Relationships between sperm DNA fragmentation, sperm apoptotic markers and serum levels of CB-153 and p,p'-DDE in European and Inuit populations

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Abstract

Persistent organochlorine pollutants (POPs) are suspected to interfere with hormone activity and the normal homeostasis of spermatogenesis. We investigated the relationships between sperm DNA fragmentation, apoptotic markers identified on ejaculated spermatozoa and POP levels in the blood of 652 adult males (200 Inuits from Greenland, 166 Swedish, 134 Polish and 152 Ukrainian). Serum levels of 2, 2', 4, 4', 5, 5'-hexachlorobiphenyl (CB-153), as a proxy of the total POP burden, and of 1,1-dichloro-2,2-bis(p-chlorophenyl)-ethylene (p,p'-DDE), as a proxy of the total DDT exposure were determined. Sperm DNA fragmentation was measured by using the TUNEL assay, whereas immunofluorescence methods were utilized for detecting pro-apoptotic (Fas) and anti-apoptotic (Bcl-xL) markers. Both TUNEL assay and apoptotic markers were statistically differed across the four populations. No correlation between neither sperm DNA fragmentation nor apoptotic sperm parameters and the large variations in POPs exposure was observed for the separate study groups. However, considering the European populations taken together, we showed that both %TUNEL positivity and Bcl-xL were related to CB-153 serum levels, whereas our study failed to demonstrate any relations between DDE and %TUNEL positivity and apoptotic sperm biomarkers (Fas and Bcl-xL) in any region or overall regions. These results suggest that CB-153 and related chemicals might alter sperm DNA integrity and Bcl-xL levels in European adult males, but not in the highly exposed Inuit men. Additional issues (genetic background, lifestyle habits and characterization of total xeno-hormonal activities) need to be investigated in order to fully assess the population variations observed.

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Introduction

The release of some synthetic chemicals into the environment with the potential to disrupt normal sexual development, differentiation and function in humans (Silvestroni & Palleschi 1999, Rozati *et al.* 2002, Sharpe &

Irvine 2004) is drawing ever-increasing attention. Many man-made chemicals, such as phenolic-like compounds and phthalate esters, but also persistent organochlorine pollutants (POPs), e.g. chlorinated pesticides and polychlorinated biphenyls (PCBs), have toxic capabilities and several possess weak hormone-like actions or can act as

endocrine disruptors (Bonefeld-Jorgensen *et al.* 2001, Sumbayev *et al.* 2005). These exogenous molecules can arise from industrial and domestic effluents or agricultural and urban runoff (Jaspers *et al.* 2005, Krauss & Wilcke 2005). The release of these compounds in the environment leads to greater pollution, in particular aquatic and marine sediments (Gustafsson *et al.* 2005, Persson *et al.* 2005). The general population continues to be exposed through ingestion of contaminated food (fish, meat and dairy products; Golec *et al.* 2003), while some areas are subjected to greater risk due to geographical and cultural reasons (Deutch & Hansen 2000, AMAP 2002). This fact is particularly true for men who are habitual consumers of sea food, for example, Swedish fishermen and Inuits.

Another particular population exposed to these pollutants is the one in Ukraine where DDT, although officially banned in the late 1970s (Kundiev 1994, Gladen *et al.* 1999), was still used until the late 1980s (Li *et al.* 2005). There are limited and somehow contradictory epidemiological evidences on whether PCBs, and possibly DDT/DDE can damage human sperm DNA (Rozati *et al.* 2002, Hauser *et al.* 2003a, Rignell-Hydbom *et al.* 2005). Reasons for this inconsistency might be different methodologies used to detect sperm DNA damage, varying exposure ranges to and mixtures of POPs, and different inclusion criteria for the studies. These results are of concern as sperm DNA integrity is essential for the accurate transmission of genetic information (Evenson *et al.* 2002, Agarwal & Said 2003, Perreault *et al.* 2003, Sakkas *et al.* 2003a). Therefore, the INUENDO study was initiated with the aim to establish if there is a relationship between exposure to selected POPs and the impairment of human semen quantity and quality in four populations: Swedish fishermen, Ukraine men and Greenlandic Inuits, known to be highly exposed and Polish people, chosen as representative of a European population with unknown exposure levels. Studies of these populations using the Sperm Chromatin Structure Assay (SCSA), an indirect test able to detect chromatin defective sperms, have shown that exposure to PCBs can damage sperm cells (Rignell-Hydbom *et al.* 2005, Spano *et al.* 2005a). Another question, which has never been investigated, regards the possible relationships between endocrine disruptors and apoptotic mechanisms in male germ cells. Male germ cell differentiation, in the adult, is known to be physiologically regulated by a finely tuned apoptotic mechanism occurring at specific stages of the spermatogenic process. Apoptosis is believed to play a key role in maintaining the correct number of germ cells so that Sertoli cells can support and eliminate abnormal cells (Blanco-Rodriguez & Martinez-Garcia 1996a, 1996b, Guo *et al.* 2000). Human ejaculated sperm cells constitute a very heterogeneous population, which have been demonstrated to contain a fraction of cells showing phenotypic features typical of apoptosis, for example, DNA fragmentation and expression of anti- and pro-apoptotic proteins, such as Bcl-xL, caspase-3 and Fas (Lee

et al. 1997, Sakkas *et al.* 1999a, 1999b, 2004, Oldereid *et al.* 2001, Cayli *et al.* 2004, Morrell *et al.* 2004). Many of these markers can efficiently be measured by flow cytometry (Wing *et al.* 1990, McVicar *et al.* 2004) and, in particular, DNA breaks can be detected by a variety of methods, such as the Comet assay (Migliore *et al.* 2002, Singh *et al.* 2003), the SCSA (Spano *et al.* 1998, Evenson *et al.* 2002) and the Terminal deoxynucleotidyl transferase-driven dUTP Nick End Labelling (TUNEL) assay (Gorczyca *et al.* 1993, Sakkas *et al.* 2002, Sergerie *et al.* 2005). The TUNEL assay functions by directly labelling the 3'-OH termini recognizing both single- and double-strand DNA breaks (Gillan *et al.* 2005). DNA fragmentation, as evaluated by the TUNEL assay, has been shown to be a negative predictor of successful pregnancy in the context of assisted reproduction technologies, and has also been related to recurrent miscarriages (Seli & Sakkas 2005).

The presence of apoptotic markers on the mature male gamete has been explained by a variety of theories, among them the 'abortive apoptosis theory' (Sakkas *et al.* 1999a, 2003b). The latter partly explains the possible coexistence of both DNA fragmentation and apoptotic-like markers in ejaculated spermatozoa and shows a distinct link to problems in spermatogenesis (Cayli *et al.* 2004). These markers have prevalently been studied in males with infertility problems, but limited knowledge is available in fertile men or in populations with unknown fertility status.

As a part of the Inuendo project, we studied ejaculated spermatozoa from men representing these different populations for sperm DNA fragmentation using the TUNEL assay and for the expression levels of apoptotic markers. We hypothesized that the serum concentrations of selected POPs could be associated with altered integrity of sperm DNA and levels of pro- and anti-apoptotic markers in human semen and therefore may provide an insight into the effect of POPs on sperm DNA integrity and vulnerability.

Materials and Methods

Study population

Pregnant couples were recruited in 19 settlements throughout Greenland, in Warsaw, Poland and in Kharkiv, Ukraine. Pregnant women and their partners were consecutively enrolled during antenatal visits at the local hospital or health clinic (Greenland), a large university hospital in Warsaw and 11 antenatal clinics or maternity hospitals in Kharkiv. Moreover, an already established cohort of Swedish fishermen possessing POPs serum concentrations spanning more than one order of magnitude (Rignell-Hydbom *et al.* 2004) were enrolled separately and independent of present pregnancy of the spouse, however, 79% out of the Swedish fishermen had fathered a child (Jonsson *et al.* 2005,

Toft *et al.* 2005b). Only men of 18 years of age or more than were born in the country were eligible. Recruitment and data collection took place from May 2002 to February 2004. The consecutive enrolment of men for the semen studies was terminated when some 200 men in each of the four cohorts had been enrolled. The participation rates were 79% for Greenland, 10% for Sweden and 30% for both Warsaw and Kharkiv. The overall participation rate in this project was 18%. Information about demographic factors, lifestyle, reproductive history and andrological factors were obtained by interview, according to a uniform protocol (Toft *et al.* 2005b).

Altogether 798 men provided a fresh semen sample: Greenland (201 men), Sweden (191 men), Warsaw (198 men) and Kharkiv (208 men). Due to a limited amount of semen and a missing sample shipment from Ukraine, we successfully analysed from 200 Greenland samples for TUNEL, 195 for Fas and 146 for Bcl-xL; from Sweden 166 samples for TUNEL, 155 for Fas and 141 for Bcl-xL; from Warsaw 134 samples for TUNEL, 123 for Fas and 99 for Bcl-xL; from Kharkiv 152 samples for TUNEL, 140 for Fas and 44 for Bcl-xL. Since there was no significant difference in exposure level, age and seminal parameters between subjects from whom apoptotic parameters were obtained and those excluded from the study, we did not consider that selection bias was of major concern.

The local ethical committees representing all participating populations approved the study and participants gave informed consent declarations.

Semen sampling

Semen samples were collected by masturbation at the participant's residence (Sweden and Greenland) or in the privacy of a collection room at the hospital (other regions). The subjects were asked to abstain from sexual activity for at least 2 days before collecting the sample and to note the actual abstinence time. All participants were supplied with an identical type of semen collection device (Sarsted 100 ml polyethylene) and instructed to keep the sample close to the body, to maintain a temperature close to 37 °C during transportation 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 (WHO 1999) and briefly vortexed, were prepared from each semen sample, coded and stored immediately in dry ice or a -20 °C freezer and within 2 weeks transferred into a -80 °C freezer.

The semen parameters considered in the selection evaluation were concentration (millions/ml), progressively motile (%motile sperm as defined by WHO category a+b) and %normal sperm as defined in the WHO (1999) manual for semen analysis (for details, see Toft *et al.* 2006).

One person performed the semen analysis in each of the four regions. All four were trained in a quality control

program set up specifically for this study. The median coefficient of variation (CV) among investigators in estimating sperm concentration and the proportion of progressive motile sperm was 8 and 11% respectively (Toft *et al.* 2005a).

Blood sampling and determination of CB-153 and p,p'-DDE

Venous blood samples were collected into 10 ml vacuum tubes and after centrifugation, the serum was stored at -80 °C until later analysis.

The levels of CB-153 and p,p'-DDE were determined by the Department of Occupational and Environmental Medicine of Lund University Hospital applying solid-phase extraction using on-column degradation of the lipids and analysis by gas chromatography mass spectrometry as previously described (Jonsson *et al.* 2005). Levels of detection, coefficients of variation and participation in quality control programs have been described in detail elsewhere (Jonsson *et al.* 2005). Serum lipids were analysed with enzymatic methods (Jonsson *et al.* 2005).

Determination of the sperm concentration in semen

The analysis of apoptosis marker expression and DNA fragmentation requires a number of sperm cells ranging between 5×10^5 and 2×10^6 . Previous testing of semen samples at known concentrations (using the CASA assay) permitted us to verify that gentle mixing of the sample and removal of only 5 µl showed a strong correlation ($P < 0.001$, data not shown) between the value of concentration obtained with cytometry and the CASA count.

Flow cytometry was used to establish an indicative value of concentration of the samples by mixing 5 µl of every sample, diluted with 595 µl of a 1 µg/ml solution of propidium iodide (PI). The samples were then left in the staining solution for 15 min on ice and then analysed by means of a flow cytometer (FCM). The instrument was adjusted to allow the use of only 20 µl of every diluted sample. Applying the correct dilution factor, we established the approximate concentration for every sample to be used to run the test samples on an equal footing. For every assay an aliquot of 2×10^6 cells from the thawed sample was centrifuged (4 °C, 700 g × 8 min) in 4 ml of 0.1% (w/v) PBS/BSA (washing solution) in a cytometry tube. The pellets obtained were used for the next three assays (TUNEL, FAS and Bcl-xL).

Determination of DNA fragmentation: in situ nick-end labelling (TUNEL) assay

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. The TUNEL technique uses dUTP-FITC conjugated for the fluorescent labelling of free 3'-OH termini (Gorczyca *et al.* 1993). The pellet obtained previously was fixed in 100 µl of 1% (w/v) paraformaldehyde (PFA) on a shaker for 1 h at 4 °C and then washed with 4 ml washing solution, centrifuged (4 °C, 700 g × 8 min) and the supernatant was discarded. The pellet was then permeabilized with 100 µl of 0.1% (w/v) Na-citrate/Triton-X 100 (permeabilization solution) for 2 min at 4 °C; subsequently, 50 µl suspension was drawn for the negative control and the two new aliquots were washed with 0.1% (w/v) PBS/BSA, centrifuged as above and the supernatant discarded.

To both tubes, we added 40 µl reaction mix (with and without the TdT enzyme for the positive and negative samples respectively) and incubated for 1 h at 37 °C in the dark.

The pellet obtained after the washing step was fixed with 100 µl of 0.5% (w/v) PFA and 1 µg/ml PI solution in PBS. Samples were stored at 4 °C in the dark overnight until the FCM analyses.

Determination of semen Bcl-xL and Fas positivity

The pellet was fixed with 100 µl PFA (2% (w/v) solution on a shaker for 20 min on ice for Bcl-xL and 1% solution for 10 min at 4 °C for Fas). Subsequently, 50 µl of the suspension was drawn for the negative control; the cells were washed and centrifuged as above.

To the Bcl-xL sample tube, we added 100 µl permeabilization solution and 0.5% (w/v) BSA with and without primary antibody (20 µg/ml) for the sample and the negative control respectively. To the Fas sample tube, we added 40 µl primary antibody diluted in 0.5% (w/v) PBS/BSA and 40 µl of 0.5% (w/v) PBS/BSA to the negative control tube.

After the incubation (1 h at 37 °C), both the samples and the negative controls were washed and the pellets resuspended in the diluted secondary antibody (for Bcl-xL 50 µl Goat anti-mouse IgG-PE conjugated 1/100 in PBS+0.3% (w/v) BSA and for Fas 100 µl Goat anti-mouse IgG-FITC conjugated; 1/100 in PBS+0.5% (w/v) BSA and incubated for 1 h at 37 °C).

After the final washing step (PBS+0.5% BSA (w/v)), the cells were fixed in 100 µl of 0.5% PFA (w/v). The Bcl-xL sample were stained with 7-aminoactinomycin D (100 µg/ml on use) and kept in the dark at 4 °C until run on the FCM machine (24 h). The Fas fixed samples were stored in the dark at 4 °C (24 h) and 15 min before the FCM analysis, the suspension was stained with 1 µg/ml PI.

Two different control ejaculates (C1 and C2), stored frozen at -80 °C in our laboratory, were thawed and processed according to the TUNEL, Bcl-xL and Fas protocol before starting every FCM run.

All reagents used were molecular biology grade. The primary and secondary antibodies were from IL

(Instrumentation Laboratory, Milano, Italy); BSA fraction V, PBS, Na-citrate, Triton-X 100 and PI from Sigma Pharmaceuticals (Sigma-Aldrich), 7-AAD from Molecular Probes (Invitrogen) and secondary antibody blocking peptide from Santa-Cruz Biotec (Santa-Cruz Biotechnology, California, CA, USA).

Flow cytometry

Flow cytometry was performed using an Epics XL (Beckman Coulter-IL, Fullerton, CA, USA). In all analyses, spermatozoa labelled with only the secondary antibody were assessed as controls. Debris was gated out based on light scatter measurements. A minimum of 10 000 spermatozoa per sample was analysed.

For quality assurance in relation to the sperm apoptotic markers, we carried out experiments aimed to estimate the intra-laboratory CV regarding the apoptotic markers utilized for the INUENDO project. To measure for variability, two different ejaculates (C1 and C2), stored frozen at -80 °C in our laboratory, were thawed and processed according to the TUNEL, Bcl-xL and Fas protocol at each start-up of the FCM. This ensured standardization and stability of the instrument from sample to sample and from day to day. The intra-laboratory CV regarding the apoptotic markers utilized for the INUENDO project was in the range from 6% for Fas to 9% for Bcl-xL. The intra-laboratory CV regarding the TUNEL assay performance was constantly under 5%.

Statistical analysis

The statistical analysis was performed with S-PLUS 6.1 and SAS 9.1 (SAS Institute, Inc., Cary, NC, USA). Normal distribution was checked by Kolmogorov-Smirnov test. Natural logarithmic transformation of the outcome variables %TUNEL positivity, Bcl-xL and Fas immunoreactions as well as of the lipid adjusted serum levels of CB-153 and p,p'-DDE improved the normal distribution. The significance threshold was set at 5%.

A general linear model was used to analyse endpoints measured on a continuous scale and all analyses were stratified by the four study groups, corresponding to the four different regions (Greenland, Kharkiv, Sweden and Warsaw). The comparisons of means between the different variables were performed by the one-way ANOVA test, whereas the *post hoc* Tukey's test was performed in order to test the difference between each pair study groups.

Since the frequency of gene polymorphisms regulating male reproduction seems to differ substantially between Inuits and Caucasians (Giwerzman Y, personal communication) and findings in this project indicate deviating levels of sperm chromatin integrity (Spano *et al.* 2005b), the analyses were stratified on Inuits and

Europeans. In this connection, we grouped Inuit and European people into three categories by serum levels of CB-153 and p,p'-DDE. Cut-off values were selected as trade offs between numbers in each category, contrast of exposure and ranges within each interval – for CB-153 (ng/g lipid): 0–50, 51–200 and >200; and for p,p'-DDE (ng/g lipid): 0–500, 501–1000 and >1000.

All analyses were performed for CB-153 and p,p'-DDE separately and we adjusted for abstinence time and age (both on a log scale) in these analyses. Furthermore, we evaluated the potential confounding effects of the following variables: season for sample collection (winter, spring or summer; yes/no); fever in the past 3 months (yes/no), spillage during semen sample collection (yes/no), current smoking status (yes/no) and self-reported urogenital infections (e.g. epididymitis, chlamydia, gonorrhoea or mumps in adulthood (yes/no) and urogenital surgery (e.g. treatment for cryptorchidism, torsio testis, testis cancer or varicose scrotal veins; yes/no). None of these potential confounders changed the estimate more than the limit of 10% suggested by Greenland (1989), when included in the model in addition to the *a priori* chosen confounders (age and abstinence time). Alcohol information was only present for three out of the four populations and only very few subjects had a high alcohol consumption (>21 drinks/week). Therefore, instead of including the variable as a potential confounder, we made a sub-analysis only including people with ≤21 drinks/week and obtained results not deviating more than 10% compared with the analysis including all subjects.

Results

Sperm DNA fragmentation (TUNEL) and apoptosis markers for the four study groups

The characteristics of the study populations are displayed in Fig. 1.

One-way ANOVA test demonstrated that the levels of sperm DNA fragmentation (evaluated by the TUNEL assay) as well as the percentage of sperm cells displaying Fas and Bcl-xL positivity were significantly different ($P < 0.001$) among the four study populations.

In particular, the complementary multiple comparison *post hoc* Tukey's method highlighted that the values of %TUNEL positivity in the Inuit group were significantly lower than all the other populations (all P values < 0.001). Moreover, the Warsaw study group had significantly higher %TUNEL positivity compared with the Kharkiv population ($P < 0.001$), whereas there were no significant differences in TUNEL between Warsaw and Sweden, and Sweden and Kharkiv respectively. The percentage of Fas positivity was significantly higher in Warsaw men compared with all others groups (all P values < 0.001), whereas Kharkiv men showed higher %Bcl-xL positivity than the other study groups (all P values < 0.001).

Association between POP and apoptotic markers

The relationships between CB-153 and p,p'-DDE (considered as continuous variables) and both %TUNEL positivity and the apoptotic markers were investigated by

Figure 1 The middle black line in each box is the median of (A) CB-153, (B) p,p'-DDE, (C) age at the date of semen sampling, (D) abstinence days, (E) the fish consuming per day, (F) percentage of TUNEL, (G) Fas, (H) Bcl-XL positivity by region (GR, Greenland; PL, Warsaw; SE, Sweden; UA, Kharkiv). The values include in the box represent the 25 and 75th percentiles. Error bars indicate 95% confidence limits.

correlation analyses, but no significant associations were observed for the separate study groups.

In addition to analyses based on unadjusted continuous exposure variables, we also analysed adjusted sperm DNA integrity and apoptotic markers as continuous variables and by categories of serum POP levels. In these analyses, we stratified the analyses on Inuit and European men. Table 1 shows that %TUNEL positivity was significantly higher (almost doubled) in Caucasians with CB-153 serum levels within the high and middle exposure groups as compared with those within the lowest group of exposure (reference group). Moreover, %Bcl-xL positive sperm was positively associated to CB-153 in Caucasians but not in Inuit's, whereas no evidence was found that Fas was related to serum levels of CB-153 in neither Inuits nor Caucasian men. Our study failed to demonstrate any relationship between p,p'-DDE and %TUNEL positivity and apoptotic sperm biomarkers (Fas and Bcl-xL) in any region or overall regions.

Discussion

The INUENDO project aimed to address the question of whether POPs can impair human reproductive function and, in particular, if highly exposed populations, such as the Inuit people, are at greater risk. The overall project was, therefore, designed to obtain a vast contrast of exposure between participating countries. The main purpose of the present study was to examine whether

DNA damage and specific apoptotic marker proteins in the ejaculated spermatozoa from individuals recruited from these different populations provided clues as to the effect of POPs on sperm DNA integrity and vulnerability. Despite the observed variations in POP exposure levels between the four study groups, we did not observe consistent differences in apoptotic sperm parameters that paralleled the differences in serum levels of POPs. However, considering the European populations taken together, we showed that both %TUNEL positivity and the anti-apoptotic marker Bcl-xL were associated to CB-153 serum levels, whereas no such associations were found for the Inuits. Moreover, our study failed to demonstrate any relation between DDE and both %TUNEL positivity and apoptotic sperm biomarkers (Fas and Bcl-xL) in any region or overall regions.

The participation rate differed among the study groups and was exceptionally high in Greenland. Although the participation rates were lower in the three European regions, however, at levels typical of many other semen studies (Bonde *et al.* 1996), selection bias is an unlikely explanation of the findings since the length of time to pregnancy did not differ between couples with men delivering an ejaculate for the study and those with men refusing to give a semen sample (G Toft, personal communication). Moreover, the clear exposure response patterns in Swedish fishermen and in men from Kharkiv are unlikely to be produced by selection of men during the recruitment phase of the study.

Table 1 Adjusted geometric mean values and linear regression coefficients of sperm DNA fragmentation, sperm apoptotic markers in Inuit and Caucasian men as a function of categorized CB-153 and DDE level. All analysis among Caucasians were adjusted for study group (Warsaw, Kharkiv and Sweden).

	Inuits			Caucasians			Inuits		Caucasians	
	CB-153 ng/g lipid			CB-153 ng/g lipid			Linear regression coefficient (95% CI)		Linear regression coefficient (95% CI)	
	0–50	51–200	>200	0–50	51–200	>200				
Sperm DNA fragmentation ^{a,b}										
In TUNEL, %	3.5	3.2	2.6	7.7	10.7	12.0	–0.1	(–0.3; 0.0)	0.2	(0.0; 0.3)
<i>n</i>	9	80	103	208	147	79	192		434	
Apoptotic marker ^{a,b}										
In fas positivity, %	22.3	16.6	17.6	17.3	16.3	21.6	0.0	(–0.1; 0.2)	–0.1	(–0.3; 0.2)
<i>n</i>	9	79	99	191	139	72	187		402	
In Bcl-XL positivity, %	12.7	12.6	10.3	16.6	16.6	20.6	–0.1	(–0.4; 7.7)	0.4	(0.1; 0.7)
<i>n</i>	7	61	72	123	86	63	140		272	
	DDE (ng/g lipid)			DDE (ng/g lipid)			Linear regression coefficient (95% CI)		Linear regression coefficient (95% CI)	
	0–500			0–500						
	0–500	501–1000	>1000	0–500	501–1000	>1000				
Sperm DNA fragmentation ^{a,b}										
In TUNEL, %	3.9	4.3	4.3	9.3	9.2	9.5	–0.04	(–0.1; 0.1)	0.0	(–0.1; 0.2)
<i>n</i>	82	49	62	208	131	95	193		434	
Apoptotic markers ^{a,b}										
In Fas positivity, %	18.3	19.0	17.7	16.9	17.4	19.9	–0.02	(–0.2; 0.1)	0.2	(–0.1; 0.4)
<i>n</i>	81	49	58	196	124	82	188		402	
In Bcl-XL positivity, %	13.1	11.5	13.1	19.2	13.9	24.1	–0.03	(–0.3; 0.1)	0.13	(–0.2; 0.5)
<i>n</i>	63	38	40	169	74	29	141		272	

^aAdjusted for the logarithm of period of abstinence (days).

^bAdjusted for the logarithm of age (years). Mean values and linear regression coefficients associated with a *P*-value < 0.05 [exposed versus reference group (CB-153 0–50 ng/g lipid)] are in bold.

It must be emphasized that the INUENDO project represents not only the largest study to date in which apoptotic sperm parameters on ejaculated spermatozoa have been analysed but, more importantly, the present study also represents the first large-scale analysis of apoptotic sperm parameters in a fertile population. On the contrary, to date, the large majority of data regarding sperm apoptosis were obtained by comparing data collected from patients affected by infertility problems compared with a variety of control subjects. These studies put in evidence a general correlation between high TUNEL positivity and low sperm concentration and poor morphology (Sakkas *et al.* 1999a), whereas TUNEL positivity and apoptotic markers do not always exist in unison (Sakkas *et al.* 2002).

The high diversity in selection strategy clearly indicates that a direct comparison between data arising from the INUENDO study and previous literature cannot be done. In relation to this, we can only speculate about any association between DNA strand breaks and exposure to the PCB proxy marker CB-153. One of the PCB congeners found in highest concentration in human serum, CB-138, was shown to act as an androgen receptor (AR) antagonist (Bonefeld-Jorgensen *et al.* 2001), which indicates that the binding of PCB congeners to the AR might affect testosterone levels. In rodents, it has recently been demonstrated that testosterone increased expression of DNA topoisomerase II, the key enzyme that nicks and ligates DNA molecules during the histone-to-protamine transition process (Marcon & Boissonneault 2004). Moreover, testosterone is required for the maintenance of topoisomerase II expression during spermatogenesis (McPherson & Longo 1993, Bakshi *et al.* 2001). A Swedish study on young males reported a weak, negative association, between CB-153 and free testosterone levels (Richthoff *et al.* 2003). Data on the effect of POP exposure on the hypothalamic–pituitary–gonadal axis in men are conflicting. Exposure to both dioxins, DDT/DDE and PCB have in several studies (Egeland *et al.* 1994, Henriksen *et al.* 1996, Ayotte *et al.* 2001, Persky *et al.* 2001, Richthoff *et al.* 2003, Dalvie *et al.* 2004), but not all (Hagmar *et al.* 2001, Cocco *et al.* 2004, Rignell-Hydbom *et al.* 2004) been associated with a decrease in testosterone levels, and with an increase of gonadotrophins in a single study (Egeland *et al.* 1994), while no convincing association with gonadotrophin levels was observed in any of the other studies (Ayotte *et al.* 2001, Hagmar *et al.* 2001, Persky *et al.* 2001, Richthoff *et al.* 2003, Cocco *et al.* 2004, Dalvie *et al.* 2004). Moreover, among the same subjects, a weak association between free testosterone levels on DNA damage evaluated by %DFI has also been observed (Richthoff *et al.* 2002). Another potential mechanism whereby PCBs may produce DNA damage is through hydroxylated PCB metabolites, which (1) have been demonstrated to show anti-estrogenic activity (Machala *et al.* 2004), (2) are extremely potent inhibitors of human estrogen sulphotransferase and can indirectly induce

estrogenic activity by increasing oestradiol bioavailability (Kester *et al.* 2000) and (3) can be further oxidized to form quinones (Schleizinger *et al.* 1999, McLean *et al.* 2000) and superoxides with the formation of reactive oxygen species leading to oxidative DNA damage and strand breaks (Oakley *et al.* 1996, Srinivasan *et al.* 2002). Moreover, PCB quinones can also inhibit topoisomerase II activity (Srinivasan *et al.* 2002).

In addition, the positive correlation in Caucasians between the anti-apoptotic factor Bcl-xL and CB-153 could be explained as a defence mechanism carried out during spermatogenesis in order to increase the number of gametes reaching the ejaculate.

Another intriguing result of our study concerns the %TUNEL positivity, which was significantly lower in the Inuit (Greenland) population, with high POP exposure levels, compared with Caucasians. We can only speculate whether these exposure levels have a direct effect on spermatogenesis. Inuit populations were fertile, hence the low levels of TUNEL positivity, as well as Fas and Bcl-xL, may be an indication that the apoptotic mechanism, at least in fertile men is unaffected by POP exposure, and therefore functions efficiently to limit the presence of abnormal spermatozoa in the ejaculate. In this respect, it must be underlined that studying the relationship between POP exposure and sperm parameters in fertile men may have some limitations since these men could be more 'resistant' to effects of xenobiotic exposures. It may also be an indication that the blood–testes barrier in fertile men acts more efficiently in limiting exposure to environmental toxicants. A similar study on males with compromised sperm parameters may yield further information about the effect of xenobiotic exposure on sperm.

The low level of spermatozoa with DNA damage in the ejaculate of Inuits, as shown by %TUNEL positivity, parallels and reinforces the data obtained with the SCSA on the same population (Spano *et al.* 2005b). In that study, Inuits showed a significantly lower fraction of sperm with denaturable DNA compared with the European populations. Moreover, the Inuits' sperm concentration and count, when adjusted for abstinence time, were the highest among the study groups (Toft *et al.* 2005b), suggesting that high levels of CB-153 and p,p'-DDE are not directly associated with severely impaired spermatogenesis. It is plausible that Inuits possess either a specific genetic background and/or are exposed to environmental conditions (for example, a high dietary consumption of seafood, including marine mammals and ambient temperature particularly favourable for correct spermatogenesis) that favour a higher sperm output compared with Caucasian men. Little is known about possible differences in gene polymorphisms between Asian (Inuit) and Caucasian (European) populations for genes involved in the metabolism of POPs (Miyoshi & Noguchi 2003) and experiments are in progress to study whether the androgen receptor (AR) gene polymorphism might

modify the biological effects of POP exposures (Giwerzman Y, personal communication).

The INUENDO project has chosen the serum concentration of CB-153 as a proxy of the whole burden of PCBs, but other compounds or mixtures of environmental toxicants might be of significance. Characterization of the xenobiotic hormone-like activity in serum cleared for endogenous steroid hormones and the integrated serum dioxin activity is in progress in a subgroup of these samples (Bonefeld-Jørgensen E, personal communication). This may provide a better understanding on the issue of population-specific variations, since the biological effect could depend on the total biological activity due to actual mixtures of POPs and other xenobiotics.

None of the markers of sperm apoptosis tested were associated with p,p'-DDE even in the presence of mean p,p'-DDE serum concentration higher than in a US study, which analysed the relationship between human semen parameters and environmental exposure to POPs (Hauser *et al.* 2003b). These data as a whole indicate that the anti-androgenic compound p,p'-DDE does not negatively affect sperm DNA integrity, at least not in the studied exposure ranges.

Our data as a whole suggest a relationship between the exposure markers CB-153 and both sperm DNA integrity and the anti-apoptotic protein Bcl-xL of European adult males but not in highly exposed Inuit people. Additional issues (e.g. differences in the genetic background and lifestyle habits, characterization of actual POP mixture and their xeno-hormonal activities) need to be investigated in order to correctly interpret these data. Finally, no associations were observed between sperm DNA fragmentation, sperm apoptotic markers and p,p'-DDE levels, neither in single study groups nor in the overall study groups.

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