RELATION BETWEEN SERUM XENOBIOTIC INDUCED RECEPTOR ACTIVITIES AND SPERM DNA DAMAGE AND SPERM APOPTOTIC MARKERS IN EUROPEAN AND INUIT POPULATIONS

Long M [1], Stronati A [2], Bizzaro D[2], Krüger T [1], Manicardi GC [3], Hjelmborg PS [1], Spanò M[4], Giwercman A[5], Toft G [6], Bonde JP [6], Bonefeld-Jorgensen EC[1]

[1]Unit of Cellular & Molecular Toxicology (CMT), Institute of Public Health, University of Aarhus, Denmark, [2]Laboratory of Applied and Molecular Genetics, Institute of Biology and Genetics, Marche Polytechnic University, Italy, [3] Laboratory of Genetics, Department of Agricultural Sciences, University of Modena and Reggio Emilia, Italy, [4]Section of Toxicology and Biomedical Sciences, BIOTEC-MED, ENEA Casaccia, Italy, [5]Fertility Centre, Malmö University Hospital, Lund University, Sweden, [6]Department of Occupational Medicine, Aarhus University Hospital, Denmark

Introduction

The development and maintenance of reproductive functions such as spermatogenesis is to a large extent controlled by steroidal hormones [1] 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 (PCDD/Fs), polychlorinated biphenyls (PCBs), organochlorine pesticides, and other compounds either can mimic and/or antagonize endogenous hormones [2-7]. Epidemiological studies have demonstrated the general association of PCBs and organochlorine pesticides and abnormal sperm motility, concentration, count and morphology in men [8] and such trends could be reinforced by other synthetic compounds [9,10]. For the toxicological assessment of xenobiotics on human health, the receptor mediated chemical 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 [11-14] and may be relatively more biologically relevant to the specific receptors than chemical analysis [15] .

The maturation and differentiation of male germ cells during spermatogenesis are regulated by hormonal systems [16]. This process is also physiologically regulated by a fine tuned apoptotic mechanism having the objective to eliminate abnormal cells minimizing the negative consequences on the fertility of a male and on the health of his progeny [17,18]. 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 [19,20]. Infertile men with poor sperm motility and morphology have increased DNA fragmentation [21]. The causes of this DNA damage are still uncertain but the major candidates are oxidative stress [22] and abortive apoptosis [23].

The EU project Inuendo (www.inuendo.dk) aimed to estimate the impact of POPs on human fertility in an epidemiological setup including Inuits from Greenland and 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 [24]. An Inuendo sub-study investigating the sperm DNA damage and apoptotic markers showed that lower DNA damage was observed for the Inuits compared to 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 [25]. 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. 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 [11-13]. Using the xenobiotic induced receptor activities as exposure markers, the aim of the present study was to explore possible relations between the integrated xenobiotic induced receptor activities in serum and sperm DNA damage and apoptotic markers.

Materials and Methods

Study Population
Subjects were male spouses of pregnant women in 19 cities and settlements in Greenland (GR), Poland (PL) and Ukraine (UA) during antenatal visits (2002-2004) [24] and Swedish fishermen (SE) [26]. The details about the inclusion/exclusion criteria for the participants have been described elsewhere [24]. Data on both serum xenobiotic induced receptor activities and sperm DNA fragmentation, apoptotic markers were obtained from 262 men in total including 54 from Greenland , 69 from Poland, 81 from Sweden and 58 from Ukraine.

Determination of serum xenobiotic induced receptor activities
The actual mixture of bio-accumulated POPs but free of endogenous hormones were extracted from serum samples by Solid Phase Extraction and High-Performance Liquid Chromatography (SPE-HPLC) for determination of ER and AR transactivity using the estrogen (ER) and androgen (AR) receptor-CALUX assays [11,12,27]. To obtain the lipophilic POPs for AhR-CALUX activity measurements the serum was extracted by ethanol and hexane and clean-up on Florisil + Na_2SO_4 column [13,28]. ER-CALUX [29], AR-CALUX [12] and AhR-CALUX [13] assays were used to determine the ER, AR and AhR transactivation of serum extract. The measured luciferase activity was expressed as relative light units (RLUs) per ml 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; 2) the competitive xenohormone and AhR activity was determined upon co-treatment with the corresponding receptor ligands (ER: 17β-estradiol (E2), AR: methyltrienolone (R1881), AhR: 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCCD) and serum extract (termed XERcomp / XARcomp / AhRcomp) 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.

The CALUX-equivalent to E2 (XER- EEQ) and to TCDD (AhR-TEQ) of the samples was obtained by interpolation of the sample response (XER/AhRag) on the corresponding receptor ligands dose-response curve [11,13].

Determination of sperm DNA fragmentation and apoptotic markers
Semen samples were collected as recommended [24] and an aliquot of 2 x 10^6 cells from the thawed sample was prepared for the analysis of DNA fragmentation and apoptosis marker expression (Fas and Bcl-xL) [25].
In Situ Nick-end Labelling (TUNEL) assay was applied for the determination of semen DNA fragmentation [25,30]. The positivity of pro- apoptotic marker (Fas) and anti-apoptotic marker (Bcl-xL) were determined by immune methods [25].

Statistical analysis
The ln-transformed data was 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 Inuits and combined European group was performed by the student t-test.
Because of the known genetic difference between Inuits and Caucasians, the subsequent analyses were thus mainly stratified on the Inuits and the combined European population. The associations between xenohormone activities, dioxin-like activity and DNA _dam, Fas and Bcl-xL were evaluated by the non-parametric Spearman's rho 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, Chicago, IL).

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

The Inuits had significantly lower median level of XER, XERcomp and AhRag/ AhR-TEQ than European men while AhRcomp and XARcomp activity were higher for the Inuits.

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. The order of the percentage of sperm cells displaying Fas positivity (pro-apoptotic marker) was PL > UA $\geq$ GR > SE and Bcl-xL (anti-apoptotic marker) positivity was UA $\geq$ SE $\geq$ GR > PL. Although not showing significant statistical difference, Inuits had lower level of the sperm apoptotic markers compared to the European men.

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 rho correlation analysis showed that the continuous XERcomp activity and XERcomp < 3.13 as well as both AhRag (AhRag $\geq$ 6.67) /AhR-TEQ and continuous AhRcomp activities (and AhRcomp $\geq$ 6.67) were negatively correlated to sperm DNA damage (r_s = -0.41~ -0.60, $p < 0.01$). In contrast, for the combined European population, significantly positive correlations were observed between the sperm DNA damage level and continuous XER as well as XERcomp $\geq$ 3.13, continuous XAR and continuous XARcomp (and XARcomp < 3.13) as well as AhRcomp < 6.67 (r_s = 0.16 ~ 0.35, $p < 0.05$).

The correlation between xenobiotic induced receptor activities and sperm apoptotic markers

Negative correlations between continuous XAR (and XAR $\geq$ 3.13) activity as well as XARcomp < 3.13 and the pro-apoptotic marker Fas were observed for the combined European populations (r_s = -0.20 ~ -0.25, $p < 0.05$), while no significant correlations between serum xenobiotic induced receptor activity and Fas were observed for the Inuits.

For the anti-apoptotic marker Bcl-xL, negative correlations were observed to continuous XAR as well as XAR $\geq$ 3.13 and XARcomp $\geq$ 3.13 for the Inuit group (r_s = -0.46 ~ -0.64, $p < 0.05$) and to XAR $\geq$ 3.13 for the combined European group (r_s = -0.31, $p < 0.01$).

The XER-EEQ and XERcomp $\geq$ 3.13 were found to be positively correlated to Bcl-xL for the Swedish fishermen (r_s = 0.57~ 0.93, $p \leq 0.01$).

Discussion

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 $\geq$ 6.67 to 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 compared to European groups in a parallel Inuendo sub-study [25], and this observation was confirmed by the finding of lower level of sperm DNA fragmentation index (DFI) for the Inuits [24]. It is known that the activation of AhR results in increased expression of enzymes involved in the metabolism of xenobiotic and endogenous compounds [31]. The relatively higher serum AhRcomp level found in Inuits [13] 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 be responsible for their lower sperm DNA damage level. However, whether genetical differences or other ethnic cofactors are involved need further studies to elucidate this phenomenon.

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 [32-36], and cause DNA damage in human sperm [37]. 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 [11,25]. Given that the serum XAR activity did not differ significantly between Inuits and European groups [12], the higher level of sperm DNA damage found in the European samples suggest that Europeans might be more sensitive to the xenobiotics. For the Kharkiv group showing similar sperm DNA damage level as the Inuit [25], a significantly positive correlation of sperm DNA damage and XAR was observed, which further support the observed differences between the Inuit and Europeans.

The reason and mechanism of the different correlations of serum xenobiotic induced receptor activities and sperm DNA damage between Inuits and 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 cytochorme 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 [22]. Whether differences of POP profiles for the Inuits and the European men has an impact on promotion or inhibition of oxidative stress is not known and need further studies.

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 suggest the potential of the bio accumulated xenobiotics to exert possible adverse effects on human sperm cells.

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