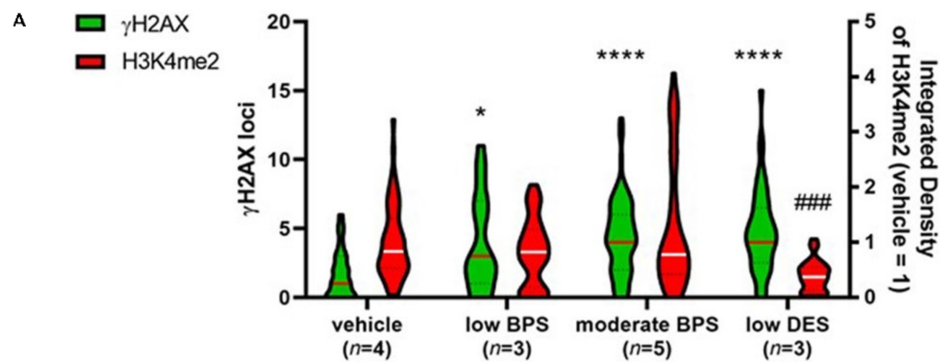
FIGURE 3 | Epigenetic quality of spermatozoa of adult males exposed to bisphenol during the nursing period. (A) Representative Western blots of dimethylation of lysine (K4) on histone H3 (H3K4me2) and phosphorylation of histone H2 (γH2AX) of sperm-head lysates from control, low bisphenol S (BPS-), moderate BPS-, and low diethylstilbestrol (DES-) exposed males. (B) Densitometry analysis of H3K4me2 and γH2AX. Band signals were normalized to tubulin. Dots represent individual males, and red lines show the median. (C) Immunocytochemistry assay of H3K4me2 and γH2AX expression in mouse spermatozoa of vehicle control and moderate BPS; the NC was prepared by omitting a specific antibody and incubated with fluorescein-conjugated antibody. NC: negative control; BPS: bisphenol S; DES: diethylstilbestrol.

significant relations are summarized in **Table 1**. For complete outputs of Spearman coefficients and confidence intervals, see **Supplementary Table 1**.

DISCUSSION

Bisphenols are widespread endocrine disruptors, and their effects on reproductive health are currently intensively disputed. Our study contributes to this issue by providing a unique method of exposure *via* breastfeeding. Although there is no evidence that the concentration of bisphenols is higher in human breast milk than in adult human urine or serum (Vandenberg et al., 2010; Dualde et al., 2019; Luo et al., 2021), nursing exposure of infants is considered dangerous due to the exclusiveness of breast milk as the source of nourishment. Moreover, newborns do not have fully developed elimination mechanisms involving detoxification in the liver and/or the glucuronidation pathway in the kidney (Matalová et al., 2016). Although the chosen dosage was extremely low in accordance with the actual exposure (González et al., 2020; Gys et al., 2020; Kim et al., 2020; Luo et al., 2021), we expected higher intake per gram of body weight of infants, leading to significant damage to germ cells and fertility while achieving adulthood, as indicated earlier in our previous findings (Nevoral et al., 2021).

We determined that nursing exposure to BPS and BPF did not affect fundamental spermiogram parameters such as motility, concentration, DFI, and HDS of spermatozoa of male mice exposed via breast milk, which is in accordance with a previous observation (Ullah et al., 2019); although direct BPS exposure of adult male mice through drinking water decreased the portion of motile spermatozoa (Řimnáčová et al., 2020). Therefore, we can assume that sperm motility affects the route of exposure and/or maturity of the exposed organism. Apparently, individual bisphenols can show different modes of action (Shi et al., 2017; Ullah et al., 2018) and, consequently, bisphenol substitutions (Eladak et al., 2015) do not seem to be a reliable approach for the elimination of endocrine disrupting effects (Žalmanová et al., 2016). We presumed that the doses of bisphenols used in our experiments influenced spermatogenesis in a more delicate way, such as through epigenetics, without any observable phenotype in terms of motility and/or sperm concentration.



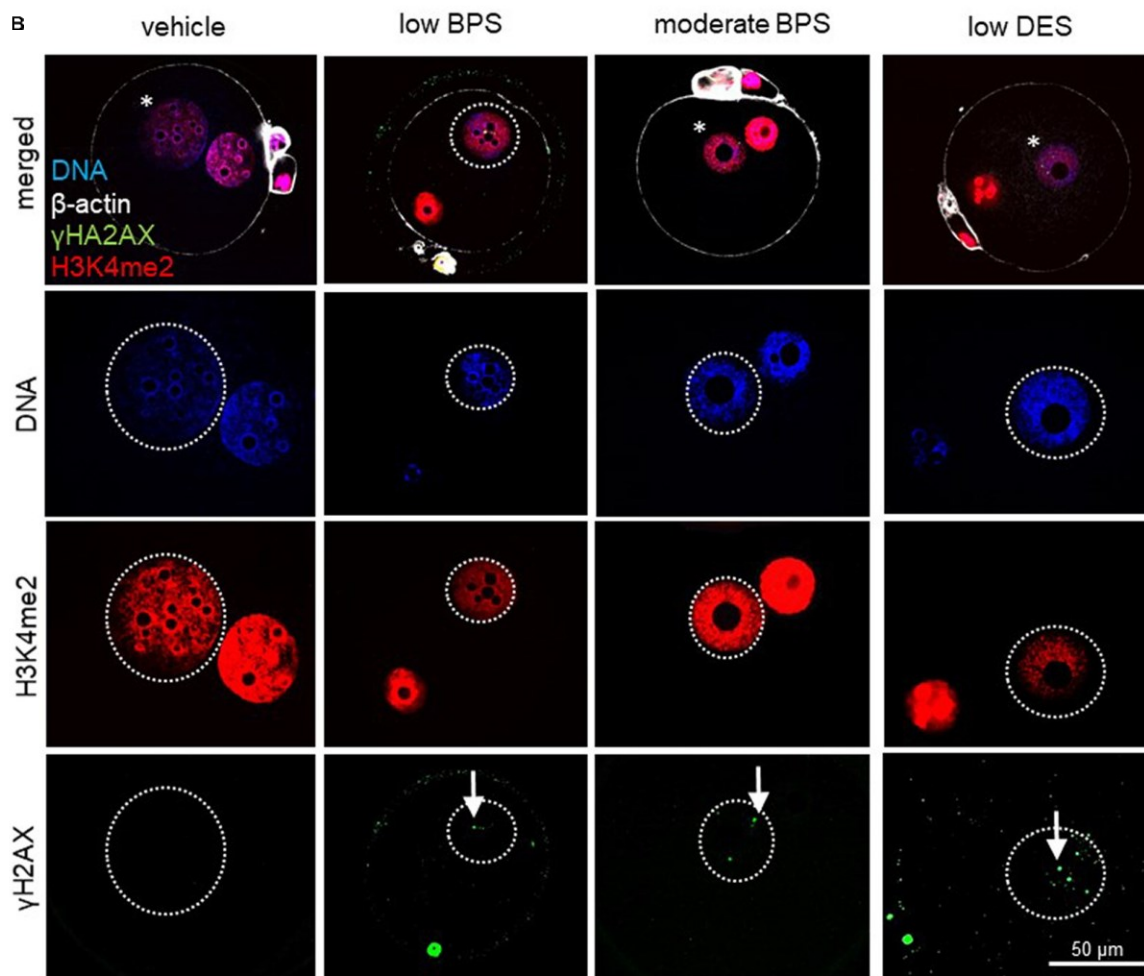


FIGURE 4 | Zygote quality after fertilization by bisphenol-affected spermatozoa. (A) Number of phosphorylation of histone H2 (γH2AX) loci in paternal pronuclei, in low and moderate BPS and low DES groups. Relative signal intensity of dimethylation of lysine (K4) on histone H3 (H3K4me2) in paternal pronuclei in zygotes of control, low and moderate bisphenol S (BPS) and low diethylstilbestrol (DES) groups. Integrated density was normalized to maternal values of the respective zygote. Violin plot shows the distribution of individual values (numbers of independent flushing sessions are indicated in brackets), lines represent the median, and dashed lines show quartiles. (B) Representative pictures of DNA, H3K4me2, and γH2AX in zygotes. Comparison between experimental groups. Asterisks indicate paternal pronucleus. Arrows indicate γH2AX loci. Differences were tested using the Kruskal-Wallis test, followed by Dunn's multiple comparisons test. Statistical differences in γH2AX loci and H3K4me2 integrated density are indicated by asterisks and daggers, respectively (*$P < 0.05$, ****$P < 0.0001$; ###$P < 0.001$). BPS: bisphenol S; DES: diethylstilbestrol.

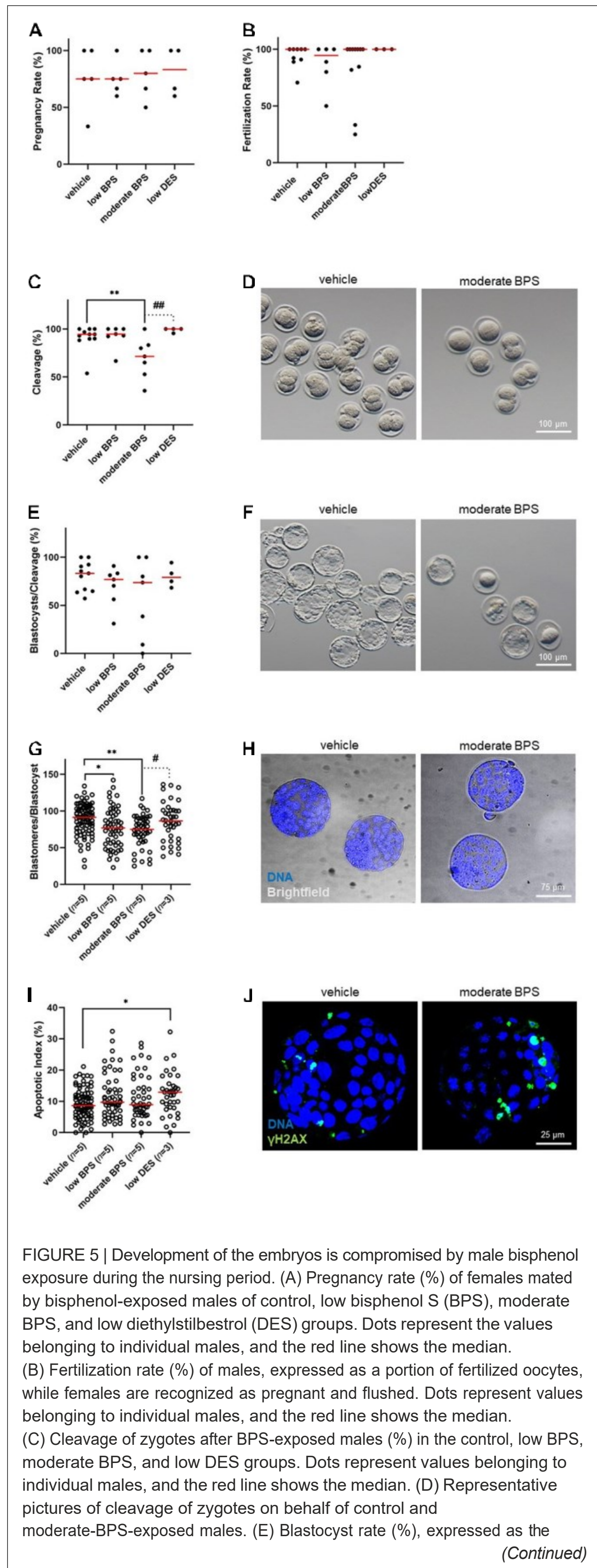


FIGURE 5 | Development of the embryos is compromised by male bisphenol exposure during the nursing period. (A) Pregnancy rate (%) of females mated by bisphenol-exposed males of control, low bisphenol S (BPS), moderate BPS, and low diethylstilbestrol (DES) groups. Dots represent the values belonging to individual males, and the red line shows the median. (B) Fertilization rate (%) of males, expressed as a portion of fertilized oocytes, while females are recognized as pregnant and flushed. Dots represent values belonging to individual males, and the red line shows the median. (C) Cleavage of zygotes after BPS-exposed males (%) in the control, low BPS, moderate BPS, and low DES groups. Dots represent values belonging to individual males, and the red line shows the median. (D) Representative pictures of cleavage of zygotes on behalf of control and moderate-BPS-exposed males. (E) Blastocyst rate (%), expressed as the *(Continued)*

FIGURE 5 | percentage of blastocysts related to cleaved embryos. Dots represent values belonging to individual males, and the red line shows the median. (F) Representative pictures of blastocysts of control and moderate BPS group. (G) Blastomeres per blastocyst in control, low BPS, medium BPS, and low DES groups. Dots represent values belonging to individual blastocysts acquired at least in three independent (*in vitro* culture) sessions, and the red line shows the median. Numbers of independent flushing sessions are indicated in brackets. (H) Representative pictures of control and moderate BPS blastocysts stained with 4,6-diamidino-2-phenylindole (DAPI). (I) Apoptotic index (%) in control, low BPS, moderate BPS, and low DES groups. Dots represent values belonging to individual blastocysts acquired at least in three independent (*in vitro* culture) sessions, and the red line shows the median. Numbers of independent flushing sessions are indicated in brackets. (J) Representative pictures of control and DES blastocysts, showing a difference in terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive blastomeres. The difference was assessed using a one-way ANOVA, followed by Tukey's multiple comparisons test. Statistical differences are indicated from the vehicle ($*P < 0.05$, $**P < 0.01$) and positive control (low DES; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$). ANOVA, analysis of variance; BPS, bisphenol S; DES, diethylstilbestrol, DAPI: 4,6-diamidino-2-phenylindole; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

Male mice were exposed to alternative bisphenols, BPS and BPF, through maternal milk in the neonatal exposure window, when the differentiation of germ cells and dynamic epigenetic changes take place (Ernst et al., 2011; Nakata et al., 2015). Hence, we used epigenetic assessment of testicular tissue by H3K4me2 and γH2AX. The H3K4me2 is a well-known marker of DNA damage in many types of body tissues (Zhang et al., 2012; Wang et al., 2020). In spermatozoa, H3K4me2 serves as a marker of immaturity and indicates aberrant histone-protamine exchange, resulting in improper chromatin decondensation (Rahman et al., 2017). Katz et al. (2009) discovered that accumulation of H3K4me2 in testicular tissue leads to sterility. Indeed, exposure to BPS increased the levels of H3K4me2 in spermatogonia and spermatocytes as shown via *in situ* immunofluorescence but not Western blot densitometry, supporting the relevance of middle stage selection for assessment. Similarly, γH2AX is a stable marker of DNA double-strand breaks and DNA integrity (Derijck et al., 2006; Kuo and Yang, 2008; Sharma et al., 2012). To the best of our knowledge, we tracked γH2AX and analyzed seminiferous tubules at the middle (VII-VIII) stages of spermatogenesis, which correspond with rendering round spermatids in the adluminal compartment (Nakata et al., 2015); round spermatids are beyond the physiological pachytene DNA breaks occurring in crossing over. Interestingly, γH2AX expression did not show any difference in testicular tissue, even in specified seminiferous tubules at stages VI-VIII. We assumed a bisphenol-modulated epigenetic shift in primary and secondary spermatocytes in seminiferous tubules, which can denote a burden transmitted to spermatozoa of adult males (Ventelä et al., 2002). We did not observe any epigenetic phenotype using the lysate of matured sperm, indicating that epigenetic damage can be repaired during further spermatogenesis. Indeed, we did not observe any correlation between H3K4me2 in testicular tissue, sperm lysate, or H3K4me2 in the male pronucleus. Neither testicular nor sperm H3K4me2 showed a relationship with blastocyst rate, which indicates sperm restoration without any impact of this sperm-born epigenetic parameter. Apparently, the

TABLE 1 | Correlations between selected parameters.

	Anogenital distance	Motility	Concentration	Testes H3K4me2	Sperm H3K4me2	PN γH2AX	Pregnancy rate	Cleavage	Blastocyst amount	Blastocyst rate	Blastomeres per blastocyst	TUNEL
Treatment				0,738					−0,498 −0,7954 to −0,007216	−0,489 −0,7912 to 0,003957		
W dam (PND 10)			−0,423 −0,7175 to −9,525e−006				−0,660 −0,8698 to −0,2485					
W% gain (PND 10)	−0,372 −0,6333 to −0,03352		−0,614 −0,8308 to −0,2350			0,687 0,2010 to 0,9015						
Average body weight (PND 21)	*0,527* 0,2646 to 0,7170											
Body weight (PND 90)		−0,550 −0,8204 to −0,07913										
Motility												−0,886
Concentration					0,746 0,3834 to 0,9093							
Testes H3K4me2								−0,650				−0,733
Sperm H2AX						0,663						
Pregnancy rate											−0,512 −0,7836 to −0,07574	
Fertilization rate									0,651			
Cleavage												0,498
Blastocyst rate (per total)										*0,874* 0,6968 to 0,9508		

Spearman coefficients are supplemented by confidence intervals. PND: post-natal day; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling. Values of Spearman correlation coefficient are significant at $P \leq 0.05$, italicized values are at $P \leq 0.01$.

method of exposure plays a crucial role, while spermatogonia are affected as possible remain of earlier damaged germ cells.

For more rigorous testing of sperm fitness, we further assessed the density of the sperm head after physiological decondensation under oocyte conditions. Accordingly, nursing-exposed males failed to show early embryonic development and showed decreased blastocyst rates in flushed zygotes. Although the impact of damaged spermatozoa on early embryonic development has been previously described (Sedó et al., 2017; Rajabi et al., 2018; Wyck et al., 2018; Casanovas et al., 2019; Middelkamp et al., 2020), our study highlights the features of early life-born sperm. Although this sperm molecular signature seems to be subtle and minor in relation to the manifestation, it is obviously responsible for the ability of sperm fertilization, in addition to other features, such as motility and/or concentration.

According to our observations, γH2AX is transferred from the spermatozoon to the zygote and then to the blastocyst; thus, we assume that corruption is transmitted, even from germ cells, through spermatogonia into the spermatid and spermatozoon (Olsen et al., 2005). DNA double-strand breaks are physiologically generated during the onset of the prophase of meiosis I to allow crossing, followed by a DNA damage response due to homologous recombination (Meng et al., 2019). Double-strand breaks persist until round spermatids if DNA damage repair is impaired (Cordelli et al., 2012). DNA damage also occurs during protamination. Godman et al. (Godmann et al., 2007) reported that elongating spermatids increases H3K4me2. After protamination, there is the last chance for spermatozoa to repair DNA damage, and H3K4me2 becomes a relevant marker of inappropriate changes in these stages of spermiation. Reparations are terminated as transcription and translation stop post-spermiogenesis; thus, spermatozoa are unable to repair further DNA damage acquired during transit through the epididymis and post-ejaculation. Paternal DNA repair is again activated after fertilization, due to the oocyte repair mechanisms. The effect of spermatozoa DNA fragmentation on early embryonic development depends on the combination of the magnitude of these DNA defects and the capacity of oocytes for reparations (González-Marín et al., 2012). In our experiment, bisphenol treatment did not show any significant effect on the presence of γH2AX in spermatozoa; however, the impact of early life-borne DNA damage of germ cells on sperm and embryo quality is noteworthy. In addition, γH2AX is transmitted from spermatogonia to spermatozoa, and our observations showed that the individual γH2AX pattern in sperm cells corresponds with γH2AX loci in paternal pronuclei and further embryonic development. In addition, H3K4me2 is transferred from the spermatozoa to the paternal pronuclei. Our findings are supported by recent studies that have shown the impact of damaged spermatozoa on early embryonic development (Sedó et al., 2017; Rajabi et al., 2018; Wyck et al., 2018; Casanovas et al., 2019; Middelkamp et al., 2020).

The first mitotic division occurs along with the fusion of the paternal and maternal pronuclei. All proteins needed for that event have to be synthesized in oocytes before fertilization, except centrioles, which are brought into the zygote by spermatozoa (Sutovsky and Schatten, 2000). Following sperm acrosomal exocytosis, penetration of the *Zona pellucida*, and oocyte cortical reaction, the first mitotic division is a crucial checkpoint for further embryonic development (Stitzel and Seydoux, 2007; Kim et al., 2008). In our study, we observed the impact of bisphenol-affected male gametes on this first mitotic division (Tesarik, 2005; Derijck et al., 2008; Gawecka et al., 2013), and moderate doses of bisphenol impaired DNA integrity in the paternal pronucleus. Accordingly, the cleavage rate was significantly decreased by 71.4% compared to that in the control group.

In a mouse two-cell embryo, the embryonic genome is activated and becomes transcriptionally active (Svoboda, 2018). This is another checkpoint for early embryonic development, and the quality of the genome of the developing embryos is crucial. Because the decreased blastocyst rate followed after cleavage was impaired, we can assume that embryonic genome activation was not affected by bisphenol. This assumption is supported by the fact that the fertilization rate is positively correlated with the blastocyst rate. Moreover, blastocysts from both low and moderate BPS groups contained fewer blastomeres than blastocysts in the control group. We also observed an increase in DNA double-strand breaks marked by TUNEL-positive blastomeres belonging to the moderate BPS group. Alongside these findings, we discovered an important correlation between γH2AX in spermatozoa and γH2AX in the paternal pronuclei. We can thus assume that γH2AX in spermatozoa is a relevant marker of zygote quality and embryonic development success (Derijck et al., 2006; Turinetto et al., 2012; Wyck et al., 2018).

To the best of our knowledge, our study provides the first evidence of the biological consequences of germ cell-borne DNA damage on embryo quality. These findings provide impactful knowledge for human reproduction and/or sperm selection for assisted reproductive therapies, challenges related to idiopathic infertility, and the failure of *in vitro* production of human embryos. We can assume that γH2AX and H3K4me2 are important indicators of sperm quality and thus, embryonic quality, because of significant correlations through whole spermatogenesis and early embryonic development.

In conclusion, this study confirms that male exposure to endocrine disruptors, such as bisphenols, during the perinatal period through maternal milk affects the quality of germ cells and thus spermatozoa, zygotes, and early embryonic development. This finding can be the answer to idiopathic infertility and the postponed burden of infertility achieved in early life.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Welfare Committee of the Ministry of Education, Youth and Sports of the Czech Republic, approval ID MSMT-11925/2016-3.

AUTHOR CONTRIBUTIONS

JN and MK: project conception. JN and PK: animal experimental design. TF, HŘ, MC, JH, and JN: execution of experiments. TF, HŘ, MC, and JN: compiling the results. JN: statistics and proofreading. TF and JN: writing the manuscript and data interpretation. All authors read and approved the final manuscript.

FUNDING

This study was supported by the Czech Health Research Council (NV18-01-00544); the Charles University Research Fund (Progress Q39), supported by the Ministry of Education, Youth and Sports of the Czech Republic, Project No. CZ.02.1.01/0.0/0.0/16_019/0000787 "Fighting Infectious Diseases;" and the grant SVV–2020-2022 No. 260 536. The sponsors had no involvement in the study design and in the collection, analysis, and interpretation of data.

ACKNOWLEDGMENTS

We would like to thank Lucie Maršálová and Markéta Šlajerová for technical support with histological analysis and Václav Rúc˘ka for enthusiastic assistance with the animal facility.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.725442/full#supplementary-material

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P5

FENCLOVÁ, Tereza, Marouane CHEMEK, Jiřina HAVRÁNKOVÁ, Yaroslav KOLINKO, Vendula SUDOVÁ, Jiří MORAVEC, Jana NAVRÁTILOVÁ, Pavel KLEIN, Milena KRÁLÍČKOVÁ a Jan NEVORAL. Effect of bisphenol S on testicular tissue after low-dose nursing exposure. Environmental Pollution. 2022 [cit. 2022-06-10]. Under review. **(IF= 8,071)**

Environmental Pollution

Effect of Bisphenol S on testicular tissue after low-dose nursing exposure

--Manuscript Draft--

Manuscript Number:	ENVPOL-D-22-03988
Article Type:	Research Paper
Keywords:	bisphenol S, idiopathic infertility, nursing exposure, testicular tissue, blood-testis barrier, oxidative stress
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Abstract:	Exposure to endocrine disruptors such as bisphenols, can lead to and be the explanation for idiopathic infertility. In our study, we assessed the effect of exposure to bisphenol S (BPS) via breast milk on the testicular tissue health of adult male mice. Milking dams were exposed to BPS through drinking water (0.216 ng.g bw/day and 21.6 ng.g bw/day) from post-natal day 0 to 15. Although there was no significant difference in testicular histopathology between the control and experimental groups, we observed an increase in the number of tight and gap junctions in the blood-testis barrier (BTB) of adult mice after nursing BPS exposure. Moreover, there was an increase in oxidative stress markers in adult testicular tissue of mice exposed during nursing. Our nursing model indicates that breast milk is a route of exposure to an endocrine disruptor that can be responsible for idiopathic male infertility through the damage of the BTB and weakening of oxidative stress resistance in adulthood.
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25.5.2022

Professor Christian Sonne, Ph.D., Professor Eddy Zeng, Ph.D.
Co-Editors-in-Chief
Environmental Pollution

Dear Co-Editors:

I wish to submit an original research article for publication in *Environmental Pollution*, titled "**Effect of Bisphenol S on testicular tissue after low-dose nursing exposure**." The paper was coauthored by Marouane Chemek, Jiřina Havránková, Yaroslav Kolinko, Vendula Sudová, Jiří Moravec, Jana Navrátilová, Pavel Klein, Milena Králíčková, and Jan Nevoral.

This study investigated translactational bisphenol exposure and its effects on newborn male mice throughout their reproductive life. We believe that what is currently diagnosed as idiopathic infertility can, in fact, be the result of exposure to endocrine disruptors during the nursing period. This study confirms that male exposure to endocrine disruptors, such as bisphenols, during the perinatal period through maternal milk affects the quality of testicular tissue and the blood-testis barrier. This finding might be useful in further research on idiopathic infertility. We believe that our study makes a significant contribution to the literature because our study is unique with respect to evaluating very low bisphenol doses in the early exposure window and related consequences in adulthood.

Further, as your journal covers research on environmental pollution and its effects on human health, we believe that this paper will be of interest to the readership of your journal.

This manuscript has not been published or presented elsewhere in part or in entirety and is not under consideration by another journal. The study design was approved by the appropriate ethics review board. We have read and understood your journal's policies, and we believe that neither the manuscript nor the study violates any of these. There are no conflicts of interest to declare.

Thank you for your consideration. I look forward to hearing from you.

Sincerely,
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Highlights

- Nursing exposure to BPS might be the cause of male idiopathic infertility.
- Nursing exposure to BPS decreases seminiferous epithelium height in adulthood.
- Volume of gap junctions in BTB increases by nursing exposure to BPS.
- Volume of tight junctions in BTB increases by nursing exposure to BPS.
- Nursing exposure to BPS increases oxidative stress in testicular tissue.

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1 # [1]Effect of Bisphenol S on testicular tissue after low-dose nursing exposure

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[1] BPs: bisphenols; BPA: bisphenol A; BPS: bisphenol S; BTB: blood-testis barrier; CX43: connexin43; DAPI: 4,6-diamidino-2-phenylindole; DES: diethylstilbestrol; DTT: 1,4-dithiothreitol; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; 8OHdG: 8-hydroxydeoxyguanosin; HSP90: heat shock protein 90; OCL11: occludin 11; PBS: Phosphate buffered saline; PND: post-natal day; PRDX6: peroxiredoxin6; ROS: reactive oxygen species; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling; ZO-1: zonula occludens 1; γH2AX: phosphorylation of histone H2

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20 **Abstract**

21 Exposure to endocrine disruptors such as bisphenols, can lead to and be the
22 explanation for idiopathic infertility. In our study, we assessed the effect of exposure to
23 bisphenol S (BPS) *via* breast milk on the testicular tissue health of adult male mice. Milking
24 dams were exposed to BPS through drinking water (0.216 ng.g bw/day and 21.6 ng.g bw/day)
25 from post-natal day 0 to 15. Although there was no significant difference in testicular
26 histopathology between the control and experimental groups, we observed an increase in the
27 number of tight and gap junctions in the blood-testis barrier (BTB) of adult mice after nursing
28 BPS exposure. Moreover, there was an increase in oxidative stress markers in adult testicular
29 tissue of mice exposed during nursing. Our nursing model indicates that breast milk is a route
30 of exposure to an endocrine disruptor that can be responsible for idiopathic male infertility
31 through the damage of the BTB and weakening of oxidative stress resistance in adulthood.

32 **Keywords:** bisphenol S, idiopathic infertility, nursing exposure, testicular tissue, blood-testis
33 barrier, oxidative stress

34

35 **Introduction**

36 In 30 to 40 % of cases of male infertility, the etiology remains unknown and is called
37 idiopathic male infertility (Bracke et al., 2018). Spermatogenesis can be significantly affected
38 by environmental factors such as endocrine disruptors; therefore, we can assume that these
39 contrived compounds are a hidden threat to male reproduction. Bisphenols are now
40 considered one of the most widespread endocrine disruptors, although their environmental
41 doses are extremely low (Gonçalves et al., 2018; Jiang et al., 2016; Kaur et al., 2018; Li et al.,
42 2016). Moreover, bisphenol S (BPS) has become a subject of interest due to a 'regrettable
43 substitution' of bisphenol A (BPA) (Žalmanová et al., 2016), which had already been
44 recognized as a reproduction-endangering compound (Eladak et al., 2015; Shi et al., 2019).
45 Therefore, we can presume the intake rather BPS than BPA, moreover, being omnipresent in
46 canned foods or drinking bottled water (Ehrlich et al., 2014; Eladak et al., 2015). In this study,
47 we used perinatal exposure *via* breast milk, an inconspicuous route of exposure aimed
48 indirectly at infants through the exposure of the mother. Indeed, nursing exposure is currently
49 underestimated although the impact seems to be significant for the following reasons: i)
50 environmental pollutants (mostly lipid-soluble) can be more concentrated in maternal milk; ii)
51 the milk renders an exclusive food for newborns; and iii) infants do not have fully developed
52 detoxification mechanisms, especially in the liver and kidneys (Matalová et al., 2016).

53 Considering the low, non-toxic doses of BPS (EFSA, 2015) and the timing of
54 exposure, we can assume that the mode of action of BPS in the damage of the gonads is subtle
55 and molecular rather than massive. Therefore, the blood-testis barrier (BTB) of the
56 seminiferous tubules is taken into consideration (Pelletier & Byers, 1992) due to the essential
57 role it plays in male reproductive health—the separation of spermatocytes from compounds
58 circulating in blood (Miething, 2010; Mruk & Cheng, 2015). The basal compartment of
59 seminiferous tubules can be impaired by blood-contained pollutants; therefore, Peroxiredoxin

60 6 (PRDX6) and Heat Shock Protein 90 (HSP90) (Clark et al., 2009; O'flaherty et al., 2020;
61 Ozkosem et al., 2016; Sable et al., 2018), which are both oxidative stress markers, were
62 assessed in the basal compartment of testicular tissue. In addition to protein markers, 8-
63 hydroxy-2'-deoxyguanosine (8OHdG) works as a marker of oxidative-stress-damaged DNA,
64 mostly affecting mitochondrial genome and describing the fitness of the cell (Amiri et al.,
65 2011; Ni et al., 1997).

66 In this study, we described the influence of nursing exposure to genuine doses of BPS
67 on the development of testicular tissue. Our study highlights the threat endocrine disruptors
68 pose and their mode of the action in the cause of idiopathic male infertility.

69 **Material and Methods**

70 **Chemicals**

71 All basic chemicals and antibodies were purchased from Sigma-Aldrich (St. Louis,
72 Missouri, USA) and Abcam (Cambridge, UK), respectively, unless otherwise noted.

73 **Ethical approval**

74 All animal experiments comply with the ARRIVE guidelines and are carried out in
75 accordance with EU Directive 2010/63/EU for animal experiments, and Act No. 246/1992
76 Coll. on the Protection of Animals Against Cruelty under the supervision of the Animal
77 Welfare Committee of the Ministry of Education, Youth and Sports of the Czech Republic
78 (approval ID MSMT-11925/2016-3).

79 **Animals**

80 Six- to seven-week-old female ICR mice were purchased from Velaz Ltd. (Czech
81 Republic) and used as mothers of F1 offspring subjected to experimental assessment.
82 Experimental design comprised four groups (described below), including six nursing mothers

83 each (*N*=24). All animals were housed in intact polysulfonate cages and maintained in a
84 facility with a 12-h light/dark cycle, a temperature of 21 ± 1 °C, and a relative humidity of
85 60%. A phyto-oestrogen-free diet (1814P; Altromin, Germany) and ultrapure water (in glass
86 bottles, changed twice per week) were provided *ad libitum*. Male offspring were weaned on
87 post-natal day (PND) 21 and housed individually under standard conditions. Alternatively,
88 several litters of sampled male pups were sacrificed at PNDs 15 and 90, and their testes were
89 sampled for histopathology, immunohistochemistry, and western blot.

90 **Bisphenol dosing**

91 Dams were administered low (0.375 ng/ml) and moderate (37.5 ng/ml) doses of BPS
92 through drinking water, while the negative control litters were exposed to the vehicle (0.1%
93 ethanol in drinking water). Diethylstilbestrol (DES) at 0.375 ng/ml was used as the estrogen-
94 like positive control. The route of exposure was used with respect to the welfare of the
95 nursing dams and the credibility of established models for environmental contamination.
96 Dams were treated from delivery until PND 15, while pups ingested breast milk solely. Doses
97 were chosen in accordance with their known biological effects (Nevoral et al., 2018;
98 Prokešová et al., 2020). We assumed that an intake of 0.2 and 20 ng.g bw/day corresponded to
99 0.216 and 21.6 ng.g bw/day, respectively, based on recorded water intake (see Supplementary
100 Fig. 1).

101 **Characterization of the route of nursing exposure**

102 BPS was extracted from the breast milk of mice, cow, and humans, and from the
103 stomach tissue of mice offspring who were breastfed with BPS-containing milk. It was also
104 extracted from the dried food, water, and bedding of mice and treated with artificial urine (see
105 Supplementary Methods). We recovered extremely low BPS doses (see Supplementary
106 Results) from the tested materials.

107 **Histology**

108 *Qualitative / histopathological analysis of testes at two ages*

109 Testes from 15- (PND15) and 90-day old (PND90) mice were fixed in Bouin's
110 fixative before dehydration, embedded in paraffin wax, and cut into 10-μm serial sections.
111 Then each 50th section was selected and stained with hematoxylin and eosin. A detailed
112 qualitative examination of the testes from both ages was performed according to the methods
113 described by the Society of Toxicologic Pathology with some modifications (Creasy, 2003;
114 Lanning et al., 2002). Histopathological evaluation of neonatal testicular section was done
115 under light microscope (Olympus BX41), equipped with UPlanSApo 20x & UPlanFL 40x
116 objectives and coupled to Promicam 3-3CC camera. More than 70 random tubules per
117 neonatal testis were evaluated blind to the experimental groups and classified as normal or
118 abnormal tubule. Seminiferous tubules were considered abnormal if they contained more than
119 three apoptotic cells and/or meiotic arrest/loss of germ cells. For the histopathological
120 evaluation of adult testes, the total volumes of normal and abnormal epithelial cells in
121 seminiferous tubules were found using a point grid approach (Gundersen et al. 1999). The
122 abnormal epithelial cells are considered cells with undergo apoptosis, vacuolization, or
123 exfoliation. The evaluation was provided using Stereologer 11 software (SRC, Biosciences
124 Tampa, FL, USA) cooperated with Nikon Eclipse Ti-U microscope (Japan) equipped with the
125 set of standard Plan Fluor objectives, XYZ motorized stage (Prior, UK) and Promicam 3-3CC
126 camera.

127 *Quantitative / histomorphometric analysis of adult testes*

128 Numerical data for seminiferous tubular diameter and epithelial height were acquired
129 using Image J software (NIH, New York, USA) from 10 cross-sections of seminiferous
130 tubules (per testis) with the most circular contour possible. Photographs were taken using
131 PlanApo 100x lens mounted on the mentioned Olympus microscope. Only seminiferous

132 tubules at spermatogenesis stages VII and VIII were chosen for quantitative measurement.
133 Spermiation stage was determined by restructuring the BTB at the stage of seminiferous
134 epithelial cycle in accordance with previously published findings (Yan et al., 2008).
135 Moreover, the density of some germ cell populations (leptotene and pachytene spermatocytes
136 and round spermatids) was counted in at least three seminiferous tubules (per testis) at the
137 stage of spermiation. Spermatogenic cells were identified by their cytoplasmic and nuclear
138 morphology as described earlier (Breucker, 1982).

139 *Analysis of the kinetics of spermatogenesis: "staging"*

140 Based on staging, a quantitative analysis of adult testes was performed to assess the
141 effects of BPS on the kinetics of spermatogenesis according to our previous research
142 (Řimnáčová et al., 2020). The seminiferous tubules were classified into three groups based on
143 their stages: stages I–VI, VII–VIII, and IX–XII. This method of classification was adopted to
144 define the different stages of seminiferous tubules (Chiou et al., 2008; Oakberg, 1956a,
145 1956b). At least 150 tubules were classified in each section. The proportion of these stages
146 was counted.

147 **Immunofluorescence**

148 For immunofluorescence staining, 10 μm- testis sections were dewaxed, rehydrated,
149 and processed as previously described (Chemek et al., 2018), with some modifications.
150 Antigens were retrieved by pressure- cooking slides for 10 min in 0.01 M citrate buffer (pH
151 6.0). Nonspecific binding sites were blocked with a solution of 10% normal goat serum
152 (NGS), 5% Bovine serum albumin and 0.1% TritonX-100, and 0.5% Tween 20 in PBS (PBS-
153 TT) for 60 min at room temperature. Subsequently, testis sections were incubated with a
154 rabbit polyclonal anti-CX43 antibody (1:1000; Abcam), a rabbit polyclonal anti-occludin11
155 (anti-OCL) antibody (1:200; Abcam), a rabbit polyclonal antibody anti-ZO1 (1:200, Abcam),
156 a rabbit polyclonal anti-8OHdG antibody (1:100; Abcam), a rabbit polyclonal antibody anti-

157 HSP90 (1:200, Abcam), a rabbit polyclonal antibody anti-PRDX6 (1:200, Abcam), and/or a
158 mouse polyclonal anti-γH2AX antibody (1:200; Abcam) before overnight incubation at 4°C.
159 After washing in a phosphate buffered saline (PBS-TT) solution containing 1% NGS, slides
160 were incubated for 40 min in peptide nucleic acid (PNA) lectin conjugated with Alexa Fluor
161 488 (1:400; Abcam), and in the appropriate secondary antibody (anti-mouse or anti-rabbit)
162 conjugated with Alexa Fluor 647 (1:200; Abcam). The slides were mounted with
163 Vectashield containing DAPI for nuclear staining. Confocal microscope with spinning disk
164 (Olympus IX83, Germany) was used for imaging, and ImageJ software (NIH, New York,
165 USA) was used for further image analysis of signal density.

166 **Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay**

167 For the detection of apoptosis, paraffin-embedded sections were stained using the
168 TUNEL kit (*In Situ* Cell Death Detection Kit, cat. no. 11684795910, Roche, Germany),
169 according to the manufacturer's protocols. To assess apoptosis in testicular cells, at least 200
170 seminiferous tubules were observed in each section at a magnification of 100×. A histogram
171 of the number of TUNEL-positive germ cells per seminiferous tubule and the percentages of
172 the number of seminiferous tubules containing TUNEL-positive germ cells was analyzed.

173 **Electrophoresis and western blot**

174 Electrophoresis and western blotting were performed on the testicular tissue of
175 experimental males. Testes samples of males at PND15 and PND90 were lysed in RIPA
176 buffer enriched with a complete mini protease inhibitor cocktail (Roche, Switzerland).
177 Thereafter, the samples were mixed with Laemli loading buffer supplemented with β-
178 mercaptoethanol. For dodecyl sulphate polyacrylamide gel electrophoresis, 4–15 % of
179 separating Mini-PROTEAN® TGX Stain-Free™ Precast Gels (Bio-Rad, France) were used;
180 30 μg of protein were loaded into each gel chamber. For western blotting, the Trans-Blot®
181 Turbo™ Transfer System (Bio-Rad, France) was used. Polyvinylidene difluoride membrane

182 was blocked in 5% bovine serum albumin in TBS with 0.5% Tween-20 for 60 min (at room
183 temperature) and incubated with specific primary antibodies (at 4°C) overnight, as follows:
184 rabbit polyclonal anti-HSP90 (1:1,000; Abcam) or anti-PRDX6 (1:1,000; Abcam). Rabbit
185 polyclonal anti-α-Tubulin (1:1,000; Cell Signaling, Massachusetts, USA) was used as the
186 internal control. Horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse or
187 anti-rabbit IgG; 1:15,000; Invitrogen, Massachusetts, USA) were applied for 1 h at room
188 temperature. The targeted proteins were visualized using ECL Select Western Blotting
189 Detection Reagent (GE Healthcare Life Sciences, UK), and membranes were scanned on a
190 ChemiDoc™ MP System (Bio-Rad, France). Images of membranes were processed using
191 ImageLab 4.1 software (Bio-Rad, France).

192 **Statistical analyses**

193 Data were analyzed using GraphPad Prism 8.1.1 (GraphPad Software Inc., San Diego,
194 California, USA). Based on Shapiro–Wilk's normality distribution tests, differences were
195 tested using an ordinary one-way analysis of variance (ANOVA), followed by Tukey's
196 multiple comparisons test. Alternatively, Kruskal–Wallis and Dunn's post-hoc tests were used
197 for non-normally distributed data. P-values ≤0.05, 0.01, 0.001, and 0.0001 were considered
198 statistically significant and are indicated with asterisks (*), (**), (***), and (****),
199 respectively. Alternatively, daggers indicate differences from the positive control of low DES
200 (#, ##, ###, ####). Normally and non-normally distributed data are expressed as means and
201 medians, respectively.

202

203 **Results**

204 **Nursing BPS exposure and testicular histopathology of infant and adult mice**

205 Very low doses of BPS exposed nursing mouse dams and, therefore, indirectly
206 suckers. We established the pipeline of sampling, sample processing, and analytical
207 assessment of the exposure model (see Supplemental Methods). In accordance with the non-
208 significant levels of BPS in maternal blood and the stomach of infants, we can consider BPS
209 doses in endocrine disrupting level succeeded in non-toxic effects, consistent with basal
210 environmental contamination (see Supplemental Materials). In the overall histological
211 assessment of the nursing exposure to either BPS or DES, estrogen-like positive control of the
212 testes in young (PND15) and adult (PND90) offspring did not show any significant impact.
213 There were no significant differences among seminiferous tubules that showed an abnormal
214 cell mass (Fig. 1A), the presence of high number of apoptotic cells (Fig. 1B), and/or germ cell
215 loss *via* meiotic arrest (Fig. 1C). Similar to PND15, there was no statistical difference among
216 the total volume of cells including normal / abnormal cells and other cells (Leydig cells) (Fig.
217 1E), total volume of normal epithelial cells (Fig. 1F), and total volume of abnormal epithelial
218 cells (Fig. 1G) at PND90.

219 **Nursing BPS exposure does not have any impact on the qualitative signs of**
220 **spermatogenesis**

221 In accordance with previous findings and the fact that nursing BPS exposure had no
222 significant impact on qualitative testicular signs at PND15 and PND90, we measured the
223 diameter of the seminiferous tubules and height of the seminiferous epithelium as quantitative
224 indicators in the testes of adult mice at PND90. A more detailed insight to the quality of
225 testicular tissue was gotten *via* the staging of spermatogenic wave and differential counting of

226 germ cells. No statistical difference in the diameter of the tubules between the control and
227 experimental groups was observed (Fig. 2A). The height of the seminiferous epithelium of the
228 tubules at the middle stages of spermatogenesis significantly decreased in the testes of males
229 exposed to moderate BPS and low DES doses (Fig. 2B). The values for the diameter of the
230 tubules and height of the seminiferous epithelium of the tubules are demonstrated in Fig. 2C.
231 The ratio of early, middle, and late stages of spermatogenesis are not affected by nursing
232 exposure to low doses of either BPS or DES (Fig. 2D). Similarly, the differential count of
233 pachytene and leptotene spermatocytes and the number of round spermatids did not show any
234 significant difference (Figs. 2F–G).

235 **DNA integrity is not affected by low doses of BPS**

236 Based on our observation, DNA integrity was affected, leading to the impactful
237 outcome of sub-/in-fertility. To assess this, we chose the phosphorylated H2AX histone
238 variant (γH2AX), a DNA double strand breaks marker (Fig. 3A). We supported these findings
239 by performing a TUNEL assay (Fig. 3B). Consequently, we can claim that BPS exposure had
240 no impact on DNA integrity, assessed by γH2AX in defined germ cells and the count of
241 TUNEL positive ($TUNEL^{+}$) cells (Figs. 3C and D). Further, the qualitative approach of
242 γH2AX analysis in pachytene (Fig. 3E), pre-leptotene (Fig. 3F), and round spermatids (Fig.
243 3G) did not show any difference.

244 **BPS increases gap junction occurrence in seminiferous tubules**

245 To assess the integrity of the seminiferous epithelium, we evaluated using
246 immunofluorescence (Gerber et al., 2016), the expression and localization of Connexin 43
247 (CX43)—a well-known marker of the gap junctions in the BTB—in tubules at the middle
248 stages (VII-VIII) of spermatogenesis in PND90 male mice. The integrated density and area of
249 seminiferous tubules in the middle stages of spermatogenesis were analyzed *via* image
250 analysis as parameters of the amount and distribution of CX43, respectively. There is an

251 increase in relative integrated density of CX43 between control and low DES of young
252 PND15 testis (Fig. 4A); however, a difference in CX43 area of expression between the
253 moderate BPS and low DES groups was observed (Fig. 4B). For adult PND90 seminiferous
254 tubules, there was a significant increase in the relative CX43 area in the moderate BPS and
255 vehicle control groups of testicular tubules (Figs. 4C and D). The representative pictures of
256 the middle stages (VII-VIII) of spermatogenesis containing the intense expression of CX43 in
257 young (PND15) and adult (PND90) male mice are shown in Fig. 4E.

258 **BPS increases the occurrence of tight junction in seminiferous tubules**

259 Since adult testes at PND90 were affected by nursing BPS exposure, their
260 seminiferous tubules were assessed. For the evaluation of the integrity of the epithelium and
261 BTB, the expression and localization of Occludin 11 (OCL11) and tight junction protein
262 (*zonula occludens,* ZO-1), both of which are well-known tight junction markers, were chosen
263 for analyses *via* immunofluorescence. There was an increase in the integrated density of OCL
264 in the control and low BPS groups (Fig. 5A). Although DES did not show any difference as a
265 positive control, an increase in area of expression of OCL in low DES was observed (Fig.
266 5B). OCL increased in the low BPS group as demonstrated in Fig. 5C. In addition, there was a
267 substantial increase in integrated density and the area of ZO-1 in the low BPS and moderate
268 BPS groups in contrast to both the vehicle and positive DES controls (Figs. 5D and E).
269 Representative pictures show the distribution of ZO-1 (Fig. 5F).

270 **BPS induces oxidative stress in testicular tissue**

271 As one of the possible modes of action of BPS in adult testes (in addition to BTB), we
272 considered oxidative stress through the proteomic analysis of adequate protein markers. We
273 evaluated the expression of HSP90, PRDX6, and 8OHdG, which are well-known markers of
274 the oxidative stress pointing out mitochondrial fitness, in the seminiferous tubules of adult
275 (PND90) mice. There was no statistical difference in integrated density of HSP90 between the

276 control and experimental groups (Figs. 6A, B, and C). Only low DES-exposed testes showed
277 increased PRDX6 compared to the vehicle control (Figs. 6D, E, and F). Both protein markers
278 were revealed based on molecular weight *via* electrophoresis and western blot (Fig. 6C, F).
279 8OHdG, accompanying mostly the damage of mitochondrial DNA, was increased in the
280 tubules of mice in the moderate BPS group (Figs. 6G and H).

281

282 **Discussion**

283 Bisphenols are well known endocrine disruptors with estrogenic activity (Rochester &
284 Bolden, 2015). Our study is novel due to the exposure *via* breastfeeding. We considered this
285 route of exposure for several reasons: newborns do not have fully developed mechanisms of
286 detoxication in the liver and/or glucuronidation pathway in the kidney (Matalová et al., 2016).
287 Moreover, breast milk is an exclusive source of nourishment that brings possible nursing
288 exposure as a burden for infants. In contrast, there is no evidence of increased concentration
289 of BPS in breast milk compared to adult human urine or blood serum (Dualde et al., 2019;
290 Luo et al., 2020; Vandenberg et al., 2010); therefore, the kinetics of endocrine disruptors are
291 yet to be elucidated. Nursing exposure to bisphenols significantly impairs germ cells and
292 compromises fertility while achieving adulthood (Nevoral et al., 2021), and the effect of
293 direct BPS exposure on the increased incidence of testicular tissue abnormalities have been
294 demonstrated (Řimnáčová et al., 2020).

295 We assessed the effect of nursing BPS exposure on qualitative parameters of the
296 testicular tissue of exposed male mice and found that BPS had no effect on the occurrence of
297 abnormalities in the seminiferous tubules of young and adult mice. These findings are similar
298 to those of a study in which rats were directly exposed to BPS (Ullah et al., 2016). Similarly,
299 this observation was supported by own measurement of BPS in the maternal blood and

300 stomach of infants, despite recent approaches seeming incapable of reliably quantifying the
301 level of an endocrine disruptor in its sub-toxic dose. Therefore, more subtle target systems,
302 such as DNA integrity maintenance, BTB, and oxidative-stress-protecting machinery, were
303 considered as alternative to coarse histopathological assessment.

304 Male offspring were exposed to BPS within the neonatal exposure window, when the
305 dynamic epigenetic changes and differentiation of germ cells take place (Ernst et al., 2011;
306 Nakata et al., 2015). Therefore, we used an epigenetic marker of DNA double strand breaks
307 and DNA integrity, γH2AX to assess testicular tissue (Derijck et al., 2006; Kuo & Yang,
308 2008; Sharma et al., 2012). Although we did not observe a BPS-induced increase in γH2AX
309 in the testicular tissue, we found that this non-physiological DNA double-strand breaks were
310 transmitted from spermatozoa into paternal pronucleus of the zygote and blastocyst (Fenclová
311 et al., 2022).

312 In addition to DNA integrity assessment, we considered the BTB, another sensitive
313 mechanism possibly affected by extremely low doses of endocrine disruptors. BTB is crucial
314 to the development of healthy spermatozoa as it restricts the paracellular flow of substances
315 from the Sertoli cells (basal) to the apical compartment where post-meiotic germ cell
316 development takes place (Yan & Mruk, 2012). The BTB is also important for spermatogonia
317 renewal and mitotic proliferation and differentiation (Yan & Mruk, 2012; Zhou & Wang,
318 2022). Spermatogonia have full access to the nutrients, hormones, and other biomolecules
319 (including toxins) released from micro vessels into the interstitial space, whereas specialized,
320 developing spermatids are under the protection of the BTB (Cheng et al., 2011; Yan & Mruk,
321 2012). It needs to be perambulated by preleptotene spermatocytes into the apical compartment
322 to initiate meiosis (Wen et al., 2018), which requires junction disassembly in stages VII and
323 VIII of the spermatogenic cycle in mice (Yan & Mruk, 2012). It is well known that gap and
324 tight junctions are the main components of the BTB. Therefore, we studied CX43, a well-

325 known marker of gap junctions in the BTB (Li et al., 2010; Pointis & Segretain, 2005; Steger
326 et al., 1999), and observed an increase in CX43 in adult males, with no difference in infants.
327 This phenomenon can be explained by the fact that the BTB begins to develop from the first
328 wave of spermatogenesis at the onset of puberty until Sertoli cells' proliferation is completed
329 (Gerber et al., 2016). Exposure to BPS can also affect key proteins (*e.g.,* connexins) that are
330 involved in BTB development and the manifestation of sexual maturity (Mruk & Cheng,
331 2015; Stanton, 2016). CX43 is also an important protein in the re-modulation of the BTB
332 during spermatogenesis in adulthood (Li et al., 2010); thus, we can assume that the increase in
333 CX43 in the moderate BPS group is associated with an increased need for the repair of gap
334 junctions in the BTB (Cheng et al., 2011; Mruk & Cheng, 2015; Yan & Mruk, 2012) CC.
335 Also an increase in the expression of tight junction markers (ZO-1 and OCL) was observed
336 (Hill et al., 2018; Li et al., 2010; Mita et al., 2011) in nursing-exposed males. Conversely, Cao
337 et al. (2020) described a decrease in tight junctions in orally-treated pubertal mice and rats
338 (Tian et al., 2017), although the damage was reversible in those exposed to low doses.

339 BPS influences the BTB and influences oxidative stress in testicular tissue. Our
340 findings did not show any increase in the expression of oxidative stress markers PRDX6 and
341 HSP90 in adulthood, but we can assume that oxidative stress is reversible when mice are still
342 growing (Dai et al., 2021; Ullah et al., 2018; Ullah et al., 2017). However, we observed that
343 8OHdG (a marker of DNA oxidative stress mostly in the mitochondrion) increased in adult
344 mice testes after nursing exposure (Nomoto et al., 2008; Richter, 1992; Suzuki et al., 1999).
345 Similarly, Kaimal et al. (2021) observed that 8OHdG increased in prenatally-exposed rats.

346 This study confirms that extremely low doses of BPS in breast milk impairs the
347 resistance of seminiferous tubules to oxidative stress and could affect testicular gap and tight
348 junctions, although other parameters are not impaired. Obviously, both non-toxic doses and
349 perinatal (*i.e.,* indirect) exposure to endocrine disruptors should be considered severe causes

350 of idiopathic infertility occurring in early life in the male individuum.

351

352 **Conflict of Interest**

353 The authors declare that the research was conducted in the absence of any commercial
354 or financial relationships that could be construed as a potential conflict of interest.

355 **Author contributions**

356 **Jan Nevoral, Milena Králíčková**: Project conception; **Jan Nevoral, Pavel Klein**:
357 Animal experimental design; **Tereza Fenclová, Marouane Chemek, Vendula Sudová,**
358 **Jiřina Havránková, Yaroslav Kolinko, Jiří Moravec, Jan Nevoral**: Execution of
359 experiments; **Tereza Fenclová, Marouane Chemek, Vendula Sudová, Jan Nevoral**:
360 Compiling the results; **Jan Nevoral**: Statistics; **Tereza Fenclová, Jan Nevoral**: Writing the
361 manuscript and data interpretation; **Jan Nevoral**: Proofreading; all authors read and approved
362 the final manuscript.

363 **Funding**

364 This study was supported by the Czech Health Research Council [grant number
365 NV18-01-00544]; the Charles University Research Fund (Progress Q39), supported by the
366 Ministry of Education, Youth and Sports of the Czech Republic, Project No.
367 CZ.02.1.01/0.0/0.0/16_019/0000787 "Fighting Infectious Diseases"; the grant SVV–2020-
368 2022 No. 260 536; and the Charles University Cooperation Program, research area
369 MED/DIAG. The sponsors were not involved in the study design and in the collection,
370 analysis, and interpretation of data.

371 **Acknowledgements**

372 We would like to thank Mrs. Lucie Maršálová and Mrs. Markéta Šlajerová for
373 technical support and Mr. Václav Rúčka for assistance in the animal facility.

374 **Conclusions**

375 According to results, we can conclude that nursing exposure to BPS decreases
376 seminiferous epithelium height in testicular tissue, affects the composition of gap and tight
377 junctions in BTB, and increases oxidative stress in testicular tissue of adult males. We can
378 thus assume that nursing exposure to BPS might be a cause of idiopathic infertility in
379 adulthood.

380

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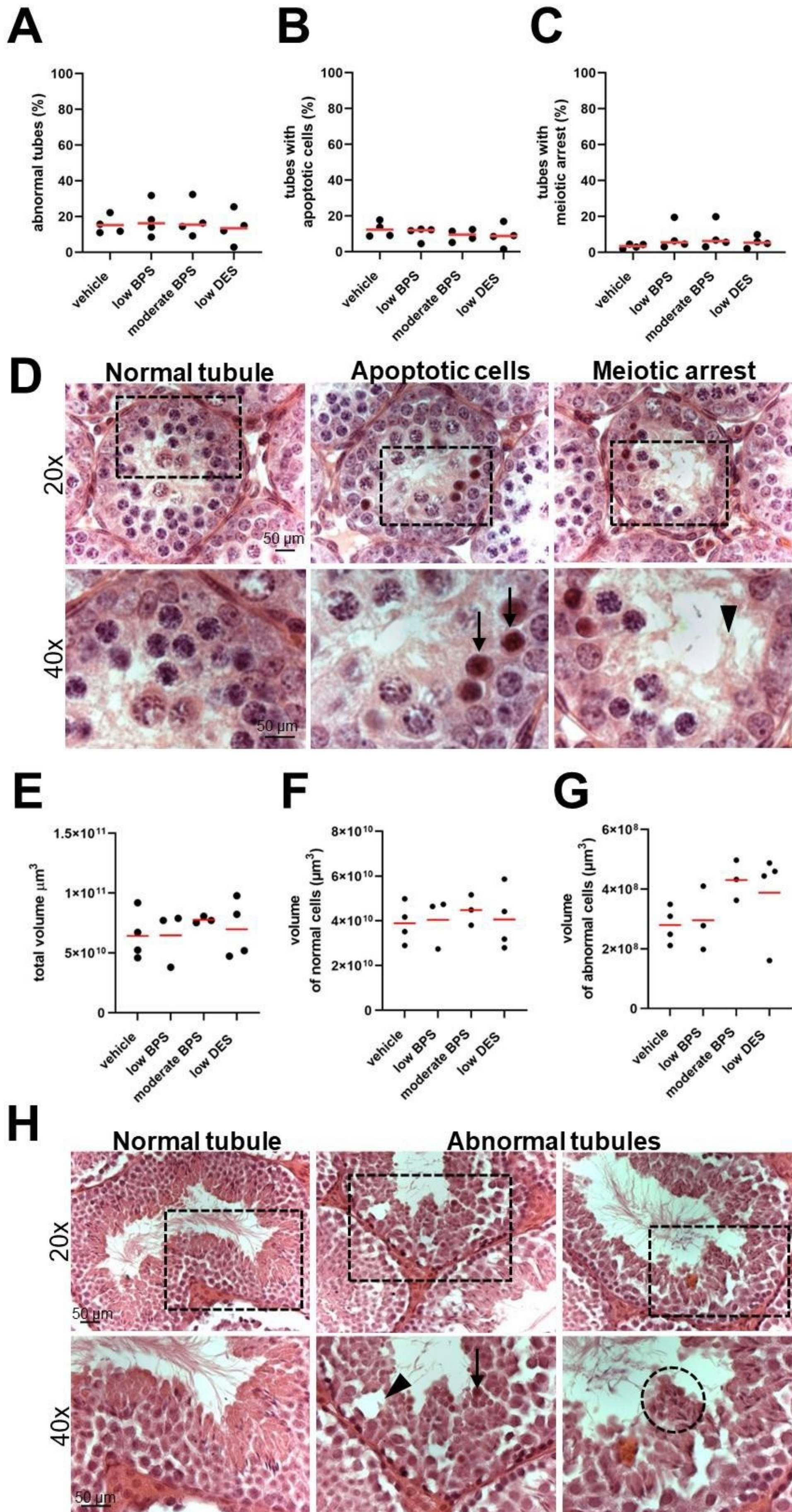
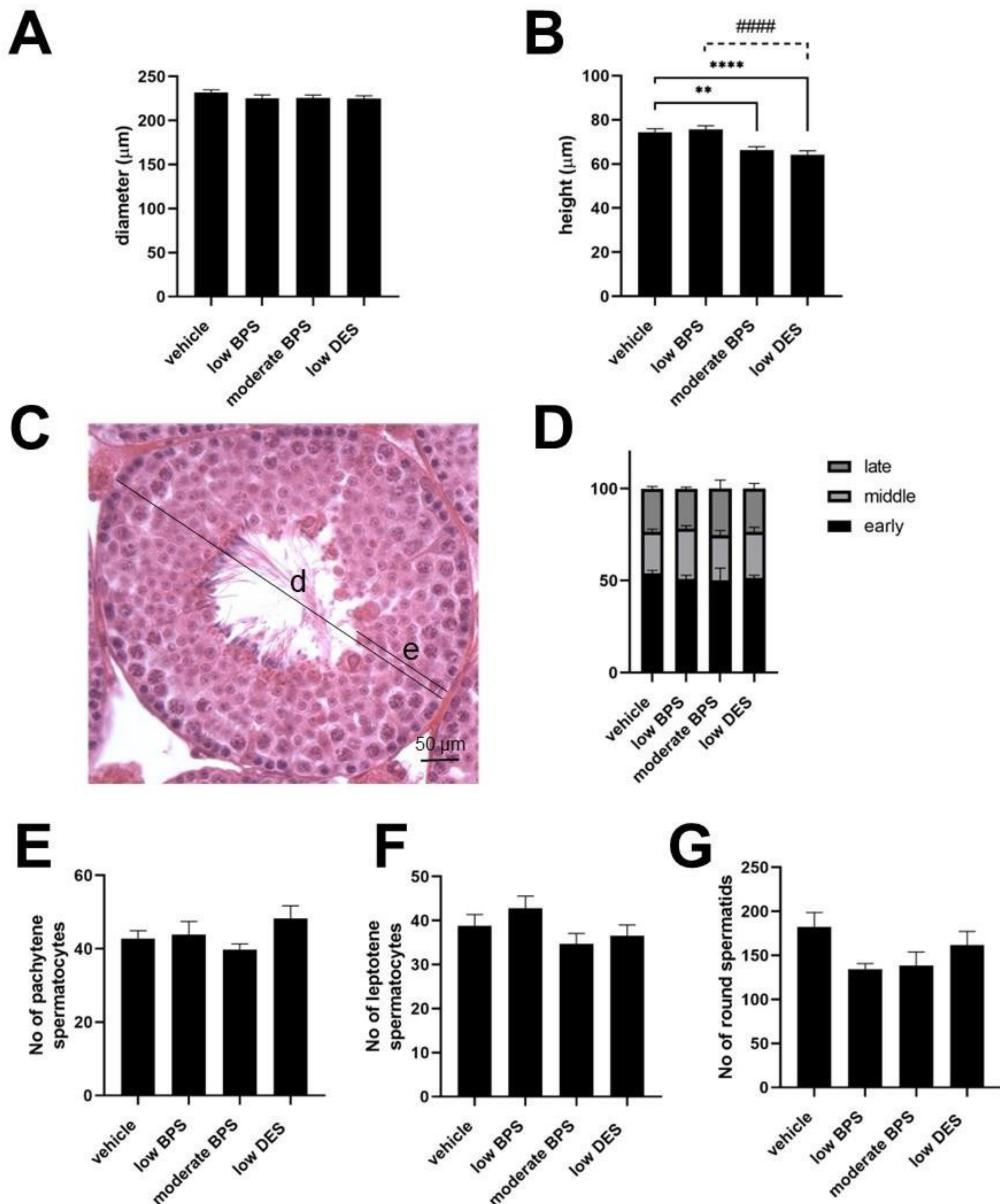

A
B
C
abnormal tubes (%)
tubes with apoptotic cells (%)
tubes with meiotic arrest (%)
100
80
60
40
20
0
vehicle
low BPS
moderate BPS
low DES
D
Normal tubule
Apoptotic cells
Meiotic arrest
20x
40x
50 µm
E
F
G
total volume µm3
volume of normal cells (µm3)
volume of abnormal cells (µm3)
H
Normal tubule
Abnormal tubules
20x
40x
50 µm

635 **Figure 1: Histopathological analysis of mice testicular tissue.** (**A**) The fraction (%) of
636 seminiferous tubules with the cross-section which contained more than three
637 pathologicaly changed cells; (**B**) percentage (%) of cross-sections of tubules containing
638 more than three apoptotic cells; and (**C**) % of tubules with cells in meiotic arrest / loss of
639 germ cells in mice testes at PND15 in the control, low BPS, moderate BPS, and low DES
640 groups. (**D**) Representative microphotographs of a normal seminiferous tubule, abnormal
641 tubules containing more than three apoptotic cells or with cells in meiotic arrest / germ
642 cells loss in mice testes at PND15. Arrows indicate apoptotic cells; arrowhead indicates
643 cells in meiotic arrest / loss of germ cells. (**E**) Total volume of the seminiferous epithelial
644 cells, (**F**) total volume of the normal seminiferous epithelial cells, and (**G**) total volume
645 of the abnormal cells in the seminiferous tubules of mice testes at PND90 in the control,
646 low BPS, moderate BPS, and low DES groups. (**H**) Representative microphotographs of
647 normal and abnormal tubules in mice testes at PND90. Arrow indicates apoptotic cells;
648 arrowhead indicates vacuolated cells; and circle indicates exfoliated cells. In dot plots,
649 dots represent individual males; red lines show the mean. The difference was tested
650 using an ordinary one-way ANOVA, followed by Tukey’s multiple comparisons test, at
651 $P \leq 0.05$.



652

653 **Figure 2: Quantitative analysis of testicular tissue issued from adult mice at PND90.** (**A**)

654 Diameter and (**B**) height of epithelium in seminiferous tubules at the middle stage of

655 spermatogenesis at PND90 in the control, low BPS, moderate BPS, and low DES groups.

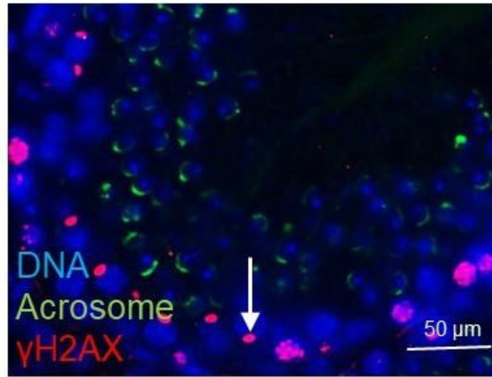
656 (**C**) A representative microphotograph of the diameter and height of the seminiferous

657 epithelium in mice testes at the middle stage of spermatogenesis at PND90

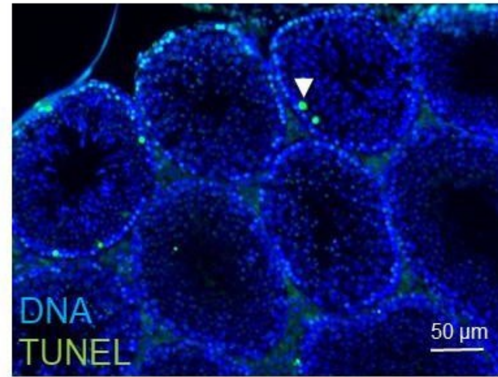
658 (magnification 20x). Line *d* indicates diameter, line *e* indicates epithelium height. (**D**)

659 Proportion (%) of the different stages of spermatogenesis in tubules as follows: early
660 stages, I –IV; middle stages, VII – VIII; and late stages, IX – XII. Number density of
661 pachytene (**E**) and leptotene (**F**) spermatocytes and round spermatids (**G**) in at least three
662 seminiferous tubules per testis in stages VII - VIII of spermatogenesis. Columns
663 represent the mean and whiskers the standard error of mean. The difference was tested
664 using the ordinary one-way ANOVA, followed by Tukey's multiple comparisons test.
665 Statistically significant differences to vehicle control are indicated with asterisks ($^{**}P$
666 ≤ 0.01, $^{****}P \leq 0.0001$); daggers indicate differences between the low DES and positive
667 control groups ($^{\#\#\#\#}P \leq 0.0001$).

A



B



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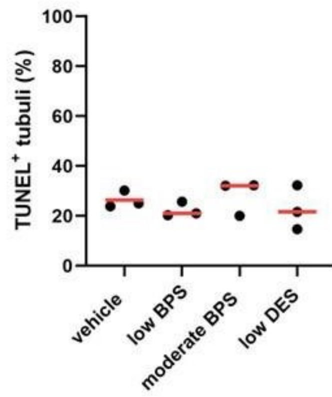

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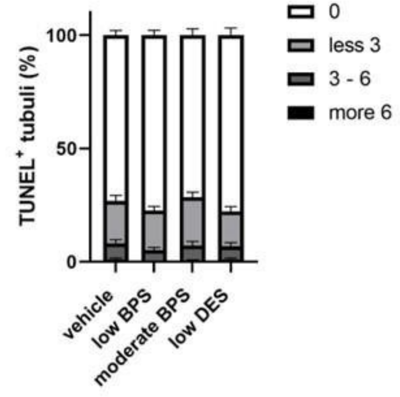
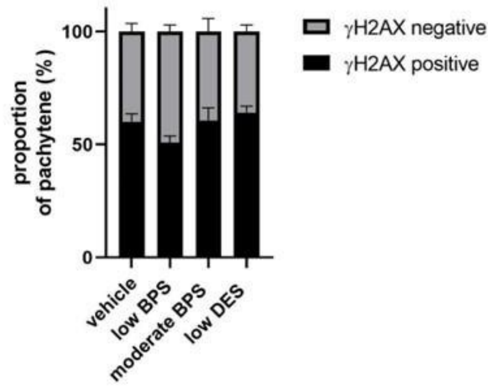
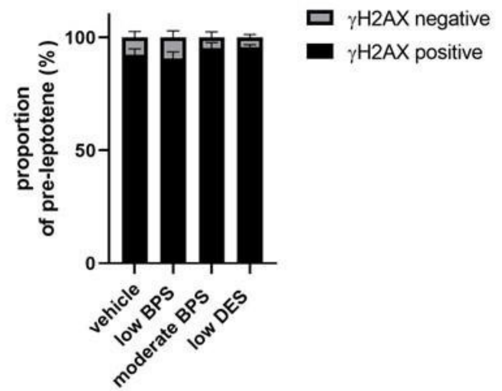


E



F



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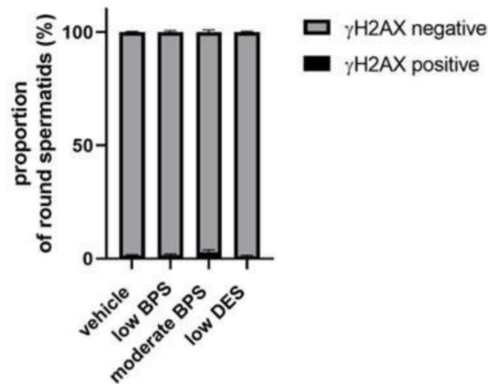

668

669 **Figure 3: The integrity of the DNA of testicular germ cells in adult mice** (**A**) A

670 representative microphotograph of γH2AX in the seminiferous tubule in mice testes at

671 middle stage (magnification 60 x). Arrow indicates γH2AX loci. (**B**) A representative

672 microphotograph of DNA double strand breaks visualized by TUNEL assay in tubules of

673 mice testis at middle stage (magnification 20 x). Arrowhead indicates TUNEL positive

674 cells ($TUNEL^+$). (**C**) Quantification of $TUNEL^+$ tubules in individual males. Dots

675 represent individual animals; red lines show the mean. (**D**) Differential counting of

676 $TUNEL^+$ tubules containing no $TUNEL^+$ cells (0), less than 3, 3–6, or more than 6

677 $TUNEL^+$ cells. Data are expressed as mean ± standard error of mean from three animals

678 in each group (one male per litter). Proportion of γH2AX-positive cells in pachytene (**E**),

679 pre-leptotene (**F**), and round (**G**) spermatocytes in middle-stage tubules at PND90 in the

680 control, low BPS, moderate BPS, and low DES groups. Columns represent mean, and

681 whiskers represent the standard error of mean. The difference was tested using the

682 ordinary one-way ANOVA, followed by Tukey's multiple comparisons test, at $P \leq 0.05$.

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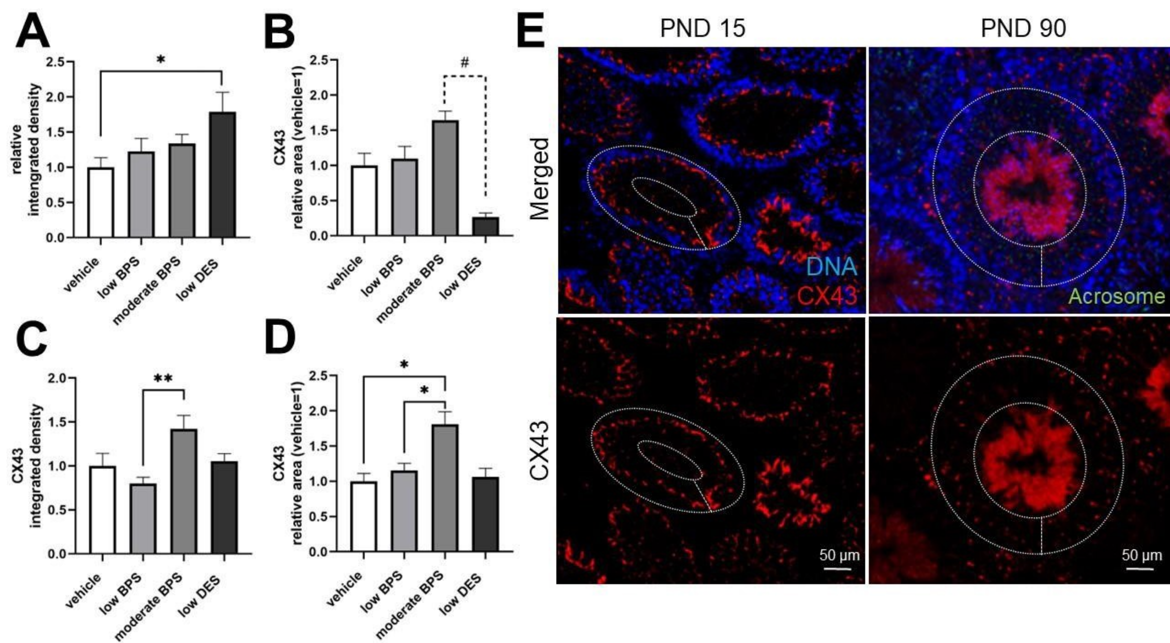
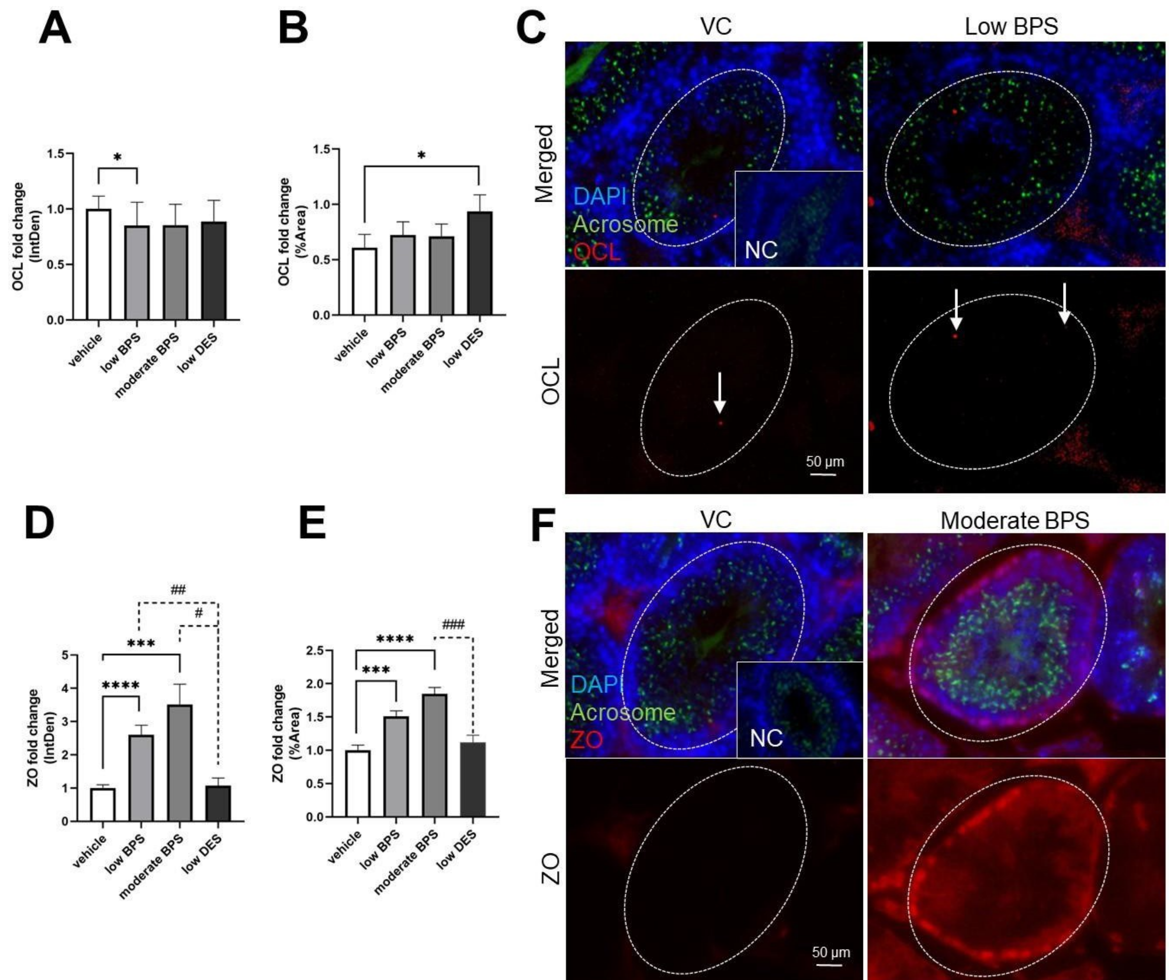


684

685 **Figure 4: Expression and localization of CX43 in testicular tissue of young**
686 **(PND15) and adult (PND90) mice.** (**A**) Relative integrated density of CX43, and (**B**) area of
687 expression of CX43 in tubules at PND15 in the control, low BPS, moderate BPS, and low
688 DES groups. (**C**) Relative integrated density of CX43, and (**D**) area of expression of CX43 in
689 middle-stage tubules at PND90 in the control, low BPS, moderate BPS, and low DES groups.
690 (**E**) Representative microphotographs of CX43 in the seminiferous tubules of mice testes at
691 PND15 (magnification 20 x) and at middle stage of spermatogenesis at PND90 (magnification
692 40 x). The dashed line indicates the measured area. Columns represent mean, and whiskers
693 represent the standard error of mean. The difference was tested using the Kruskal–Wallis test,
694 followed by Dunn's multiple comparisons test. Asterisks indicate statistical differences (*P
695 ≤0.1, **P ≤0.01), and daggers indicate differences from the positive (low DES) control ($^{\#}P$
696 ≤0.1).



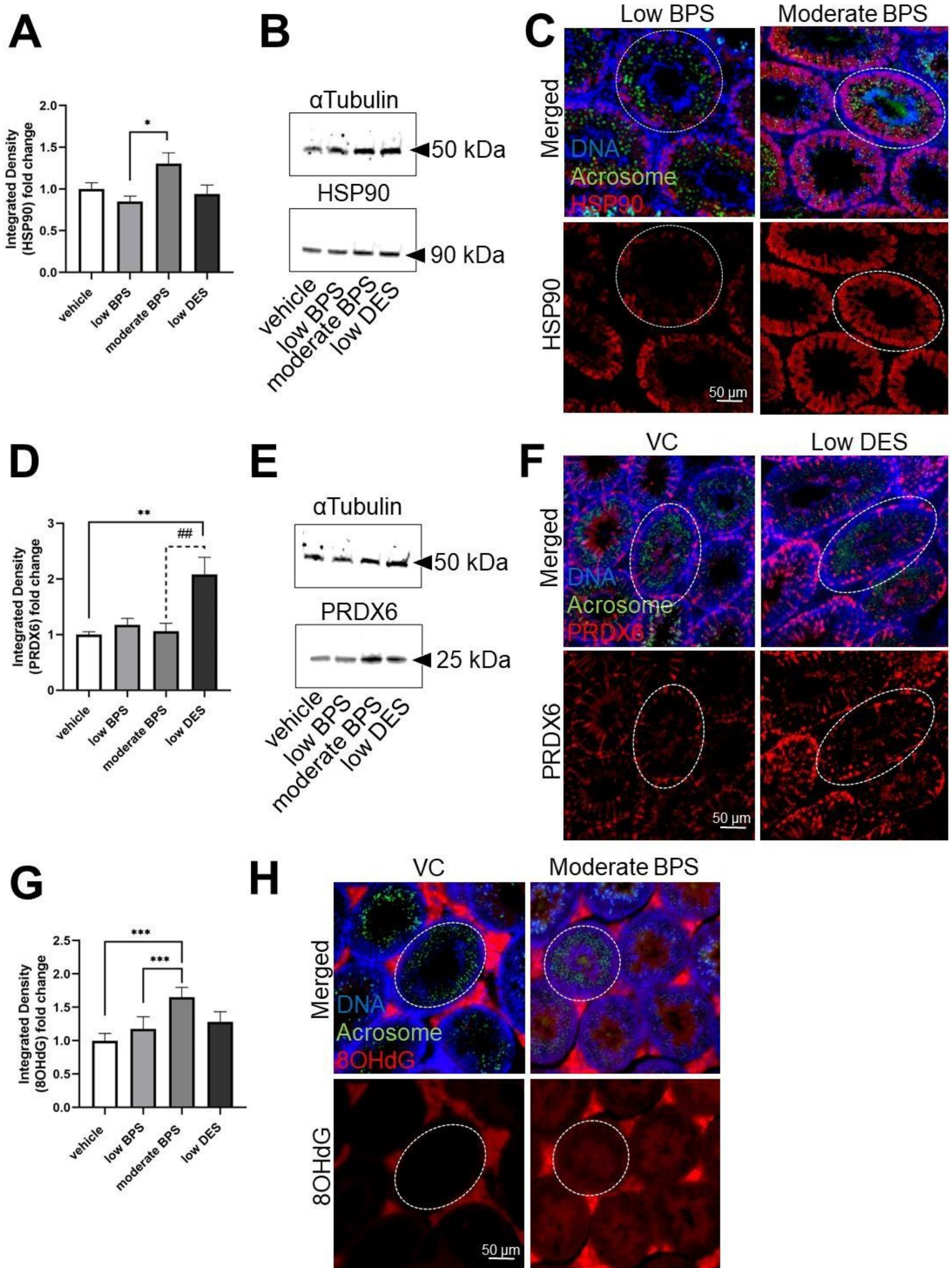
697

698 **Fig. 5: Expression and localization of tight junction proteins in the testicular tissue of**
699 **adult mice. (A)** Integrated density of occludin11 (OCL), and **(B)** %area of OCL in
700 tubules at middle stage (VII-VIII) at PND90 in the control, low BPS, moderate BPS, and
701 low DES groups. **(C)** Representative microphotograph of OCL in tubules in the control
702 and low BPS groups. Arrows indicate OCL. Dashed lines indicate measured area.
703 Magnification 40 x. **(D)** Integrated density of ZO1 tight junction protein (ZO), and **(E)**
704 %area of ZO-1 in tubules at middle stage at PND 90 in the control, low BPS, moderate
705 BPS, and low DES groups. **(F)** Representative microphotograph of ZO in tubules in the
706 control and moderate BPS groups. Dashed lines indicate measured area. Magnification
707 40 x. Columns represent mean, and whiskers represent the standard error of mean. The
708 difference was tested using the Kruskal–Wallis test, followed by Dunn's multiple

709 comparisons test. Asterisks indicate statistical differences (*P ≤0.1, **P ≤0.01, ***P

710 ≤0.001, ****P ≤0.0001), and daggers indicate differences from the positive (low DES)

711 control ($^{\#}P$ ≤0.1, $^{\#\#}P$ ≤0.01, $^{\#\#\#}$ P ≤0.001).



712

713 **Fig. 6: Expression and localization of oxidative stress proteins in the testicular tissue of**
714 **adult mice. (A)** Integrated density of Heat Shock Protein 90 (HSP90) in the
715 seminiferous tubules of mouse testis at middle stage at PND90 in the control, low BPS,
716 moderate BPS, and low DES groups. **(B) A** representative microphotograph of HPS90 in
717 tubules at middle stage in the low and moderate BPS groups. Dashed lines indicate
718 measured area. Magnification 40 x. (**C)** HSP90 in testicular tissue. **(D)** Integrated density
719 of Peroxiredoxin 6 (PRDX6) in seminiferous tubules at middle stage in the control, low
720 BPS, moderate BPS, and low DES groups. **(E)** Representative microphotograph of
721 PRDX6 in tubules at middle stage in the control and low DES groups. Dashed lines
722 indicate measured area. Magnification 40 x. **(F)** PRDX6 in testicular tissue. **(G)**
723 Integrated density of 8OHdG in seminiferous tubules at middle stage in the control, low
724 BPS, moderate BPS, and low DES groups. **(H)** Representative microphotograph of
725 8OHdG in tubules at middle stage in the control and moderate BPS groups. Dashed lines
726 indicate measured area. Magnification 40 x. Columns represent mean, and whiskers
727 represent the standard error of mean. The difference was tested using the Kruskal–Wallis
728 test, followed by Dunn's multiple comparisons test. Asterisks indicate statistical
729 differences (*P ≤0.1, **P ≤0.01, ****P ≤0.0001), and daggers indicate differences from
730 the positive (low DES) control ($^{\#\#}$P ≤0.01).

Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Author Statement

☒ The authors declare that they contributed to the work on this paper as described below:

Jan Nevoral, Milena Králíčková: Project conception; **Jan Nevoral, Pavel Klein**: Animal experimental design; **Tereza Fenclová, Marouane Chemek, Vendula Sudová, Jiřina Havránková, Yaroslav Kolinko, Jiří Moravec, Jan Nevoral**: Execution of experiments; **Tereza Fenclová, Marouane Chemek, Vendula Sudová, Jan Nevoral**: Compiling the results; **Jan Nevoral**: Statistics; **Tereza Fenclová, Jan Nevoral**: Writing the manuscript and data interpretation; **Jan Nevoral**: Proofreading; all authors read and approved the final manuscript.

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