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BDE-209 and DBDPE induce male reproductive toxicity through telomere-related cell senescence and apoptosis in SD rat

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ABSTRACT

Decabrominated diphenyl ether (BDE-209) and decabromodiphenyl ethane (DBDPE) are common flame retardants utilized in many kinds of electronic and textile products. Due to their persistence and bioaccumulation, BDE-209 and DBDPE extensively exist in the surrounding environment and wild animals. Previous studies have indicated that BDE-209 could induce male reproductive toxicity, whereas those of DBDPE remains relatively rare. In this study, we investigated the effects of both BDE-209 and DBDPE on reproductive system in male SD rats, and explored the potential mechanisms under the reproductive toxicity of BDE-209 and DBDPE. Male rats were orally administered with BDE-209 and DBDPE (0, 5, 50 and 500 mg/kg/day) for a 28-day exposure experiment. The current results showed that BDE-209 and DBDPE led to testicular damage in physiological structure, decreased the sperm number and motility, and increased the sperm malformation rates in rat. Moreover, BDE-209 and DBDPE could damage the telomeric function by shortening telomere length and reducing telomerase activity, which consequently caused cell senescence and apoptosis in testis of rat. This could contribute to the decline of sperm quality and quantity. In conclusion, BDE-209 and DBDPE led to reproductive toxicity by inducing telomere dysfunction and the related cell senescence and apoptosis in testis of SD rat. Comparatively, BDE-209 had more severe effects on male reproduction. Our findings may provide new insight into the potential deleterious effects of BFRs on male reproductive health.

1. Introduction

Decabromodiphenyl ether (BDE-209) and decabromodiphenylethane (DBDPE), the main active ingredients of additive brominated flame retardants (BFRs), are extensively added to various commercial products such as plastics, electronics, textiles, and building materials. However, they can easily volatilize from these products and leak into the surrounding environment due to no covalent bonding to the polymeric compounds (Stubbings and Harrad, 2014). Polybrominated diphenyl ethers (PBDEs) have been widely detected in multiple environmental media and biological samples ranging from plankton to mammals (Ikonomou et al., 2011; Shi et al., 2018). Commercial deca-BDE mixtures, one type of the PBDEs used as BFRs, are mainly composed of BDE-209. Because of the potential hazard to environment and human health, several types of commercial PBDEs including deca-BDE have been listed as persistent organic pollutants (POPs) under the Stockholm Convention. Nevertheless, the production and utilization of BDE-209 are still continued in China (http://chm.pops.int/TheConvention/ThePOPs). Now DBDPE are brought into the market as a novel BFR, which has lower bioavailability than BDE-209 (Alaee et al., 2003). As an alternative to BDE-209, DBDPE has been widely used around the world (Vuong et al., 2015).

Bioaccumulation of BFRs was associated with reproductive toxicity in human and wild life (Chen et al., 2018a). BDE-209 was the most dominant congener of PBDEs in settled dust of urban regions in China, which contributed to the main health risks resulting from indoor PBDE exposure (Bu et al., 2020). It was also reported that BDE-209 could accumulate in organisms and may link to negative health effects, including reproductive diseases, endocrine disorders, neurotoxicity and cardiovascular toxicity (Chen et al., 2018b; Chevrier et al., 2016; Jing et al., 2019; Sarkar and Singh, 2017). Our previous study has indicated that BDE-209 could induce reproductive toxicity by causing DNA

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damage and cell apoptosis in male mice (Li et al., 2019). Similar to BDE-209, DBDPE can also volatilize from products and leak into the surrounding microenvironments, and has the definite potential for long-range environmental transport (Vorkamp et al., 2015). In addition, exposure to DBDPE could also lead to multiple adverse effects to human and wildlife, such as proliferation inhibition, cytotoxicity and apoptosis induction (Nakari and Huhtala, 2010; Sun et al., 2012, 2014, 2018). However, few studies have investigated the reproductive toxicity induced by DBDPE exposure.

Notably, some POPs are known to have the potential to cause DNA damage and chromosomal instability (Letcher et al., 2010; Sletten et al., 2016), thereby causing dysfunctions in cells and tissues. Telomeres are DNA-protein complexes located at the end of chromosomes, which play an important role in maintaining the genomic integrity (Blackburn, 1991). Telomere erase with aging, and can be accelerated by increased oxidative stress and inflammation (Fouquerel et al., 2019). Nevertheless, the abnormal telomere attrition could eventually result in chromosomal instability and trigger DNA damage response pathways, which may in turn induce cell senescence and apoptosis (Carneiro et al., 2016; d'Adda di Fagagna et al., 2003). Cellular senescence is a distinctive stress-response phenomenon that enforces an essentially irreversible cell-cycle arrest and cell dysfunction, which was accompanied by increased senescence-associated β-galactosidase (SA-β-gal) activity (Campisi, 2001). Therefore, the functional telomere and its prolonged replicative capacity are important for cells to ensure the normal life activities. The maintenance of telomere length mainly depends on the telomerase and shelterin complex (Turner et al., 2019). Telomerase is composed of telomerase reverse transcriptase (TERT) catalytic subunit and its telomerase RNA component (TERC), which synthesize the new telomere repeat sequences (Greider and Blackburn, 1989; Xin et al., 2008). The TERT holds the dominant position in maintaining the normal function of telomere. The dysfunctional of TERT has been shown to induce DNA damage, cell senescence and apoptosis in male reproduction (Ling et al., 2018).

Cellular telomere length may be accelerated under the influence of many factors. Multiple environmental pollutants are particularly sensitive inducements of telomere attrition (Zhang et al., 2013). As reported that exposure to oxychlordane was associated with shorter telomeres (Blevin et al., 2016), and PCBs congeners were associated with telomerase activity reduction and shortened telomere length of cells in vitro (Senthilkumar et al., 2011). Therefore, telomere could be considered as a sensor of organic health maintenance under environmental stress (Guzzardi et al., 2016). However, it remains uncommon if BDE-209 and DBDPE may result in telomere dysfunction, which may turn into one of the potential incentives of reproductive toxicity.

In this study, the toxic effects of BDE-209 and DBDPE on male reproductive system and telomere were investigated and generally compared. In detail, we estimated the changes of telomeric function in testes of SD rats after oral exposure to BDE-209 and DBDPE. By determining the consequent performance of cell senescence and apoptosis, we investigated the association between telomeric dysfunction and spermatogenesis disorder. This might provide new evidence for revealing the underlying mechanism of male reproductive toxicity induced by BDE-209 and DBDPE in SD rats.

2. Materials and methods

2.1. Animals and experimental design

Eighty-four SD male rats (6 weeks old, weighing 180 to 220 g) were employed in this study, which were purchased from Vitalriver Experimental Animal Center (Beijing, China). The rats were housed in standard plastic cages (specified for 475*350*200 mm; 3 rats per cage) and bred under hygienic laboratory conditions with a room temperature of 20 ± 2 °C, 12-h photoperiod and relative humidity of 60 ± 10%. Animals were fed freely on sterilized food and fresh water.

In this study, the powder of BDE-209 (>98% purity) and DBDPE (>98% purity) were purchased from Acros Organics (NJ, USA). After one-week's acclimation period, animals were randomly divided into seven groups (n = 12 per group) including control group, BDE-209 and DBDPE treatment groups. The BDE-209 or DBDPE exposure mixture were both prepared with corn oil. The control group was treated with corn oil only; in BDE-209 treatment groups, rats were dosed daily by gavage administration with BDE-209 at 5, 50, and 500 mg/kg/d during a 28-day exposure period; DBDPE treatment groups were dosed by the same dosages and method as the above treatment of BDE-209. During the experiment, the body weights of all animals were recorded weekly until the final exposure. The doses of BDE-209 and DBDPE were designed based on an oral reference dose (RfD) of 0.007 mg/kg bw/day of deca-BDE recommended by the U.S. Environmental Protection Agency (https://nepis.epa.gov/Exe/ZyPDF.cgi/P1009FYF.PDF?Dockey=P1009FYF.PDF). In detail, the low exposure dose of 5 mg/kg/d was determined with dose conversion coefficient of 6.25, and a 100-fold uncertainty factor; then the medium and high exposure doses were determined as 50 and 500 mg/kg/d by 10 and 100 fold amplification, respectively. The dose conversion coefficient of 6.25 was determined by using the Body Surface Area Method, which was described in the book of Experimental Methodology of Pharmacology (Xu et al., 2002). After the last exposure, animals were euthanized under tribromoethanol anesthesia. After blood samples collection, testes and epididymides were dissected from rats of all groups. Unilateral testes from five random animals of each group were chosen for the examination of testicular morphological, and the rest of them were weighted and then kept frozen at −80 °C until further use.

The animal experimental process is commonly performed by the method for the sub chronic toxicity evaluation of certain chemicals, following the principles of 28-day repetitive-dose oral toxicity test. All of the animal experiments were conducted according to institutional guidelines and approved by the Animal Experimental Ethics Committee of Capital Medical University (Ethical review number: AEEI-2019-003).

2.2. Observation of testicular histopathology

After dissected, testes were then fixed immediately in 4% paraformaldehyde and embedded in paraffin wax. The sections of testis were stained with hematoxylin and eosin (HE), then observed under a light microscope (Olympus BX53, Japan). The histopathological examination of testis was performed under the same stage of seminiferous tubules identification according to Russell et al. (1993).

2.3. Evaluation of body weight and testicular organ coefficient

The daily growth of body weight was determined by the initial and final weights of rats recorded in the 28-day exposure experiment. The testis organ coefficient (the ratio between organ weight and body weight) was determined by the specific value of testis and final body weight, which were calculated by using the formula: Testicular organ coefficient (g/100 g BW) = W_t/W_b, where W_t and W_b represented the testis and body weight of rat, respectively.

2.4. Determination of sperm quality and quantity

In this study, the parameters of sperm number, motility and malformation rate were detected for the assessment of sperm quality and quantity. After dissected from the rats, the epididymides were cut into small pieces, and then maintained in Dulbecco's Modified Eagle Medium (DMEM) at 37 °C for 10 min to ensure the extrusion of spermatozoa. The sperm suspension was then collected for the determination of sperm number and motility, which was implemented by an automatic sperm analyzer (Hamilton TOX IVOS, USA).

For the assessment of sperm malformation rate, sperm suspension was then centrifuged at 1200 revolutions per minute (rpm) for 10 min, then re-suspended by phosphate buffer saline (PBS). Sperm smears were

made by dropping 20–30 μL of mixed sperm suspension on an anti-stripped glass slide, then covering with another one to make the sperm distributing between two slides. After dried and fixed with 95% ethanol for 30 min, sperm smears were stained with Eosin for 1 h, then rinsed with water for 2 min. To observe the morphology of abnormal sperm, the smears were imaged under a light microscopy (Olympus BX53, Japan). Then six samples of each group were chosen randomly to estimate the sperm malformation rate by calculating 1000 spermatozoon including the numbers of both normal and abnormal sperm in each sample. Sperm malformation rate was determined by the following formula: Sperm malformation rate (%) = $n/1000 \times 100\%$, where n representing the number of abnormal sperm recorded in each sample.

2.5. Estimation of MDA level and SOD activity in testis homogenate

Oxidation product MDA and the total SOD (T-SOD) were detected to evaluate the oxidative stress level of testis in rat. The testis tissue was homogenized (1:10, w:V) with PBS (pH7.2–7.4). Then the homogenate was centrifuged at 4 °C for 10 min. The MDA level and T-SOD activity were detected with the commercially available kits (Nanjing Jiancheng, China) by following the manufacturer's instructions.

2.6. Assessment of cell apoptosis in testis

The level of cell apoptosis was examined by using a TUNEL assay kit (KeyGen, China). In this experiment, the apoptotic cells in testis sections were marked by fluorescein isothiocyanate (FITC)-labeled streptavidin after treated with biotin-streptavidin. Then the positive cells with green fluorescence could be observed by using a fluorescence microscope. Briefly, after the conventional dewaxing and hydration process, testis sections were treated and stained with dyeing working solution by following the manufacturer's instructions. The TUNEL-positive cells (apoptotic cells) in seminiferous tubules were then imaged under a laser scanning confocal microscope (LSCM 710; Zeiss, Germany). For the evaluation of cell apoptosis levels, ten samples from the images in each group were chosen randomly to analyze the apoptotic index (the average numbers of apoptotic cells per seminiferous tubule) in testis.

2.7. Detection of cell senescence in testis

The senescence levels of spermatogenic cells in testes were detected by a β-Galactosidase Staining Kit (Solarbio, China). According to the conventional method, the paraffin sections of testis were treated by dewaxing and hydration. Then the testis sections were stained by following the manufacturer's protocols. With the up-regulated activity of SA - β - gal (sense associated β-galactosidase), the aging cells in testis sections were stained, and observed under an ordinary optical microscope (Olympus BX53, Japan). The level of cell senescence in testis of each group was then assessed by calculating the average number of aging cells per seminiferous tubule.

2.8. Assessment of telomerase activity in homogenate

The telomerase activity of testis was determined by using a Telomerase (TE) ELISA Kit (CUSABIO, China). Firstly, testis tissue (100 mg) was rinsed with 1 × PBS buffer, then homogenized (1:10, w:V) with PBS (pH7.2–7.4) and stored overnight at −20 °C. Two freeze–thaw cycles were performed to ensure the complete fragmentation of testicular cells. Then the homogenates were centrifuged for 5 min at 5000 rpm. The supernatant was used to assess the telomerase activity according to the manufacturer's protocols as described in the study of Pawełczyk et al. (2018). Briefly, 100 μL standard or homogenate sample was added to each well coated by the antibody specific for TE, and incubated for 2 h at 37 °C. The liquid of each well was removed, and separately reacted with biotin-antibody and HRP-avidin for 1 h at 37 °C followed by three times washing. Each well was then incubated with 90 μL TMB substrate for 30 min at 37 °C. The reaction was terminated by adding 50 μL stop solution. The optical density of each well was read at 450 nm by a microplate reader, which was used to determine the telomerase activity of each sample.

2.9. Detection of telomere length in testis of rats

In this experiment, the method of real time fluorescence quantitative PCR (qPCR) was conducted for the detection of the telomere average length by using a SYBR FAST qPCR Kit (KAPA, USA). This experiment measured the relative telomere length of reproductive cells in testis by determining the ratio of telomere (T) copy number to the single-copy gene 36B4 (S) copy number (T/S ratio) as described in previous studies (Cawthon, 2002; Ling et al., 2018). Firstly, the genomic DNA of testis was extracted for qPCR analysis by a DNA Rapid Extraction Kit (Beyotime, China). The forward and reverse primers for telomere were 5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′ and 5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3′, respectively. The forward and reverse primers for 36B4 were 5′-ACTGGTCTAGGACCCGAGAAG-3′ and 5′-TCAATGGTGCCTCTGGAGATT-3′, respectively.

The qPCR analysis was performed in a reacting volume of 20 μL containing 15 ng of genomic DNA. The qPCR protocol was used by the following steps of 95 °C for 3 min, 95 °C for 3 s and 60 °C for 20 s followed by 40 cycles, and a melting curve analysis was performed to check for amplification specificity. Meanwhile, a standard curve was performed by using a series of dilutions from a reference DNA sample. With the standard curve, the values of T and S copy numbers were determined by the Ct (cycles to threshold) values obtained from qPCR assay (Lin et al., 2019). The T/S ratio was then quantified to determine the relative telomere length in each sample.

2.10. Western blot analysis

Total protein extracts of testis from each group were used to exam the expression of factors related to telomere function and cell senescence related signaling pathway. The quantification of all proteins was determined by using the bicinchoninic acid (BCA) protein assay (Dingguo Biotechnology, China). The equal amount of protein (35 μg) was loaded and electrophoresed in 12% SDS-polyacrylamide gels, then transferred to NC membranes (Millipore Corporation, USA). The membranes were then block with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) at room temperature for 1 h, and then incubated with the primer antibodies of TERT (1:1000, Bioss), and P53/P21/P16 (1:1000, CST) over night at 4 °C. The internal control was Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:1000, CST). The membranes were washed with Tris-buffered saline Tween 20 (TBST), and then incubated with the fluorescent anti-rabbit Ig G secondary antibody (1:15000, CST) for 1 h at room temperature. After washing with TBST again, the antibody binding proteins were detected by using LiCor Odyssey system (Li-Cor Biosciences, USA). The densitometric value of the protein bands were analyzed by using ImageLab™ Software.

2.11. Statistical analysis

All data were analyzed by using SPSS 21.0 software. The significant difference between the control group and BDE-209 or DBDPE groups was separately determined by One-way analysis of variance (ANOVA) followed by least significant difference (LSD). The independent-sample t test was performed to investigate the statistical difference between BDE-209 and DBDPE group at the same dose. The data of daily growth of body weight was analyzed by covariance analysis, in which the initial body weight was considered as the covariate. The level of statistical significance was considered as $P < 0.05$. All the analytical data were presented by mean ± standard deviations (S.D.).

Table 1
Effects of BDE-209 and DBDPE on the body weight and testicular organ coefficient of rat. Data was described as means ± S.D. (n = 10 per group).

Exposure doses (mg/kg/d)	Daily growth of body weight (g)		Testicular organ coefficient (g/100 g BW)	
	BDE-209	DBDPE	BDE-209	DBDPE
0	6.01 ± 0.86	6.01 ± 0.86	0.83 ± 0.12	0.83 ± 0.12
5	5.89 ± 1.20	5.46 ± 0.81	0.78 ± 0.12	0.82 ± 0.13
50	5.60 ± 1.04	6.35 ± 1.11	0.87 ± 0.67	0.86 ± 0.06
500	6.35 ± 0.72	5.61 ± 0.96	0.80 ± 0.90	0.81 ± 0.12

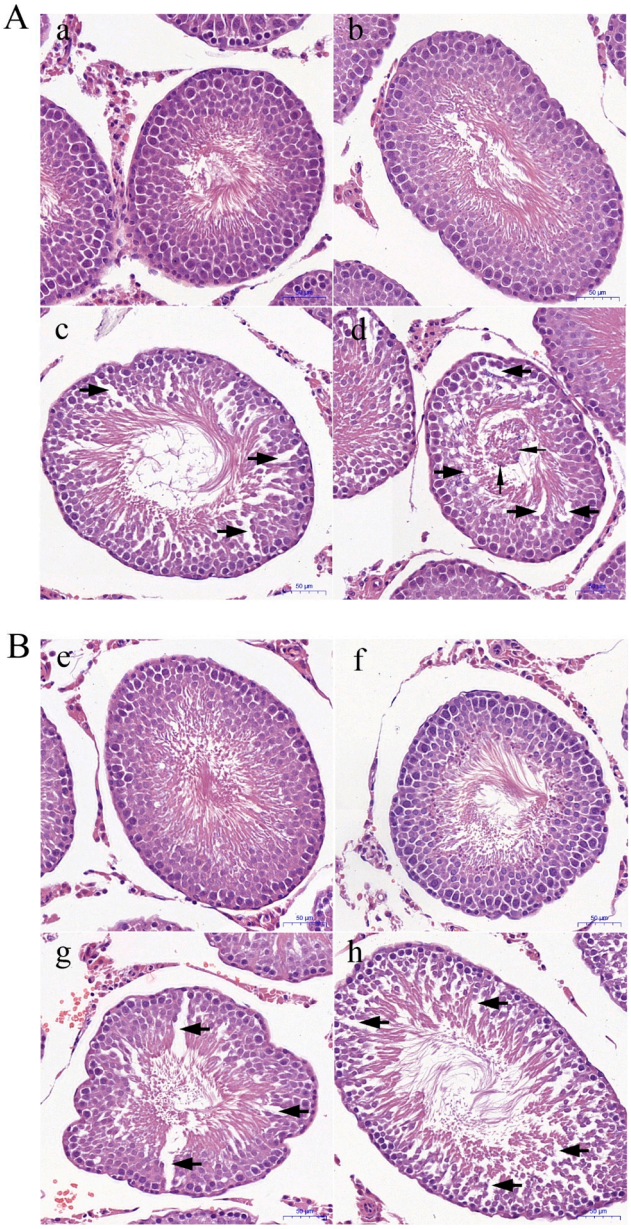


Fig. 1. Testis histopathological changes in rat induced by BDE-209 and DBDPE. (A) BDE-209 treatment groups. (B) DBDPE treatment groups. (a-d) represent the 0, 5, 50 and 500 mg/Kg/d BDE-209 groups, respectively. (e–h): represent the 0, 5, 50 and 500 mg/Kg/d DBDPE groups, respectively. The thick black arrows denote the seminiferous epithelium deletion and intraepithelial vacuolation; the thin black arrows denote the exfoliated cells.

3. Results

3.1. Changes of body weight and testicular organ coefficient

No significant differences were observed in the daily growth of body weight between the control and BDE-209 or DBDPE treatment groups. Compared with the control, the testicular organ coefficients had no change in the BDE-209 or DBDPE treatment group (Table 1).

3.2. Histopathological changes of testis induced by BDE-209 and DBDPE

The histological structure of testis in the control group exhibited largely normal morphology, and the spermatogenic cells in seminiferous tubules arranged regularly and tightly. Compared with the control group, no histopathological change was found in 5 mg/kg/d BDE-209 group. However, in the 50 and 500 mg/kg/d BDE-209 groups, obvious reduction of sperm cells and pathological changes in seminiferous tubules were observed, which performed as seminiferous epithelium deletion, intraepithelial vacuolation, and even cell exfoliation (Fig. 1A). No apparent morphological differences were noted between the 5 mg/kg/d DBDPE group and the control group. In the 50 and 500 mg/kg/d DBDPE treatment groups, the seminiferous epithelium showed significant deletion in the testes of rats compared with the control group (Fig. 1B).

3.3. Changes of sperm quality and quantity

As shown in Fig. 2, the sperm number was significantly decreased in 50 and 500 mg/kg/d BDE-209 groups ($P < 0.05$), while no significant difference was observed between 5 mg/kg/d BDE-209 group and the control group ($P > 0.05$). Similarly, in the DBDPE treatment group, the sperm number decreased significantly at the doses of 50 and 500 mg/kg/d ($P < 0.05$) (Fig. 2A). Compared with the control group, the sperm motility decreased in 50 and 500 mg/kg/d BDE-209 treatment groups ($P < 0.05$), but no significant change was found in the 5 mg/kg/d group. In addition, the sperm motility decreased in all DBDPE treatment groups ($P < 0.05$) (Fig. 2B).

The treatment of 5 mg/kg/d BDE-209 had no influence on sperm malformation rate, while exposure to 50 and 500 mg/kg/d BDE-209 significantly increased the sperm malformation rate in rats ($P < 0.05$). Exposure to DBDPE led to an increase of sperm malformation rate only in the 500 mg/kg/d group ($P < 0.05$) (Fig. 2C). Furthermore, the sperm malformation rate in 500 mg/kg/d BDE-209 treatment group was substantially higher than that in 500 mg/kg/d DBDPE group. Meanwhile, the abnormal sperms can be observed obviously in the 50 and 500 mg/kg/d BDE-209 and DBDPE treatment groups. The different types of abnormal sperm morphology were mainly identified by coiled tail, bent neck and irregularly shaped head, which were denoted by different types of arrows in Fig. 2D and E.

3.4. Changes of MDA level and SOD activity

The MDA content and T-SOD activity were measured to evaluate the oxidative stress in testis after BDE-209 and DBDPE treatment. As shown in Fig. 3, although no change was found in 5 mg/kg/d group, the 50 and 500 mg/kg/d BDE-209 exposure significantly increased the MDA content in testis of rats. The T-SOD activity decreased obviously in 500 mg/kg/d BDE-209 treatment group ($P < 0.05$), while no change was observed at the doses of 5 and 50 mg/kg/d. However, in the DBDPE treatment groups, the MDA content increased significantly only at the dose of 500 mg/kg/d ($P < 0.05$). The T-SOD activity decreased significantly in 500 mg/kg/d DBDPE treatment group ($P < 0.05$), but there was no change in the 5 and 50 mg/kg/d group. Moreover, the increase of MDA content and decrease of T-SOD activity were more severe in 500 mg/kg/d BDE-209 group than those in 500 mg/kg/d DBDPE group ($P < 0.05$).

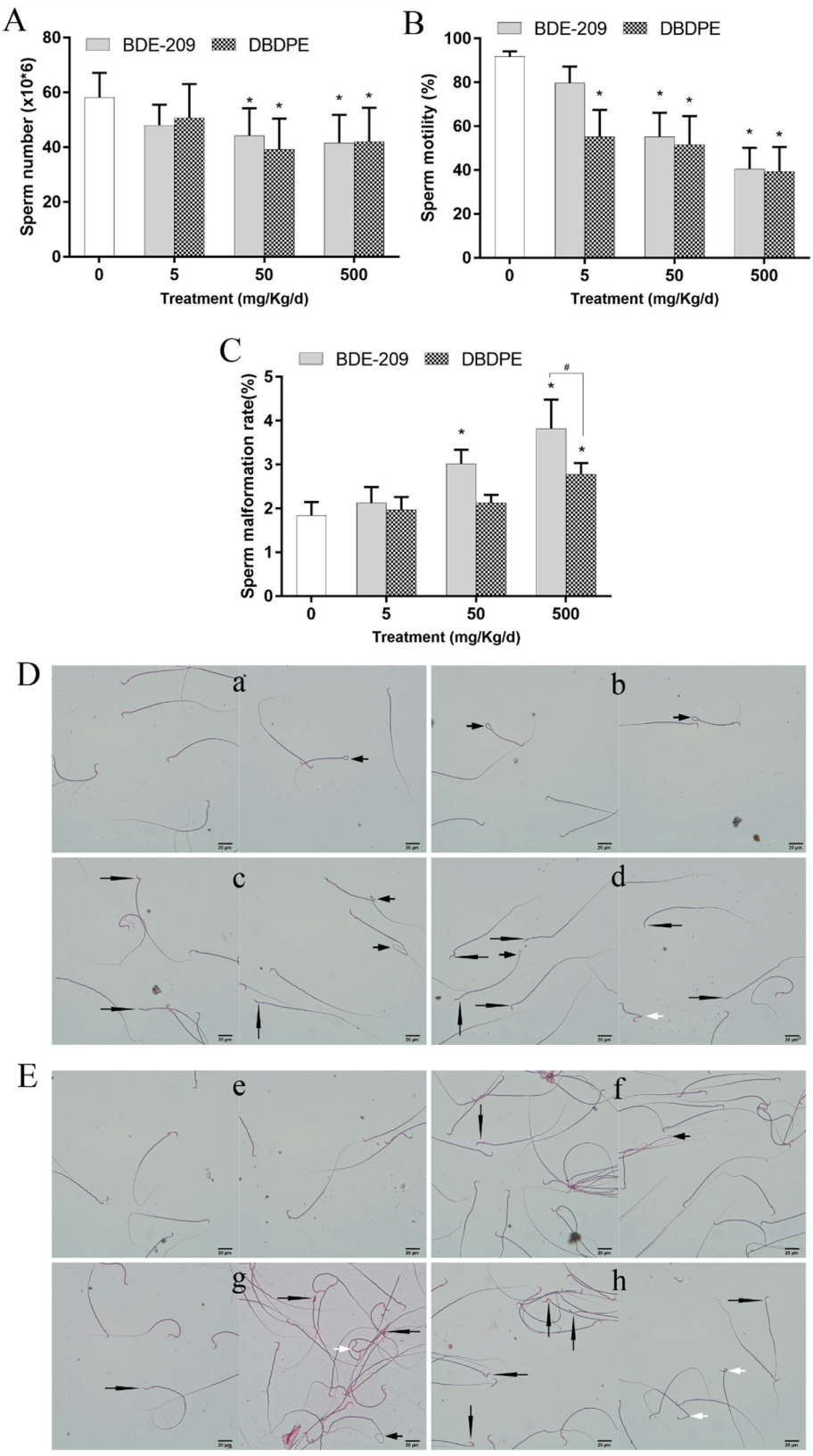


Fig. 2. Effects of BDE-209 and DBDPE on sperm quality and quantity. (A) Changes of sperm number (n = 10 per group). (B) Changes of sperm motility (n = 10 per group). (C) Changes of sperm malformation rate (n = 6 per group). (D and E) Sperm morphological changes in BDE-209 and DBDPE treatment groups, respectively. (a-d) represent the 0, 5, 50 and 500 mg/Kg/d BDE-209 groups, respectively; (e–h) represent the 0, 5, 50 and 500 mg/Kg/d DBDPE groups, respectively. The thick black arrows denote the sperm abnormality of coiled tail; thick white arrows denote bent neck; thin black arrows denote irregularly shaped head. Data was described as means ± S.D. * indicates $P < 0.05$ compared with 0 mg/Kg/d group; # indicates $P < 0.05$ compared with previous group in the bar graph.

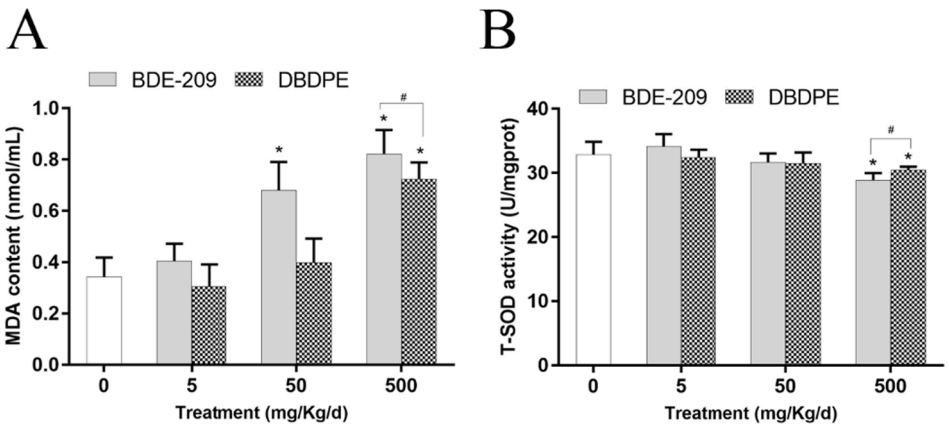


Fig. 3. BDE-209 and DBDPE induced oxidative stress in testis of rat. (A) The MDA level in testis of SD rat. (B) The total SOD activity in testis of SD rat. Data was described as means ± S.D. ($n = 6$ per group). * indicates $P < 0.05$ compared with 0 mg/Kg/d group; # indicates $P < 0.05$ compared with previous group in the bar graph. The experiments were repeated for at least three times independently.

3.5. Testicular cell apoptosis induced by BDE-209 and DBDPE

TUNEL staining experiment was used to assess the apoptotic level of spermatogenic cell in testis. With the increasing of BDE-209 and DBDPE exposure doses, the apoptotic cells with green fluorescence could be obviously observed in the seminiferous tubules as shown in Fig. 4A and B. Compared with the control group, BDE-209 exposure significantly increased the numbers of TUNEL-positive cells per seminiferous tubule in 5, 50 and 500 mg/kg/d groups. DBDPE exposure led to significant increase of TUNEL-positive cells in the 50 and 500 mg/kg/d groups ($P > 0.05$) (Fig. 4C). Moreover, compared with the DBDPE, BDE-209 exposure caused more severe cell apoptosis in testis at the dose of 50 and 500 mg/kg/d groups.

3.6. Testicular cell senescence induced by BDE-209 and DBDPE

As shown in Fig. 5A and B, the β-galactosidase positive cells was clearly observed in testis of BDE-209 and DBDPE treatment groups. The data analysis showed that the numbers of senescent cells per seminiferous tubule were increased significantly in the 5, 50 and 500 mg/kg/d BDE-209 treatment groups, and also increased in the 5, 50 and 500 mg/kg/d DBDPE groups ($P < 0.05$) (Fig. 5). Moreover, BDE-209 induced a higher level of cell senescence in the 500 mg/kg/d group compared with the 500 mg/kg/d DBDPE group.

3.7. Effects of BDE-209 and DBDPE on telomerase in testis

To explore the effects of BDE-209 and DBDPE on telomerase, the expression of TERT in testis was firstly detected by western blot. Compared with the control group, BDE-209 exposure decreased TERT expression in the 5, 50 and 500 mg/kg/d group. But DBDPE exposure downregulated the expression of TERT only in the 500 mg/kg/d group (Fig. 6A and B). Additionally, no difference of telomerase activity was found between 5 mg/kg/d BDE-209 group and the control ($P > 0.05$), but significant reductions were noted in the 50 and 500 mg/kg/d BDE-209 groups ($P < 0.05$). In addition, the testicular telomerase activities were also decreased in the testes of rats after treated with 50 and 500 mg/kg/d DBDPE ($P < 0.05$), but no change was observed in the 5 mg/kg/d DBDPE group ($P > 0.05$) (Fig. 6C).

3.8. BDE-209 and DBDPE induced telomere length shortening in testis

The relative telomere length was significantly reduced in 500 mg/kg/d BDE-209 treatment groups ($P < 0.05$), while no change was observed in 5 and 50 mg/kg/d group compared with the control group ($P > 0.05$). Similarly, a significant decrease was observed in the 500 mg/kg/d DBDPE treatment group ($P < 0.05$), but no obvious change was found in the 5 and 50 mg/kg/d DBDPE groups compared with the control group ($P > 0.05$) (Fig. 7).

3.9. Changes of cell senescence signals induced by BDE-209 and DBDPE

Compared with the control group, the expressions of p53 and p21 increased significantly in the 50 and 500 mg/kg/d BDE-209 treatment groups ($P < 0.05$), while no change in the 5 mg/kg/d group ($P > 0.05$). Similarly, p53 and p21 was increased in the 50 and 500 mg/kg/d DBDPE treatment groups ($P < 0.05$). BDE-209 or DBDPE exposure resulted in the increase of p16 in the 50 and 500 mg/kg/d groups ($P < 0.05$), but there is no difference in the 5 mg/kg/d group compared with the control group ($P > 0.05$). Moreover, BDE-209 exposure had a greater effect than DBDPE on the upregulation of p53 and p16 in the 500 mg/kg/d group, and on that of p21 in the 50 mg/kg/d group (Fig. 8).

4. Discussion

It has been reported that BDE-209 could induce toxic effects on male reproductive health (Sarkar et al., 2016; Sarkar and Singh, 2018). Our previous results also indicated that BDE-209 was able to induce spermatogenesis disorder through DNA damage and consequent cell apoptosis in mice. In this study, we found that both of BDE-209 and DBDPE exposure could lead to negative effects on male reproduction by inducing telomere dysfunction and the consequent cell apoptosis and senescence in testis of SD rat. Although the testicular weights were neither affected, exposure to different doses of BDE-209 or DBDPE damaged the physiological structure of testis by varying degrees in male rat. Both of BDE-209 and DBDPE caused the reduction of the sperm quality and quantity. This result is generally consistent with our previous study that sperm number and motility were decreased by a 28-day oral exposure to BDE-209 in ICR mice.

Epidemiological studies indicated that POPs have long-term consequences for male semen quality by interfering spermatogenesis (Brokken et al., 2014; Vested et al., 2014). BDE-209 and DBDPE are both common flame retardants (Alaee et al., 2003), which might impact functional spermatogenesis. Yan et al. (2018) found that perinatal exposure to DBDPE could induce toxic effects on male offspring, but it was unclear if DBDPE could affect the male F0 generation. In this study,

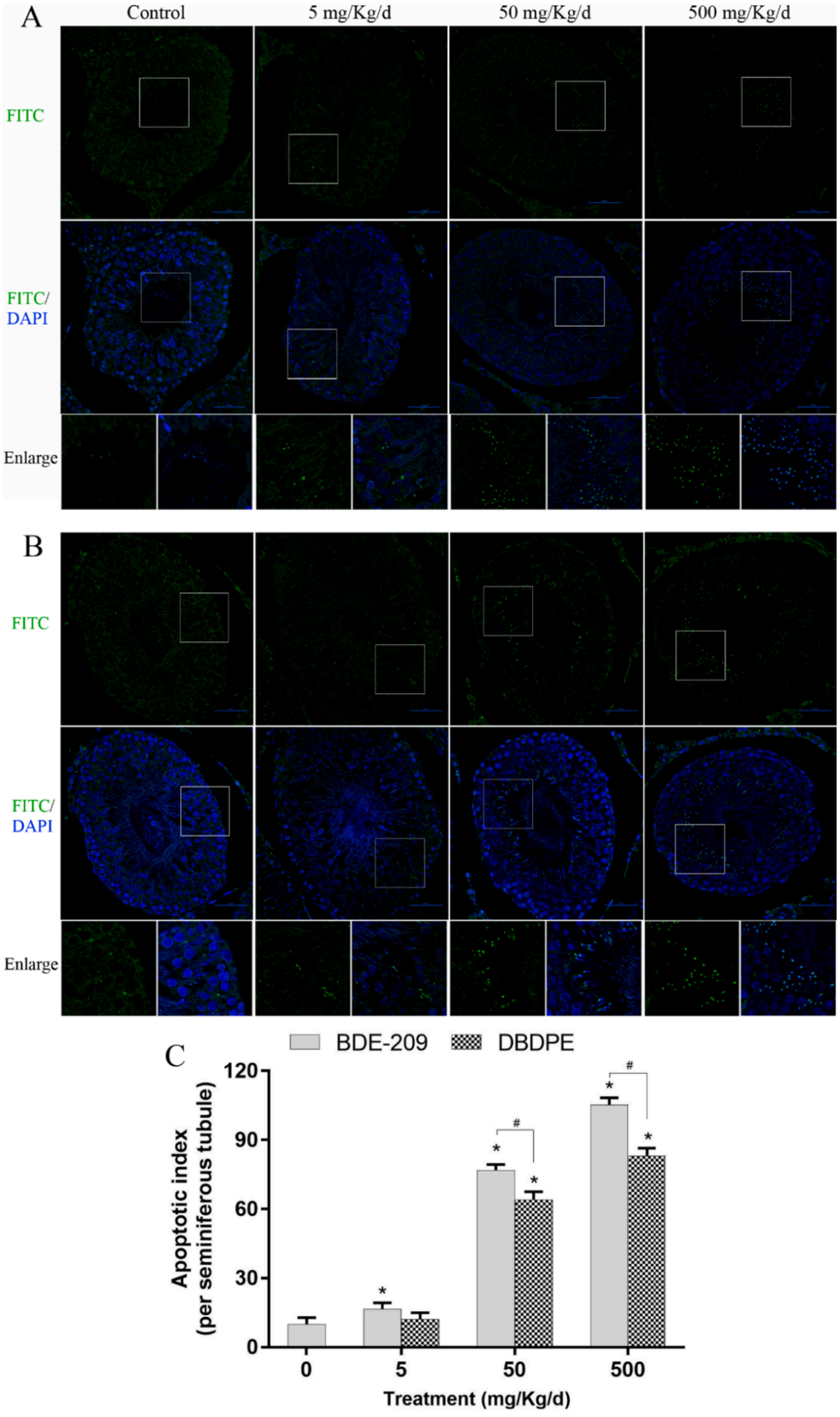


Fig. 4. BDE-209 and DBDPE induced cell apoptosis in testis of rat. (A) BDE-209 treatment groups. (B) DBDPE treatment groups. (C) The apoptotic index was analyzed by the number of TUNEL-positive cells per seminiferous tubule. Data was described as means ± S.D. ($n = 6$ per group). * indicates $P < 0.05$ compared with 0 mg/Kg/d group; # indicates $P < 0.05$ compared with previous group in the bar graph. The experiments were repeated for at least three times independently.

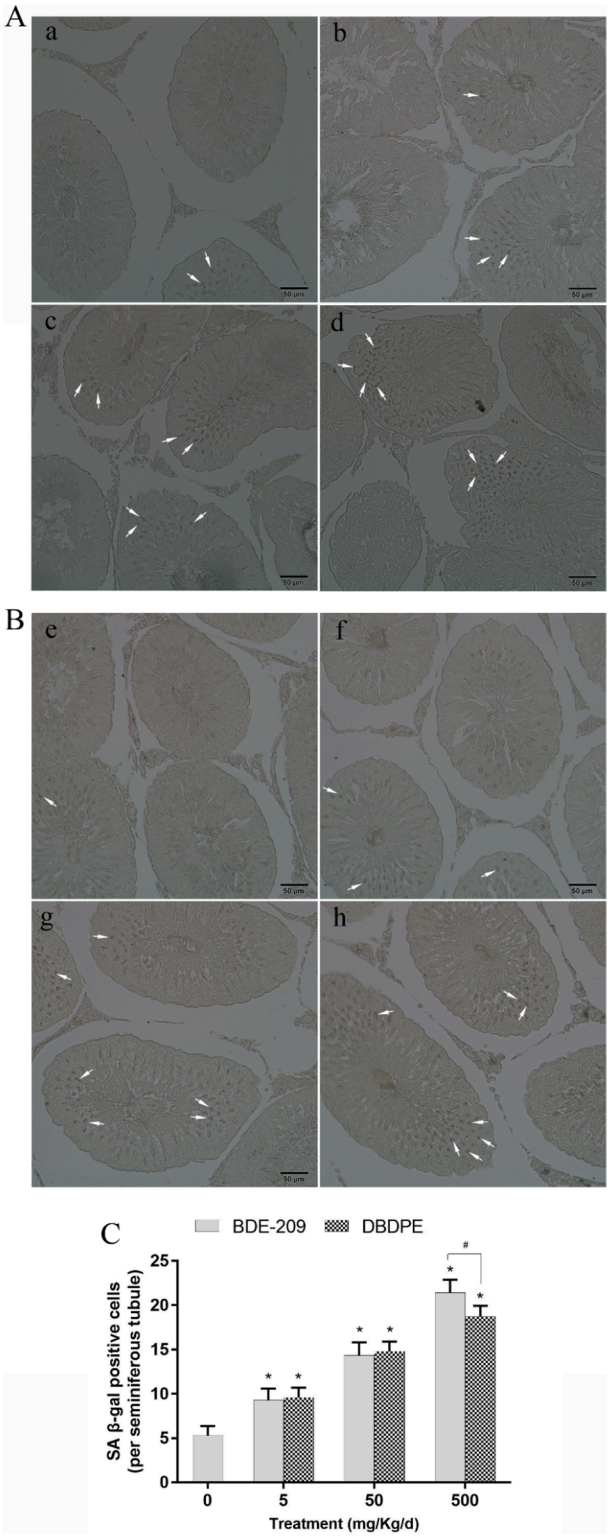


Fig. 5. BDE-209 and DBDPE induced cell senescence in testis of rat. (A) BDE-209 treatment groups. (B) DBDPE treatment groups. (a–d) represent the 0, 5, 50 and 500 mg/Kg/d BDE-209 groups, respectively; (e–h) represent the 0, 5, 50 and 500 mg/Kg/d DBDPE groups, respectively. (C) The cell senescence level was determined by the number of SA β-gal positive cells per seminiferous tubule. The white arrows denote the senescent cells in testis. Data was described as means ± S.D. ($n = 6$ per group). * indicates $P < 0.05$ compared with 0 mg/Kg/d group; # indicates $P < 0.05$ compared with previous group in the bar graph. The experiments were repeated for at least three times independently.

TUNEL examination indicated that BDE-209 and DBDPE both led to cell apoptosis in the testis of rat, which was probably considered as a reason for the decrease of sperm number and motility. In addition, exposure to BDE-209 and DBDPE also caused elevated sperm abnormity in epididymis, which might contribute to the decline of sperm quantity. Meanwhile, the adverse effects of BDE-209 on sperm malformation seemed to be more severe than those of DBDPE. This might have been originally owing to the different mechanisms and effective stages of spermatogenesis in testis of rat after exposed to BDE-209 and DBDPE. Thus, the potential mechanisms still need further researches.

It has been reported that long-term POPs exposure could induce telomere dysfunction and elevate the risk of aging-associated diseases (Yuan et al., 2018). Telomeres are composed of TTAGGG repeats bound by shelterin protein complex (Liu et al., 2004), which were considered as an important role in genome stability and cell proliferation. Considering the potentiality of POPs to cause DNA damage (Letcher et al., 2010), we wondered if BDE-209 and DBDPE could produce injures in telomere sequence. The present results showed that both of BDE-209 and DBDPE resulted in the shortening of telomere length. Telomere length are essential for normal cellular process, such as DNA repair, mitosis and even meiosis (Giardini et al., 2014; Pendlebury et al., 2017). Shorter or dysfunctional telomeres are recognized as the signals of DNA damage response, cell aging and apoptosis (Blasco, 2007; Srinivas et al., 2020). Study from Selvam et al. (2018) has reported that telomere shortening and telomerase inhibition mediated rapid cell senescence or apoptosis with the participation of p16 and caspase-3 proteins. In the present study, cell senescence and apoptosis were obviously observed in testes of rats, which might be associated with the erosion of telomere length induced by BDE-209 and DBDPE.

Telomere sequence with insufficient repeats is considered to be such an “uncapped” chromosomal end, which can be regarded as DNA double-strand break (Capper et al., 2007). Thus, the shortening of telomere length will initiate the DNA damage response (DDR), which activates ataxia-telangiectasia mutated (ATM) and p53 (Lansdorp, 2017). In this study, we found that the signaling pathway of p53/p21/p16 were activated in testis after exposure to BDE-209 and DBDPE. The p53/p21 signaling is generally involved in the response of DNA damage, and p16 is an essential cyclin-dependent kinase inhibitor; these are both important pathways regulating cell-cycle arrest and cell senescence (Sung et al., 2017; Yang et al., 2018). Our previous works have found the upregulation of ATM and p53 in testis of mice after BDE-209 exposure. Thus, we concluded that telomere dysfunction could lead to the dysregulation of p53/p21/p16 by the bridge of DNA damage and ATM activation, along with the inhibited cell proliferation in the testis of rats. Altogether, these results proved the role of telomere-related aging pathway, leading to mitosis stagnate and cell senescence in male germ lines after BDE-209 and DBDPE exposure.

Exposure to different BFRs could lead to multiple consequences including cell apoptosis or senescence, which is dependent on the different toxicity patterns. A similar study suggested that BDE-47 had more adverse effects than BDE-99 and -209 on the DNA damage and cell apoptosis (Montalbano et al., 2020). Study of Pereira et al. (2017) found that BDE-209 caused mitochondria associated apoptosis with cytochrome *C* release and caspase-9/-3 activation in HepG2 cells. Exposure to BDE-154 and BDE-47 could also result in the mitochondrial damage and apoptotic cell death in HepG2 (Souza et al., 2016). Combined with the present results of cell apoptosis induced by BDE-209 and DBDPE, there are evidence for the potential involvement of mitochondrial apoptosis pathway in the reproductive toxicity. Here we found the worse effects of BDE-209 exposure on testicular cell senescence and apoptosis in this study. This might reveal that BDE-209 can cause higher toxicity in male reproduction. But it still needs further research in future study.

Additionally, telomerase plays important role in maintenance and regulation of telomere length (Giardini et al., 2014). TERT, the catalytic subunit of telomerase, dominates an important position in the synthesis of new telomeric repeats (Greider and Blackburn, 1989). Study reported

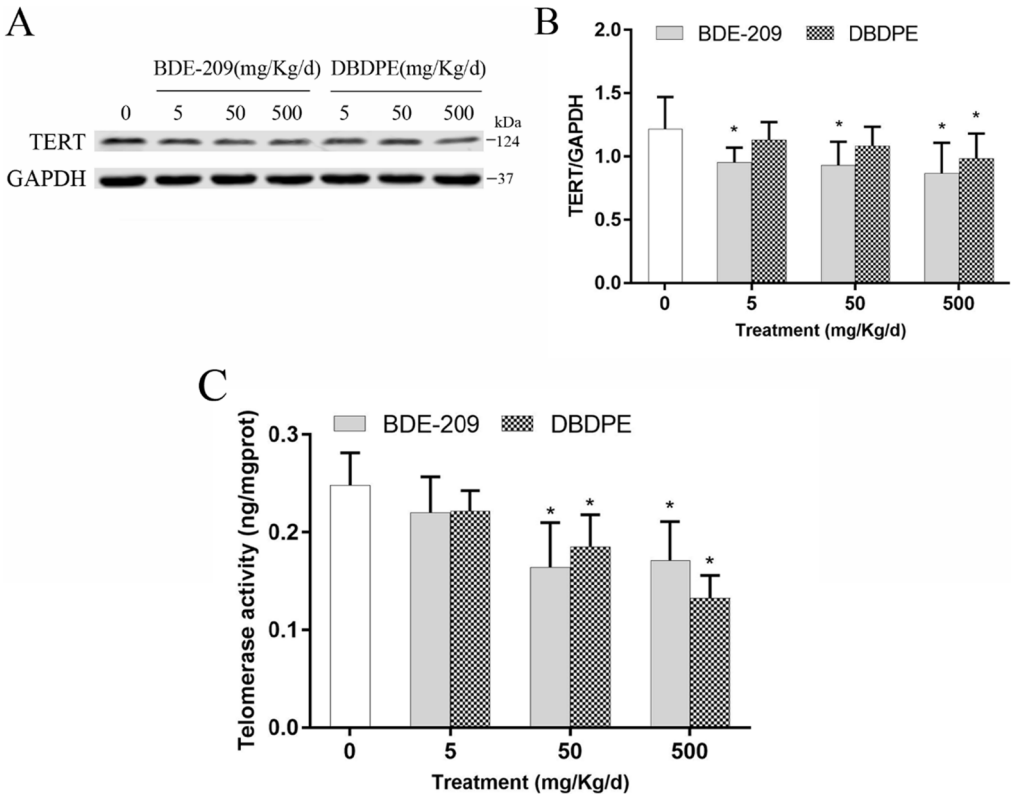


Fig. 6. Effects of BDE-209 and DBDPE on telomerase in testis. (A) The expression of TERT in testis after BDE-209 and DBDPE exposure. (B) Relative densitometric analysis of the protein band versus GAPDH. (C) The telomerase activity changes in testis after BDE-209 and DBDPE exposure. Data was described as means ± S.D. (n = 6 per group). * indicates $P < 0.05$ compared with 0 mg/Kg/d group. The experiments were repeated for at least three times independently.

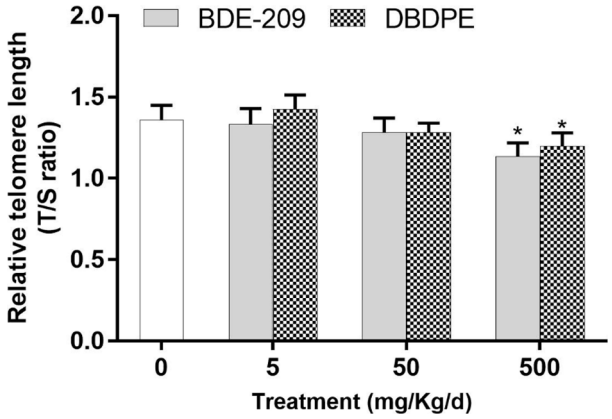


Fig. 7. Effects of BDE-209 and DBDPE on telomere length in testis. The T/S ratio was determined to analyze the relative telomere length in testis. Data was described as means ± S.D. (n = 6 per group). * indicates $P < 0.05$ compared with 0 mg/Kg/d group. The experiments were repeated for at least three times independently.

that oxidative damage could impact telomere sequence and telomerase activity, thereby causing telomere dysfunction (Ahmed and Lingner, 2018). Here, we found that BDE-209 and DBDPE exposure led to an increase of oxidative stress, and caused the reduction of telomerase activity with TERT downregulation. There were evidences indicated that the correspondence of oxidative adducts to the end of telomeric DNA was the impediment of telomere extension (Ahmed and Lingner, 2018). Additionally, DBDPE has lower capacity for the induction of oxidative damage, which is consistent with the results that BDE-209 was able to induce more severe oxidative stress in rat liver and heart (Jing et al., 2019; Sun et al., 2020). This may explain why DBDPE can be used continuously as the alternative to BDE-209. However, the molecular mechanism still remains unclear about how oxidative stress interferes with telomere length maintenance or telomerase activity. It still requires further investigation whether these mechanisms are involved in the reproductive toxicity induced by BDE-209 and DBDPE. All these together revealed that BDE-209 and DBDPE were able to interfere with telomere elongation, which thereby led to telomere-related cell dysfunction in testis of male rat.

5. Conclusion

In summary, BDE-209 and DBDPE could induce oxidative stress and telomere dysfunction, which consequently caused reproductive toxicity via cell senescence and apoptosis in testis. BDE-209 and DBDPE seemed to be similar in the induced-reproductive toxicity in male rats, although it has reported that DBDPE has lower toxicity on human and wild life. This might be attribute to the sensibility of reproductive system as the target organ of environmental pollutants. In general, our findings reveal that environmental BDE-209 and DBDPE could be the risk factors for male infertility, and might provide new insight into the biological mechanisms for BFRs-exposure hazard on male reproductive health. However, more researches are still needed to estimate the safety of BDE-209 and DBDPE in their administration and application.

Declaration of Competing Interest

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.

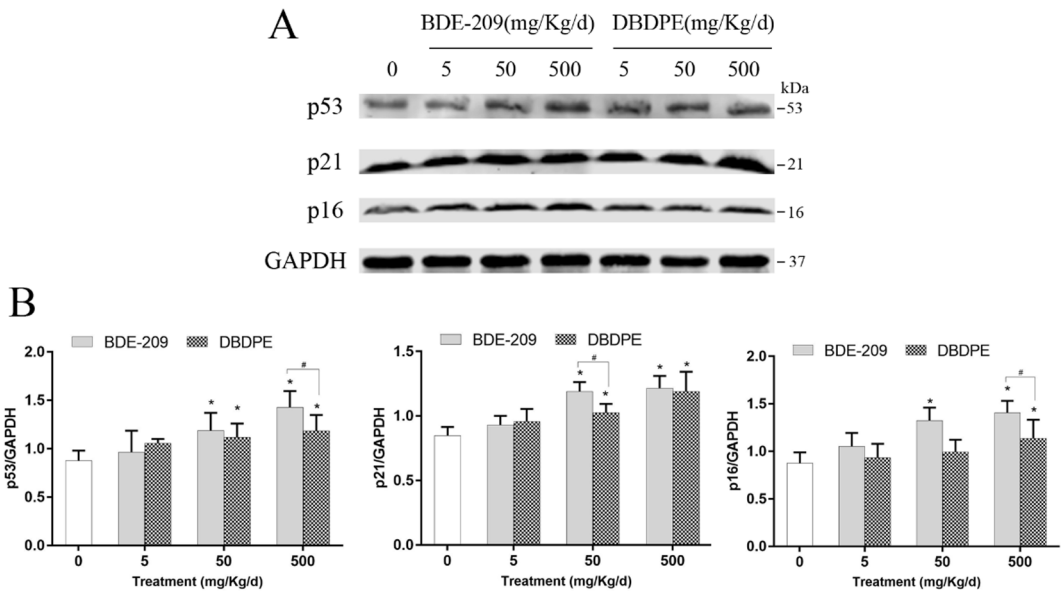


Fig. 8. Changes of cell senescence signaling pathway induced by BDE-209 and DBDPE in testis. (A) Expressions of p53, p21 and p16 in testis. (B) The relative densitometric analysis of those protein bands versus GAPDH. Data was described as means ± S.D. ($n = 6$ per group). * indicates $P < 0.05$ compared with 0 mg/Kg/d group; # indicates $P < 0.05$ compared with previous group in the bar graph. The experiments were repeated for at least three times independently.

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