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Postnatal exposure of the male mouse to 2,2′,3,3′,4,4′,5,5′,6,6′-decabrominated diphenyl ether: Decreased epididymal sperm functions without alterations in DNA content and histology in testis

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Abstract

2,2′,3,3′,4,4′,5,5′,6,6′-Decabrominated diphenyl ether (PBDE 209) is the second most used brominated flame retardant (BFRs) in constructed materials because it is considered less toxic than others, though other fire retardants, some congeners of PBDE 209, are reported to be toxic. This combined the fact that PBDE 209 has been found in high levels in human milk, blood, indoor environments as well as in foodstuffs has led us in this study attempt to find out whether PBDE 209, also known as decaBDE and decabrominated diphenyl oxide (DBDPO), has an adverse effect on this histology of testes and sperm in CD-1 male mice. The mice we studied were divided into groups and gavaged with 10, 100, 500 and 1500 mg/kg PBDE 209 in corn oil per day between postnatal Days 21 and 70. On Day 71, the mice were anesthetized and sperm function, testis DNA content, and histopathology were studied. We found in the 500- and 1500-mg/kg/day groups that neonatal exposure to PBDE 209 reduced sperm epididymal sperm mitochondrial membrane potential (MMP), reduced amplitude of the lateral head displacement (ALH) and induced the generation of hydrogen peroxide (H_2O_2) in the sperm of sexually mature male mice, without affecting the sperm count, motility, morphology, curvilinear velocity (VCL), angular progressive velocity (VAP), straight-line velocity (VSL), beat-cross frequency (BCF), sperm chromatin structure assay (SCSA), superoxide anion ($O_2^{-\bullet}$) generation, DNA content in testis cells, or testicular histopathology. ALH was positively associated with an increase in MMP and negatively associated with generation of sperm H_2O_2. The reduction of MMP was negatively associated with an increase in generation of sperm H_2O_2. The presence of the relationships between sperm ALH, MMP, and generation of H_2O_2 indicate toxic action possibly resulting from PBDE 209-induced oxidative stress. In conclusion, this is the first study to report the lowest-observed-adverse-effect level (LOAEL) for sperm function to be 500 mg/kg of PBDE 209 in male mice. Decreased epididymal sperm MMP and ALH as well as induced generation of sperm H_2O_2 were some of the most

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serious effects of postnatal PBDE 209 exposure. Future investigations should be performed to study the effects of prenatal exposure of PBDE 209 and the mechanism behind PBDE 209-related oxidative stress in the fetal and pubertal stages of development.

Keywords: Polybrominated diphenyl ethers; Brominated flame retardants; Postnatal exposure; Sperm function; Testis DNA content

1. Introduction

Polybrominated diphenyl ethers (PBDEs) are extensively used as brominated flame retardants (BFRs) in polymer products and can leak into the environment (Renner, 2000). Several PBDEs have also been shown to accumulate in animal life (Haglund et al., 1997; Darnerud et al., 2001; Norstrom et al., 2002). PBDEs have also been found to have increased in human milk lipids from 0.07 ng/g in 1972 to 4.02 ng/g in 1997 in Sweden (Meironyté et al., 1999). In America, concentrations of PBDEs in breast milk lipids have been reported to range from 6.2 to 419 ng/g (Schecter et al., 2003). Over the past few years, increased concentrations of PBDEs have been emerging as an environmental problem (Lindström et al., 1999; She et al., 2002; Ikonomou et al., 2002).

2,2′,3,3′,4,4′,5,5′,6,6′-Decabrominated diphenyl ether (PBDE 209), also known as decaBDE and decabrominated diphenyl oxides (DBDPO), is a fully brominated (e.g. 10 bromine atoms) diphenyl oxide with a molecular weight of 959.17. It is the second most used BFR in constructed materials next to tetrabromobisphenol A (TBBPA). PBDE 209 accounted for about 75% of the 40,000 tons of PDBE produced in 1990. Today it accounts for approximately 82% of total PBDE production (BSEF, 2003). In 1999, the demand for PBDE 209 in the United States, Europe and Asia was 24,300, 7500 and 23,000 metric tons, respectively (BSEF, 2003). PBDE 209 is mostly used in high impact polystyrene used for electronic enclosures such as television set cabinet backs (Hardy, 2002). A comparatively minor, but important, use of PBDE 209 is in upholstery textiles where it is applied in a polymer back coat to the fabric (Hardy, 2002).

Exposure to PBDEs, however, mainly comes through the consumption of foodstuffs such as in freshwater fish, marine species, chickens, eggs and mixed meat products (Sjödin et al., 2000; Domingo, 2004). They have also been discovered in indoor dust, which, if inhaled, could also expose people, particularly children, to significant amounts (Wilford et al., 2005). With regard to the work environment, Sjödin et al. (1999) have reported that the dismantling and disposal of electronic goods in factories in Sweden co-occurs with higher levels of BFRs in factory air, resulting in high blood BFRs levels their workers. In a follow-up study, they found serum concentrations of PBDE 209 to be significantly higher in electronics dismantling workers than in those not occupationally exposed to PBDEs (Sjödin et al., 2001). In another Swedish study conducted by a different group of researchers monitoring personal air in an electronic recycling facility, the most abundant of the PBDE congeners was PBDE 209 (<0.7–61 ng/m^3) (Pettersson-Julander et al., 2004).

In animals, PBDE 209 has been reported to be irreversibly neurotoxic and neonatal exposure has been found to result in cognitive deficiencies (Viberg et al., 2003). Recently, female mice fed a single low dose of pentabrominated diphenyl ether (PBDE 99), a level similar to that found in some humans, produced offspring with decreased sperm counts and neurobehavior deficiencies (Kuriyama et al., 2005). It is, however, not known if PBDE 209, the world's second most produced BFR has a similar effect on male reproductive toxicology. To explore this possibility, we fed five groups of recently weaned CD-1 male mice different daily doses of PBDE 209 or corn oil for 50 days and then studied its affect on sperm function, DNA content, and histopathology of testes.

2. Materials and methods

2.1. Chemicals

PBDE 209 (98%, CAS no. 1163-19-5) was obtained from Aldrich-Chemie. The PBDE 209 solution was prepared by mixing PBDE 209 compound with corn oil and sonicating the mixture at room temperature for 30 min. The desired dosing solutions (10, 100, 500 or 1500 mg/ml) were prepared weekly. For each dosing solution, seven parts were prepared at the beginning of the week, with only one part used per day. The suspensions were vortexed during dosing for at least 30 min. Corn oil, eosin Y and propidium iodide (PI) stain were purchased from Sigma (St. Louis, MO, USA). Other stains including 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl benzimidazolyl carbocyanine iodide (JC-1), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), and hydroethidine (HE) were acquired from Molecular Probes Inc. (Eugene, OR, USA). Phosphate-buffered saline (PBS) and human tubule fluid (HTF) medium consisting of 105.6 mM NaCl, 5.06 mM KCl,

0.73 mM $CaCl_2$, 1.17 mM KH_2PO_4, 1.01 mM $MgSO_4 \cdot 7H_2O$, 25.3 mM $NaHCO_3$, 0.27 mM sodium pyruvate, 21.6 mM sodium lactate, 5.56 mM glucose, 1000 IU/ml penicillin, and 50 μg/ml streptomycin were purchased from Gibco Life Technologies Ltd. (New York, USA). All the other reagents were obtained from Sigma–Aldrich (St.Louis, MO, USA).

2.2. Animals and experimental design

The pregnant CD-1 mice were purchased from the Animal Center of National Cheng Kung University Medical Center (Tainan, Taiwan) and were housed individually in plastic cages in an animal chamber maintained at 22 ± 2 °C with a constant humidity of 60 ± 10%. Lighting period was maintained at 12/12 h light and dark cycle. Diet (Laboratory Rodent Diet 5001, LabDiet, Richmond, IN, USA) and distilled water were provided ad libitum. Day of birth was designated postnatal day 0 (PND 0). Mice were weaned at PND 21. A total number of 50 male pups were divided into five groups: one control and four exposure groups: those gavaged with 10, 100, 500 or 1500 mg/kg of PBDE 209 dissolved in corn oil per day until PND 70. All mice were anesthetized by CO_2 on PND 71. The reason for treating the animal from post-weaning period to puberty is to simulate the exposure of infants and young children to these chemicals and the associated risk of adverse effects during early stages of development. Therefore, the neonatal period of Day 21 to Day 70 was chosen to represent the time self-feeding and full maturity was reached, respectively.

2.3. Body and reproductive organ weights

At PND 71, the animals were anesthetized by CO_2 and the testis, epididymis as well as seminal vesicles were removed and weighed. Relative organ weight was calculated by calculating the ratio between organ weight and body weight. The left and right testis was used for histological and DNA content analysis, respectively. The left cauda epididymis was used to perform sperm chromatin structure analysis (SCSA). The right cauda epididymis was used to prepare sperm suspension for measuring sperm count, motility, morphology, mitochondrial membrane potential (MMP), and reactive oxygen species (ROS) generation.

2.4. Sperm motility and motion analysis

The right cauda epididymis of each control and treated male mouse was removed and placed in a medium of 1 ml HTF maintained at 34 °C in an environment saturated with 5% CO_2. After 5 min, cauda epididymis was minced with curved scissors and the sperm was dispersed. After 20 min, the sperm was collected and transferred to a fresh tube. A volume of 10 μL aliquots of sperm suspension was placed on a pre-warmed Makler chamber (10 μm depth; Sefi-Medical Instruments, Haifa, Israel). Sperm motility was expressed as the ratio between the number of motile sperm and total number of sperm. The Makler chamber was put under a phase-contrast microscope (Olympus CH-2, Tokyo, Japan) connected to a video recorder (Sony, Tokyo, Japan) and recorded. Sperm motion parameters were analyzed from videotaped images using computer-assisted sperm analysis (CASA) obtained for velocity indices with a Hamilton Thorn Research motility analyzer (version HTMIVOS Specification, Beverly, MA, USA). We also measured motion parameters, including curvilinear velocity (VCL, μm/s), angular progressive velocity (VAP, μm/s), straight-line velocity (VSL, μm/s), lateral head amplitude (ALH, μm), and beat-cross frequency (BCF, Hz).

2.5. Sperm morphology analysis

The sperm suspension was diluted with phosphate-buffered saline then mixed with 1% aqueous eosin Y (10:1). The resulting mixture was set aside and left still for 30 min. After that, a drop of the mixture was placed on a clear slide and a uniform smear was made. Two samples were made for each mouse. After air drying, the slides were briefly rinsed in methanol to remove excess stain. They were air dried again, and cover-slipped with mounting medium. At least 600 cells from each mouse were examined for morphological abnormalities under a light microscope (400×) (Zeiss, Axioskop2, Germany). Abnormalities were classified as amorphous, truncated, calyculate, collapsed, triangular, giant, and hammer (Wyrobek and Bruce, 1975).

2.6. Flow cytometry (FCM) sperm chromatin structure assay (SCSA)

The procedure of FCM SCSA is described in detail elsewhere (Evenson and Jost, 1994). Briefly, the left cauda epididymis was removed from each mouse and placed in TNE buffer solution (0.15 M NaCl, 0.01 M Tris–HCL, 1 mM disodium EDTA, pH 7.4) in a Petri dish and minced. After allowing the tissue fragments to settle, the sperm suspension was filtered through a 0.2 mm nylon mesh into 2-ml cryogenic vials and mixed with glycerol to form a final solution at a concentration of 10% (v/v). The solution was then frozen at −80 °C. When we were ready to perform SCSA analysis, the frozen samples were diluted to a concentration of 1–2 × 10^6 sperm/ml with TNE buffer solution. 200 μL of the diluted sample was put into a Falcon tube (BD Immunocytometry Systems, San Jose, CA, USA) and added with 400 μL low-pH detergent solution (0.1% Triton X-100, 0.15 M NaCl, and 0.08 N HCl, pH 1.2). After 30 s, the cell was stained with 1.2 ml of acridine orange (AO) staining solution containing 6 μg/ml of AO in staining buffer (0.1 M citric acid, 0.2 M Na_2HPO_4, 1 mM EDTA, 0.15 M NaCl, pH 6.0). After 3 min of staining, the sample was analyzed using a FACScan FCM (BD Immunocytometry Systems, San Jose, CA, USA). A total of 10,000 spermatozoa were collected and analyzed at a flow rate of 100–200 cells/s.

AO is a metachromatic fluorochrome used to stain differentially doubles/single stranded nucleic acids. When excited by blue light at 488 nm, AO intercalates into double-stranded DNA fluoresces green (FL1, native DNA). If, however, DNA is

damaged and exposed single-stranded DNA, AO will attach to single-stranded nucleic acids and exhibit a red fluorescence (FL3, denatured, single stranded DNA). In SCSA analysis, the metachromatic shift from green to red fluorescence is expressed in αT, as a ratio between red and total fluorescence (red + green). SCSA variables in the present study were mean (XαT), standard deviation (S.D.αT), and the percentage of cells outside the main population of αT (COMPαT).

Data were calculated with use of the ListView software (Phoenix Flow Systems, San Diego, CA, USA).

2.7. Sperm mitochondrial membrane potential

The lipophilic cation with dual emission fluorophore JC-1 was used to measure the MMP (Gravance et al., 2001). When excited by blue light at 488 nm, if the JC-1 molecule remains in its monomeric form. After passing through the mitochondrial membrane, it will exhibit green fluoresce at 530 nm, representing low potential (inactivity or death). If JC-1 molecule transforms into the J-aggregate form, it will exhibit an orange fluoresce at 590 nm, indicating high membrane potential (high activity). Concentrations of 1.53 mM JC-1 stock solution were prepared in DMSO. The sperm suspension was diluted to 1–2 × 10^6 sperm/ml and stained with JC-1 to a final stain concentration of 2.0 μM. The samples were set aside for 10 min at 34 °C, and then analyzed using a FACScan FCM. A total of 10,000 spermatozoa were collected and analyzed at a flow rate of ∼200 cells/s. The percentage of orange stained (mitochondrial-active sperm) cells were recorded.

2.8. Sperm ROS generation

Sperm hydrogen peroxide (H_2O_2) level in most cells can be measured using a fluorescent probe, DCFH-DA. To do this, H_2O_2 is incorporated into hydrophobic lipid regions of the cell (Bass et al., 1983). The acetate moieties are cleaved off leaving the nonfluorescent 2′,7′-dichlorofluorescin (DCFH). DCFH-DA is a stable compound that passively diffuses into cells and is hydrolyzed by intracellular esterase to yield DCHF, which is trapped inside cells. H_2O_2 produced by cells oxidize DCHF to the highly fluorescent compound, 2′,7′-dichlorofluorescein (DCF) which is fluorescent at 530 nm. The DCFH-DA stock can dissolve in absolute alcohol at 2.5 mM (1.2 mg/ml). We modified the method used by Fisher et al. (2005) to evaluate H_2O_2 level in sperm. Sperm cells at a concentration of 1–2 × 10^6 sperm/ml were added into 2.5 mM DCFH-DA to a concentration of 12.5 μM. The mixture was set aside and maintained at 34 °C for 30 min.

Sperm superoxide anion ($O_2^{-\bullet}$) level was measured using a modification of a previously described method (Marchetti et al., 2002). The HE stock dissolves in DMSO at 0.33 mM, and can be directly oxidized into ethidium bromide by $O_2^{-\bullet}$ produced by sperm. Sperm cells at a concentration of 1–2 × 10^6 sperm/ml were added into 0.33 mM HE to a concentration of 2 μM. The mixture was set aside and maintained at 34 °C for 30 min. Intracellular H_2O_2 and $O_2^{-\bullet}$ levels were measured using a FACScan FCM with excitation and emission settings at 488 and 530 nm, respectively.

2.9. FCM DNA content analysis of testis cells

Testis monocellular suspensions were prepared according to the procedure described by Spanò et al. (1996). Briefly, the right testis was minced and treated with 1 ml 0.1% pepsin–HCl solution gently mixed by magnetic stirring at room temperature for 10 min. These monocellular suspensions were then filtered through a 37 μm nylon mesh to separate the cells from tissue. The resulting monocellular suspensions were stabilized with 4 ml of 95% ethanol and stored at −20 °C for several weeks. For the FCM analysis, fixed samples were added to 0.5% pepsin–HCl solution to a 1:1 volume. After 10 min of gentle magnetic stirring, cells were immediately added to 50 μg/ml propidium iodide (PI) in 0.1 M Tris–HCl buffer solution (pH 7.5) containing 0.04 mg/ml DNAse-free RNAse.

The fluorescence intensity of DNA content of the testis cells was measured using a FACScan FCM. PI fluorescent emissions were monitored using a 620 nm band-pass filter. A total of 2 × 10^4 cells were collected for each sample. Based on the DNA content, main germ cell peaks can be classified into four categories: (1) mature haploid (elongated spermatids; stages X-XIV), (2) immature haploid (round and elongating spermatids; stages I-IX), (3) diploid (spermatogonia, secondary spermatocytes, tissue somatic cells), and (4) tetraploid (mostly primary spermatocytes). The region between the diploid and tetraploid peaks, called the S-phase, is comprised of cells that actively synthesize DNA (Spanò et al., 1996). The relative proportions of haploid, diploid, S-phase and tetraploid cell types were calculated.

2.10. Histopathology of testis

The left testis was fixed in Bouin's solution for at least 48 h. Each was processed in an automatic tissue processor and embedded in paraffin. Thin sections were cut at 3–4 μm thickness, stained with hematoxylin and eosin, and examined under a light microscope. Each germ cell stage in the seminiferous epithelium was categorized according to a classification system used by Oakberg (1956).

2.11. Statistics

Results are expressed as mean ± standard error of the mean (S.E.M.). All statistical comparisons were performed using the JMP statistical package (SAS Institute Inc., Gary, NC, USA) for the control and treated groups. The body weights, reproductive organ weights, sperm count, motility, velocity of motion, morphology, FCM SCSA, MMP, generation of H_2O_2 and $O_2^{-\bullet}$ as well as DNA content of testis cells of the groups exposed to PBED 209 and the control group were compared using one-way analysis of variance (ANOVA), followed by the Tukey–Kramer honestly significant difference (HSD). $p < 0.05$ was considered

statistically significant. The relationships among ALH, MMP and H_2O_2 were measured using linear regression analysis.

3. Results

3.1. Body and reproductive organ weights

The data showed no significant differences in mean body weight and daily weight gain between PBDE 209 exposed and control groups (Fig. 1). There were no significant differences in absolute and relative weights of body, testis, epididymis, cauda epididymis, and seminal vesicles among the four groups exposed to PBDE 209 exposed and the controls (Table 1).

3.2. Sperm motility, velocity and morphology

In our motion analysis of sperm velocity, average displacement of ALH was found to be significantly more decreased in the groups gavaged with 500 and 1500 mg/kg of PBDE 209 than in controls ($p < 0.05$). In addition, although there was a dose-dependent decrease in the VCL, VAP and VSL in study groups, there were no significant differences in measures found between any study group and the controls (Table 2). There were also no significant differences in sperm count, motility and morphology between the groups exposed to PBDE 209 exposed and the controls (Table 2).

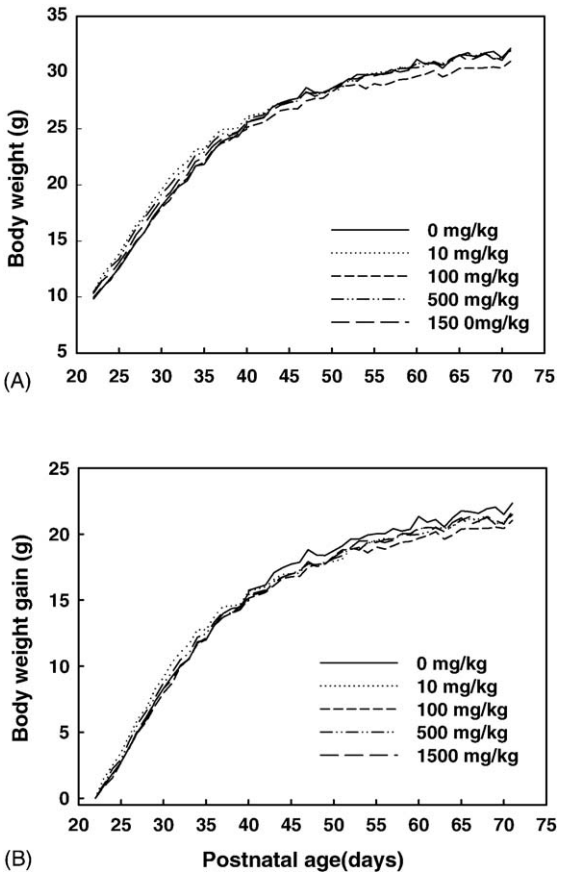

Fig. 1. Lack of effect of postnatal exposure to PBDE 209 on body weights (A) and gain body weights (B). Data was presented as group means (g).

3.3. FCM analysis of SCSA, MMP and ROS generation

Cauda epididymal sperm was analyzed by FCM SCSA to investigate whether there was any PBDE 209 induced changes in sperm chromatin DNA integrity. There were no significant differences in α, XαT, S.D.αT, or COMPαT between the PBDE 209 groups and the controls (Table 3). The group exposed to 1500 mg/kg was found to have a significantly fewer high MMP sperm than 0 and 10 mg/kg groups. The 500 mg/kg group was also found to have a significant decrease in high MMP sperm compared with 10 mg/kg group alone.

To evaluate sperm ROS generation, we measured H_2O_2 and $O_2^{-\bullet}$ in the groups treated with PBDE 209. While we found a significant increase in the generation of sperm H_2O_2 in the 500 and 1500 mg/kg/day groups compared to that of the control group, we did not find such a difference in the generation of $O_2^{-\bullet}$ (Table 3).

3.4. Relationships among ALH, MMP, and ROS

We performed a regression analysis of sperm motion of ALH, MMP, and H_2O_2 (Fig. 2). The sperm motion of ALH was positively and significantly associated with MMP ($p < 0.0001$) and negatively associated with the generation of sperm H_2O_2 ($p < 0.0001$). The MMP was negatively and significantly associated with the generation of sperm H_2O_2 ($p = 0.0021$).

3.5. DNA content analysis of testis cells

The cell frequencies of various testis subpopulations were expressed as percentage of total histogram area (Fig. 3). There were no significant changes in testis DNA content of the four cell types (mature haploid, immature haploid, diploid, and tetraploid) as well as that of the S-phase cell.

3.6. Histopathology of testis

Histopathological examinations were performed on the testicular sections of both PBDE 209-treated groups

Table 1
Body and tissue weight between mice postnatally exposed to 2,2,3,3,4,4,5,5,6,6-decabrominated diphenyl ether (PBDE 209) (10, 100, 500, and 1500 mg/kg) and unexposed controls

Parameters	Treatment of PBDE 209 (mg/kg)				
	Control ($n=10$)	10 ($n=10$)	100 ($n=10$)	500 ($n=10$)	1500 ($n=10$)
Absolute					
Body weight (g)	32.1 ± 0.7	32.1 ± 0.7	30.9 ± 0.2	31.7 ± 0.6	31.8 ± 0.5
Testis (g)	0.21 ± 0.01	0.23 ± 0.01	0.20 ± 0.01	0.21 ± 0.01	0.21 ± 0.01
Epididymis (g)	0.080 ± 0.002	0.086 ± 0.003	0.080 ± 0.002	0.081 ± 0.002	0.078 ± 0.002
Cauda epididymis (g)	0.032 ± 0.001	0.032 ± 0.001	0.031 ± 0.001	0.031 ± 0.002	0.031 ± 0.001
Seminal vesicles (g)	0.22 ± 0.01	0.21 ± 0.01	0.20 ± 0.01	0.20 ± 0.01	0.21 ± 0.01
Relative					
Testis (%)	0.65 ± 0.03	0.71 ± 0.03	0.65 ± 0.02	0.67 ± 0.04	0.65 ± 0.03
Epididymis (%)	0.25 ± 0.01	0.27 ± 0.01	0.26 ± 0.01	0.25 ± 0.01	0.25 ± 0.56
Cauda epididymis (%)	0.099 ± 0.005	0.100 ± 0.004	0.101 ± 0.004	0.098 ± 0.006	0.096 ± 0.003
Seminal vesicles (%)	0.69 ± 0.04	0.67 ± 0.04	0.64 ± 0.03	0.63 ± 0.04	0.67 ± 0.02

Data was expressed as means ± S.E.M.

Table 2
Sperm quality of mice postnatally exposed to 2,2′,3,3′,4,4′,5,5′,6,6′-decabrominated diphenyl ether (10, 100, 500, and 1500 mg/kg) and unexposed controls

Parameters	Treatment of PBDE 209 (mg/kg)				
	Control ($n=10$)	10 ($n=10$)	100 ($n=10$)	500 ($n=10$)	1500 ($n=10$)
Sperm count (10^6/ml)	12.0 ± 0.9	11.3 ± 0.8	11.1 ± 0.8	11.1 ± 1.4	11.3 ± 1.2
Sperm motile (%)	63.5 ± 4.8	67.9 ± 7.6	62.3 ± 6.1	60.7 ± 4.7	54.7 ± 6.7
Abnormal sperms head (%)	6.5 ± 0.4	6.5 ± 1.3	6.0 ± 1.1	6.8 ± 2.3	6.4 ± 0.8
Velocity of motion					
VCL[a] (μm/s)	153.7 ± 6.1	149.4 ± 6.0	140.6 ± 12.7	127.8 ± 9.8	124.6 ± 11.4
VAP[b] (μm/s)	84.9 ± 3.4	82.4 ± 3.1	79.4 ± 7.1	72.9 ± 5.3	72.2 ± 7.0
VSL[c] (μm/s)	63.0 ± 2.7	59.2 ± 1.7	58.7 ± 5.8	52.4 ± 4.3	51.9 ± 5.4
ALH[d] (μm)	7.3 ± 0.2	7.0 ± 0.3	6.4 ± 0.6	6.0 ± 0.3*	5.7 ± 0.5*
BCF[e] (Hz)	20.2 ± 0.9	21.1 ± 0.7	20.3 ± 0.9	20.1 ± 0.8	19.7 ± 1.5

All data was expressed as means ± S.E.M.
[a] VCL: curvilinear velocity.
[b] VAP: average path velocity.
[c] VSL: straight line velocity.
[d] ALH: amplitude of lateral head displacement.
[e] BCF: beat-cross frequency.
* $p<0.05$ as compared with control group.

and the control group (Fig. 4). There were no apparent morphological differences between the exposed groups and the controls. Spermatogenesis was normal as indicated by the presence of normal seminiferous tubules undergoing spermatogenic cycle, from spermatogonia, to spermatocytes, to round spermatids, and to elongated spermatids.

4. Discussion

This study had shown that postnatal exposure to high dose PBDE 209 can reduce displacement of sperm ALH and MMP, and induce the generation of H_2O_2. We have found no significant changes sperm count, motility, morphology, VCL, VAP, VSL, BCF, SCSA, or generation of $O_2^{-\bullet}$. We also found no changes in DNA content in testis cells and testicular histopathology related to the exposure to PBDE 209. Body weight, weight gain, the testis, epididymis, cauda epididymis, and seminal vesicles weights of our groups treated with PBDE 209 were not significantly different from our control group. This report is the first to show the possibility that postnatal exposure to PBDE 209 might affect the displacement of ALH displacement, reduce MMP and increase generation of sperm H_2O_2.

We found a significant relationship between ALH displacement, MMP, and generation of sperm H_2O_2. Other studies, though not specifically focusing on PBDE

Table 3
Epididymal sperm chromatin structure assay (SCSA), mitochondrial membrane potential (MMP) and reactive oxygen species (ROS) generation in mice postnatally exposed to 2,2′,3,3′,4,4′,5,5′,6,6′-decabrominated diphenyl ether (10, 100, 500, and 1500 mg/kg) and unexposed controls

Parameters	Treatment of PBDE 209 (mg/kg)				
	Control ($n=10$)	10 ($n=10$)	100 ($n=10$)	500 ($n=10$)	1500 ($n=10$)
MMP[a] (%)	93.8 ± 0.9	94.7 ± 0.6	95.1 ± 0.5	90.8 ± 1.2[#]	90.2 ± 2.2[#,*]
Sperm H_2O_2 generation[b] (arbitrary units)	75.7 ± 8.0	90.3 ± 12.3	90.2 ± 8.9	133.7 ± 27.6[*]	160.4 ± 25.0[*]
Sperm $O_2^{-\bullet}$ generation[b] (arbitrary units)	257.17 ± 30.75	305.39 ± 28.01	279.72 ± 23.75	326.56 ± 24.14	345.21 ± 27.29
COMPαT[c] (%)	7.3 ± 2.2	5.7 ± 1.5	8.0 ± 1.6	7.2 ± 1.5	10.1 ± 2.5
XαT[d] (arbitrary units)	203.5 ± 2.5	199.0 ± 2.5	203.0 ± 2.5	204.1 ± 2.8	207.8 ± 3.6
S.D.αT[e] (arbitrary units)	41.2 ± 2.6	39.3 ± 2.0	40.6 ± 2.8	43.9 ± 5.7	44.2 ± 4.5

All data was expressed as means ± S.E.M.
[a] Values represent percentages of sperm population with high mitochondrial membrane potential as assessed by flow cytometric analysis with the JC-1 probe.
[b] Fluorescence intensity.
[c] COMPαT is the percentage of sperm with abnormal chromatin DNA structure.
[d] XαT expressed in fluorescence channel units.
[e] S.D.αT expressed in fluorescence channel units.
[*] $p<0.05$ as compared with control group.
[#] $p<0.05$ as compared with 10 mg/kg group.

209, have produced similar findings. Reistad et al. (2005) found another BFR but not a PBDE, TBBPA able induce the generation of ROS in human neutrophil granulocytes through the activation of respiratory burst (Reistad et al., 2005). Two other studies of TBBPA have found that it significantly induced ROS generation in the livers of *Carassius auratus* and rainbow trout (Shi et al., 2005; Ronisz et al., 2004). With regard to PBDEs specifically, one in vitro study by Reistad and Mariussen (2005) exposing human neutrophil granulocytes to pentabrominated diphenyl ether mixture (DE-71) and tetrabrominated diphenyl ether (BDE-47) found the two PBDEs able to increase the production of ROS in a dose-dependent manner, though this induction was not found with octabrominated diphenyl ether (OBDE), decabrominated diphenyl ether (DBDE or PBDE 209), or non-brominated diphenyl ether (NBDE).

In one recent study by Mörck et al. (2003), when a single oral dose of 14C-labeled decaBDE (PBDE 209) was administered to rats, at least 10% of the dose was absorbed. In that study, they found PBDE 209 underwent oxidative debromination and the metabolites have five to seven bromine atoms and a guaiacol structure (a hydroxy and a methoxy group). They also found trace amounts of nonabrominated diphenyl ethers and monohydroxylated metabolites. They hypothesized that metabolic pathway involved a reactive intermediate metabolite. Recent studies had proven that PBDE 209 was detected in Swedish peregrine falcon eggs (<20–430 ng/g lipid weight) and freshwater fish (median 48 ng/g lipid weight) (Burreau et al., 2004; Lindberg et al., 2004). It was also found in 18 of 21 eggs of peregrine falcons (Falco peregrinus) from northern and southern Sweden (Sellström et al., 2001). The presence of PBDE 209 in eggs suggested that some limited uptake may occur and that PBDE 209 might concentrate to a small extent within some food and indicated that PBDE 209 is bioavailable. There was also evidence for the metabolism of PBDE 209 formulations to penta-octa BDE congeners in fish (Stapleton et al., 2004a,b). These studies indicated that higher brominated PBDEs were absorbed and metabolized to lower brominated PBDEs, which were highly bioaccumulative and toxic.

We further hypothesized that this metabolite may cause generate ROS. We found that at the end of our 50-day exposure period, the generation of sperm H_2O_2 was significantly greater in our groups treated with 500 and 1500 mg/kg of PBDE 209 than the controls. In this study, we isolated and analyzed epididymal sperm for the loss of MMP, and found a significant loss of MMP in 500 (compare with 10 mg/kg/day) and 1500 mg/kg/day (compare with 0 and 10 mg/kg/day) groups, possibly indicating that exposure of PBDE 209 might affect sperm MMP. In fact, one study by Fisher et al. (2005), in which, not a PBDE, but 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was injected into C57BL/6 male mice found a loss in MMP and an increase in ROS production in epididymal sperm. That study suggested exposure to that dioxin might lead to increases of ROS production and the loss of MMP (Fisher et al., 2005).

Except for displacement of ALH, we found no significant differences in sperm motion parameters, though there was a slight dose-dependent decrease in VAP, VCL, and VSL, suggesting that a decrease in sperm

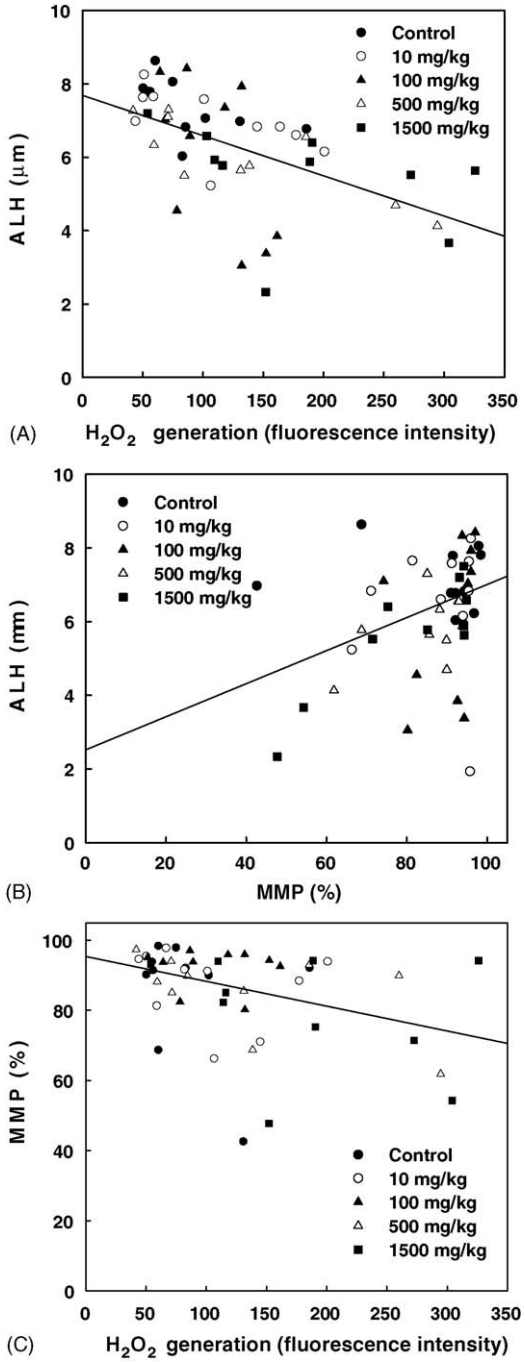

Fig. 2. Relationships among amplitude of the lateral head displacement (ALH), mitochondrial membrane potential (MMP), and hydrogen peroxide (H_2O_2) generation by general linear model. (A) Decreased ALH in relation to sperm H_2O_2 generation. ALH = 7.74 − 0.01 × (sperm H_2O_2 generation) ($R = -0.57$, $p < 0.0001$); (B) increased ALH in relation to MMP. ALH = −0.60 + 0.08 × MMP; $R = 0.64$, $p < 0.0001$; (C) decreased MMP in relation to sperm H_2O_2 generation. MMP = 96.75 − 0.07 × (sperm H_2O_2 generation) ($R = -0.46$, $p = 0.0021$).

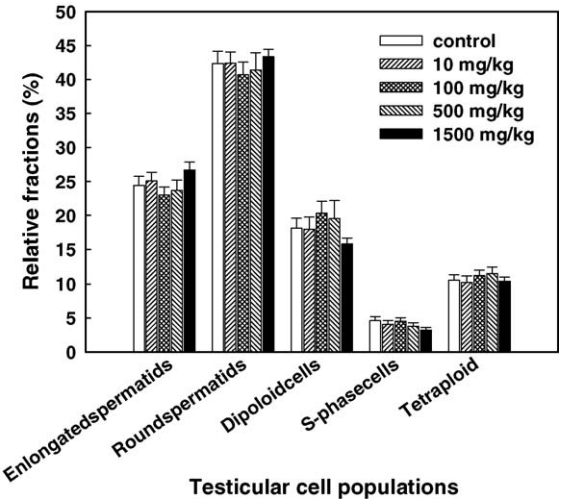

Fig. 3. Relative fractions (means ± S.E.M.) of the various testis subpopulations, calculated as percentage of the histogram area between mice postnatally exposed to PBDE 209 (10, 100, 500, and 1500 mg/kg) and unexposed controls.

MMP might lead to a decrease of sperm motion parameters and fertility potential. We compared the relationships among ALH displacement, generation of H_2O_2, and the decrease of MMP. We found that in mice exposed to PBDE 209, the generation of H_2O_2 affected MMP and the displacement of ALH, though further research is needed elucidate the mechanism behind its influence.

Norris et al. (1975) found that concerning the effects of 77% pure PBDE 209 did not have an adverse effect on reproduction or unborn fetus in rats exposed to less than 100 mg/kg/day. Similarly, a more recent experiment by Hardy et al. (2002) has reported that exposure to 97% pure PBDE 209 also did not have an adverse effect on the development of offspring in female rats exposed to 1000 mg/kg/day throughout gestation. They found the no-observable-effect level (NOEL) for maternal and developmental toxicity to be about 1000 mg/kg of PBDE 209 per day. Our study, however, has found that high concentrations of PBDE 209 might damage sperm function in male mice. We found the lowest-observed-adverse-effect level (LOAEL) for reproductive toxicity was found to be oral 500 mg PBDE 209/kg/day on postnatal Days 21–70 in the male mice. This study represents the first indication of the adverse effect of PBDE 209 on sperm function.

In recent years, regulatory agencies have raised concerns about detection of certain PBDE 209 and PBDE congeners in the environment and in human adipose tissue, blood serum, and/or breast milk. PBDE 209 was one of the three commercial PBDEs to be banned in

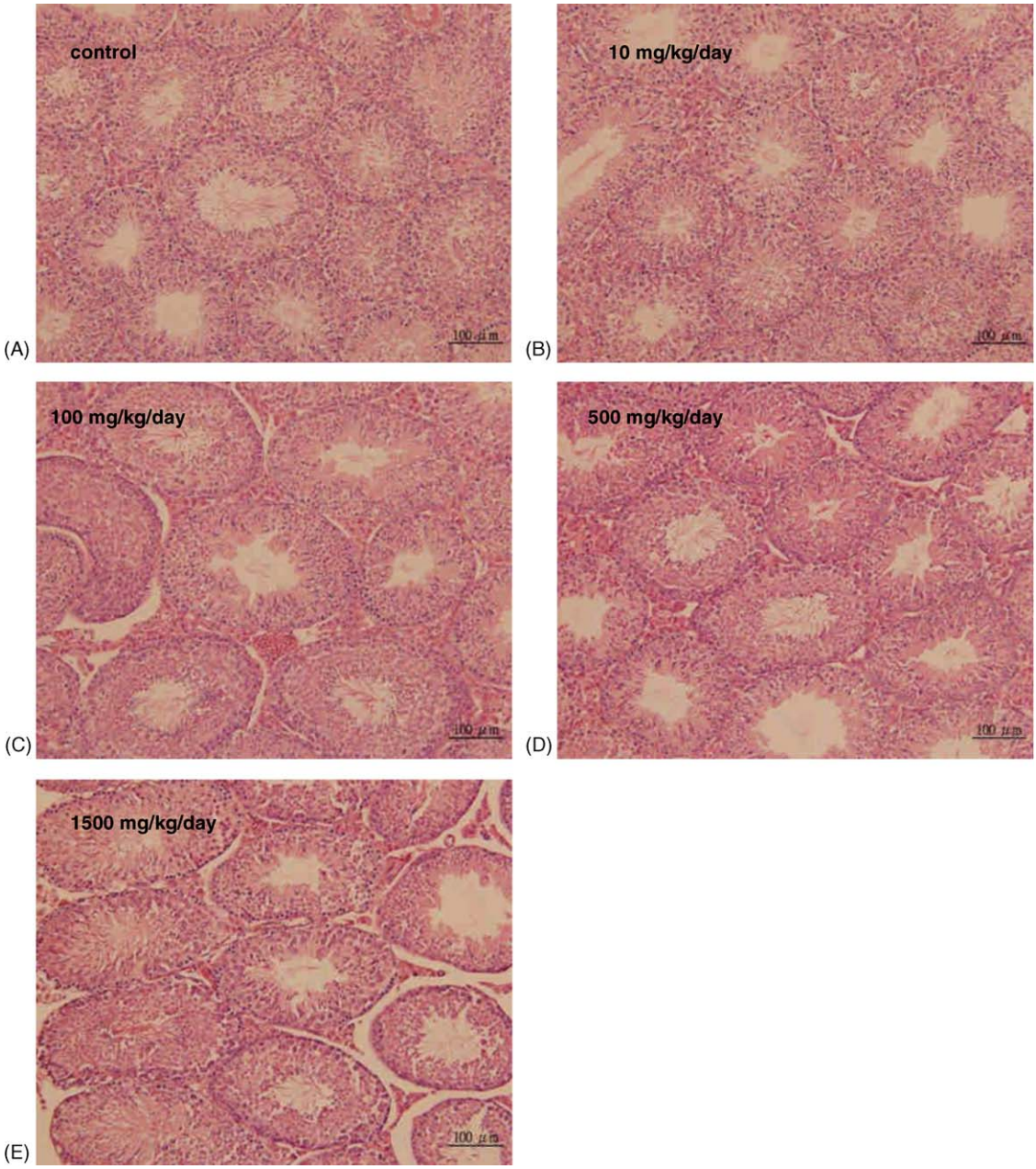

Fig. 4. Photomicrographs of H&E stained sections of mouse testis. (A) Controls showing normal testicular histology with evidence of spermatogenesis; (B) 10 mg PBDE 209/kg treated group; (C) 100 mg PBDE 209/kg treated group; (D) 500 mg PBDE 209/kg treated group; and (E) 1500 mg PBDE 209/kg treated group. Morphology of the testicular tissues appeared normal in all the treated groups as compared with that of the control group. Scale bar, 100 μm.

new E&E products from July 2006. From the reproduction toxicity point of view, the chance of humans for exposing at a dose of 1500 mg/kg/day is very small. However, findings in mice postnatally exposed to high levels of PBDE 209 provide valuable information on male reproductive effects of PBDE and related chemicals in human. The mice exposed to such chemicals at high levels provide unique situation where human effects in sperm dysfunction can be studied. Besides, comparing with the toxicity of other PBDEs, PBDE 209 seems to be much safer. However, the pollutions of PBDE 209 might have been existed in environment for a long time and it may biotransfer from environment to organisms. The non-specific effects associated with exposure to high

doses and the male reproductive toxicity of the related metabolites warrant further investigation.

The alteration of sperm function might reflect an indirect effect of the well being of the animals. Study with other PBDE (such as DE-71) suggested that postnatal exposure to these chemicals rendered the rodents hypothyroid (Stoker et al., 2004). They found that the delay in preputial separation and reduction in seminal vesicle and ventral prostate weights indicated a modification or inhibition of endogenous androgenic stimulation directly by DE-71 or a secondary effect that occurs in response to a PBDE-induced change in thyroid hormones. Whether the subtle effects in sperm function reflect thyroid hormonal imbalance and other metabolic alterations is unclear and will be a subject for future studies.

In conclusion, our study has found that exposure to PBDE 209 reduces sperm ALH and MMP and induces H_2O_2 generation in male mice. These changes in ALH, MMP and generation H_2O_2 indicate a toxicity possibly resulting from PBDE 209-induced oxidative stress. Future investigations are needed to study the effects of prenatal exposure to PBDE 209 on fetal and pubertal stages of development and adult reproduction and the mechanism behind the generation of ROS and how it affects the displacement of ALH and MMP.

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