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Effects of bisphenol A exposure on DNA integrity and protamination of mouse spermatozoa

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ABSTRACT

Background: Bisphenol A is widely used in the manufacture of polycarbonate plastics and has caused increasing concern over its potential adverse impacts on spermatogenesis. However, the effect of bisphenol A on spermiogenesis is yet to be explored.

Objectives: To evaluate whether bisphenol A has adverse effects on DNA integrity and protamination of spermatogenic cell.

Materials and methods: Newborn male mice were subcutaneously injected with bisphenol A (0.1, 5 mg/kg body weight, $n = 15$) or coin oil (control group, $n = 20$) daily from post-natal day 1 until 35. At post-natal day 70, epididymis caudal spermatozoa and testes were collected. Sperm count, sperm motility, and sperm morphology were analyzed. The sperm chromatin structure assay was performed to examine the sperm DNA fragmentation. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method was used to assess apoptosis of spermatogenic cells. The ultrastructural features of testicular sections were examined under a transmission electron microscope. Western blot and RT-PCR were used to detect the expression levels of transition protein (Tnp) 1 and Tnp2, protamine (Prm) 1 and Prm2 protein, and mRNA in mice testes.

Results: Bisphenol A significantly reduced sperm counts, impaired sperm motility, and increased the percentage of malformed spermatozoa. Poor sperm chromatin integrity and increased TUNEL-positive spermatogenic cells were also observed in mice exposed to bisphenol A. Ultrastructural analysis of testes showed that bisphenol A exposure caused incomplete chromatin condensation, retention of residual cytoplasm, and abnormal acrosome formation. In addition, the relative expression levels of Tnp2 and Prm2 in mice testes decreased significantly in bisphenol A groups.

Discussion and conclusion: Our findings identified that neonatal bisphenol A exposure may negatively contribute to the sperm quality in adult mice. Mechanistically, we showed that bisphenol A reduced sperm chromatin integrity along with increased DNA damage, which may be due to poor protamination of spermatozoa.

INTRODUCTION

In recent decades, declining semen quality and increasing male infertility caused public health concerns (Shine *et al.*, 2008). Endocrine disrupting chemicals (EDCs) are identified to have deleterious effects on the development of male reproductive system (Vitku *et al.*, 2016). Bisphenol A (BPA) is one of the most common EDCs to which we are exposed daily because of its wide availability in the environment (Ikezuki *et al.*, 2002; Welshons *et al.*, 2006). BPA is widely used as a common ingredient in the manufacture of plastic products such as food packaging materials, mineral water bottles, toys, and dental filling materials (Kubwabo *et al.*, 2009; Hope *et al.*, 2016). Plastics are broadly integrated into today's lifestyle and BPA can be detected in more than 90% of human urine samples (Calafat *et al.*, 2008; Chen *et al.*, 2011). Epidemiological studies conducted among groups of BPA-exposed human males showed that urine BPA levels were associated with declined semen quality (Li *et al.*, 2011). Human BPA exposure presented negative effects on the development and function of male reproductive system (Maffini *et al.*, 2006; Peretz *et al.*, 2014; Minguez-Alarcon *et al.*, 2016). Animal experiments also confirmed that environmentally relevant doses of BPA significantly impaired spermatogenesis (Okada & Kai, 2008; Salian *et al.*, 2011).

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Spermatogenesis is a lengthy and complex process of cell differentiation that undergoes three stages including mitosis, meiosis, and spermiogenesis. Dysregulations in any of these processes can lead to impaired spermatogenesis. Emerging evidence supports that BPA could interfere with different stages of spermatogenesis. Sheng *et al.* (2013) reported that BPA (10^{-9} M) boosted mouse spermatogonial cell proliferation in vitro by inducing the G protein-coupled receptor 30 expression. Curved seminiferous tubule of rats treated with BPA in vitro presented partial meiosis arrest with increased leptotene spermatocyte percentage and decreased diplotene spermatocyte percentage (Ali *et al.*, 2014). In vivo BPA (20 μg/kg·bw/day) exposure also led to delayed meiosis initiation in the early meiotic stage and accumulation of chromosomal abnormalities and DNA double-strand breaks (DSBs) in the late meiotic stage (Liu *et al.*, 2014). Spermatogenic cells with abnormal mitosis or meiosis usually result in apoptosis and will not produce spermatozoa. In addition, damaged sperm chromatin, including DNA damage, may impair the ability of spermatozoa to fertilize. Human clinical study showed that sperm DNA damage was highly correlated with pregnancy outcomes (Evenson *et al.*, 1999). It has been shown that DNA damage could reduce the success of in vitro fertilization (IVF) (Wdowiak *et al.*, 2015) and intracytoplasmic sperm injection (ICSI) (Avendaño *et al.*, 2010), weakens pregnancy (Carlini *et al.*, 2017), and even affects the offspring's health (Jin *et al.*, 2015). Comparative studies of spermatozoa from clinical patients and laboratory animals found that BPA may be associated with declined semen quality and increased sperm DNA damage (Toyama & Yuasa, 2004; Meeker *et al.*, 2010; Vitku *et al.*, 2016; Fawzy *et al.*, 2018; Omran *et al.*, 2018). However, the underlying mechanism for the impacts of BPA on sperm DNA damage is still unclear.

Spermiogenesis is the final stage of spermatogenesis, which converts round spermatids into spermatozoa including elongation and condensation of nuclei, formation of the acrosome and flagellum, and removal of excess cytoplasm. The mammalian spermatozoa is a specialized cell with a condensed nucleus, in which the majority of the DNA is tightly packaged in toroidal structures by protamines (Oliva & Dixon, 1991; Oliva & Ballesca, 2012). Protamines, with two major forms as Prm1 and Prm2, are arginine-rich DNA-binding proteins. Protamine replacement occurs in maturation of male haploid germ cells during spermiogenesis. Histones are first transiently supplanted by transition proteins and subsequently replaced with protamines as the major nuclear proteins (Steger *et al.*, 1998). Protamines provide substantial compaction to DNA structure and protection against DNA damage (Schneider *et al.*, 2016). Clinical studies found that sperm DNA damage was mostly correlated with abnormal protamine replacement (Nili *et al.*, 2009; Ni *et al.*, 2016).

Declining semen quality and increasing incidence of male reproductive disorders in adulthood may result from early exposure. Our previous studies found that neonatal BPA exposure could inhibit the expression of Boule in mouse testes (Xie *et al.*, 2016). It was revealed that Boule had a novel role in spermiogenesis (VanGompel & Xu, 2010). Based on that, we found that BPA could interfere with spermiogenesis by impairing sperm DNA integrity. However, it is still unknown whether the sperm DNA damage induced by BPA is related to the aberrant protamination of spermatozoa. Therefore, in this study, we further demonstrated that BPA exposure disturbed the protamination pathway via decreasing the expression of Tnp2 and Prm2, which could reduce chromatin integrity, increase DNA damage, and finally interfere with spermatogenesis.

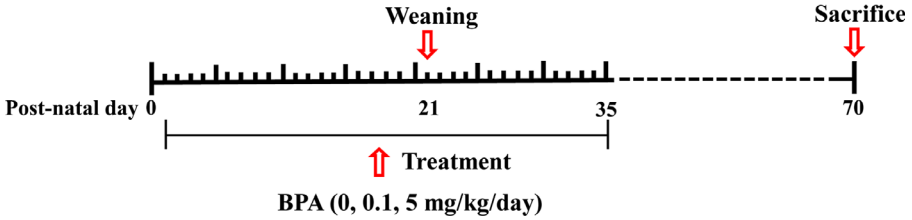
MATERIALS AND METHODS

Animals and BPA treatment

SPF *mus muculus* (ICR strain, 10 weeks of age) were obtained from the Beijing Vital River Laboratory Animal Technology Co., Ltd, China. All experimental procedures and handling of animals were approved by the ethics committee for experimental laboratory animals at Weifang Medical University (2017016) and according to the Guide for the Care and Use of Laboratory Animals (Eighth edition). Animals were housed in polypropylene cages under a 12-h light/12-h dark cycle and acclimatized to an environmentally controlled room (room temperature 24 ± 2 °C, relative humidity 40–50%, frequent ventilation). A commercial pellet diet (BPA-free, Beijing Vital River Laboratory Animal Technology Co., Ltd, China) and drinking water (in glass bottles) were fed ad libitum. A week later, female mice were mated with males and checked daily; once vaginal plug was formed, female mice were separated into new polypropylene cages till delivery.

After delivery, the pups were sexed by anogenital distance, female pups were removed and each dam was left with 4 to 5 male pups within 24 h of delivery. Newborn male pups were assigned to one of three neonatal treatment groups with 15–20 pups/group: negative control (corn oil) group (n = 20), BPA (0.1 mg/kg, an environmentally relevant exposure dose) group (n = 15), and BPA (5 mg/kg, a dose equal to the no observed adverse effect level) group (n = 15). And the pups were subcutaneously injected daily with BPA (4 μL/g·bw, purchased from Sigma-Aldrich, dissolved in absolute ethanol and then diluted in corn oil) or with vehicle from PND 1 until 35 to cover the first spermatogenesis wave, with the day of birth designated as PND 0. All mice in a litter received the same treatment. At the time of weaning (PND 21), male offspring mice were separated from their mothers. At PND 70, male offspring were euthanized by CO_2 inhalation (Fig. 1). Testes were collected immediately after

Figure 1 Schematic of animal treatment procedure. The day of parturition was designated as PND 0, and BPA treatment started at PND 1. BPA was administered subcutaneously at doses of 0.1 and 5 mg/kg/day till up to PND 35. At PND 70, mice were euthanized and tissues were collected.



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euthanasia and weighed, either stored at −80 °C for further molecular analyses or immediately fixed in 4% paraformaldehyde, embedded in paraffin.

Sperm count, sperm motility, and sperm morphology analyses

Spermatozoa were acquired by dissecting caudal epididymides in 37 °C pre-warmed enriched Krebs-Ringer bicarbonate medium (EKRB medium; 120.1 mM NaCl, 4.8 mM KCl, 25.2 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, supplemented with 11.1 mM glucose, 2 mM glutamine, 1 × essential amino acids, 1 × non-essential amino acids, 100 mg/mL streptomycin, 100 U/mL penicillin) (Gou *et al.*, 2017). Then, sperm motility, quantified by the percentage of motile spermatozoa, was evaluated visually. The inactive sperm count was firstly determined by counting the number of non-linear spermatozoa (including dead spermatozoa, spermatozoa that oscillates and rotates) on a hemocytometer under microscope and calculated the number of spermatozoa per milliliter. Then, the hemocytometer was incubated at 120 °C for 5 min and the number of spermatozoa was counted and calculated the total number of spermatozoa per milliliter. Sperm motility = (total sperm count − non-linear sperm count)/total sperm count. For sperm morphological analysis, seminal smear was made on glass slides with the sperm sample, fixed in 4% paraformaldehyde and stained with 4′, 6-diamidino-2-phenylindole (DAPI). Sperm morphology was observed under a laser scanning confocal microscope (LSCM) (100 × oil objective), and the percentage of malformed spermatozoa was calculated. Sperm malformations are mainly manifested in the head. According to Wyrobeks classification criteria, the main types are as follows: no hook, banana shape, amorphous, fat head, tail fold, double head, and double tail. Spermatozoa without tail, overlapping heads, or the entire overlap with the other were not counted (Gatimel *et al.*, 2017).

Sperm chromatin structure assay

The sperm chromatin structure assay (SCSA) was carried out followed by the description of Evenson (Evenson *et al.*, 2002; Evenson, 2013) using an acridine orange (AO) fluorescence staining kit (Genmed Scientifics Inc., Wilmington, DE, USA). Briefly, sperm samples from epididymis were washed and diluted with cleaning buffer to obtain the concentration of 10^6 sperm/mL, and then treated with an acidic solution (pH 1.20) for 30 sec to denature the DNA at the sites of strand breaks. 0.2 mL of AO staining solution was added to 2 × 10^5 sperm and incubated for 5 min. The spermatozoa were washed with cleaning buffer twice. A total of 10,000 cells were analyzed by flow cytometry (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ, USA) per sample, with a flow rate of 200–250 cells/sec. The FL1-H (530 ± 30) and FL3-H (695 ± 40) were adjusted to thereafter proceed with all the samples to be analyzed at one time. A reference sample was established consisting of spermatozoa from control mice for the flow cytometer setup and calibration. A positive control sample was prepared by incubating caudal spermatozoa from control mice in DNase buffer (200 U/mL) with 1% Triton X-100 at 37 °C for 1 h. Reference and positive control samples were stored at −80 °C until use. By stained with the fluorescent dye AO, double-stranded native DNA emits green fluorescence, while fragmented single-stranded DNA emits red fluorescence. The extent of DNA denaturation is expressed as DNA fragmentation index (DFI), which is the ratio of red (level of denatured DNA) to total fluorescence intensity (the total DNA).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method was used to assess DNA fragmentation in the cells. TUNEL assay was performed using In Situ Cell Death Detection Kit, POD (Roche, Germany) following manufacturer's instructions. After dewaxing and rehydration, the tissues were treated with proteinase K solution, successively incubated with TUNEL reaction mixture and converter POD, and then covered with 3, 3′-diaminobenzidine (DAB) solution. Finally, the sections were counterstained with hematoxylin, dehydrated, mounted, and observed under a light microscopy (Olympus, Japan). Apoptosis (positive TUNEL staining) of testicular cells was assessed with randomly selected seminiferous tubules (100 tubules per mouse). Apoptosis index (AI) was defined as: the number of positive cells/the total number of counted cells × 100%.

Ultrastructure analysis of testes

Small fragments (1 mm^3) of dissected testes were removed and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), post-fixed in 1% OsO_4. The following protocol referred to the standard procedures (Tian *et al.*, 2017). The ultrastructural features of testicular sections were examined under a transmission electron microscope (TEM) (Hitachi, HT7700, Japan). The numbers of abnormal and normal spermatozoa were counted: More than 300 spermatozoa were randomly selected from each group.

RNA extraction and RT-PCR

Total RNA from the testes was extracted using Trizol reagent solution (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed according to the HiFiScript cDNA synthesis kit instructions (ComWin Biotech, Beijing, China). Reverse transcriptase was replaced by RNase-free water in RT negative control. The polymerase chain reaction system was as following: 2 μL cDNA, 12.5 μL 2 × Es Taq MasterMix (Dye) (ComWin Biotech, Beijing, China), 10 μL RNase-free water, and 5 pmol of each primer in a total volume of 25 μL under the following conditions: 2 min at 94 °C; annealing for 30 sec (Tm of *Tnp1*, *Tnp2,* and *M-Actb* were 56 °C, that of *Prm1* and *Prm2* were 57 °C), 30 sec at 72 °C, followed by 30 cycles; 2 min at 72 °C. The PCR negative control was supplemented with RNase-free water instead of cDNA.

Table 1 Sequences of gene primer sets for RT-PCR

Gene	Primer sequences		Product size (bp)
M-Actb	Forward	5′-GGGACGACATGGAGAAGATCT-3′	177
	Reverse	5′-CCTGGATGGCTACGTACATG-3′	
Tnp1	Forward	5′-CAAGCTAAAGACTCATGGCATG-3′	186
	Reverse	5′-TGAAGACCACCAGGGCAG-3′	
Tnp2	Forward	5′-CAAAGTCACACCAGTGTCCAGTG-3′	187
	Reverse	5′-GTCTTGCAGGTGAGTGTCGAG-3′	
Prm1	Forward	5′-GAAGATGTCGCAGACGGAG-3′	120
	Reverse	5′-ATGGACTTGCTATTCTGTGCATC-3′	
Prm2	Forward	5′-CTCTCGTAAGAGCTACATAGGATC-3′	162
	Reverse	5′-GCTTAGTGATGGTGCCTCCTAC-3′	

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Primer pairs for genes are given in Table 1. PCR products were electrophoresed on 2% agarose gels in 1 × TAE buffer and visualized by ethidium bromide staining. Image analysis of the bands obtained was performed using image analysis system (Image J 1.44p, National Institutes of Health, Bethesda, MD, USA).

Western blotting analysis

Total testicular protein lysates were prepared by RIPA lysis buffer (ComWin Biotech, Beijing, China) containing PMSF (Solarbio Science & Technology, Beijing, China) and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Protein concentrations were confirmed using the bicinchoninic acid (BCA) protein assay (ComWin Biotech, Beijing, China). The 50 μg proteins were loaded onto each lane of a 15% denatured polyacrylamide gel. The proteins were separated and transferred onto Immobilon-P PVDF membranes (Merck Millipore, Tullagreen, Ireland) at 300 V, 200 mA, 4 °C for 110 min using the mini-protean tetra system (Bio-Rad, USA). Membranes were blocked in 5% non-fat dry milk for 1 h at room temperature and then incubated with primary antibodies overnight at 4 °C, and primary antibodies include rabbit anti-PRM1 polyclonal antibody (1:1000) (Briar Patch Biosciences, USA), rabbit anti-PRM2 polyclonal antibody (1:1000) (Briar Patch Biosciences, USA), mouse anti-TNP1 polyclonal antibody (1:1000) (Santa Cruz, CA, USA), mouse anti-TNP2 polyclonal antibody (1:1600) (Santa Cruz, CA, USA), and mouse anti-β-actin polyclonal antibody (1:1000) (Santa Cruz, CA, USA). The membranes were washed 3 times for 10 min each (TBS and 0.1% Tween-20) and further incubated with horseradish peroxidase-conjugated secondary antibody (1:5000) (Santa Cruz, CA, USA) for 2 h at room temperature. The membranes were washed with TBS containing 0.1% Tween-20. Immunoblots were visualized and finally developed with electrochemiluminescence (ECL). The protein signal was quantified by densitometry with image analysis system (Image J 1.44p, National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

The experimental data were presented as the mean ± SD. Statistical significance was determined by two-tailed, unpaired Student's *t*-test. A p value of less than 0.05 was considered significant.

RESULTS

Body weight and relative testis weight

Neonatal exposure to BPA showed no effect on litter survival, and all animals survived to the end of the experiment. The body weight and relative testis weight were similar, within normal ranges among different groups ($p > 0.05$, Fig. 2). The weight of bilateral testes in each mouse was normalized by its body weight measured prior to euthanasia.

Figure 2 Effects of neonatal BPA exposure on the changes in body weight from PND 1 to PND 70 (left panel) and testes relative weight at PND 70 (right panel) of mice. Data are shown as the mean ± SD.

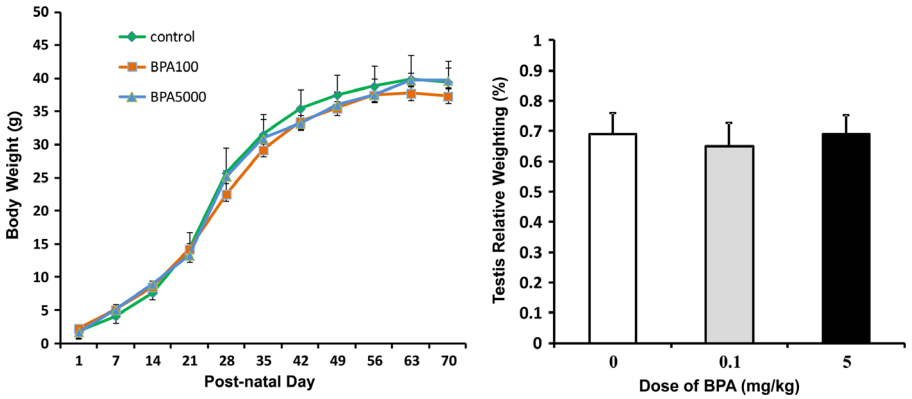

Figure 3 Effects of neonatal BPA exposure on epididymal sperm count and sperm motility. Data are shown as the mean ± SD. * represents $p < 0.05$ as compared to control.

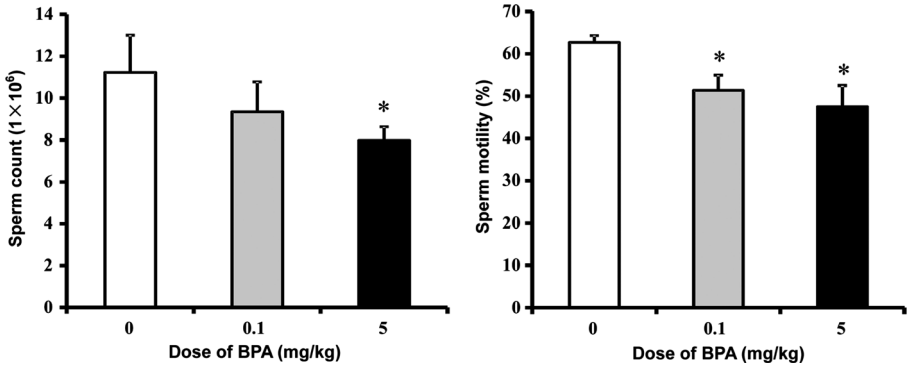

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Figure 4 Epididymal spermatozoa from BPA-treated mice showed abnormal morphology. Representative images of control (A) and BPA-treated mice (B–D). (E) The quantification of malformed spermatozoa in different doses of BPA-treated mice. Spermatozoa with bent tails (arrowhead-indicated), sperm nuclear vacuole (asterisk-indicated), and spermatozoa with bent apex (arrow-indicated) were present in BPA-treated groups. Bars = 5 μm. ** represents $p < 0.01$ as compared to control.

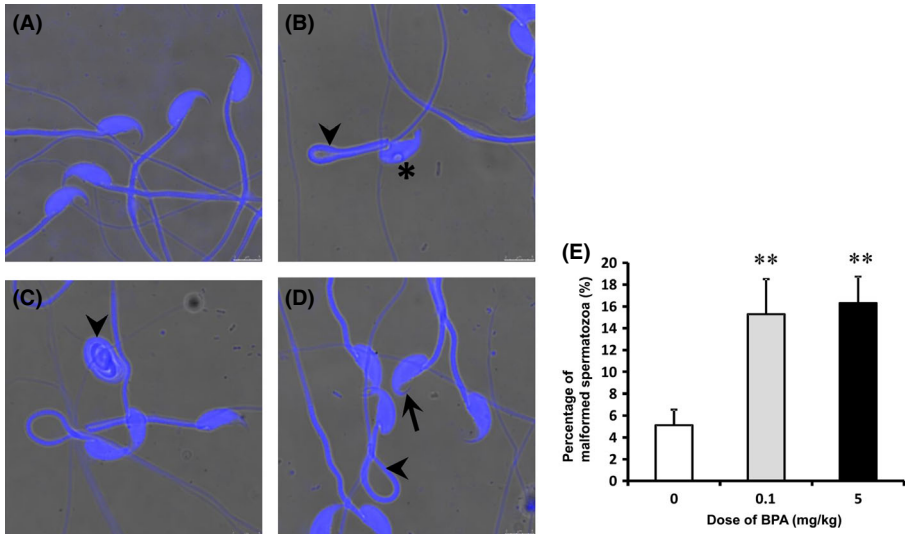
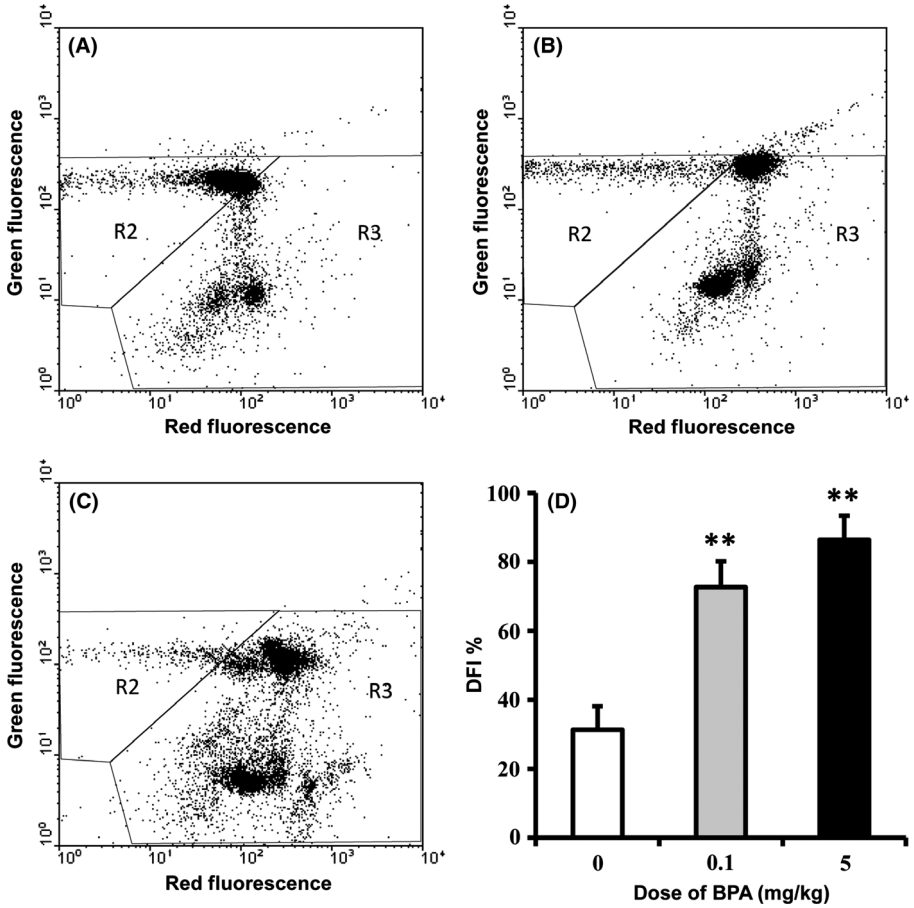


Figure 5 Effects of Bisphenol A on the sperm DNA integrity detected by flow cytometry. (A–C) Representative images of control, BPA 0.1 mg/kg and BPA 5 mg/kg groups. Each dot represents a spermatozoa characterized by the amount of double-stranded DNA (green fluorescence) or single-stranded DNA (red fluorescence). R2 population show normal sperm population; R3 population show DNA denatured sperm population. (D) DNA fragmentation index. Bars represent the mean ± SD per group ($n = 15$). ** represents $p < 0.01$ as compared to control.



BPA exposure induced sperm defects

BPA exposure adversely affected the sperm quality of adult male offspring mice. Average sperm counts in caudal epididymis dramatically decreased in high-dose BPA-treated group ($p < 0.05$, Fig. 3), and sperm motility impaired in both BPA-treated groups ($p < 0.05$, Fig. 3). The morphological deformities in the head and tail of spermatozoa were observed in BPA groups (Fig. 4B–D). Nuclear vacuoles (Fig. 4B), bent apexes (the sharp point of the sperm nucleus, Fig. 4D) in the heads, and bent tails (head folding back onto the flagellum or curly flagellum, Fig. 4B–D) were found in spermatozoa from BPA-treated mice. Statistically significantly increased frequencies of malformed spermatozoa were recorded in BPA groups ($p < 0.01$, Fig. 4E).

BPA exposure impaired DNA integrity in spermatozoa

Effects of BPA on sperm chromatin structure were examined using SCSA on sperm cells from caudal epididymis. Examination of the fluorescence spectra of spermatozoa from the untreated mice mostly emitted green fluorescence, whereas the fluorescence spectra of spermatozoa from the BPA-treated mice mostly emitted red or orange fluorescence (Fig. 5A–C). Sperm DNA fragmentation (evaluated by DFI) was compared between different groups, and the statistical analysis of data showed that BPA had a significant effect on DFI ($p < 0.01$, Fig. 5D). After BPA treatment, both groups showed increased levels of DFI in a dose-dependent manner. According to our results, neonatal BPA exposure caused higher sperm DNA fragmentation.

Effects of BPA on apoptosis of spermatogenic cells

We performed TUNEL staining to confirm cell apoptosis in mouse testis. As shown in Fig. 6, the apoptotic nuclei were stained yellow brown or dark brown. Many TUNEL-positive cells were detected in testicular sections of mice treated with BPA (Fig. 6B,C), while only a small number of TUNEL-positive cells were observed in the control group (Fig. 6A). The TUNEL-positive cells in control group were predominantly early stage spermatogenic cells including spermatogonia, spermatocytes, and a few round spermatids (Fig. 6A). Besides the abovementioned cell types, considerable elongated spermatids were TUNEL-positive in BPA-treated groups (Fig. 6B,C). Quantitative analysis showed that BPA exposure caused a significant increase in apoptosis index of spermatogenic cells (including spermatogonia, spermatocytes, round spermatids, and elongated spermatids) ($p < 0.05$, Fig. 6D).

BPA exposure induced DNA decondensation and acrosome abnormalities

TEM was used for further investigation of ultrastructural injury of spermatogenic cells induced by BPA. Testicular sections of control group showed normal ultrastructure for well-organized different stages of spermatogenic cells (Fig. 7A,B). Conversely, disappearance of nucleus content or sparse nucleus content was observed in BPA-treated spermatocytes (Fig. 7C). In the lumen of the seminiferous vesicles, the sperm chromatin was tightly compacted in the control group as indicated by the density of staining observed under TEM (Fig. 7B), whereas heterogeneous electron density of nucleus and clear vacuoles was observed in nuclei of spermatozoa from mice treated with BPA (Fig. 6E,F). Besides, swollen acrosomes, shrunken subacromial space, and retention of residual cytoplasm were observed in spermatozoa from BPA-treated group (Fig. 7D). The percentage of abnormal spermatozoa

Figure 6 Effects of Bisphenol A on apoptosis of spermatogenic cells detected by TUNEL assay. (A–C) Representative images of control, BPA 0.1 mg/kg and BPA 5 mg/kg groups. Arrows show TUNEL-positive staining cells. (D) Apoptosis index. Shown is the number of TUNEL-positive cells per seminiferous tubule from 100 tubule cross-sections from each mouse. Bars represent the mean ± SD per group ($n = 15$). * represents $p < 0.05$ as compared to control.

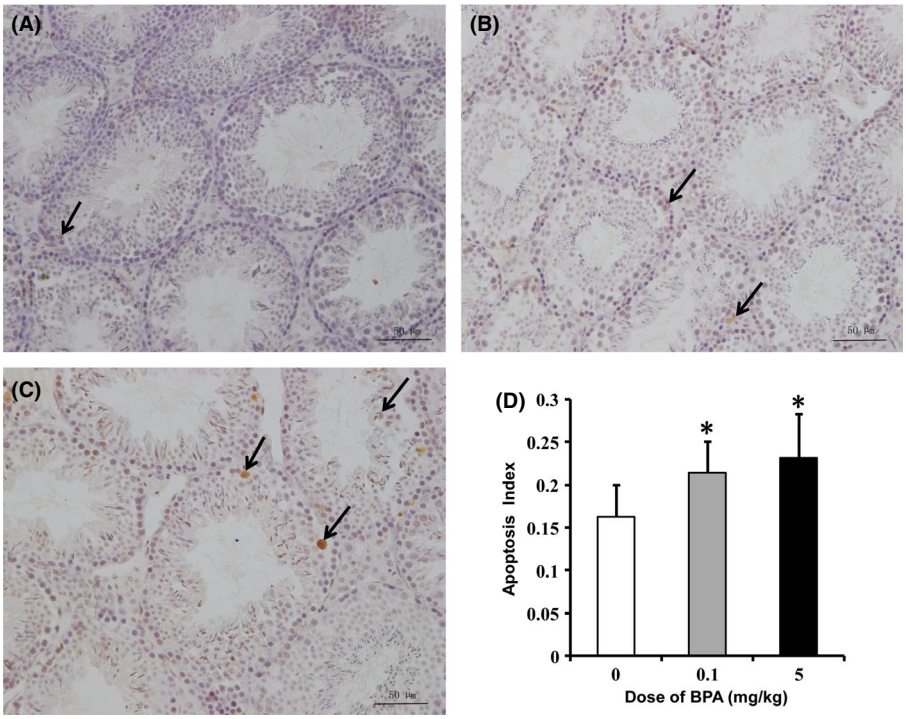


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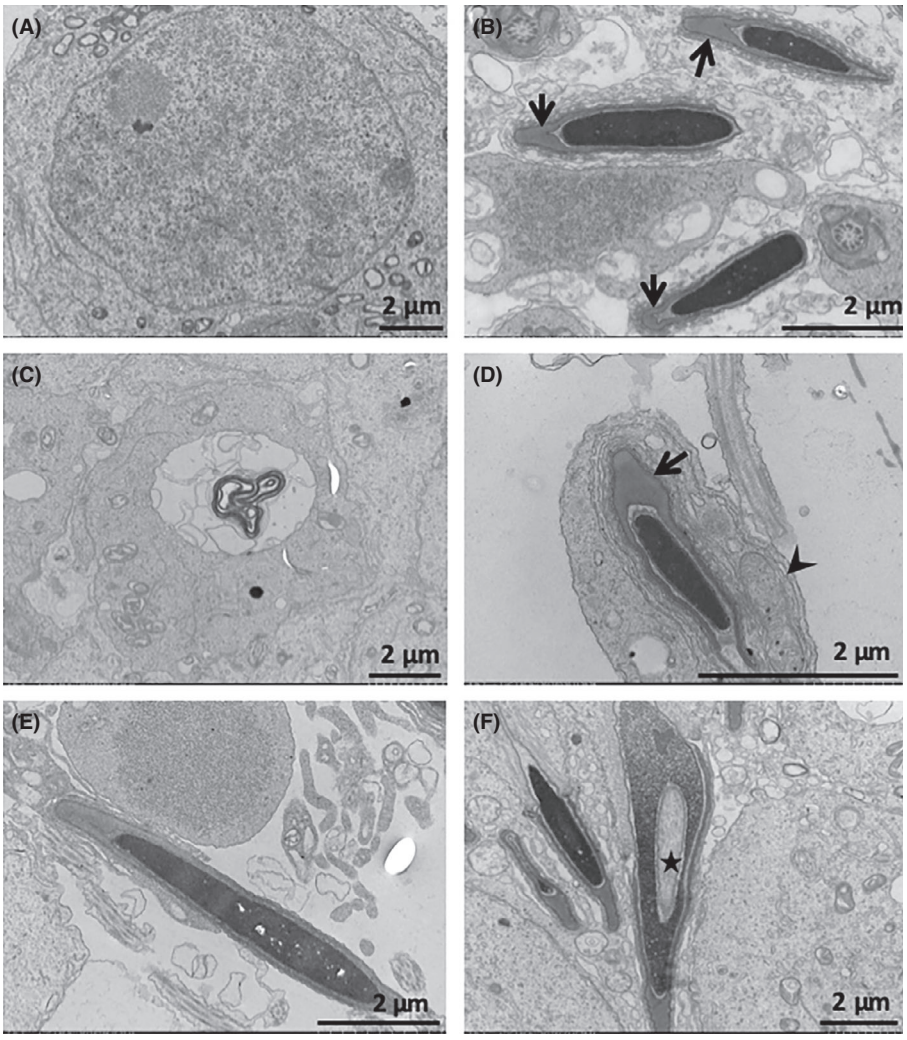
Figure 7 Ultrastructural examination of the testes from control and BPA groups. (A,B) Representative images of a normal spermatocyte and spermatozoa from control groups. (C–F) Representative images of an abnormal spermatocyte and spermatozoa from BPA-treated groups. (A) Spermatocyte with normal microtubule structure. (B) Spermatozoa with normal acrosomes (arrow-indicated) and nucleus showing normal electron density. (C) Spermatocyte without nucleus content. (D) Spermatozoa with swollen acrosome (arrow-indicated), shrunken subacrosomal space and residual cytoplasm (arrowhead-indicated). (E) Vacuole in the nucleus of spermatozoa. (F) Nucleus of spermatozoa showing heterogeneous electron density (asterisk-indicated).



reached more than 60% in high-dose BPA group and 30% in low-dose BPA group, which were higher than the percentage of malformed spermatozoa seen by LSCM.

BPA exposure changed Tnp2 and Prm2 expression

To evaluate whether the presentation of DNA decondensation and sperm abnormalities induced by BPA was associated with the alterations of chromatin reorganization-related proteins, the relative protein expression of Tnp1, Tnp2, Prm1, and Prm2 was determined in testes. Results of Western blotting showed that BPA exposure significantly decreased the protein expression level of Tnp2 and Prm2 in the adult testes in a dose-dependent manner ($p < 0.05$, Fig. 8). However, BPA caused no effects on the expression of Tnp1 and Prm1. Further investigation was performed to analyze the relative transcript level of *Tnp1, Tnp2, Prm1,* and *Prm2* in the testes. The relative transcript level of *Tnp1, Tnp2, Prm1,* and *Prm2* did not show apparent change in testes upon BPA treatment ($p > 0.05$, Fig. 9).

DISCUSSION

Neonatal stage is a critical developmental period that is susceptible to chemical exposure (Kuwada *et al.*, 2002). Previous studies found that BPA exposure at this stage was speculated to affect spermatogenesis of mouse (Aikawa *et al.*, 2004; Xie *et al.*, 2016). In the present study, BPA was administered to male neonatal mice via subcutaneous injection to identify the effect of BPA on the spermiogenesis of mature male mouse. Subcutaneous injection is an appropriate route for BPA delivery into the neonatal mice to assess its effects on testicular development. The reason why we chose subcutaneous injection as the route of BPA administration was explained in another paper (Xie *et al.*, 2016).

In the present study, BPA exposure has been shown to have adverse effects on sperm motility and count in a dose-dependent fashion. Spermatozoa from BPA-treated mice displayed severe morphological defects concerning the nuclear, acrosomal shape and residual bodies. TEM analysis of testis represented a further confirmation of these alterations. During spermiogenesis, the

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Figure 8 Quantification of relative protein level of Tnp1, Tnp2, Prm1, and Prm2 by Western blotting in mice testes. Representative blots show protein expression in the left panel, and results from densitometry analysis are shown in the right panels. Bars represent the mean ± SD per group (n = 15). * represents $p < 0.05$ as compared to control.

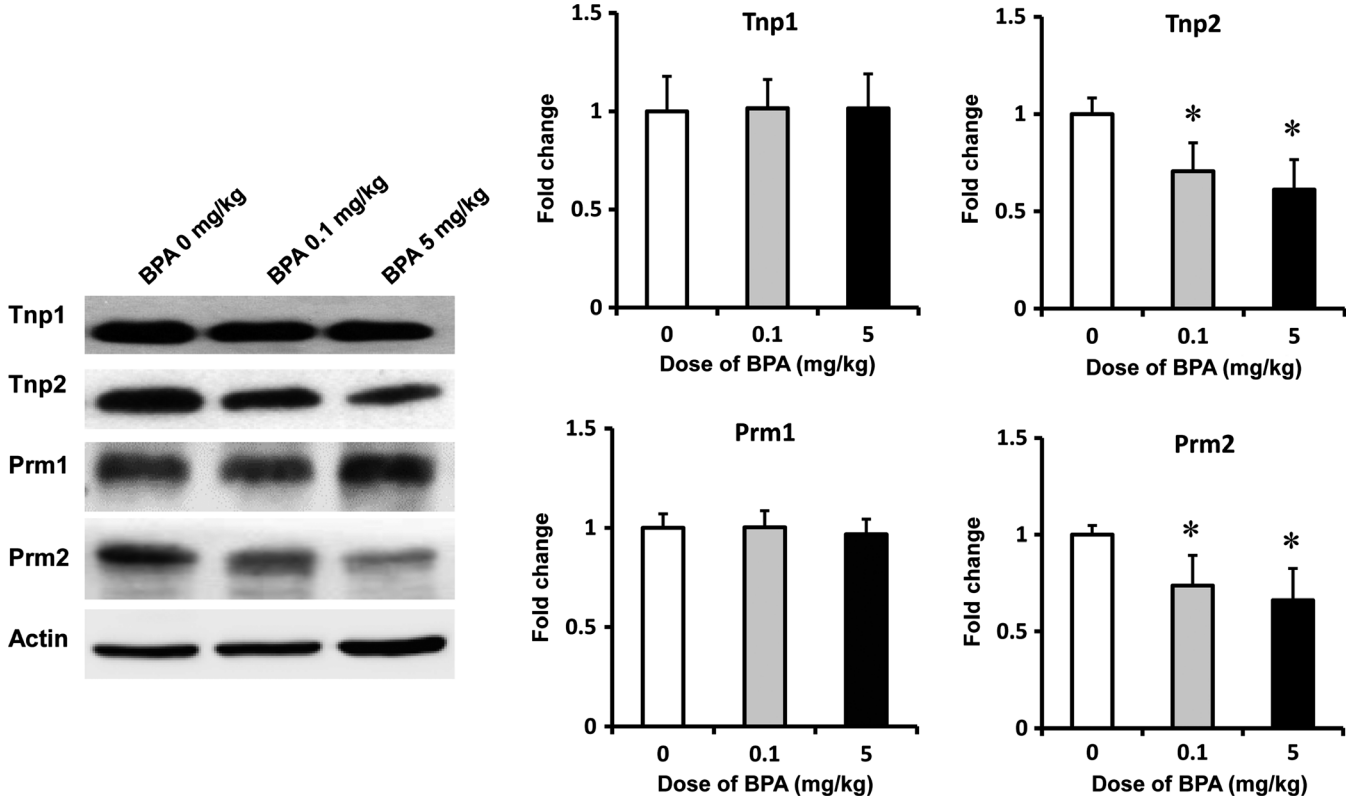
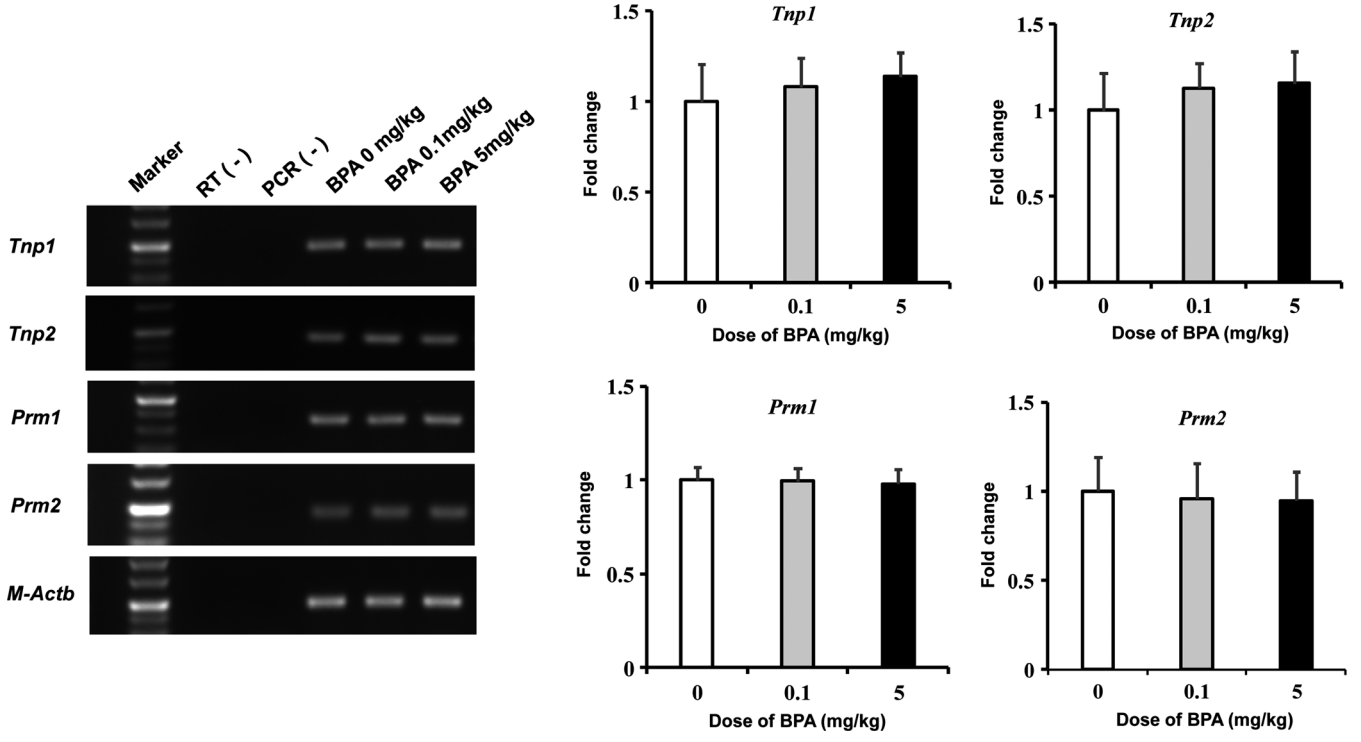


Figure 9 Quantification of relative mRNA level of *Tnp*1, *Tnp*2, *Prm*1, and *Prm*2 genes by RT-PCR in mice testes. Representative gels show mRNA level in the left panel, and results from densitometry analysis are shown in the right panels. Bars represent the mean ± SD per group (n = 15).



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Golgi apparatus surrounds the condensed nucleus to form the acrosome and the excess cytoplasm (known as residual bodies) is removed from the spermatozoa. The defects of acrosome formation and the retention of residual cytoplasm could affect spermatid head shaping (Liska *et al.*, 2009; Tokuhiro *et al.*, 2009; Kierszenbaum *et al.*, 2011). Abnormal head shapes represent a form of teratozoospermia that can result in defective sperm function and impair fertilization capacity (Keating *et al.*, 1997).

Until now, the condition of childlessness affects about 90 million couples worldwide, and one out of seven couples (one out of four in Western countries) could seek treatment via assisted reproduction technology (ART), including IVF and ICSI (Inhorn & Patrizio, 2015). Semen collected for ART is usually only evaluated on the basis of conventional seminal parameters. However, the classical semen parameters, such as sperm density, morphology, and motility, are not sufficient to effectively assess sperm fertility. DNA damage in the male spermatogenic line has been linked with a variety of adverse clinical outcomes including impaired fertility, an increased incidence of miscarriage, and birth defects in the offspring (Aitken *et al.*, 2009; Aitken & De Iuliis, 2010). Therefore, a method for sorting high-quality sperm samples from samples with normal semen parameters is highly needed. Sperm DNA integrity can be evaluated by some methods, one of which being the SCSA, that is a high-precision flow-cytometric test (Evenson *et al.*, 1980, 2002). Particularly, it is the only sperm DNA fragmentation method of testing both DNA strand breaks and chromatin structure. In our finding, increased sperm DNA damage induced by BPA was confirmed via SCSA, which was also mentioned in other studies from human and rodent (Rochester, 2013; Wu *et al.*, 2013). Herein, we found that DNA integrity was positively associated with sperm quality and once again reminded the necessity of DNA integrity to evaluate semen parameters. In parallel, abnormal condensed chromatin of sperm nuclei was observed in BPA groups under the transmission electron microscopy. Nuclear packaging plays important roles in maintaining the integrity and transcriptional inaction of DNA (Kocer *et al.*, 2015). The increased apoptosis index and DNA fragmentation index induced by BPA may be due to the poor DNA package.

Chromatin remodeling toward protamine incorporation is of outstanding importance for spermatid differentiation (de Boer *et al.*, 2015). Protamines are fundamental to compaction of the sperm nucleus, maintaining the integrity of chromatin and protecting the DNA from damage (Braun, 2001; Rathke *et al.*, 2014). Therefore, the changes in chromatin reorganization-related genes (protamine and transition protein) were further evaluated. Our data further indicate that the protein expression levels of Tnp2 and Prm2 in the testes from BPA groups were reduced compared with control group, whereas we have not detected any correlation between the Tnp2 and Prm2 alterations and the *Tnp2* and *Prm2* transcript level in the testes. Therefore, the Tnp2 and Prm2 alterations detected at the protein level in our study are not due to a transcriptional alteration in the corresponding genes, but must originate through post-translational processing and/or protein stabilization. Previous studies showed that low expression of Prm2 affected the effective combination of protamines and nuclear DNA, resulting in incomplete chromatin agglutination and damaged sperm DNA in mice (Cho *et al.*, 2001, 2003). According to the DNA protection hypothesis for protamines, the prediction would be that the detected increase in spermatogenic DNA damage would correlate with decreased protamination.

The association of abnormal protamine expression with low sperm count, decreased sperm motility and morphology, diminished fertilization ability, and increased sperm DNA damage has been reported (Aoki *et al.*, 2005; Carrell *et al.*, 2007). Protamine metabolism has also been proposed to play an important role in governing the sperm head development during spermiogenesis (Cho *et al.*, 2003). Poor protamination and the retention of excess residual cytoplasm are commonly encountered in defective human spermatozoa (Aitken & De Iuliis, 2010), but the relationship between these two processes needs to be clarified.

CONCLUSION

Our findings indicated that BPA exposure showed strong associations with semen quality parameters and sperm DNA integrity. Then, the possible mechanism may be that BPA exposure disturbed the protamination pathway via decreasing the expression of Tnp2 and Prm2, which could reduce chromatin integrity, increase DNA damage, and finally interfere with spermatogenesis. However, the precise molecular mechanism by which BPA affects the protamination of sperm cell requires further study.

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CONFLICT OF INTEREST

There were no competing interests among the authors.

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