Relation between serum xenobiotic-induced receptor activities and sperm DNA damage and sperm apoptotic markers in European and Inuit populations

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Abstract

Persistent organic pollutants (POPs) can interfere with hormone activities and are suspected as endocrine disrupters involved in disorders, e.g. reproductive disorders. We investigated the possible relation between the actual integrated serum xenoestrogenic, xenoandrogenic and aryl hydrocarbon receptor (AhR) activities, and the sperm DNA damage and sperm apoptotic markers of 262 adult males (54 Inuits from Greenland, 69 from Warsaw (Poland), 81 from Sweden, and 58 from Kharkiv (Ukraine)) exposed to different levels of POPs. Xenobiotic-induced receptor activities were determined by receptor-mediated luciferase reporter gene expression. Sperm DNA damage was measured using terminal deoxynucleotidyl transferase-driven dUTP nick labeling assay (TUNEL) and pro- (Fas) and anti-apoptotic (Bcl-xL) markers were determined by immune methods. Different features of xenobiotic-induced receptor activity in serum and sperm DNA fragmentation and apoptotic markers existed between the Inuits and the European Caucasians. Negative correlations between xenobiotic-induced receptor activities and DNA damage were found for Inuits having relatively lower xenoestrogenic, lower dioxin-like activity, and lower sperm DNA damage, but higher xenoandrogenic activity. In contrast, in the European groups, xenobiotic-induced receptor activities were found to be positively correlated with the DNA damage. Further research must elucidate whether altered receptor activities in concerted action with genetic and/or nutrient factors may have protecting effect on sperm DNA damage of the Inuit population.

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Introduction

The development and maintenance of reproductive functions, such as spermatogenesis, is to a large extent controlled by steroidal hormones (Sharpe 1993, Sharpe & Skakkebaek 1993) and may therefore be influenced by endocrine disrupting compounds (EDCs). *In vitro* and *in vivo* studies have demonstrated that EDCs, such as persistent organic pollutants (POPs) including polychlorinated dibenzo-*p*-dioxins/furans, polychlorinated biphenyls (PCBs), organochlorine pesticides, and other compounds either can mimic and/or antagonize endogenous hormones (Soto *et al.* 1994, Kelce *et al.*

1995, Bonefeld-Jorgensen *et al.* 1997, 2001, Connor *et al.* 1997, Routledge *et al.* 1998, Andersen *et al.* 2002, Rozati *et al.* 2002, Bonefeld-Jorgensen & Ayotte 2003, Fisher 2004). Exposure to EDCs was reported to associate with rat testicular atrophy and reduced male fertility in mammals (Sager *et al.* 1987, Mably *et al.* 1992, Peterson *et al.* 1993, Kelce *et al.* 1995). Epidemiological studies have demonstrated the general association of PCBs and organochlorine pesticides and abnormal sperm motility, concentration, count and morphology in men (Hauser *et al.* 2002), and trends that could be reinforced by other synthetic compounds like phthalates (Paigen 1999, Hauser *et al.* 2005). However, whether the exposure to

low level of EDCs has impact on human reproduction remains inconclusive (Toft *et al.* 2004, 2005).

The toxicological assessment of xenobiotics on human health is complicated since individuals are exposed to a complex mixture of contaminants throughout life in particular during critical developmental windows. In addition, crosstalks between receptors might cause initiation of cascade changes in cellular processes if one receptor is affected, e.g. the aryl hydrocarbon receptor (AhR) is known to have crosstalks with the estrogen (ER) and/or the androgen receptor (AR) (Lanzino *et al.* 2005, Pocar *et al.* 2005). The analytical chemical approach for the detection of all xenobiotics is practically impossible especially on large-scale surveys; even in this case, it is impossible to derive the complete spectra of biological effects since the various pollutants can interfere with various hormonal receptors. Therefore, the assessment of the integrated biological effect of the actual chemical mixture found in human blood represents a Herculean task. The receptor-mediated chemically activated luciferase gene expression (CALUX) bioassay has been introduced and proven to be a sensitive and effective tool for *ex vivo* measurement of the integrated biological effect of chemical mixture interfering with the AhR, ER, and AR functions (Ziccardi *et al.* 2000, Bonefeld-Jorgensen *et al.* 2006, Long *et al.* 2006, Krüger *et al.* 2007) and may be relatively more biologically relevant to the specific receptors than the chemical analysis (Brouwer *et al.* 1995).

The maturation and differentiation of male germ cells during spermatogenesis are regulated by hormonal systems (Liu 2005). This process is also physiologically regulated by a fine-tuned apoptotic mechanism having the objective to eliminate the abnormal cells minimizing the negative consequences on the fertility of a male and on the health of his progeny (Rodriguez *et al.* 1997, Oldereid *et al.* 2001). The two systems, the hormonal and the apoptotic ones, are closely linked since germ cell apoptosis is a hormonally regulated process (Sinha Hikim & Swerdloff 1999) evidenced by 17 β -estradiol (E2) inhibited germ cell apoptosis *in vitro* (Pentikainen *et al.* 2000), and activation of AR by testosterone caused initiation and maintenance of the spermatogenous process and inhibition of germ cell apoptosis (Dohle *et al.* 2003).

Human ejaculated sperm cells include a fraction of cells showing phenotypic features of an apoptotic cell, such as DNA fragmentation, together with expression of both pro- and anti-apoptotic proteins like Fas and Bcl-xL (Cayli *et al.* 2004, Morrell *et al.* 2004). Infertile men with poor sperm motility and morphology have increased DNA fragmentation (Irvine *et al.* 2000). The causes of this DNA damage are still uncertain but the major candidates are oxidative stress (Lewis & Aitken 2005) and abortive apoptosis (Sakkas *et al.* 1999, 2002).

The EU project Inuendo (www.inuendo.dk) aimed to estimate the impact of POPs on human fertility in an epidemiological setup including the Inuits from

Greenland and the Caucasians from three European countries using the serum level of 2,2',4,4',5,5'-hexachlorobiphenyl (CB-153) and 1,1-dichloro-2,2-bis (*p*-chlorophenyl)-ethylene (*p,p'*-DDE) as proxy markers for the body burden of POPs (Toft *et al.* 2005). An Inuendo sub-study investigating the sperm DNA damage and apoptotic markers showed that lower DNA damage was observed for the Inuits when compared with the European study groups, and no consistent regional difference in DNA damage and apoptotic sperm parameters paralleled with the difference in the two POP markers (Stronati *et al.* 2006). Thus, the variation in the profiles of the chemical mixtures found in the blood must be taken into account when assessing the risk of environmental compounds on human reproductive function. Therefore, in order to bypass the limitations of only measuring two proxies of POPs, we estimated the integrated xenohormone and dioxin-like activity in serum on a subset of male volunteers from the main Inuendo project. A clear difference between the Inuits and the European males in the integrated xenobiotic-induced receptor activities as well as heterogeneity in correlation with the proxy markers was observed (Long *et al.* 2006, Bonefeld-Jorgensen *et al.* 2007, Krüger *et al.* 2007). Using the xenobiotic-induced receptor activities as exposure markers, the aim of the present study was to explore possible relations between integrated xenobiotic-induced receptor activities in serum and sperm DNA damage and apoptotic markers.

Materials and Methods

Study population

Subjects were recruited among pregnant women and their male spouses from May 2002 through February 2004 in 19 cities and settlements in Greenland, in Warsaw, Poland, and in Kharkiv, Ukraine during antenatal visits (Toft *et al.* 2005). In addition to the pregnant couples, an already established cohort of fishermen from Sweden (Rignell-Hydbom *et al.* 2004) was included in the study. The details about the inclusion/exclusion criteria for the participants have been described elsewhere (Toft *et al.* 2005). Briefly, in all countries, the participating man should be >18 years of age, born in the country where the study was performed and demonstrated fertility by having a pregnant or recently pregnant wife, except for the Swedish study group where fishermen were eligible if the first two criteria were fulfilled. However, 80% of the Swedish fishermen had fathered a child (Rignell-Hydbom *et al.* 2004). In total, 798 men provided fresh semen sample with participate rate of 79% in Greenland, 7% in Sweden, 29% in Warsaw, and 33% in Kharkiv. The blood samples were collected from the participating men within 1 week of the semen sample collection, except for a subgroup of 116 men from

Greenland who had their blood sample collected up to 1 year in advance.

Due to limited amount of semen and missing sample shipment from Ukraine together with limited amount of serum available for the determination of all receptor activity for each sample, data on both serum xenobiotic-induced receptor activities and sperm DNA fragmentation, apoptotic markers could be obtained from 262 men, in total, including 54 from Greenland (Sisimiut and Tasiilaq), 69 from Warsaw, Poland, 81 from Sweden, and 58 from Kharkiv, Ukraine.

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

Determination of serum xenobiotic-induced receptor activities

Collection of blood samples

Venous blood samples were collected into 10 ml vacuum tubes and after centrifugation the serum was transferred to brown glass tubes and stored at -80°C for later analysis (Jonsson *et al.* 2005, Toft *et al.* 2005).

SPE-HPLC fractionation of the serum samples for determination of ER and AR activity

Similar to the described methods (Sonnenschein *et al.* 1995, Rivas *et al.* 2001, Rasmussen *et al.* 2003), POPs were extracted from 3.6 ml serum samples by solid phase extraction (SPE) using Oasis HLB cartridges from Waters. The crude serum extract was then further processed using high-performance liquid chromatography (HPLC) in order to obtain the serum fraction (F1) containing the actual mixture of bio-accumulated POPs but free of endogenous hormones for the determination of the xenoestrogen and xenoandrogen receptor transactivity using the ER and AR-CALUX assays (Bonefeld-Jorgensen *et al.* 2006, Hjelmberg *et al.* 2006, Krüger *et al.* 2007).

Hexane:ethanol serum extraction for AhR-CALUX determination

The extraction of serum to obtain the lipophilic POPs for AhR-CALUX activity measurements was performed at a certified laboratory, Le Centre de Toxicologie, Sainte Foy, Quebec, Canada, using ethanol and hexane, followed by cleaning on Florisil + Na_2SO_4 column using 2 ml serum as described previously (Ayotte *et al.* 2005, Long *et al.* 2006).

Receptor chemically activated luciferase gene expression (CALUX) assays

The CALUX bioassay was used to determine the ER-, AR-, and AhR-mediated activities. Briefly, determination of ER transactivation was carried out in the stable p-vit-kt-luc-Neo transfected human breast adenocarcinoma MCF-1 cell line called MVLN (kindly provided by M Pons,

Montpellier, France), carrying the endogenous *ER α* and *ER β* genes and estrogen responsive element-luciferase (ERE-luc) reporter vector (Grunfeld & Bonefeld-Jorgensen 2004, Hofmeister & Bonefeld-Jorgensen 2004, Bonefeld-Jorgensen *et al.* 2005, 2006). The AR activity was determined in the Chinese hamster ovary cells (CHO-K1) by transient co-transfection with the mouse mammary tumour virus promoter-luciferase (MMTV-luc) reporter vector (kindly provided by Dr Ronald M Evans, Howard Hughes Medical institute, La Jolla CA, USA) and the AR expression plasmid pSVAR0 (kindly provided by Dr A O Brinkmann, Erasmus University, Rotterdam, The Netherlands) (Krüger *et al.* 2007). The AhR-mediated dioxin-like activity was determined as the transactivation of AhR using the stable transfected mouse hepatoma cell line Hepa1.12cR carrying the AhR-luciferase reporter gene kindly provided by M S Denison (University of California, Davis, CA, USA) (Long *et al.* 2006). The luciferase activity was measured in a LUMIstar luminometer (BMG Lumistar, RAMCON, Birkerød, Denmark) and corrected for cell protein by fluorometric measurements in the WALLAC VICTOR2 (Perkin-Elmer, Wellesley, MA, USA) at 355/460 nm wavelength as described previously (Andersen *et al.* 2002, Bonefeld-Jorgensen *et al.* 2005). The measured luciferase activity was expressed as relative light units (RLUs) per milliliter of serum. The values of the solvent controls were 3.13, 3.13, and 6.67 RLU/ml serum for ER, AR, and AhR respectively.

In each assay, all samples were tested in triplicate in two sets: (1) the effect of serum extract alone (termed XER/XAR/AhRag) designed to test primarily for agonistic effect, but if the response was below the reference level a decreased effect is indicated and (2) the competitive xenohormone and AhR activity were determined upon co-treatment with the corresponding receptor ligands and serum extract (termed XERcomp/XARcomp/AhR-comp) designed to test primarily for antagonistic effects on ligand-induced receptor activity, but if the response was higher than the reference values an additive or synergistic effect is indicated.

No cell toxicity was determined upon exposure of the cells to either SPE-HPLC F1 or hexane-ethanol extracts determined by CellTiter 96 cell proliferation assay from Promega (Bonefeld-Jorgensen *et al.* 2005).

Controls, equivalents, and half-maximum effect concentration of ligands

For each of the receptor analyses, a dose-response control (ER, E2, 0.05–500 pM; AR, methyltrienolone (R1881), 5–500 pM; AhR, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2–5000 pM) was analyzed in parallel. The half-maximum effect concentration (EC_{50}) of the respective receptors ligands (ER, EC_{50} –E2 = 33 pM; AR, EC_{50} –R1881 = 25 pM; AhR, EC_{50} –TCDD = 60 pM) was calculated by fitting the dose-response data to the sigmoid curve using Sigma Plot (SPSS, Chicago, IL, USA).

The CALUX-equivalent to E2 (XER-EEQ) and to CALUX-equivalents to TCDD (AhR-TEQ) of the samples was obtained by interpolation of the sample response on the sigmoid Sigma Plot curve (Bonefeld-Jorgensen *et al.* 2006, Long *et al.* 2006).

In addition, in each assay, parallel control analyses of the receptor ligand in the concentration used for the determination of the competitive effect of serum extracts (ER, EC_{40} –E2 = 25 pM; AR, EC_{50} –R1881 = 25 pM; AhR, EC_{50} –TCDD = 60 pM) upon cell co-treatment were used as positive control. The solvent controls ($\pm$ EC_{40} –E2, EC_{50} –R1881, EC_{50} –TCDD) consisted of sample solvent treated like the SPE–HPLC F1 or hexane–ethanol extracts but without adding the serum extract. For ER and AR activity measurements, SPE–HPLC F1 fractions of the combined blood bank serum from male control serum sample (KHM) and female control serum sample (KHF) were analyzed in parallel frequently as described previously (Bonefeld-Jorgensen *et al.* 2006, Krüger *et al.* 2007).

The intra-coefficient variations (CVs) of serum extracts and inter-CV values of solvent controls with or without ligand added in a concentration of 40–50% maximum activity were below 11 and 18% respectively and the combined blood bank serum was below 18 and 31% respectively (Bonefeld-Jorgensen *et al.* 2006, Hjelmberg *et al.* 2006, Long *et al.* 2006, Krüger *et al.* 2007).

Determination of sperm DNA fragmentation and apoptotic markers

Semen sampling

Semen samples were collected by masturbation after at least 2 days of sexual abstinence and the actual abstinence time was noted. All participants were supplied with a special device and instructed to keep the sample close to 37 °C when transported to the laboratory immediately after collection. The undiluted raw semen, collected 30 min after liquefaction, was prepared and directly put into a box with dry ice or a –20 °C freezer and within 2 weeks transferred into a –80 °C freezer (Spano *et al.* 2005b).

Determination of the sperm cell concentration

The analysis of DNA fragmentation and apoptosis marker expression requires a number of sperm cells ranging between 5×10^5 and 2×10^6 . Flow cytometry was used to establish sperm concentration of the samples by staining 5 µl raw semen sample in 595 µl 1 µg/ml propidium iodide (PI) solution for 15 s on ice. For each assay, an aliquot of 2×10^6 cells from the thawed sample was centrifuged (4 °C, 700 g × 8 min) in 4 ml PBS with 0.1% BSA as described previously (Stronati *et al.* 2006). The subsequent sperm pellets were used for the determination of DNA fragmentation and apoptosis markers.

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

A slightly modified TUNEL assay (Gorczyca *et al.* 1993) was performed. The detail has been described elsewhere (Stronati *et al.* 2006). Briefly, the sperm pellet was fixed in 1% paraformaldehyde (PFA) for 1 h and then permeabilized with 100 µl 0.1% (w/v) Na-citrate/Triton X-100. This suspension was divided into two aliquots of 50 µl each (negative control and test sample) and the two aliquots were washed with 0.1% (w/v) PBS/BSA, centrifuged, and the supernatant was discarded. After that, 40 µl reaction mix (dUTP-fluorescein isocyanate (FITC), dTTP, $CoCl_2$ terminal deoxynucleotidyl transferase (TdT) cacodylate buffer, with the TdT enzyme in the test sample and without the enzyme in the negative control sample) was added and incubated for 1 h at 37 °C in the dark. The washed pellet obtained was fixed with 100 µl of 0.5% PFA and 1 µg/ml PI solution in PBS. Samples were stored at 4 °C in the dark overnight until the flow cytometry (FCM) analyses.

Determination of sperm Bcl-xL and Fas positivity

The sperm cells (2×10^6) were fixed in 100 µl PFA (2% for 20 min for Bcl-xL and 1% for 10 min for Fas on a shaker at 4 °C). Fifty µl of the suspension was then drawn for the negative control and the fixative was washed out (Stronati *et al.* 2006). The Bcl-xL sample and its negative control were added to 100 µl of 0.1% (w/v) Na-citrate/Triton X-100 and 0.5% BSA with and without primary antibody. Forty microliters of primary antibody diluted in 0.5% PBS/BSA were added to the Fas sample and 40 µl of 0.5% PBS/BSA to the Fas negative control tube. After incubation for 1 h at 37 °C, the cells were washed and the pellets were then resuspended in the diluted secondary antibody (Bcl-xL, goat anti-mouse IgG-PE; Fas, goat anti-mouse IgG-FITC) and incubated for 1 h at 37 °C as described previously (Stronati *et al.* 2006). After the final washing step, the cells were fixed in 100 µl of 0.5% PFA. The Bcl-xL sample was stained with 7-aminoactinomycin D (7-AAD) and kept in the dark for 24 h at 4 °C until the FCM analysis. The Fas samples were stored in the dark at 4 °C for 24 h and 15 min before the FCM analysis the suspension was stained with 1 µg/ml PI (Stronati *et al.* 2006).

Flow cytometry

Flow cytometry was performed using an Epics XL (Beckman Coulter-IL, Fullerton, California, USA). In all analyses, spermatozoa labeled only with the secondary antibody were assessed as controls. Debris was gated out based on light scatter measurements. A minimum of 1×10^4 spermatozoa per sample were analyzed.

The intra-laboratory CV values calculated on reference sperm samples regarding TUNEL assays were constantly under 5%, whereas for the apoptotic markers it was in the range from 6% for Fas to 9% for Bcl-xL.

All reagents used were of molecular biology grade. The primary and secondary antibodies were from IL (Instrumentation Laboratory, Milan, 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).

Statistical analysis

The measured parameters and their potential effects on the male reproductive function are displayed in Table 1. The distribution of data was checked by Q–Q plots followed by confirmation of Kolomogorov–Smirnov test. After natural logarithmic transformation of both exposure and outcome variables, the normality and homogeneity of variance was improved and the ln-transformed data were used for the statistical analysis. The comparisons of serum xenobiotic-induced receptor activities (xenohormone activities, AhR-mediated dioxin-like activity), sperm DNA fragmentation (DNA_{dam}) and sperm apoptotic outcomes (Fas and Bcl-xL) between different groups were performed in one-way ANOVA. Comparison of the variables between the Inuits and the combined European group was performed by the Student's *t*-test.

The overall association between the exposure variables (xenobiotic-induced receptor activities) and the outcomes (sperm DNA damage, sperm apoptotic markers) across the four study groups (combined data) was assessed by comparing the regression lines for each study group using multiple regression analysis. Significant heterogeneity among the study groups was observed for some associations (Supplementary Table 1 which can be viewed online at <http://www.reproduction-online.org/supplemental/>). However, when the Inuits group was excluded, these heterogeneities were no longer statistically significant except for serum XAR ≥ 3.13 and sperm DNA damage, XER-EEQ as well as XERcomp and sperm Bcl-xL (Supplementary Table). Owing to the known genetic difference between the Inuits and the Caucasians, the subsequent analyses were thus mainly stratified on the Inuits and the combined European population for all associations except for the correlations of XAR ≥ 3.13 and sperm DNA damage, XER-EEQ as well as XERcomp and sperm Bcl-xL. The correlation analyses were also performed in the single study groups. However, in this analysis, we cannot exclude confounders such as age and abstinence time, which are well established determinants of sperm chromatin anomalies (Spano *et al.* 1998, Giwercman *et al.* 2003). Nevertheless, similar pattern of associations was observed after adjustment for these potential confounders.

Since the response of natural and synthetic ligands to the receptors is seldom linear (Long *et al.* 2003, Bonfeld-Jorgensen *et al.* 2005, Krüger *et al.* 2007), the

associations between xenohormone activities, dioxin-like activity and DNA_{dam}, Fas, and Bcl-xL were evaluated by the non-parametric Spearman's rank correlation. To allow for analysis of non-monotonic response across the whole range of receptor activity, the correlation analyses in the receptor subgroups eliciting activities below or above the respective solvent reference levels were additionally performed.

All the statistical analysis was performed in SPSS 13.0 (SPSS Inc).

Results

The levels of serum xenobiotic-induced receptor activities and sperm DNA fragmentation, sperm apoptotic markers

The serum xenobiotic-induced receptor activities differed among the study groups. The Inuits had significantly lower median level of XER, XERcomp, and AhRag/TCDD equivalent (AhR-TEQ) than the European men, while AhRcomp and XARcomp activity were higher for the Inuits (Table 2).

The European men included in this sub-study showed at least two times higher sperm DNA damage level than the Greenlandic Inuits with the order of PL $\geq$ SE $>$ UA $>$ GR (Table 2). The order of the percentage of sperm cells displaying Fas positivity (pro-apoptotic marker) was PL $\geq$ UA $\geq$ GR $>$ SE and Bcl-xL (anti-apoptotic marker) positivity was UA $\geq$ SE $\geq$ GR $>$ PL (Table 2). Although not showing significant statistical difference, Inuits had lower level of the sperm apoptotic markers when compared with the European men (Table 2).

The correlation between xenobiotic-induced receptor activities and sperm DNA damage

The background levels of xenobiotic-induced receptor activity and dioxin-like activity were 3.13 and 6.67 RLU/ml serum respectively. For the Greenlandic Inuits, Spearman's rank correlation analysis showed that the continuous XERcomp activity and XERcomp < 3.13 as well as both AhRag (AhRag ≥ 6.67)/AhR-TEQ and continuous AhRcomp activities (and AhRcomp ≥ 6.67) were negatively correlated with sperm DNA damage (Table 3, Fig. 1). In contrast, for the combined European population, significantly positive correlations were observed between sperm DNA damage level and continuous XER as well as XERcomp ≥ 3.13 , continuous XAR and continuous XARcomp (and XARcomp < 3.13) as well as AhRcomp < 6.67 (Table 3, Fig. 2).

For each single European group, sperm DNA damage was found to be negatively correlated with XAR ≥ 3.13 in Swedish group and positively correlated with the continuous XAR (XAR ≥ 3.13) in Warsaw and Kharkiv group respectively (Table 3).

Table 1 The measured effect parameters of lipophilic xenobiotics* and their potential effect on the male reproductive health.

Parameter	Interpretation	Potential effect on male reproductive health
XER ^a	Xenobiotic effect on ER activity; serum F1 extract alone	By mimic or blocking the effect of endogenous estrogens on ERs, the xenobiotics may disrupt the function of endogenous estrogens on the development and/or the maintenance of the male hypothalamo–pituitary–testis axis, stimulation of spermatozoa and thus influence spermatogenesis
XER < 3.13 RLU/ml serum	Decreased ER activity by xenobiotics	Xenobiotics can act as androgens or anti-androgens and affect the function of endogenous androgens to disrupt the homeostasis of androgens <i>in vivo</i> and impair the development and maintenance of male reproductive health
XER > 3.13 RLU/ml serum	Agonistic effect of xenobiotics on ER activity	
XER-EEQ	Estradiol equivalent of XER > 3.13 RLU/ml serum	
XERcomp ^a	Competitive effect of xenobiotics on E2-induced ER activity	
XERcomp < 3.13 RLU/ml serum	Antagonistic effect of xenobiotics on E2-induced ER activity	
XERcomp > 3.13 RLU/ml serum	Further increase by xenobiotics of the E2-induced ER activity	
XAR ^a	Xenobiotic effect of on AR activity; serum F1 extract alone	Xenobiotics acting via AhR can increase the production of reactive oxygen species and/or affect the steroid hormone receptors due to the cross-talk and disrupt the development and physiological male reproductive function
XAR < 3.13 RLU/ml serum	Decreased AR activity by xenobiotics	
XAR > 3.13 RLU/ml serum	Agonistic effect of xenobiotics on ER activity	
XARcomp ^a	Competitive effect of xenobiotics on R1881-induced AR activity	
XARcomp < 3.13 RLU/ml serum	Antagonistic effect of xenobiotics on R1881-induced AR activity	
XARcomp > 3.13 RLU/ml serum	Further increase by xenobiotics of the R1881-induced AR activity	
AhRag ^a	Xenobiotic effect on AhR activity; serum extract alone	Xenobiotics acting via AhR can increase the production of reactive oxygen species and/or affect the steroid hormone receptors due to the cross-talk and disrupt the development and physiological male reproductive function
AhRag < 6.67 RLU/ml serum	Decreased AhR activity by xenobiotics	
AhRag > 6.67 RLU/ml serum	Agonistic effect of xenobiotics on AhR activity	
AhR-TEQ	TCDD equivalent of AhRag > 6.67 RLU/ml serum	
AhRcomp ^a	Competitive effect of xenobiotics on TCDD-induced AhR activity	
AhRcomp < 6.67 RLU/ml serum	Antagonistic effect of xenobiotics on TCDD-induced AhR activity	
AhRcomp > 6.67 RLU/ml serum	Further increase by xenobiotics of the TCDD-induced AhR activity	Impairment of sperm genomic integrity and male fertility and potential transmissible DNA damage to the offspring
DNA _{dam}	Sperm DNA strand break	
Fas	Pro-apoptotic marker: initiate an intrinsic cell death pathway within the Fas-bearing cell by triggering apoptosis	
Bcl-xL	Anti-apoptotic marker: protect cells from apoptosis	

*Xenobiotics are suspected to have a negative effect on male reproduction. In this study, the term xenobiotics means the lipophilic compounds, including POPs, extracted from serum. For XER and XAR analyses, the endogenous hormones have been removed, for the AhR analyses the un-fractionated lipophilic serum extract is applied.

^aContinuous data include below and above the reference value.

The correlation between xenobiotic-induced receptor activities and sperm apoptotic markers

Negative correlations between continuous XAR (and XAR ≥ 3.13) activity as well as XARcomp < 3.13 and the pro-apoptotic marker Fas were observed for the combined European populations, while no significant correlations between serum xenobiotic-induced receptor activity and Fas were observed for the Inuits (Table 3).

For the anti-apoptotic marker Bcl-xL, negative correlations were observed to continuous XAR as well as XAR ≥ 3.13 and XARcomp ≥ 3.13 for the Inuit group and to XAR > 3.13 for the combined European group (Table 3).

The XER-EEQ and XERcomp ≥ 3.13 were found to be positively correlated with Bcl-xL for the Swedish fishermen (Table 3).

The distribution of demographic and life-style factors including age, abstinence time (Table 2), sperm

Table 2 Serum xenobiotic-induced receptor activities and sperm DNA damage, apoptotic markers and potential confounders of the study populations.

N	Greenlandic Inuit (GR) 54	European (PL + SE + UA) 208	Warsaw (PL) 69	Sweden (SE) 81	Kharkiv (UA) 58	All (GR + PL + SE + UA) 262
Exposure variables						
XER (RLU/ml serum) ^a	2.9 (1.0; 6.0)	3.09 (2.6; 12.0)	3.1 (2.4; 6.5)	3.0 (2.4; 12)	3.2 (1.0; 8.0)	3.1 (1.0; 12)
XER-EEQ (pg/g lipid) ^b	— ^c	132 (44.0; 580)	103 (44; 516)	76 (50; 364)	139 (80; 580)	114 (44; 580)
XERcomp (RLU/ml serum) ^a	2.7 (2.0; 3.8)	2.92 (1.0; 7.0)	3.0 (1.8; 7.0)	2.9 (1.0; 6.8)	2.9 (1.1; 4.5)	2.9 (1.0; 7.0)
XAR (RLU/ml serum) ^d	3.8 (1.7; 6.1)	3.66 (1.9; 10.8)	3.5 (1.8; 14)	3.7 (1.9; 7.4)	3.5 (2.2; 5.9)	3.6 (1.7; 14)
XARcomp (RLU/ml serum) ^d	3.9 (2.5; 7.8)	2.77 (1.6; 4.6)	3.0 (1.7; 4.5)	2.9 (1.6; 5.3)	2.2 (1.1; 4.1)	2.8 (1.1; 7.8)
AhRag (RLU/ml serum) ^e	22.9 (6.6; 257)	31.0 (8.7; 102)	35.6 (11; 118)	33 (8.0; 102)	27 (8.8; 57)	29 (6.6; 257)
AhR-TEQ (pg/g lipid) ^b	197 (38; 1188)	317 (130; 925)	312 (72; 1054)	428 (104; 1261)	337 (110; 781)	310 (38; 1261)
AhRcomp (RLU/ml serum) ^e	8.32 (3.9; 16.4)	6.26 (1.6; 10)	6.5 (3.2; 9.1)	6.2 (1.6; 10)	6.7 (1.5; 12)	6.8 (1.5; 16)
Outcome variables						
%DNA_dam	2.6 (0.3; 15.5)	11.8 (6.1; 73.7)	13 (1.8; 80)	11 (0.5; 74)	5.4 (1.5; 66)	7.4 (0.3; 80)
%FAS	13.2 (0; 51)	16.5 (0; 96.6)	51 (0; 97)	4.6 (0; 96)	20 (0; 98)	17 (0; 98)
%Bcl-xL	17.6 (0; 95)	26.6 (0; 99.2)	8.3 (0; 77)	36 (0; 89)	55 (0.2; 99)	21 (0; 99)
Confounders						
Abstinence time (days)	2.5 (0.5; 240)	3.0 (1.0; 3.0)	3.0 (0.1; 90)	3.0 (0.5; 21)	3.0 (1.0; 7.0)	3.0 (0.1; 240)
Age (years)	30 (18; 43)	33 (16; 68)	29 (25; 46)	47 (24; 68)	25 (18; 45)	29 (18; 68)

Values given are median (min; max).

^aFrequency of samples with XER agonistic and antagonistic effect 1 and 71% (GR), 21 and 7% (PL), 12 and 19% (SE), and 14 and 30% (UA) respectively. ^bCalculated on data from samples with agonistic effects only. Agonistic activity calculated as samples (triplicates) that differed significantly from the solvent control values using Student's *t*-test. For details of XER/XERcomp, XAR/XARcomp, and AhRag/AhRcomp see Bonefeld-Jorgensen *et al.* (2006), Long *et al.* (2006), Krüger *et al.* (2007). ^cOnly one serum sample from Greenland had agonistic XER activity, thus no XER-EEQ was given. ^dFrequency of samples with XAR agonistic and antagonistic effect 35 and 3% (GR), 25 and 21 (PL), 34 and 8% (SE), and 26 and 50% (UA) respectively. ^eFrequency of samples with AhR agonistic and antagonistic effect 92 and 3% (GR), 100 and 8% (PL), 95 and 12% (SE), and 100 and 34% (UA) respectively.

concentration, sperm morphology, season for sperm collection, fever last 3 months, spillage, urogenital infection/surgery and BMI, seafood intake and smoking status as well as sperm DNA fragmentation and apoptotic markers of the 262 adult men in this study were similar to the main Inuendo study population as previously reported (Jonsson *et al.* 2005, Toft *et al.* 2005, Stronati *et al.* 2006).

Discussion

The present study evaluate the relations between serum xenobiotic-induced receptor activities (xenoestrogenic, xenoandrogenic, and dioxin-like activity) and sperm DNA damage and apoptotic markers of males exposed to different levels of POPs (Jonsson *et al.* 2005). Heterogeneous associations between the Inuits and the European males were observed. The most markedly observed feature was the negative correlations between serum xenobiotic-induced receptor activities (e.g. XERcomp, AhRag/AhR-TEQ, and AhRcomp) and sperm DNA damage in the Inuits, whereas for the European males, positive correlations of XER/XERcomp, XAR/XARcomp, and AhRcomp with the sperm DNA damage were observed. Associations of xenobiotic-induced receptor activity with the apoptosis markers were found only for the XAR activities being inversely to both Fas and Bcl-xL in the European males, and for the Inuits inverse correlation was found only to Bcl-xL.

The rationale of detecting xenobiotic-induced receptor activity using CALUX is to bypass the problem

that single POP markers at a given level may not fully represent different POP profiles and their different toxicological effects. In addition to analyzing the serum xenobiotic-induced receptor activity as continuous data (across the whole range), the subgroups representing low and high activities when compared with the respective background reference level (xenohormone, 3.13 and AhR, 6.67) were also assessed. This strategy was used to determine the enhanced or inhibited effects on the respective receptors, which may result in different outcomes (Table 1).

The observed inverse correlation between serum XERcomp < 3.13 and sperm DNA damage found for Inuits suggests that a xenobiotic-antagonized E2-ER activity increases sperm DNA damage, which might be normalized getting closer to the reference level mimicking physiological E2-induced ER activity.

The negative correlations of AhRag/AhR-TEQ and AhRcomp ≥ 6.67 with sperm DNA damage observed for the Inuits suggest that higher serum AhR-mediated activities tend to result in lower sperm DNA damage level. The Inuits were reported to have lower sperm DNA damage level when compared with the European groups in a parallel Inuendo sub-study (Stronati *et al.* 2006), and this observation was confirmed by the finding of lower level of sperm DNA fragmentation index (DFI) for the Inuits (Spano *et al.* 2005b). It is known that the activation of AhR results in increased expression of enzymes involved in the metabolism of xenobiotic and endogenous compounds (Nebert *et al.* 2000). The relatively

Table 3 The Spearman's correlation coefficients of serum xenobiotic-induced receptor activity and sperm DNA fragmentation and apoptotic markers in males of Inuit and European.

		Inuit			European ^a			Warsaw			Sweden			Kharkiv		
		<i>n</i>	<i>r_s</i>	<i>P</i>	<i>n</i>	<i>r_s</i>	<i>P</i>	<i>n</i>	<i>r_s</i>	<i>P</i>	<i>n</i>	<i>r_s</i>	<i>P</i>	<i>n</i>	<i>r_s</i>	<i>P</i>
DNA_dam	XER	51	0.12	0.40	206	0.16	0.02	67	0.19	0.16	81	0.30	0.006	58	0.02	0.91
	XER < 3.13	40	0.32	0.05	122	0.15	0.10	41	0.14	0.38	51	0.23	0.10	30	0.07	0.72
	XER ≥ 3.13	11	−0.03	0.94	84	0.13	0.22	26	0.42	0.03	30	−0.06	0.74	28	−0.02	0.93
	XER-EEQ	1	–	–	30	0.12	0.52	17	0.17	0.51	6	0.60	0.21	7^b	−0.86^b	0.01^b
	XERcomp	51	−0.42	0.002	198	0.04	0.55	65	0.03	0.91	75	−0.05	0.70	58	−0.04	0.79
	XERcomp < 3.13	47	−0.41	0.004	140	0.07	0.43	42	−0.11	0.49	56	0.08	0.54	42	0.06	0.70
	XERcomp ≥ 3.13	4	0	1.0	58	0.29	0.03	23	0.18	0.40	19	0.44	0.06	16	−0.52	0.04
	XAR	27	−0.08	0.69	158	0.18	0.02	60	0.28	0.04	45	−0.16	0.31	53	0.42	0.002
	XAR < 3.13	4	0.80	0.20	46	0.04	0.79	21	0.18	0.43	11	−0.02	0.96	14	0.17	0.56
	XAR ≥ 3.13	23	−0.21	0.34	^c	^c	^c	39	−0.01	0.96	34	−0.42	0.01	39	0.43	0.007
	XARcomp	27	−0.22	0.27	158	0.25	0.002	60	0.09	0.43	45	0.04	0.82	53	−0.08	0.59
	XARcomp < 3.13	1	–	–	116	0.24	0.01	35	0.26	0.13	33	0.05	0.77	48	−0.11	0.47
	XARcomp ≥ 3.13	26	−0.32	0.11	42	0.28	0.07	25	0.11	0.61	12	0.24	0.46	5	0.70	0.19
	AhRag	54	−0.45	0.001	186	0.11	0.16	67	0.05	0.59	63	−0.11	0.40	56	0.24	0.08
	AhRag < 6.67	1	–	–	0	–	–	0	–	–	0	–	–	0	–	–
	AhRag ≥ 6.67	53	−0.49	<0.001	186	0.11	0.16	67	0.05	0.67	63	−0.11	0.40	56	0.24	0.08
	AhR-TEQ	52	−0.60	<0.001	180	0.03	0.73	67	0.10	0.44	61	−0.15	0.25	52	0.14	0.31
	AhRcomp	54	−0.49	<0.001	186	0.10	0.16	67	0.19	0.16	63	0.03	0.82	56	−0.01	0.92
	AhRcomp < 6.67	7	0.29	0.54	104	0.35	<0.001	37	0.28	0.09	36	0.24	0.16	31	0.20	0.29
	AhRcomp ≥ 6.67	47	−0.43	0.003	82	−0.11	0.32	30	−0.13	0.50	27	0.29	0.14	25	−0.08	0.71
Fas	XER	47	−0.21	0.26	187	0.38	0.19	59	−0.09	0.26	65	0.20	0.08	45	0.11	0.56
	XER < 3.13	36	−0.20	0.25	110	0.05	0.58	37	−0.16	0.34	40	0.18	0.25	25	0.10	0.63
	XER ≥ 3.13	11	0.20	0.56	77	0.15	0.20	22	0.05	0.84	25	0.13	0.80	20	0.003	0.99
	XER-EEQ	1	–	–	29	0.30	0.12	17	0.50	0.04	6	0.12	0.83	6	−0.09	0.87
	XERcomp	48	−0.03	0.84	180	0.01	0.91	57	−0.19	0.18	62	0.04	0.55	45	−0.18	0.34
	XERcomp < 3.13	45	0.001	0.70	128	0.14	0.11	37	−0.05	0.78	50	0.10	0.44	33	0.05	0.75
	XERcomp ≥ 3.13	3	−1.0	0.80	52	0.04	0.79	20	−0.16	0.51	12	−0.50	0.095	12	−0.14	0.66
	XAR	25	−0.05	0.43	145	−0.24	0.004	52	−0.42	0.001	37	0.02	0.53	42	0.06	0.99
	XAR < 3.13	4	0.60	0.40	42	−0.11	0.50	20	−0.46	0.04	10	0.15	0.68	10	0.43	0.21
	XAR ≥ 3.13	21	−0.20	0.22	103	−0.25	0.01	32	−0.49	0.005	27	−0.02	0.94	32	−0.14	0.45
	XARcomp	25	0.07	0.92	145	0.02	0.85	52	−0.08	0.69	37	0.10	0.36	42	−0.13	0.60
	XARcomp < 3.13	1	–	–	105	−0.20	0.04	31	−0.09	0.64	25	−0.20	0.35	37	−0.26	0.13
	XARcomp ≥ 3.13	24	0.03	0.96	40	0.010	0.96	21	−0.18	0.44	12	0.34	0.29	5	0	10.0
	AhRag	50	0.21	0.16	169	0.09	0.25	59	−0.08	0.25	51	0.06	0.26	43	0.02	0.87
	AhRag < 6.67	1	–	–	0	–	–	0	–	–	0	–	–	1	–	–
	AhRag ≥ 6.67	49	0.20	0.17	169	0.9	0.25	59	0.08	0.54	51	0.06	0.69	42	0.02	0.92
	AhR-TEQ	49	0.21	0.15	164	−0.05	0.56	59	0.10	0.44	51	0.12	0.37	42	0.12	0.45
	AhRcomp	50	0.05	0.83	169	−0.01	0.86	59	−0.04	0.70	51	−0.13	0.78	43	−0.003	0.65
	AhRcomp < 6.67	7	0.75	0.05	92	−0.15	0.16	32	−0.19	0.30	26	−0.31	0.13	24	−0.30	0.16
	AhRcomp ≥ 6.67	43	0.10	0.52	77	0.14	0.21	27	0.06	0.78	25	0.41	0.04	19	−0.05	0.84
Bcl-xL	XER	38	−0.14	0.15	128	−0.06	0.51	42	−0.15	0.40	70	−0.11	0.59	12	−0.33	0.30
	XER < 3.13	31	0.07	0.70	74	−0.13	0.27	24	−0.17	0.34	41	−0.16	0.55	6	−0.31	0.54
	XER ≥ 3.13	7	0.11	0.23	54	0.05	0.72	18	−0.08	0.77	29	0.25	0.12	6	0.03	0.96
	XER-EEQ	1	–	–	^c	^c	^c	11	−0.28	0.40	7^b	0.93^b	0.003^b	0	–	–
	XERcomp	39	−0.12	0.98	^c	^c	^c	40	−0.10	0.61	65	0.01	0.74	12	−0.20	0.54
	XERcomp < 3.13	35	−0.18	0.79	^c	^c	^c	24	0.13	0.55	46	0.10	0.50	9	−0.37	0.33
	XERcomp ≥ 3.13	4	−0.40	0.60	^c	^c	^c	16	−0.40	0.12	19	0.57	0.01	3	0.50	0.67
	XAR	23	−0.46	0.03	90	−0.10	0.35	37	−0.13	0.25	42	−0.01	0.96	10	−0.13	0.73

Figure 1 The scatter plot relating serum xenobiotic-induced receptor activity and sperm DNA damage in Greenlandic Inuits.

higher serum AhRcomp level found in Inuits (Long *et al.* 2006) indicate the existence of compounds further enhancing the dioxin-induced AhR activity. Therefore, we speculate that the observed inverse correlation between serum AhRag/AhRcomp and sperm DNA damage in the Inuits might indicate a protective effect due to the metabolism of compounds, potentially stimulating sperm DNA damage, and thus partly

XAR<3.13	4 ^b	1.0 ^b	—	22	0.29	0.19	11	−0.32	0.34	9	0.38	0.31	2	1.0	—
XAR≥3.13	19	−0.64	0.003	68	−0.31	0.009	26	−0.22	0.27	33	−0.20	0.27	8	−0.29	0.49
XARcomp	23	−0.35	0.10	90	−0.11	0.29	37	−0.11	0.61	42	0.004	0.98	10	−0.33	0.35
XARcomp<3.13	1	—	—	63	−0.10	0.44	22	−0.30	0.17	30	0.10	0.60	10	−0.33	0.35
XARcomp≥3.13	22	−0.44	0.04	27	−0.04	0.85	15	−0.10	0.73	12	−0.34	0.29	—	—	—
AhRag	40	−0.12	0.95	113	−0.18	0.06	42	0.11	0.59	57	−0.47	<0.001	12	0.11	0.75
AhRag<6.67	1	—	—	0	—	—	0	—	—	0	—	—	2	—	—
AhRag≥6.67	39	−0.16	0.82	113	−0.18	0.06	42	0.11	0.50	57	−0.47	<0.001	10	0.11	0.75
AhR-TEQ	39	−0.09	0.58	109	0.06	0.53	42	0.06	0.70	56	−0.31	0.02	10	−0.07	0.86
AhRcomp	40	−0.17	0.41	113	0.03	0.74	42	0.10	0.50	57	0.16	0.14	12	0.25	0.43
AhRcomp<6.67	6	−0.71	0.11	65	−0.08	0.54	21	−0.28	0.22	34	0.30	0.09	8	0.31	0.46
AhRcomp≥6.67	34	0.08	0.42	48	−0.17	0.25	21	−0.53	0.01	23	−0.08	0.71	4	−0.40	0.60

Correlation coefficients marked in bold indicate significant ($P<0.05$) correlations between serum xenobiotic-induced receptor activities and sperm DNA fragmentation or apoptotic markers. ^aPooled data composed of Warsaw, Sweden, and Kharkiv. ^bSample size was very small and bias may exist. ^cAnalyzed in each single group because of heterogeneity.

Figure 2 The scatter plot relating serum xenobiotic-induced receptor activity and sperm DNA damage in European men.

responsible for their lower sperm DNA damage level. However, whether genetic differences or other ethnic cofactors are involved need further studies to elucidate this phenomenon.

Interestingly, in the Inuits, similar but weaker correlations of xenobiotic-induced receptor activity and sperm DFI assessed by flow cytometric sperm chromatin structure assay (SCSA) were observed in a parallel Inuendo sub-study (Krüger *et al.* 2007). The SCSA and TUNEL techniques, established extensively for use in evaluating the human sperm chromatin anomalies, were previously reported to be highly correlated (Chohan *et al.* 2006). In the present study, the sperm DNA fragmentation evaluated by TUNEL and SCSA was found to be moderately correlated in the Inuits ($r_s=0.39$, $P<0.001$), suggesting that the sperm DNA damage and the chromatin integrity are parallel but not equivalent. This fact could be explained when considering that they mirror two different aspects of sperm abnormalities, since TUNEL evidences the presence of DNA strand breaks, whereas SCSA highlights sperm with highly denaturable DNA also influenced by DNA packaging alterations (Spano *et al.* 2005a). Thus, it can be expected that the associations of serum xenobiotic-induced receptor activity and sperm DNA fragmentation evaluated by TUNEL and SCSA are similar but not equal in the same population. This finding suggests that both sperm DNA damage and chromatin integrity may be affected by similar mechanisms, e.g. through interference with relevant receptors.

In contrast to the Inuits, positive correlations of the serum XER/XERcomp or XAR/XARcomp activities as well as AhRcomp < 6.67 and sperm DNA damage level were observed in combined European population. Previous studies reported that exposure to non-physiological concentrations of estrogen, testosterone, and estrogen-like chemicals could stimulate the apoptotic pathway in animal germinal cells (Zhou *et al.* 2001, Kim *et al.* 2003, Nair & Shaha 2003, Jung *et al.* 2004, Mishra & Shaha 2005), and cause DNA damage in human sperm (Anderson *et al.* 2003). These reports support the correlations we observed for the European men, e.g. the positive correlation of XER and sperm DNA damage. Furthermore, this is also in accordance with the reports of higher level of serum XER and sperm DNA damage found in the European study groups (Bonfeld-Jorgensen *et al.* 2006, Stronati *et al.* 2006). Given that the serum XAR activity did not differ significantly between the Inuits and the European groups (Krüger *et al.* 2007), the higher level of sperm DNA damage found in the European samples suggests that Europeans might be more sensitive to the xenobiotics. For the Kharkiv group showing similar sperm DNA damage level as the Inuit (Stronati *et al.* 2006), a significantly positive correlation of sperm DNA damage and XAR was observed, which further supports the observed differences between the Inuit and the Europeans.

Since the sperm DNA damage assessed by TUNEL and the apoptotic markers do not always exist in unison (Sakkas *et al.* 2002), it is not surprising that the correlation between the xenobiotic-induced receptor activities and the apoptotic markers, Fas and Bcl-xL, was less and dissimilar with that for DNA fragmentation. However, the observed inverse correlations of the serum xenoandrogenic activities and Fas and Bcl-xL for European men, and for Bcl-xL in Inuits, support the report that high (Tohda *et al.* 2001) or low (Bakalska *et al.* 2004) level of testosterone can induce apoptosis in the spermatozoa, suggesting that the exposure to compounds mimicking androgens or enhancing the androgen ligand-induced AR activity may have impact on the programmed sperm death. The fact that the correlations between serum xenobiotic-induced receptor activities and apoptotic markers for European population were stronger than for DNA damage suggests that the xenobiotics act more at testicular levels and indicates that sperm DNA damage is the result of both abortive apoptosis (in the testis) (Sakkas *et al.* 1999, 2002) and other factors that may play a role after release from the testis.

The reason and mechanism of the different correlations of serum xenobiotic-induced receptor activities and sperm DNA damage between the Inuits and the European Caucasians, and low level of sperm DNA damage in the Inuits are not clear at this stage. Different POP composition, lifestyle, and/or genetic factors may be involved in a concerted action. Scientific evidences showed that high detoxifying activity (generally involving cytochrome P450 system mainly initiated by AhR activation) cause an increase of reactive oxygen species (ROS)-mediated oxidative stress which is suggested as one of the main causes of DNA damages in spermatozoa (Lewis & Aitken 2005). However, it is known that the Ah gene battery has the capacity not only to promote oxidative stress but also to prevent it by increasing the levels of oxidative stress-detoxifying enzymes, and thus the total action of AhR and the Ah gene battery represents a pivotal upstream event in the apoptosis cascade (Nebert *et al.* 2000). Whether differences of POP profiles for the Inuits and the European men have an impact on promotion or inhibition of oxidative stress is not known and need further studies.

Human spermatozoa are particularly susceptible to oxidative stress owing to high content of polyunsaturated fatty acids. The docosahexaenoic acid (DHA) was reported to improve the progressive sperm motility of stallions (Brinsko *et al.* 2005). Antioxidants such as vitamin E, vitamin C, and carotenoids can restore a proper pro-oxidant-antioxidant balance and maintain the integrity of sperm cells. Selenium (Se) and vitamin E supplementation seem to improve sperm quality and fertility (Hansen & Deguchi 1996, Wong *et al.* 2000, Beckett & Arthur 2005). The intake of Se and n-3 fatty acids (n-3 FA) including DHA was much higher (up to ten times) for the Inuits than the Caucasians (Van Oostdam & Tremblay 2003, Hansen *et al.* 2004). Whether the higher

intake of these factors contribute to the relative better sperm quality in Inuits deserve further research.

Thus, the lower level of sperm DNA damage found for the Inuits when compared with the European men (Stronati *et al.* 2006) probably also relate to their food items and/or unknown genetic factors possessing a high efficient system able to compensate for the elevated production of ROS generated by the AhR activation. Significant ethnical as well as geographical differences in semen quality and incidence of hypospadias have been reported (Giwerzman *et al.* 2006). Little is known about the possible differences in gene polymorphisms between the Asian (Inuit) and the Caucasian (European) populations for genes involved in metabolism of POPs (Miyoshi & Noguchi 2003). Nevertheless, a part of the Inuendo study evaluating the AR gene CAG repeats and the effect of POP markers on semen characteristics such as DFI indicated an inverse correlation of POP exposure and sperm DFI for long AR gene CAG repeat, whereas a positive correlation was observed for short AR CAGs. The Inuits have longer AR CAGs than the European Caucasians (Giwerzman *et al.* 2007). Due to significant correlation of SCSA and TUNEL in this study, the findings of AR CAGs modifying the effect of POP exposure on sperm DFI may also fit with the observed correlations of xenobiotic-induced receptor activity and sperm DNA damage assessed by TUNEL. Therefore, the role of genetic factors attributable to the low level of sperm DNA damage of the Inuits may be taken into account.

In summary, the present study found some correlations between serum xenobiotic-induced receptor activity and sperm DNA damage and apoptotic markers, suggesting that xenobiotic compounds can interfere with the steroid receptor activities and the apoptotic pathway. However, the direct biological consequences of these associations are difficult to identify since a large number of other factors, most likely related to intrinsic population difference, also influence the outcome. Since this study for the first time explores the correlation of serum xenobiotic-induced receptor activity and sperm DNA damage and apoptosis in human beings, it is a primary impression of unknown effects. These statistically significant but moderate associations need to be confirmed in future studies before any strong conclusion can be made. The inverse correlation of xenobiotic-induced receptor activities to sperm DNA damage in Inuits suggest that the receptor activities may be involved in the protection of sperm DNA in concerted action with genetic and/or diet and life-style factors. Furthermore, the weakly positive association between serum xenobiotic-induced receptor activities and sperm DNA damage for the European groups suggests the potential of the bio-accumulated xenobiotics to exert possible adverse effects on human sperm cells.

Finally, it must be considered that the crosstalks between AhR, ER, and AR (Pocar *et al.* 2005) may result in other responses on the receptors *in vivo*, when

compared with the *ex vivo* tests used in the present study, where the response on the single receptor was tested. This may further complicate the interpretation of the associations of the xenobiotic activities and sperm apoptotic markers. Although, we might get closer to the integrated effects *in vivo* by evaluating the combined responses on the different receptors.

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