Toxicology and Applied Pharmacology 329 (2017) 301–308

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

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

Maternal exposure to a mixture of persistent organic pollutants (POPs) affects testis histology, epididymal sperm count and induces sperm DNA fragmentation in mice

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ARTICLE INFO

Article history:
Received 13 December 2016
Revised 14 June 2017
Accepted 20 June 2017
Available online 20 June 2017

Keywords:
Persistent organic pollutants (POPs) mixture
Reproductive toxicity
Testis histology
Sperm count
Sperm chromatin structure assay (SCSA)

ABSTRACT

Persistent organic pollutants (POPs) are widespread throughout the environment and some are suspected to induce reproductive toxicity. As animals and humans are exposed to complex mixtures of POPs, it is reasonable to assess how such mixtures could interact with the reproductive system. Our aim is to investigate how maternal exposure to a mixture of 29 different persistent organic pollutants, formulated to mimic the relative POP levels in the food basket of the Scandinavian population, could alter reproductive endpoints. Female mice were exposed via feed from weaning, during pregnancy and lactation in 3 exposure groups (control (C), low (L) and high (H)). Testicular morphometric endpoints, epididymal sperm concentration and sperm DNA integrity were assessed in adult male offspring. We found that the number of tubules, proportion of tubule compartments and epididymal sperm concentration significantly decreased in both POP exposed groups. Epididymal sperm from both POP exposed groups showed increased DNA fragmentation. It is concluded that maternal exposure to a defined POP mixture relevant to human exposure can affect testicular development, sperm production and sperm chromatin integrity.

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1. Introduction

Persistent organic pollutants (POPs) refer to groups of toxic environmental chemicals with a carbon-based structure, which are resistant to environmental degradation and are widely distributed via soil, water and air (Hung et al. 2016). Because of their lipophilic nature, POPs tend to biomagnify through the food chain and bioaccumulate from lower organisms to top predators and humans (Daley et al. 2014). Among different classes of POPs, chlorinated, brominated and perfluorinated compounds are the most persistent compound classes, widely detected in human adipose tissue, breast milk and blood samples from all over the world (Knutsen et al. 2008; Polder et al. 2008; Linderholm et al. 2010; Kim et al. 2013; Pumarega et al. 2016).

The lipophilic properties of some POP families makes them capable of passing through biological barriers such as the blood-brain barrier (Rasinger et al. 2014) as well as the placenta (Vizcaino et al. 2014), a process which leads to accumulation of POPs in the fetus. Parental exposure to POPs has in epidemiological studies been associated with adverse effects in fetuses and neonates such as lower birth weight (Casas et al. 2015; Robledo et al. 2015), Attention Deficit Hyperactivity Disorder (ADHD) and depression (Strom et al. 2014), decreased mental development (Gascon et al. 2012), immune-related diseases and lung dysfunction (Cao et al. 2016). In contrast, some classes of POPs like perfluorinated compounds do not accumulate in adipose tissue, but can bind to proteins and interfere with normal endocrine function (Jones et al. 2003; Zhang et al. 2013b).

Reproductive toxicity upon exposure to some of the POP members including decabromodiphenyl ether (BDE 209), polychlorinated biphenyl (PCB 101), PCB 118 and perfluorononanoic acid (PFNA) has been reported in both females and males in different animal models including mice (Tseng et al. 2013; Fiandanese et al. 2016), rats (Zhou et al. 2013) and zebrafish (Nourizadeh-Lillabadi et al. 2009; Zhang et al. 2016). It has been shown that some POPs induce reproductive toxicity via the disruption of steroidogenesis, interference with normal hormonal balance by interaction with carriers/receptors, induction of oxidative stress and/or interference with epigenetic mechanisms (Sharpe and

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http://dx.doi.org/10.1016/j.taap.2017.06.019
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Irvine 2004; Jeng 2014; Vested et al. 2014). Furthermore, some POP families are able to disrupt the blood-testis barrier, accumulate in the testis and impact testicular function (Li et al. 2009; Qiu et al. 2013; Lu et al. 2016). Moreover, some of the POPs have been associated with cancer in reproductive organs. For instance, human studies have shown that concentrations of PCBs, HCB and chlordanes are higher in mothers of patients with testicular cancer (Hardell et al. 2003; Hardell et al. 2004).

Although several methods have been developed in order to study male fertility, testis histology assessment is still considered a gold standard to assess testicular toxicity. The sperm chromatin structure assay (SCSA) provides a measure of sperm DNA fragmentation and compaction and is a valuable method to assess sperm quality (Evenson and Wixon 2005). Sperm DNA integrity is a key factor for the proper transmission of genetic material, and sperm chromatin abnormalities or DNA damage may cause male infertility (Agarwal and Said 2003). It has been suggested that POP exposure could induce sperm DNA damage and decrease the DNA integrity (Rozati et al. 2002; Rignell-Hydbom et al. 2005; de Jager et al. 2009).

The majority of toxicology studies have focused on the effects of single compounds only, whereas in reality we are exposed to complex mixtures of pollutants (Frederiksen et al. 2009; Ni et al. 2013). How such mixtures interact on toxicological endpoints is relatively unknown, but is a significant concern as several studies have demonstrated the potential of different compounds to have additive effects. For instance, it has been shown that co-exposure to PCB 153 and methyl mercury enhances developmental neurotoxic effects in mice (Fischer et al. 2008). Further, exposure to a mixture of lead, arsenic and organic mercury in pregnant mice enhanced maternal toxicity compared to individual exposure (Bellés et al. 2002). Recently we developed a complex mixture based on the POPs found in Scandinavian food basket surveys, as well as human blood and breast milk samples (Berntsen et al. 2016a) and preliminary unpublished data indicates that our POP mixture affects the mice behavior and stress responses in a statistically significant way.

A key aspect in reproductive toxicity studies is that the exposure scenario must be translatable to a human scenario. Therefore, investigating the effects of environmentally relevant POPs in a mixture formula would be more realistic than investigating effects of single POPs. In the current experiment, we investigated effects on testis histology, sperm production and sperm DNA integrity in male mice exposed in utero and through mother's milk to a complex POP mixture reflecting POP ratios found in Scandinavian food.

2. Material and methods

2.1. Animals, housing and husbandry

The study was performed at the Section for Experimental Biomedicine at The Norwegian University of Life Sciences in Oslo, Norway. The unit is licensed by the Norwegian Food Inspection Authority (NFIA) and accredited (2017/58763) by the Association for Assessment and Accreditation of Laboratory Animal Care (www.aaalac.org). The study (fots5583/2013/39783-2) was approved by the unit's animal ethics committee (Institutional Animal Care and Use Committee/IACUC) and NFIA.

All animals were group housed in open type III cages (Tecniplast, Buguggiate, Italy), containing standard aspen bedding (Scanbur BK, Nittedal, Norway) and cellulose nesting material. The animals had free access to their assigned feed. Tap water was available from standard drinking bottles (Tecniplast, Buguggiate, Italy). The animal room was on a 12:12 light–dark cycle, with a room temperature of 21 ± 2 °C as well as 20 air changes per hour and 45 ± 5% relative humidity. The cages, bedding, nesting material and water bottles were changed once a week. In-house bred 129:C57BL/6 females (which parents were obtained from Jackson Laboratory, Maine, USA), were mated with non-brother males of the same hybrid and generation to produce the male specimens used in the current study.

2.2. Feed design, chemicals and exposure scenario

The design and preparation of the POP mixture is described in Berntsen et al. (2016a) In brief, compounds occurring at the highest levels in Scandinavian food, blood and breast milk were selected for the mixture, and estimated daily intake (EDI) levels were used as a basis for feed concentration ratios of the different compounds. However, the clearance rates for many toxicants have shown to be higher in mice than in humans (Walton et al. 2001). When determining doses to use, factors such as interspecies differences in compound metabolism and limitations in numbers of distinct doses due to high costs were taken into consideration. In addition, there was also a risk of background exposure, as low levels of POPs often tend to be present in commercial mice feed. Therefore, the doses were set higher, and two doses with a factor of 20 of difference were selected at levels 5000 times and 100,000 times the EDIs calculated for the basic mixture.

All polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyl (PCBs) and other organochlorines were originally purchased from Chiron As (Trondheim, Norway). Hexabromocyclododecane (HBCD) and all perfluorinated compounds (PFAAs), except perfluorohexane sulfonic acid (PFHxS), which was from Santa Cruz (Dallas, USA), were obtained from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were dissolved in an appropriate solvent and added to corn oil (Asko, Norway), intended for human consumption. In all diets, Soybean oil in the original feed recipe was exchanged with the same corn oil used for preparations of the mixture. Four different diets were made, three exposure diets for pregnant mice, including control (containing corn oil from which solvents had been evaporated), low dose (5000× EDI) and high dose (100,000× EDI), and one reference diet for males and pups after weaning (containing untreated corn oil only). The females used for breeding were randomly assigned to either the high concentration exposure group (H) (16 females), low concentration exposure group (L) (16 females) or control group (C) (15 females) and were exposed to the mixture of POPs through the feed. The exposure started at weaning and continued through breeding and lactation until necropsy. The offspring from the exposed mothers were only exposed to POPs (6 weeks) via the placenta, mother's milk and by nibbling of their mothers' feed before weaning. After weaning, pups were given the reference diet with no added POPs.

2.3. Sample collection

In order to assess general toxicity, offspring mice were weighed at weaning and prior to necropsy. Nine week old mice (15 pups per exposed group) were anesthetized by isoflurane inhalation (4,5% ISO at 700 ml airflow), euthanized by exsanguination and decapitation under deep anesthesia. During exsanguination, in order to measure the actual concentration of chemicals in plasma, a blood sample was collected using 1 ml EDTA coated syringes with a 23 G needle (Terumo, Norway), transferred into a 1 ml EDTA coated microvette tube (Sarstedt, Norway) and kept at 4 °C. Later on, samples were centrifuged at 5000 rpm/4 °C for 10 min; plasma was collected and stored at −80 °C until analyzes. In order to collect the testis and epididymis, the abdominal cavity was opened. After collecting the internal organs, the testes were pushed forward from the scrotal sac. The left testis was dissected and fixated in Davidson solution (30% v/v formaldehyde, 15% v/v ethanol, 5% v/v glacial acetic acid and 50% v/v distilled water) for 24 h at 4 °C for histological studies. The right testis and the epididymis compartments (including cauda epididymis, vas deferens and caput) from both sides were stored at −80 °C until further analyzes.

2.4. POPs plasma concentration measurement

The plasma concentrations of POPs were measured at the Norwegian University of Life Sciences, Department of Food Safety and Infection Biology, Laboratory of Environmental Toxicology as more thoroughly described by (Berntsen et al. 2016b). One pooled sample from each exposure group was measured. These pools also included samples of females used for other studies in the project. For the lipophilic groups of chemicals, extraction of POPs from biologic samples has been described by (Polder et al. 2014). Separation and detection of the pesticides and PCBs were performed on a high resolution gas chromatograph (HRGC) (Agilent 6890 Series, Agilent Technologies, PA, USA). Detection of PBDEs (except from PBDE 209) and HBCD was performed on a high-resolution gas chromatograph-low resolution mass spectrometer (HRGC-LRMS) (Agilent 6890 Series, Agilent Technologies, PA, USA). Perfluorinated compounds were analyzed according to (Bytingsvik et al. 2012) and references therein. The samples were analyzed by separation of compounds on a high-performance liquid chromatographer (HPLC) with a Discovery C18 column, connected to a C18 pre-column (Supelco, Sigma-Aldrich) and detection with liquid chromatography tandem mass spectrometry (MS-MS) (API 3000, LC/MS/MS System). Since perfluorinated compounds do not accumulate in lipids, they were not measured based on ng/g lipid weight in plasma.

2.5. Absolute measures and proportions of seminiferous tubules

Fixed testes were dehydrated, using 70% ethanol, and embedded in paraffin. Subsequently, 5-μm sections were mounted on slides and stained with hematoxylin and eosin (H&E) according to the local protocol. Images were taken using a digital camera (color view XC30, Olympus) and software for image capture (CellSens Dimension v1.6, Olympus). Images were captured from testis areas where round cross-sections of the seminiferous tubules were clear. In total, 15 samples per exposure group and 20 round seminiferous tubules per sample were captured and measures of the seminiferous tubules were taken using ImageJ v.1.51 software (National Institutes of Health, Bethesda, MD, USA) for windows according to (Montoto et al. 2012). Briefly, mean diameter of the seminiferous tubule and lumen diameter were calculated across the minor and major axes. The distance from the basal membrane to the luminal border was considered as epithelial thickness and the final value was calculated as the mean value of measurements in each cross-section. The area occupied by seminiferous tubules and lumen was measured and epithelium area was calculated by subtracting the lumen area from the seminiferous area. The relative number of seminiferous tubules was calculated as described in fig. 1.

2.6. Epididymal sperm count

One dissected cauda epididymis of each animal was weighed, and the sperm cells were gently squeezed out into 800 μl of ice-cold TNE buffer (pH 7.4; 0.01 M Tris-HCl, 0.15 M NaCl, 1 mM EDTA). The cell suspension was carefully pipetted and 50 μl of cell suspension was fixed by adding 50 μl of 0.2% paraformaldehyde and kept at 4 °C for cell counting the next day whereas the remaining suspension was used for the SCSA analyzis. A volume of 10 μl of each sperm suspension was diluted 1:1 with trypan blue and counted in a Bürker chamber under light microscopy.

2.7. Sperm chromatin structure assay (SCSA)

Cauda epididymis and vas deferens sperm were analyzed by SCSA following the standardized procedures described by (Evenson and Jost 2000) to determine sperm chromatin integrity. Briefly, sperm cells from both vas deferens and cauda epididymis (after a few incisions) were separately and gently squeezed out using an L shape needle into 800 μl of cold TNE buffer and passed through a 160-μm nylon filter. Prior to analyzis, sperm samples were denatured for 30 s by adding an acid solution (pH 1.2; 0.08 N HCl, 0.15 M NaCl, 0.1% Triton-× 100), and subsequently loaded with acridine orange (AO) staining buffer (pH 6.0; 0.1 M citric acid, 0.2 M Na2PO4, 1 mM EDTA, 0.15 M NaCl

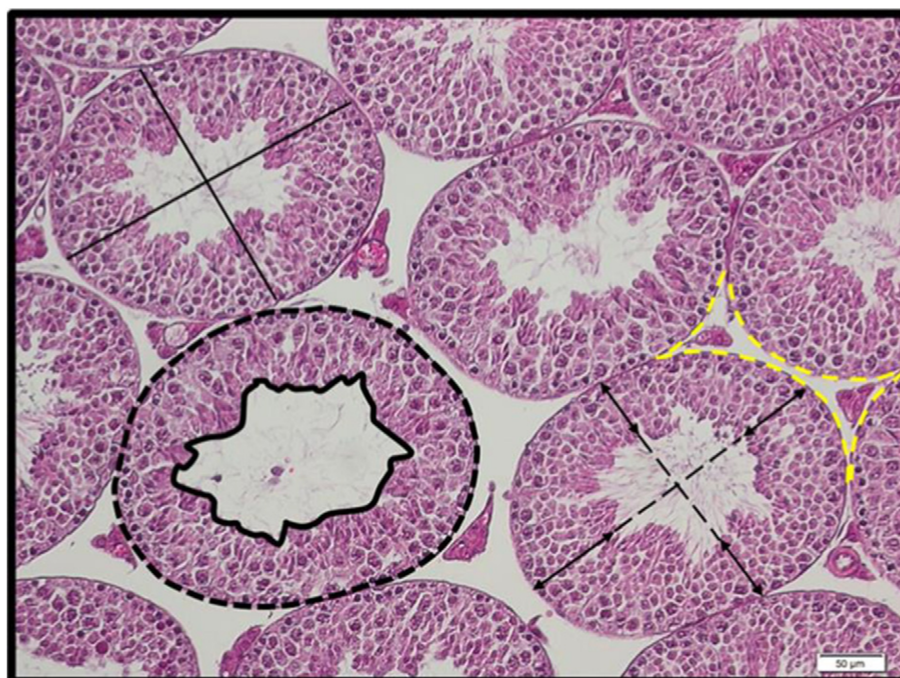

Fig. 1. Measurements of components of the seminiferous tubules. The thick dotted and continuous black lines define the circumference of the seminiferous tubule and lumen, respectively. The double-headed arrows, dotted and continuous black narrow lines show the epithelium thickness, lumen diameter and seminiferous tubule diameter, respectively. The percentage of total interstitial area in the testis was obtained by measuring the area occupied by the sum of all interstitial space (dotted yellow line). The percentage of total area occupied by seminiferous tubules was calculated by subtracting the obtained area occupied by interstitial area from the image area (bold black borderline).

and 0.6 μg/ml of AO). After 3–5 min incubation at 4 °C, ten thousand cells per sample were analyzed by flow cytometry (LSRII; BD Bioscience, CA, USA). Three parameters from the SCSA diagram were analyzed: DNA fragmentation index (DFI), which is the relationship between sperm cells with red fluorescence and total (red and green) fluorescence. The % DFI or percentage of cells with elevated DFI, was calculated from the DFI frequency histogram, and represents cells with an increased level of single-stranded DNA and is defined as the percentage of sperm that falls outside the main population in a sample. The main population was defined based on the DFI distribution of a control sample. The highly DNA stainable (% HDS) represents sperm cells with an elevated incorporation of AO into double-stranded DNA and is visualized as an increased green fluorescence. HDS cells are considered sperm cells with an incomplete chromatin condensation. For the flow cytometer set-up and calibration, a reference sample was established consisting of batches of control cauda epididymis sperm cells stored at −80 °C. A positive control sample was prepared by incubating the cauda epididymis sperm 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. Flow data were obtained and SCSA parameters were calculated using The BD FACSDiva software (v. 6.1.2) and FCS express (DeNovoSoftware, CA, USA; v.3), respectively.

2.8. Statistical analyzes

Data were analyzed using R Studio (R Studio Team 2015, version 0.99.473 for windows). To test the effect of the POP mixture on endpoints, a linear mixed effect model (LME) was employed. Morphometric measures, sperm count and SCSA data were introduced as dependent variables, exposure group as a categorical independent variable, and mothers as a random effect. Examination of the residual plots verified that no systematic patterns occurred in the errors (e.g. q-q plots). To assess individual doses to the controls, we used the contrast results provided within R. Prior to correlation analyzes; the normal distribution of data was tested using the Shapiro-Wilk test. If necessary, the data were log-transformed to meet the requirements for a parametric test. Correlations between endpoints were determined by Pearson correlation coefficient method in case of normal distribution and by Spearman's rank correlation coefficient in case of non-normal distribution. The limit of significance was set at $p < 0.05$. At the end, data were plotted using GraphPad Prism version 7.02 for Windows, (GraphPad Software, San Diego, California, USA).

Table 1
POP plasma levels in offspring mice.

	Control		Low		High	
	ng/g wet weight	ng/g lipid weight	ng/g wet weight	ng/g lipid weight	ng/g wet weight	ng/g lipid weight
Lipid content (%)	–	0.4	–	0.3	–	0.4
Chlorinated compounds						
PCB 28	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PCB 52	n.d.	n.d.	0.12	40.0	2.805	766.4
PCB 101	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PCB 118	0.282	72.0	0.369	123.3	11.829	3232.0
PCB 138	1.158	296.2	3.984	1332.5	49.756	13,594.6
PCB 153	1.160	296.8	2.891	967.0	48.939	13,371.3
PCB 180	0.330	84.4	0.774	258.8	14.000	3825.1
p,p'-DDE	n.d.	n.d.	0.299	99.9	1.707	466.5
HCB	0.274	70.0	0.747	249.8	9.512	2599.0
α - chlordane	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
oxy - chlordane	n.d.	n.d.	1.285	429.7	13.659	3731.8
trans-nonachlor	n.d.	n.d.	0.359	119.9	6.220	1699.3
α-HCH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
β-HCH	n.d.	n.d.	0.149	50.0	10.000	2732.2
γ-HCH (Lindane)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Dieldrin	n.d.	n.d.	1.355	453.0	16.829	4598.2
Brominated compounds						
BDE 47	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BDE 99	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BDE 100	n.d.	n.d.	0.090	30.1	0.61	166.6
BDE 153	n.d.	n.d.	n.d.	n.d.	1.098	299.9
BDE 154	n.d.	n.d.	n.d.	n.d.	1.951	533.1
BDE 209	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BDE 202[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BDE 196[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BDE 208[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BDE 207[a]	n.d.	n.d.	0.159	53.3	n.d.	n.d.
BDE 206[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
HBCD	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Perfluorinated compounds						
PFHxS	7.574	n.m.	16.385	n.m.	341.659	n.m.
PFOS	18.799	n.m.	30.443	n.m.	684.585	n.m.
PFOA	13.676	n.m.	27.558	n.m.	644.490	n.m.
PFNA	15.458	n.m.	24.799	n.m.	506.993	n.m.
PFDA	16.799	n.m.	29.038	n.m.	542.347	n.m.
PFUnDA	3.730	n.m.	6.571	n.m.	123.064	n.m.
PFDoDA[a]	n.d.	n.m.	n.d.	n.m.	n.d.	n.m.
PFTrDA[a]	n.d.	n.m.	n.d.	n.m.	n.d.	n.m.

(n.d) not detected; (n.m) not measured.Table was adapted from (Berntsen et al. 2016b).
[a] Break down products.

3. Results

3.1. POP levels in mice plasma

POP levels in dam and offspring plasma levels, adipose tissue and brain levels were reported in (Berntsen et al. 2016b). Here only offspring plasma levels are given. As demonstrated in Table 1, plasma POP levels (ng/g ww) in high exposed mice were almost 5 to 32 times higher than in low exposed mice except for β-HCH. The levels of all perfluorinated compounds and to a lesser degree some PCBs turned out to be positive in the control offspring plasma.

3.2. POP exposure affected testicular histology

Body mass was not affected by exposure neither at weaning nor at necropsy. However, exposure to POPs altered the structure of the seminiferous tubules. The POP mixture in both low and high concentrations significantly reduced and increased the relative total number of seminiferous tubules and the interstitial space, respectively (Fig. 2A). Our findings showed that the seminiferous tubule diameter decreased in both low and high exposed mice. However, this trend was statistically significant only in high exposed mice and lumen diameters as well as epithelial thickness were not affected significantly by POP exposures (Fig. 2B). Using area measures we found a statistically significant reduction in both lumen and epithelium area. As shown in Fig. 2C, POP exposure in both groups significantly decreased the area occupied by seminiferous tubules and lumen. Epithelium area was also reduced significantly in low exposed mice. Our results showed that the area measures correlated significantly with diameter measures (r = 0.911, 0.886 and 0.739, for tubules, lumen and epithelium area, respectively).

3.3. The POP mixture affected the number of epididymal sperm cells

POP exposure in both low and high groups resulted in a significant reduction in epididymal sperm count (Table 2). However, there was no significant correlation between number of epididymal sperm cells and total number of seminiferous tubules as well as epithelium thickness/area.

3.4. The POP mixture reduced sperm DNA integrity

Sperm cells collected from POP exposed mice showed increased DNA fragmentation. As demonstrated in Fig. 3A and B, POP exposures increased both DFI and % DFI in vas deferens sperm cells, but this trend was statistically significant only for the % DFI in high exposed

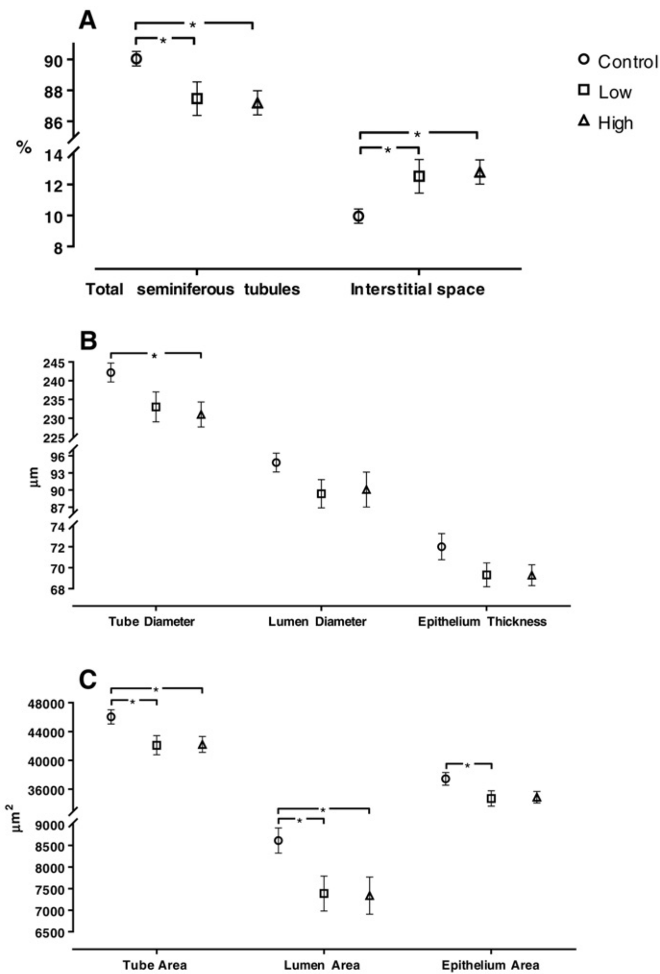

Fig. 2. Testis histomorphological measures in offspring mice after exposure to a mixture of POPs. (A) Percentage of total seminiferous tubules and interstitium in the testes; (B) Diameter of seminiferous compartments and (C) Circumference of tubules compartments. Data presented as mean ± SE. Asterisk showing results significantly different from control by linear mixed effect analyzes (* = $p < 0.05$).

group. Vas deferens sperm cells in exposed mice showed a higher degree of DNA condensation, but this trend was statistically significant only in low exposed mice (Fig. 3C). On the other hand, in cauda epididymis sperm cells, DFI and the % DFI were significantly increased in the low exposure group (Fig. 3A and B) and the % HDS was not statistically different compared to control (Fig. 3C).

4. Discussion

In this study, we demonstrate that our POP mixture, which made to mimic the realistic human exposure scenario, changed the area occupied by different parts of seminiferous tubules, reduced the number of sperm cells and induced sperm DNA fragmentation in the offspring of exposed mothers in a statistically significant manner.

Table 2
Epididymal sperm count in offspring mice after exposure to low and high dose of POP mixture. Data presented as mean ± SE. Asterisk showing results significantly different from control by linear mixed effect analyzes.

	Control	Low	High
Sperm count (10^4/mg cauda epididymis)	56.06 ± 4.95	42.58 ± 2.73*	36.39 ± 3.78**

* $p < 0.05$.
** $p < 0.005$.

Although some brominated and chlorinated compounds, which are normally detected in human blood, were not detected in exposed offspring mice, most of the POPs in the low exposed group were found in the range of 1–20 times human blood levels. Oxy-chlordane, dieldrin, BDE 100, PFNA and perfluorodecanoic acid (PFDA) were found at concentrations up to 70 times higher than in humans (Berntsen et al. 2016b). In this study, the plasma level (ng/g ww) of perfluorinated compounds in high exposed mice was close to what has previously been reported in polar bears (Bytingsvik et al. 2012). We noticed that some of the chlorinated and all of the perfluorinated compounds were found in the control group. This can probably be explained by airborne contamination through inhalation of feed dust. However, offspring were only exposed during fetal life and the suckling period, after which all animals were given the same feed and placed in another room separated from the dams. The control dams had lower levels of these same compounds than their offspring for unknown reasons (Berntsen et al. 2016b).

To date, little attention has been paid to how mixtures of POPs can affect male reproduction. However, previous investigations revealed that in utero exposure to a mixture of endocrine disrupting pesticides induced reproductive toxicity in rat male offspring (Hass et al. 2012; Jacobsen et al. 2012). Previous work suggested that the results of single compounds are not fully translatable to mixture scenarios, mainly because of unknown interactions between different chemicals in complex mixtures (Groten et al. 2001). In this study, gestational and lactational exposure to a mixture of POPs significantly decreased the seminiferous compartments area, while no such significant trend was found when we applied diameter measurement; hence measuring the area occupied by seminiferous compartments was more sensitive than measuring the compartments diameter. These differences can be explained in part by the proximity of diameter measures because not all of the seminiferous tubules were completely round and we measured diameter only in two axes. Although we found a close correlation between the values in both approaches, most of the studies reported the tubular diameter as an endpoint and our results further support the relationship between the effect of environmental pollutants and testicular structure. For instance, it has been reported that mice exposed to Bis(2-ethylhexyl) phthalate (DEHP) or PCBs (alone and in combination), showed smaller seminiferous tubule diameter, while the epithelial thickness was not affected (Fiandanese et al. 2016). Similar results were found in mice offspring upon lactation exposure to a mixture of PCBs (101 + 118) (Pocar et al. 2012). Another study reported that in utero exposure to PCB (118 + 153) mixtures in a range equal to 3 times higher (ng/g lipid weight) than the levels in this study, exerted subtle effects on developing fetal testis proteome but did not significantly disturb testis development in sheep (Krogenaes et al. 2014). There is little published data on the effect of perfluorinated compounds on seminiferous tubule structure. It has been documented that perfluorooctanoic acid (PFOA) disrupted the blood-testis barrier (Lu et al. 2016), and accumulated in mice testis (Zhang et al. 2014). Perfluorinated compounds like perfluorooctane sulfonate (PFOS) could induce histopathological lesions such as vacuolations in mice testis (Qu et al. 2016). Further studies need to be carried out in order to address the role of perfluorinated compounds on testicular structure.

Our results indicated that developmental exposure to a mixture of environmental pollutants affected the epididymal sperm count in a statistical significant way. Our finding is in agreement with previous studies where in utero exposure to PFOA was associated with a reduction in sperm concentration and total sperm count in humans (Vested et al. 2013). Furthermore, studies suggest that exposure to both PFOA (Zhang et al. 2014) and PFOS may impair sperm quality in mice (Wan et al. 2011). On the other hand, it has been shown that sperm concentration in mice was not affected by maternal exposure to BDE 209 (Tseng et al. 2013) or a mixture of PCB (101 + 118) (Pocar et al. 2012; Fiandanese et al. 2016). Furthermore, PCBs or *p,p'*-DDE levels in human plasma were not associated with the number of produced sperm cells (Toft et

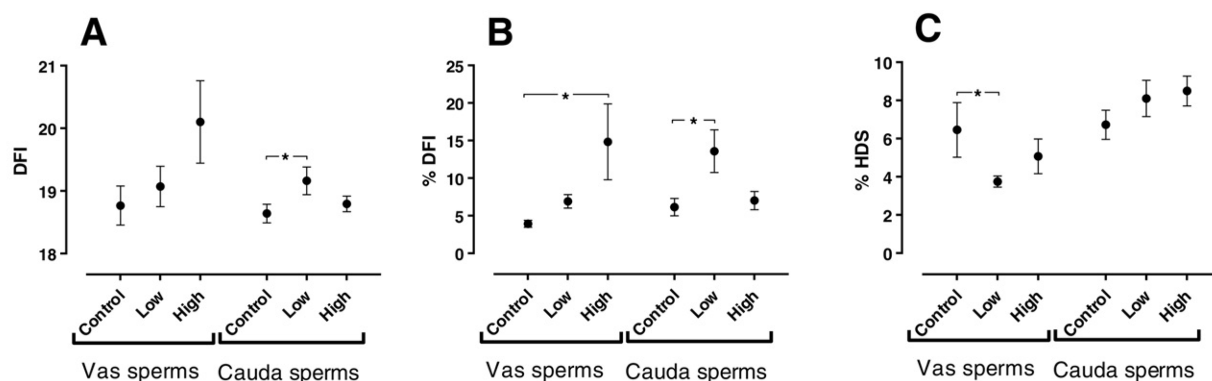

Fig. 3. Sperm chromatin structure assay of sperm cells collected from vas deferens (vas sperms) and cauda epididymis (cauda sperms) in offspring mice after exposure to a mixture of POPs. (A) DNA fragmentation index; (B) percentage of sperm cells with damaged DNA and (C) percentage of sperm cells with immature DNA condensation. Data presented as mean ± SE. Asterisk showing results significantly different from control by linear mixed effect analyzes (* = $p < 0.05$).

al. 2006; Jurewicz et al. 2009; Haugen et al. 2011). It has been suggested that the number of seminiferous tubules are correlated with the number of produced sperm cells (Montoto et al. 2012). Our results revealed no significant relationship between produced sperm cells and number of seminiferous tubules, which would indicate that, POPs could interfere with the spermatogenesis process via other pathways. It has been shown that some of the environmental chemicals cause meiotic and mitotic arrest in spermatogenesis process, a mechanism that might explain the reduction in sperm cell numbers. For instance, it has been shown that Bisphenol A (BPA) exposure increased the morphological abnormalities at all stages of the meiotic prophase, induced meiotic arrest and apoptosis of spermatogenic cells in both rat and mice (Ali et al. 2014; Xie et al. 2016). Moreover, DEHP induced mitotic arrest during spermatogenesis and increased sperm DNA fragmentation in zebrafish (Corradetti et al. 2013). Similarly, it has been reported that PFOS promotes apoptosis in germ cells and thereby decreases the number of sperm cells (Qu et al. 2016). The fact that we in the present study detected a high level of different compounds even in low exposed mice plasma, advocate for more research to determine the combined toxicities of perfluorinated compounds and the classical POPs in the spermatogenesis process.

Our SCSA analyzes suggest that the POP mixture could impair the cauda epididymis and vas deferens sperm DNA integrity. The induction of DNA fragmentation in the high dose group was only statistically significant when analyzed in vas deferens sperm. In addition, cauda epididymis sperm cells in exposed mice showed a lower degree of DNA condensation (% HDS) compared to vas deferens sperm cells. The reasons for this finding are not clear but could be explained by the differences in cauda epididymis and vas deferens luminal fluid composition. A higher level of DNA damage in sperm cells after incubation with vas deferens than epididymal luminal fluid has been reported suggesting that a program of abortive apoptosis occurs to a higher degree in vas deferens than in cauda epididymis sperm (Gawecka et al. 2015). Effects of POPs on mice sperm DNA integrity are not consistent across the literature. It has been reported that maternal exposure to BDE 209 in mice, increased the % DFI (Tseng et al. 2013) whereas exposure to PCB 153 (75 times higher than the level in our high exposed group), did not affect the sperm DNA integrity in mice (Oskam et al. 2004). On the other hand, PCB 153 at a level equal (ng/g ww) to our high exposed mice, increased the % DFI in 40 week old goats (Oskam et al. 2005), these results may be explained by the different developmental timing of the exposures and complexity of the POP mixture. To our knowledge, the only epidemiological study investigating the association between perfluorinated compounds and sperm DNA integrity, revealed no associations in men from three geographical regions (Specht et al. 2012). It is well documented that oxidative stress and reactive oxygen species (ROS) are both involved in the induction of sperm DNA fragmentation (Lopes et al. 1998; Wright et al. 2014). It has been shown that *p, p'*-DDE exposure, which was a part of our mixture, could affect the sperm DNA fragmentation via ROS production and mitochondrial dysfunction (Pant et al. 2014). Another study reported that exposure to PFOS and PCB 153 increased ROS production in Sertoli cells (Zhang et al. 2013a) and BDE 209 resulted in oxidative stress and increased the level of H_2O_2 in testis tissue (Tseng et al. 2006)… More research is needed to evaluate the role of POP mixtures in ROS induction in reproductive organs as well as in the different phases of spermatogenesis.

The main goal of the current study was to determine whether a human POP mixture based on the Scandinavian food basket could induce reproductive toxicity in male mice following maternal exposure. Our results showed that the POP mixture had a statistically significant effect on the seminiferous tubule compartments, sperm production and sperm DNA fragmentation. The current findings highlight the potential for reproductive toxicity following in utero and lactational exposure to a human relevant POP mixture. Further experiments are needed to investigate possible mechanisms of action and effects on male fertility.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgment

The authors acknowledge the Research Council (Funding number: NFR 21307/H10) of Norway for funding the project. We would like to thank students for labeling the sampling tubes and staff at experimental biomedicine at the Norwegian University of Life Sciences for excellent care for the animals during the weekends. The authors are grateful to Ruth Hansen and Gunn Østby, Kristine Hansen, Christer Johnsen, Cesilie Bjørklund at Norwegian institute of public health and Norwegian university of life science for their great support in preparing the testis slides and flow cytometry analyzes as well as technical support in mice handling. We gratefully acknowledge Dr. Thomas Fraser for proofreading the manuscript.

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