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Sperm DNA integrity in relation to exposure to environmental perfluoroalkyl substances – A study of spouses of pregnant women in three geographical regions

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ABSTRACT

Perfluoroalkyl substances (PFASs) can interfere with male reproductive function, but evidence in humans is limited. Six hundred four fertile men (199 from Greenland, 197 from Poland and 208 from Ukraine) were enrolled in the study. We measured four PFASs in serum (PFOS, PFOA, PFNA and PFHxS) and concurrent DNA damage in spermatozoa by sperm chromatin structure assay (SCSA) and in situ terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay, apoptotic markers in semen (Fas-receptor and Bcl-xL), and reproductive hormones in serum. No association between PFASs and SCSA, apoptotic markers or reproductive hormones emerged. We observed a slight increase in SHBG and TUNEL-positivity with increased PFOA exposure in men from Greenland. Thus, consistent evidence that PFAS exposure interferes with sperm DNA fragmentation, apoptosis or reproductive hormones was not found.

1. Background

Perfluoroalkyl substances (PFSAs) are man-made surfactants widely used in consumer and industrial products due to their water and lipid resistant properties [1]. They are identified as global pollutants and are suggested to pose health risks to humans and wildlife [1,2]. Of the PFASs, perfluorooctane sulfonate (PFOS) is found at the highest concentrations in humans as well as in the environment, followed by perfluorooctanoic acid (PFOA) [3,4]. In Europe, median serum concentrations in the adult non-occupationally exposed population spans from 8 to 62 ng/mL for PFOS [5] and from 0.5 to 40 ng/mL for PFOA [6]. Human half-lives have been estimated in geometric means to 4.8 (95% CI: 4.0;5.8) and 3.5 (95% CI: 3.0;4.1) years, respectively [7].

PFASs bind to blood proteins and accumulate mainly in liver and bile [6]. Animal studies demonstrate immunotoxicity, hepatotoxicity and developmental toxicity of these compounds [1], in addition to adverse reproductive effects. Thus decreased testosterone and increased estradiol serum levels were seen in male rats following administration of PFASs [8,9]. In mice, a recent study found reduced serum concentrations of testosterone and reduced epididymal sperm counts after daily exposure to 10 mg PFOS/kg for 21 days, but not to 5 and 1 mg PFOS/kg/day [10]. Moreover, expression of testicular receptors for gonadotrophin, growth hormone, and insulin-like growth factor 1 was considerably reduced in mice exposed daily to 10 or 5 mg PFOS/kg [10]. Mouse serum concentrations were approximately 30–300 folds higher than expected in the general human population; these findings might therefore not be of direct relevance for human toxicity. Moreover, two-generation rat studies did not observe changed mating or fertility after exposure

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to 3.2 mg PFOS/kg/day and 30 mg PFOA/kg/day [11,12]. Apoptosis in testicular cell populations possibly arise secondarily to effect on male reproductive hormones, as testosterone and gonadotrophin withdrawal from the testis have been shown to increase germ cell apoptosis in rats [3]. In a study of male rats exposed to perfluorododecanoic acid (PFDoA) testosterone was markedly decreased with Leydig, Sertoli and germ cells showing apoptotic features including condensed chromatin after doses of 5 or 10 mg PFDoA/kg/day [9]. Apoptosis of spermatogenic cells have also been observed in rats exposed to 5 mg kg/day of perfluorononanoic acid (PFNA), probably associated with the Fas receptor-dependent apoptotic pathway [13]. DNA strand breaks in the spermatozoa are suggested to be a sign of sperm apoptosis [14]. In vitro, PFOA have been observed to induce DNA damage, measured as DNA strand breaks, and oxidative stress in HepG2 cells and Vero cells [15,16]. However, a recent study found no statistically significant increase in oxidative DNA damage in rat testicular cells studied in vitro by the comet assay after exposure to 100 μM or 300 μM of four PFASs (PFOA, PFNA, 8:2 FTOH and 6:2 FTOH) [17].

A study of 256 men presenting at a clinic for fertility assessment found no adverse associations between plasma and seminal concentrations of PFOS and PFOA on volume, sperm concentration, percent motility, swim-up motility and concentration or directional motility [18]. However, a Danish study of 105 young men from the general population found a higher proportion of morphologically abnormal spermatozoa in men with higher levels of combined PFOS and PFOA [19], which may reflect higher levels of DNA damage in the spermatozoa [20,21].

DNA strand breaks can be detected by a variety of methods like the sperm chromatin structure assay (SCSA) [22] and the terminal deoxunocleotidyl transferase-driven dUTP nick end labeling (TUNEL) assay [23]. The TUNEL assay measures apoptosis by providing a direct measure of DNA strand breaks of the spermatozoa whereas SCSA indirectly reflects DNA damage through assessment of chromatin susceptibility to acid denaturation. DNA damage of the spermatozoa as measured by SCSA is a strong and reliable predictor of male fertility [24–26]. In a study of 229 male partners of infertile couples, DNA fragmentation index correlated positively and significantly with follicle stimulation hormone indicating that impaired spermatogenesis is associated with DNA damage [27]. Furthermore, sperm DNA damage is subject to less inter-individual variation over time than sperm concentration [22,28] To our knowledge, no epidemiologic studies have investigated sperm DNA damage or apoptotic markers in human spermatozoa in relation to environmental PFAS exposure. The aim of this study was to investigate the reproductive toxicity of PFASs by examining DNA fragmentation and apoptotic markers of spermatozoa in a large group of fertile men. Since some experimental studies indicated changes in testosterone levels as mediators of association between PFAS exposure and sperm DNA integrity, we also assessed serum levels of reproductive hormones.

2. Materials and methods

2.1. Study population

In the present study male partners of pregnant women were enrolled until at total of approximately 200 men had provided semen samples in each region [29]. In the original study altogether 3833 pregnant women and their male partners were encouraged to participate when presenting at the first antenatal care visit at one of three locations: (1) local hospitals in 19 cities and settlements throughout Greenland, (2) a large central hospital in Warsaw, Poland, and (3) three hospitals and eight antenatal clinics in Kharkiv, Ukraine. In all countries, it was required that both partners were 18 years or more of age and born in the country of the study. The participation rate among all couples who were asked to participate was 26% (640/2478) in Kharkiv, 68% (472/690) in Warsaw, and 90% (598/665) in Greenland [30]. In total 1710 pregnant women (45%) were included in the study.

The low participation rate in Kharkiv was a consequence of the recruitment procedure where contact between potential participants and the project team was managed by approximately 30 medical doctors at the three hospitals and eight antenatal clinics. With this large organization a high level of information and encouragement to participate was not possible.

Male partners were interviewed regarding lifestyle, occupational and reproductive history. The interview included questions regarding occupational factors, urogenital disorders, abstinence and issues regarding delivery of a semen sample. Information about smoking habits and diet (seafood intake, caffeinated drinks and alcohol consumption) was obtained as well, but with reference to the period when the couple tried to become pregnant. The questionnaires were translated to the native language and back translated to English for correction of errors that occurred during the translation process. All questionnaires were typed by hand twice to minimize errors during the typing process. If inconsistencies occurred between the two sets of typing, the original data was consulted and the typed data was corrected.

The local ethical committees representing all participating populations approved the study and all subjects signed an informed consent.

2.2. Collection of semen and blood samples

We collected semen and blood samples between May 2002 and February 2004 in 604 of the male spouses as described in [31].

Participants were instructed to collect a semen sample by masturbation at their residence after at least two days of sexual abstinence, and the duration of sexual abstinence was recorded. The sample was kept close to the body to maintain a temperature close to 37 °C when transported to the laboratory immediately after collection. Two nunc cryotubes (VWR International, Roskilde, Denmark) with 0.2 mL aliquots of undiluted raw semen, collected 30 min after liquefaction, were prepared from each semen sample, coded and directly transferred into a −80 °C freezer for later assessment of DNA fragmentation and expression of apoptotic markers. After liquefaction, semen samples were analyzed for motility and concentration of spermatozoa, according to recommendations by the World Health Organization [29]. Results of the conventional semen analysis will be the subject of another paper.

Venous blood samples were collected within 1 week of semen collection, except for 116 of the Greenlandic blood samples, which were collected up to 1 year in advance. The blood samples were centrifuged immediately after collection and sera were stored at −80 °C for later analysis [29].

2.3. Determination of perfluoroalkyl substances

All serum samples were analyses at the department of Occupational and Environmental Medicine in Lund, Sweden. Analysis for PFHxS, PFOS, PFOA, PFNA, PFDA, perfluoroundecanoic acid (PFUnDA) and perfluorododecanoic acid (PFDoDA) was performed using liquid chromatography tandem mass spectrometry (LC–MS/MS). Aliquots of 100 μL serum were added 25 μL of a water:acetonitrile (50:50) solution containing ^{13}C- or ^{18}O-labeled internal standards for all evaluated compounds (Wellington Laboratories Inc., Ontario, Canada; supplementary data). The proteins were precipitated with 175 μL acetonitrile and vigorously shook for 30 min. Samples were thereafter centrifuged and analyzed using a LC (UFLCXR, SHIMADZU Corporation, Kyoto, Japan) connected to a hybrid triple quadrupole linear ion trap mass spectrometer (QTRAP 5500, Applied Biosystems).

SRM transitions used in the analysis are described in supplementary data published online. Reported results represent the average of two measurements from the same sample worked-up and analyzed on different days. These results were also used to calculate the reproducibility of the method, determined as the coefficient of variation (CV) of duplicate samples worked-up and analyzed on different days. These data and the limits of detection (LOD) are also shown in supplementary data.

In all sample batches, the quality of the measurements was controlled by analyzing chemical blanks and in-house quality control samples. The method will be described in detail elsewhere.

In all sample batches, the quality of the measurements was controlled by analyzing chemical blanks and in-house quality control samples. The method will be described in detail elsewhere. Samples below LOD were included in the analyses by half the value reported from the mass spectrometer. PFDA, PFUnDA and PFDoDA were not detected in 112, 357 and 409 samples respectively, and due to the high prevalence of samples below detection limits, analysis were not performed for these compounds.

The analyses of PFOS and PFOA are part of the Round Robin inter-comparison program (Professor Dr. med. Hans Drexler, Institute and Out-Patient Clinic for Occupational, Social and Environmental Medicine, University of Erlangen-Nuremberg, Germany) with results within the tolerance limits.

2.4. Determination of DNA fragmentation by sperm chromatin structure assay (SCSA)

DNA damage was measured by SCSA following a strict and standardized procedure described by Evenson et al. [22].

All coded frozen semen samples from the three study regions were shipped on dry ice to the flow cytometry facility of the Section of Toxicology and Biomedical Sciences (ENEA Casaccia, Rome, Italy), for SCSA analysis. The percentage of sperm with detectable DNA fragmentation index (%DFI) was analyzed using a flow cyto-

metric (FCM) technique which identifies the spermatozoa with abnormal chromatin packaging, envisioned by increased susceptibility to acid-induced DNA denaturation in situ. Measurements were stopped when a total of 20,000 sperms had been accumulated for each sample. Acridine orange staining distinguished between denaturated (red fluorescence, single stranded) and native (green fluorescence, double stranded) DNA regions in sperm chromatin. %DFI was calculated from the DFI frequency histogram obtained from the ratio between the red and total (red plus green) fluorescence intensity using a dedicated software (SCSASoft; SCSA Diagnostic, Brookings, SD, USA) [22,29]. Flow cytometer set-up and calibration were standardized using a reference semen sample as described in Spanò et al. [29]. Inter-day SCSA variability, evaluated after 216 flow sessions and considering the coefficients of variation (CV) of the %DFI was 6.0%. In addition, 358 randomly chosen samples (50.6% of the total) were measured twice in independent FCM sessions. Results from the two measurements were highly correlated (DFI, $r = 0.96$).

Finally, the reliability and stability of SCSA measurements has also been challenged by an external quality control study that were carried out within the framework of this project, based on the blind analysis of three aliquots from seven different donors not selected among the participants in this study. The median inter-sample variability for %DFI, expressed as CV, was 1.5% [29].

2.5. Determination of DNA fragmentation by in situ terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay

All coded frozen semen samples from the three study regions were shipped on dry ice to the flow cytometry facility of Polytechnic University of March (Ancona, Italy) for TUNEL analysis. The presence of free 3′-OH termini in the DNA is indicative of a strand break. This fragmentation is detectable using an appropriately modified fluorescent nucleotide in an enzymatic reaction driven by terminal deoxynucleotidyl transferase. The TUNEL technique uses dUTP-FITC conjugated for the fluorescent labeling of free 3′-OH termini and is analyzed using flow cytometry, as described in details in [32]. Measurements were stopped when a total of 20,000 spermatozoa had been accumulated for each sample. The intra-laboratory CV regarding the TUNEL assay performance was constantly under 5%

2.6. Determination of sperm Fas and Bcl-xL positivity

As for the TUNEL assay, all coded frozen semen samples from the three study regions were shipped on dry ice to the flow cytometry facility of Polytechnic University of March (Ancona, Italy) for Fas and Bcl-xL analysis. To detect pro- (Fas) and anti- (Bcl-xL) apoptotic markers on spermatozoa, incubation with anti-Fas primary monoclonal antibodies was followed by a goat anti-mouse IgG-FITC conjugated secondary antibodies treatment, an indirect immunofluorescence. Whereas for Bcl-xL assessment, the primary monoclonal antibody anti-Bcl-xL was detected by a goat anti-mouse IgG-PE conjugated. Details are described in depth elsewhere [32]. 20,000 sperm cells were analyzed using flow cytometry. Two different control ejaculates, stored frozen at −80 °C in the laboratory, were thawed and processed according to the Bcl-xL and Fas protocol before starting every FCM run which ensured standardization and stability of the instruments. The intra-laboratory CV regarding the apoptotic markers was in the range from 6% for Fas and 9% for Bcl-xL [32].

The results are given as percent Fas- and Bcl-xL-positive sperm cells.

2.7. Determination of reproductive hormones

Serum concentrations of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and estradiol were analyzed with immunofluorometric techniques using the UniCel DxI 800 Beckman Access Immunoassay system (Chaska, MN, USA). The lower limits of detection (LODs) for the assays were 0.2 IU/L, 0.2 IU/L, and 8.0 pmol/L, respectively. The total assay coefficients of variation were 2.9%, 2.6%, and 8.1%, respectively. Serum testosterone levels were measured by means of a competitive immunoassay (Access; Beckman Coulter Inc., Fullerton, CA, USA) with an LOD of 0.35 nmol/L and a total assay CV of 2.8% at 2.9 nmol/L and 3.2% at 8.1 nmol/L. Sex hormone-binding globulin (SHBG) concentrations were measured using a fluoroimmunoassay (Immulite 2000; Diagnostic Products Corporation, Los Angeles, CA, USA). The LOD was 0.02 nmol/L. The total assay coefficients of variation were 5.5% and 4.6%, respectively. Inhibin B levels were assessed using a specific immunometric method, with a detection limit of 15 ng/L and intra-assay and total assay coefficients of variation <7%, as previously described [33,34]. All assays were performed at Malmö University Hospital (Sweden) after completion of sample collection.

2.8. Statistical analysis

General linear models (Proc GLM) were used to analyze uncorrected and adjusted associations, as well as trend analyses, between serum concentrations of PFASs and sperm DNA damage, apoptotic markers and reproductive hormones, respectively. The trend statistics were based on analysis of continuous outcome and exposure variables. Serum levels of the four exposure PFASs (PFOS, PFOA, PFNA and PFHxS) were divided into tertiles in each of the three study regions, Greenland, Poland and Ukraine. The outcome variables, %DFI, TUNEL-positivity, Fas, Bcl-xL, testosterone, estradiol, inhibin-B, FSH, LH and SHBG were analyzed on a continuous scale.

All analyses were stratified by study region. In the multiple general linear models we adjusted for known and possible confounders, namely, sexual abstinence period [log(days)], age (years), body mass index (BMI, kg/m^2), caffeinated drinks (cups/day), cotinine in serum [log(ng/mL)], fever during the past three month (yes/no), self reported genital infections (yes/no) and testicular disorders (yes/no), as well as for spillage of semen sample (yes/no) [35]. Alcohol consumption was not included in the analyses because these data were missing for 64% in Greenland, 14% in Poland and 26% in Ukraine.

Furthermore, we examined interactive effects of PFOS (dichotomized by the median) and the following covariates: age (dichotomized at age 35), smoking status when wife became pregnant (yes/no), cotinine levels in serum (dichotomized at the median), and PFOA (dichotomized by the median). The main effect, PFOS, was multiplied with each the covariates mentioned above. The outcome variables (%DFI, TUNEL-, Fas- or Bcl-xL-positivity) of the interaction analysis were on a continuous scale. Interaction effects were only examined for PFOS because it was the PFAS with the highest exposure level in the three study regions.

Correlations between the seven PFASs were examined by the non-parametric method Spearman's rank correlation.

Residuals were checked for normality and where appropriate, continuous variables were transformed by natural logarithm: DFI (%), TUNEL-positivity (%), Fas-positivity (%), Bcl-xL-positivity (%), testosterone, estradiol, inhibin-B, FSH, LH, SHBG, sexual abstinence time, cotinine and PFASs. Mean, median, adjusted mean and 95% confidence interval (95% CI) for DFI, Fas and Bcl-xL were back transformed for presentation in Tables 3–5.

Statistical analysis of the data was performed with SAS software, version 9.1 for Windows (SAS Institute Inc., Cary, NC, USA).

3. Results

Demographic and semen characteristics of the 604 adult men from Greenland, Poland and Ukraine with complete data are shown in Table 1. The number of missing values for Bcl-xL and Fas from Poland (49.7% and 37.6%, respectively) and Ukraine (78.4% and 32.7%, respectively) were high due to accidental loss of samples during shipment and insufficient number of cells for analysis. In Greenland, 27.6% Bcl-xL and 3.0% Fas samples were missing due to insufficient cell numbers. For reproductive hormone samples, 32% 41% and 0.1% were missing in Greenland, Poland and Ukraine, respectively.

Serum concentrations of the seven measured PFASs differed significantly among regions with the highest concentrations observed in Greenland (Table 2). PFOS occurred at the highest concentrations in all countries followed by PFOA. Serum levels of the individual PFASs were highly and significantly correlated, with correlation coefficients ranging from $r = 0.4$ (PFUnDA and PFOA, $p < 0.001$) to $r = 0.9$ (PFDoDA and PFUnDA, $p < 0.001$) (data not shown). No seasonal variations were observed for the PFASs. No significant interactions for PFOS and the covariates (age, smoking status, cotinine and PFOA) on DFI, TUNEL, Fas and Bcl-xL were revealed.

3.1. Sperm chromatin structure assay

An association of PFOS, PFOA, PFNA or PFHxS with sperm DNA fragmentation was not found in any of the three regions in uncorrected analyses, and similar results were obtained after adjustment for potentially confounding effects (results for PFOS and PFOA are given in Tables 3 and 4, respectively).

3.2. TUNEL assay

Serum PFOA was associated with increased percentage TUNEL-positive sperm cells in men from Greenland but only in the trend analysis [uncorrected $p = 0.077$, uncorrected trend $p = 0.026$ and adj. $p = 0.10$, adj. trend $p = 0.033$; adjusted means (95% CI) for %TUNEL-positive sperm cells in each PFOA tertile: 12.3% (5.2;28.9), 14.8% (6.1;35.9), 17.3% (7.2;41.32)]. No such associations were observed in Poland or Ukraine, nor were PFOS, PFHxS and PFNA associated with TUNEL-positivity.

Table 1
Characteristics of the study populations.

	Greenland (*N* = 199)					Poland (*N* = 197)					Ukraine (*N* = 208)				
	n	Percent[a]	Mean	Median	Range	*n*	Percent[a]	Mean	Median	Range	*n*	Percent[a]	Mean	Median	Range
Population characteristics															
Period of abstinence (days)	199		5.3	3.0	0.5–240	152		7.7	4.0	0.1–90	208		3.9	3.0	0.5–11
Seafood intake (week)	190		2.1	1.8	0–7	178		1.2	1.0	0–6	NA		NA	NA	NA
Age (years)	197		31.0	30.6	19–51	196		30.3	29.6	20–46	204		26.5	25.1	16–45
Body mass index (kg/m^2)	197		26.1	25.5	12–58	194		25.8	25.4	19–38	205		24.2	24.0	18–36
Caffeinated drinks per day	187		6.8	6.0	0.5–30	188		4.4	4.0	1–13	195		3.3	3.0	1–14
Cotinine (ng/mL)	196		165.1	143.5	0.3–570	190		39.9	0.31	0.3–446	205		153.5	119.7	0.3–799
Current smokers	198	72.7				196	27.2				206	66.7			
>14 alcoholic drinks a week	71	26.8				169	11.2				154	0.7			
Mother smoked during pregnancy	122	62.3				170	14.7				182	1.7			
Fever the last three month	196	13.3				195	8.7				203	9.9			
Employed	153	85.9				91	87.4				156	76.4			
Genital infection[b]	199	82.4				197	5.1				208	4.9			
Testicular disorders[c]	199	0.5				197	4.1				208	3.4			
Semen parameters															
DFI (%)	198		9.1	7.5	1–38	143		12.2	9.7	3–49	207		13.3	10.5	2–50
TUNEL positive (%)	198		4.5	2.9	0.3–45	134		15.2	13.1	0–80	133		9.3	6.7	0.02–50
Fas (%)	193		25.0	19.0	0–91	123		48.6	42.3	0–98	140		28.2	17.3	0–98
Bcl-xL (%)	144		26.5	10.7	0–97	99		18.1	9.3	0–90	45		66.0	75.5	0.2–99
Reproductive hormones															
Testosterone (nmol/L)	135		14.8	14.2	3–27	116		13.1	12.7	5–24	207		18.0	17.7	6–37
Estradiol (pmol/L)	135		65.5	65.3	33–113	116		75.8	69.0	36–297	206		84.1	79.1	33–160
FSH (IU/L)	135		4.9	4.3	0–15	116		4.0	3.6	0.7–17	207		4.2	3.4	1–21
LH (IU/L)	135		4.4	4.1	1–13	116		4.1	3.7	1–9	207		4.2	4.0	1–13
Inhibin B (ng/L)	135		184.2	181.0	48–470	116		157.9	153.0	22–338	207		194.6	186	55–390
SHBG (nmol/L)	135		28.2	27.9	11–55	116		23.6	21.7	6–64	207		27.7	26.8	9–64

[a] Percent if the variable is dichotomous.
[b] Genital infections: epididymitis, gonorrhea, Chlamydia or mumps in adulthood.
[c] Testicular disorders: treatment for retracted testis, surgery for varicose veins, torsio testis or testis cancer.
NA: not analyzed

3.3. Apoptotic markers

In all regions, the higher the PFOS serum concentration, the higher were the percentage of sperm cells positive for the pro-apoptotic marker Fas (Table 3). The trend was statistically significant for Polish men only (p trend = 0.03), but the association between the tertile means were not significant (Table 3), and an overall analysis including all three regions did not reach statistical significance (data not shown). No other associations between PFOA (Table 4), PFNA and PFHxS and apoptotic markers were consistent across regions or in models within regions.

3.4. Reproductive hormones

Serum concentrations of PFOA were associated with higher SHBG in Greenland and Poland, although this tendency was not significant after adjustment in Greenland (Table 5). PFOS, PFNA and PFHxS were not consistently related to SHBG concentrations (data not shown) and associations between PFASs and testosterone, estradiol and gonadootrophins were not consistent across regions (data not shown).

4. Discussion

The large majority of previous studies of alterations in sperm chromatin compared data from patients affected by infertility problems with a variety of control subjects. Overall, these studies indicate correlation between sperm DNA integrity and human fertility [13,36]. Data in the present paper represent, to our knowledge, the first study addressing effects of PFASs on sperm DNA fragmentation and apoptotic markers in semen collected from spouses of pregnant woman, representing all degrees of sub-fecundity except

Table 2
PFAS serum concentrations in men from Greenland, Poland and Ukraine.[a,b]

PFAS (ng/mL)	Greenland (*N* = 199)				Poland (*N* = 197)				Ukraine (*N* = 208)			
	Mean	Median	Range	%>LOD	Mean	Median	Range	%>LOD	Mean	Median	Range	%>LOD
PFOS	51.9	44.7	12–161	100	18.6	18.5	8–40	100	8.1	7.6	3–30	100
PFOA	4.8	4.5	2–14	100	5.1	4.8	2–16	100	1.8	1.3	0.3–35	92.1
PFNA	2.3	1.4	0.5–12	100	1.3	1.2	0.5–6	100	1.1	1.0	0.2–4	100
PFHxS	2.8	2.2	1–21	100	1.2	1.2	0.4–4	100	0.4	0.3	0.03–3	99.5
PFDA	1.1	0.9	0.1–6	99	0.4	0.4	0.1–1	96.3	0.3	0.2	0.1–1	51.2
PFUnDA	1.7	1.3	0.2–13	94.4	0.3	0.3	0.2–0.7	11.6	0.3	0.3	00.2–0.5	12.3
PFDoDA	0.2	0.1	0.04–2	80.6	0.1	0.1	0.04–0.2	8.4	0.1	0.1	0.04–0.1	3.0

[a] Missing values: there were 3 missing values for each PFAS in Greenland, 7 for each PFAS in Poland and 3 for each PFAS in Ukraine.
[b] The mean of PFOS, PFOA, PFNA, PFHXS, PFDA, PFUnDA and PFDoDA differs significantly among regions ($p < 0.0001$, ANOVA).

Table 3
DNA fragmentation index (DFI), Fas and Bcl-xL according to PFOS in serum by study populations.[a]

PFOS (ng/mL)	DFI (%)					Fas (%)					Bcl-xL (%)				
Tertiles	*n*	Median	Mean	Adj. mean[b]	(95% CI)	*n*	Median	Mean	Adj. mean[b]	(95% CI)	*n*	Median	Mean	Adj. mean[b]	(95% CI)
Greenland															
0.1–35.5	66	7.1	8.4	11.6	(6.2;21.7)	65	18.8	22.7	16.7	(5.8;48.2)	45	11.3	25.9	10.3	(5.2;20.5)
35.5–62.6	66	7.2	8.9	11.7	(6.1;22.3)	64	21.2	26.4	22.9	(7.7;68.4)	51	15.0	31.0	14.9	(7.7;28.9)
62.6–160.6	66	8.4	10.0	13.2	(6.9;25.1)	64	19.4	25.9	23.4	(7.9;69.3)	48	8.5	22.3	10.6	(5.7;9.7)
Poland															
0.1–14.9	49	9.8	12.2	14.4	(8.5;20.4)	38	31.1	44.1	13.7[c]	(5.5;34.2)	31	12.2	19.7	50.4	(8.4;301.9)
14.9–20.8	52	10.3	12.0	12.1	(8.7;16.9)	37	50.5	50.6	15.5	(6.3;38.3)	36	10.1	19.4	55.7	(13.5;229.4)
20.8–40.2	42	8.4	12.3	13.7	(10.0;18.7)	38	49.7	50.6	19.3	(8.2;45.9)	32	7.8	14.9	39.8	(7.6;208.3)
Ukraine															
0.1–5.9	67	11.5	14.0	14.4	(10.1;20.4)	47	17.6	28.1	13.7	(5.5;38.3)	12	74.8	64.6	50.4	(8.4;301.9)
5.9–8.5	69	8.8	12.5	12.1	(8.7;16.9)	45	14.8	25.8	15.5	(6.3;38.3)	17	88.0	70.0	55.7	(13.5;229.4)
8.5–29.9	69	11.8	13.6	13.7	(10.0;18.7)	46	20.3	30.8	19.4	(8.2;45.9)	14	75.4	62.3	39.8	(7.6;208.3)

CI = confidence interval.
[a] Non of the analyzed differences were statistical significant.
[b] Adjusted for age, body mass index, caffeinated drinks, cotinine, fever, spillage, abstinence time, genital infections and testicular disorders.
[c] *p*-Trend = 0.03 for adjusted Fas means according to PFOS in Polish men.

Table 4
DNA fragmentation index (DFI), Fas and Bcl-xL according to PFOA in serum by study populations.[a]

PFOA (ng/mL)	DFI (%)					Fas (%)					Bcl-xL (%)				
Tertiles	*n*	Median	Mean	Adj. mean[b]	(95% CI)	*n*	Median	Mean	Adj. mean[b]	(95% CI)	*n*	Median	Mean	Adj. mean[b]	(95% CI)
Greenland															
1.5–4.2	64	6.9	8.6	11.6	(6.2;21.6)	63	18.5	23.7	17.9	(6.1;52.2)	44	11.2	22.6	11.5	(5.7;23.2)
4.2–5.5	66	7.0	8.9	11.9	(6.2;22.5)	63	18.8	24.9	19.8	(6.6;59.6)	48	6.5	25.7	10.3	(5.2;20.6)
5.3–13.7	65	8.6	9.8	12.9	(6.8;24.4)	64	23.0	26.6	20.7	(7.0;61.5)	51	10.9	29.4	12.5	(6.9;22.8)
Poland															
1.5–4.2	52	8.5	12.1	15.4	(8.9;26.5)	41	50.6	50.6	33.8	(11.2;102.1)	41	50.6	50.6	2.1	(0.4;10.9)
4.2–5.7	40	9.6	12.3	15.4	(8.9;26.8)	35	40.5	45.7	35.8	(11.9;109.1)	35	40.5	45.7	1.9	(0.4;9.1)
5.7–16.0	50	10.3	12.1	14.7	(7.9;27.3)	46	44.7	49.7	43.4	(12.6;149.2)	46	44.7	49.7	2.0	(0.3;11.8)
Ukraine															
0.3–1.0	68	9.7	13.4	13.4	(9.6;18.8)	48	22.0	34.2	18.5	(7.6;45.4)	14	57.0	58.1	36.1	(7.0;84.5)
1.0–1.6	69	10.2	12.9	12.9	(9.3;17.9)	44	16.4	26.9	12.7	(5.0;32.2)	15	91.5	77.7	86.7	(18.4;407.8)
1.6–35.0	68	11.9	13.7	13.6	(9.8;18.9)	47	13.1	22.5	18.5	(7.5;45.6)	16	81.7	66.0	50.0	(13.4;187.0)

CI = confidence interval.
[a] Non of the analyzed differences were statistical significant.
[b] Adjusted for age, body mass index, caffeinated drinks, cotinine, fever, spillage, abstinence time, genital infections and testicular disorders.

sterility. The results are reassuring with no indications of adverse effects, except for a slight increase in %TUNEL positive sperm cells and SHBG associated with PFOA exposure in men from Greenland.

A previous investigation of relationships between serum levels of PFASs and semen quality in Danish young men (median age, 19 years) from the general population, found that a combination of high PFOS and PFOA levels were associated with lower numbers of normal sperm cells [19].

Associations of environmental chemical exposures like polychlorinated biphenyls and dichlorodiphenyldichloroethylene with DNA fragmentation in fertile populations have previously been identified by SCSA and TUNEL assay [29,37], but in the present study

Table 5
Sex-hormone binding globulin (SHBG) according to PFOA in serum by study populations and across regions.

PFOA (ng/mL)	SHBG (nmol/L)								
Tertiles	*n*	Median	Mean	p_{crude}	p_{trend}	Adj. mean[a]	(95% CI)	$p_{adj.}$	p_{trend}
Greenland									
1.5–4.2	44	24.3	25.3	0.03	0.01	30.5	(22.3;41.7)	0.07	0.08
4.2–5.5	45	30.1	29.4			35.6	(25.6;49.3)		
5.3–13.7	45	29.7	30.6			34.7	(25.2;47.7)		
Poland									
1.5–4.2	43	19.0	21.5	0.19	0.12	15.0	(10.7;21.0)	0.003	0.07
4.2–5.7	33	22.9	25.4			21.5	(15.3;30.2)		
5.7–16.0	40	23.4	24.5			17.8	(12.3;25.8)		
Ukraine									
0.3–1.0	69	25.9	27.6	0.93	0.74	26.8	(22.2;32.3)	0.77	0.82
1.0–1.6	69	27.6	28.0			27.6	(32.0;33.1)		
1.6–35.0	69	26.5	27.5			26.4	(22.0;31.7)		
Across regions									
0.3–2.4	188	26.9	27.8	0.09	0.14	24.7	(21.3;28.6)	0.06	0.39
2.4–4.7	144	23.5	25.4			24.2	(21.2;27.7)		
4.7–35.0	125	25.6	27.2			27.0	(23.6;31.0)		

CI = confidence interval.
[a] Adjusted for age, body mass index, caffeinated drinks, cotinine, fever, spillage, abstinence time, genital infections and testicular disorders.

Greenlandic, Polish and Ukrainian populations seemed unaffected or only marginally affected by the present level of PFAS exposure.

In rats, perfluorononanoic acid (PFNA) has been observed to induce sperm cell apoptosis at a dose level of 3 and 5 mg PFNA/kg/day, examined by changes in Fas mRNA expression levels in testis [13]. In our study, higher PFOS serum concentrations were associated with higher percentage of sperm cells positive for the pro-apoptotic marker Fas, but only for Polish men (p trend = 0.03) whereas no other associations between PFASs and apoptotic markers were consistent across regions or in different models within regions, which might be because of the low exposure levels.

Some animal studies of PFAS exposure and male fertility report increased estradiol and decreased serum testosterone in male rats and mice as well as changes in testicular cell populations [1,10]. However, another study of rats orally exposed up to 30 mg PFOA/kg/day found no evidence of altered testicular structure and function [12]. We found no consistent associations between PFOS and reproductive hormones except SHBG. Although decreased testosterone levels in testis can increase germ cell apoptosis in rats [38], the levels of PFASs might be too low to causally affect testosterone production in the adult human testis. Some epidemiological studies have addressed male reproductive toxicity of PFOS and PFOA and reported no significant associations with estradiol and testosterone levels [18,19,39]. One US occupational study of 782 men employed at a polymer plant in West Virginia reported a significant association between PFOA (median serum levels were 494 ng/mL) and serum estradiol and testosterone in linear regression models (regression coefficients = 22.3 pmol/L and 0.6 nmol/L, respectively) [40]. An occupational study of workers in a PFOA plant also observed significant and positive association between serum estradiol in men with the highest PFOA levels (>30000 ng/mL), however this observation was likely confounded by body mass index [39]. A study by Raymer et al. found positive correlation of LH with plasma PFOS and PFOA, but no associations with seminal parameters were observed [18]. Finally, a recent study of age of puberty found a relationship of reduced odds of having reached puberty, using total testosterone as an puberty indicator, with increased serum levels of PFOS in boys [41]. This indicates later onset of puberty or postponement of the testosterone surge in boys when highly exposed to PFOS (>19.4 ng/mL), but the association did not prove significant for PFOA [41] We observed a slight increase in SHBG with increased PFOA exposure in men from Greenland. SHBG is produced in the liver and PFOA have been found to be hepatotoxic in animal studies [1]. We speculate that high concentrations of PFOA may activate the peroxisome proliferator-activated receptor α (PPARα) that reduces lipids in the liver and insulin in the blood [42] and thereby up-regulates SHBG. However, this does not fit observations of decreased testosterone after PFAS exposure in rodents [2]. Furthermore, two cross-sectional studies of chemical workers with serum PFOA concentrations between 0 and 80,000 ng/mL (n = 111) and between 0 and 115,000 ng/mL (n = 80), respectively found no association between PFOA exposure and SHBG despite of higher exposure levels than observed in the present study [39].

The median PFOS level in the Greenlandic study population (45 ng/mL) was almost twice as high as in Danish (25 ng/mL) and American populations (21 ng/mL) [19,43], but at the same time the Greenlandic population considered in the present study is characterized by the lowest level of DNA fragmentation detected by SCSA [29,32]. In comparison, the highest level of DNA fragmentation was in the Ukrainian study populations, which had the lowest serum concentrations of PFASs. Overall, the %DNA fragmentation levels in the two European countries and in Greenland are in range with levels observed in other European populations [44,45].

The analysis of serum concentrations of PFASs, reproductive hormones, SCSA and TUNEL were analyzed in the same laboratories for all three study regions. Collection of semen and blood samples was done according to the same procedures in the three countries. The substantial variations in PFAS levels, %DFI, %TUNEL-positive and apoptotic markers between regions can therefore not be due to inter-laboratory variations or variations in procedures but are most likely explained by other factors like genetics, disease patterns, different food habits or unknown concomitant exposures.

Cross sectional studies of semen quality suffer often of low participation rates which may bias the internal validity of a study. In occupational semen studies, sub-fertility seems to be a stronger motivation for participation among referents than among exposed workers, who may have an interest to have potential harmful exposures documented [31]. Such a differential selection may, however, be less important in this environmental study where the participants may know little or nothing about their individual exposure level. Thus, low participation rates are not expected to bias the relation between exposure and semen characteristics [31]. Furthermore, selection bias is unlikely since the length of time to pregnancy did not differ among couples where men provided a semen sample in comparison with couples where men declined to deliver semen [32]. The participation rate was low in Ukraine (26%) due to the recruitment procedure. Demographic and reproductive information was obtained from a sample of 605 of those Ukrainian women that declined participation in the study. Only average age was slightly lower among non-participating women [22.8 (SD 2.4) versus 24.9 (SD 2.8) years] [30].

In Greenland 116 of the 199 blood samples were collected approximately a year before the semen samples. Since half-lives for PFASs in humans are in the range of years [7] and no seasonal variations in PFAS exposure was observed, bias because of skewed sampling is unlikely.

5. Conclusion

Our data do not suggest that exposure to PFOS, PFOA, PFHxS and PFNA in men of pregnant women is associated with DNA damage or apoptosis in sperm cells or changes in serum levels of reproductive hormones (estrogen, testosterone, FSH, LH). We did observe a slight increase in SHBG and TUNEL-positivity with increased PFOA exposure in men from Greenland, though not consistent or in line with previous study results.

Conflict of interest

The authors have no financial interest or other conflicts of interest in the publication of these results.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.reprotox.2012.02.008.

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