Exposure to persistent organochlorine pollutants and seminal levels of markers of epididymal and accessory sex gland functions in Swedish men

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BACKGROUND: A major exposure route for persistent organochlorine pollutants (POPs) in Sweden is through consumption of fatty fish from the Baltic Sea. Endocrine disruptors, such as POPs, may have a negative impact on sperm quality. The present study aimed to investigate whether exposure to 2,2',4,4',5,5'-hexachlorobiphenyl (CB-153) and 1,1-dichloro-2,2-bis (p-chlorophenyl)-ethylene (p,p'-DDE) affects epididymal and accessory sex gland function. METHODS: 157 fishermen from the coastal stretches of Sweden, aged 27–67 years, provided semen samples which were analyzed for prostate-specific antigen (PSA), neutral α-glucosidase (NAG), fructose and zinc levels. Serum levels of CB-153 and p,p'-DDE were determined. RESULTS: The median CB-153 serum level was 189 ng/g lipid (range 40–1460) and a median p,p'-DDE serum level 231 ng/g lipid (range 40–2252). There was a significant linear association between CB-153 and total amount of PSA (slope [β] = −2.5, 95% confidence interval [CI] −4.0, −0.9; P = 0.02). With age, abstinence time and smoking included in the model the association became non-significant (β = −1.4, 95% CI−3.0, 0.1; P = 0.07). There were no significant associations between CB-153 and zinc, fructose and NAG. As for the exposure variable p,p'-DDE and the outcome variables, no significant associations were found. CONCLUSIONS: The study gives only very limited support of an association between CB-153 in serum and total PSA, and a random finding cannot be excluded.

Key words: accessory sex glands/epididymis/polychlorinated biphenyls/p,p'-DDE/prostate-specific antigen

Introduction

Since the 1930s, polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and the insecticide dichlorodiphenyl trichloroethane (DDT) have been released into the environment. These persistent organochlorine pollutants (POPs) are highly lipophilic, bioaccumulate in the food chain and are still detected in animals and humans all over the world (Longnecker et al., 1997), even though they were banned in most countries during the 1970s and 1980s.

There has been concern that exposure to POPs, of which some act as endocrine disruptors, may affect male fertility. Several studies have demonstrated estrogenic, antiestrogenic and androgen competing properties in various POPs (Kelce et al., 1995; Danzo, 1997; Bonefeld-Jörgensen et al., 2001). In some epidemiological studies, from both our group and others, POP exposure was associated with decreased sperm motility, decreased sperm concentration, and increased sperm DNA damage (Guo et al., 2000; Dallinga et al., 2002; Hauser et al., 2003; Hsu et al., 2003a; Richthoff et al., 2003; Rignell-Hydbom et al., 2004; Rignell-Hydbom et al., 2005). Although these studies indicate an effect of POP exposure on sperm parameters, the underlying mechanisms remain unsolved. One explanation could be a direct effect on spermatogenesis mediated through testicular aryl hydrocarbon receptors (AhR), androgen receptors (AR) or estrogen receptors α and β (ER α, ER β). However, it has been shown that epididymal and accessory gland functions are among factors affecting sperm motility and sperm DNA integrity (Elzanaty et al., 2002; Richthoff et al., 2002). These organs are strongly regulated by sex hormones and were also shown to express AhR, AR as well as ER α and β (Enmark et al., 1997) and are thereby potential targets of POP action. However, there is very limited information on how POPs may interact with epididymal and accessory sex gland function. Epididymal neutral α-glucosidase (NAG), prostatic zinc as well as prostate-specific antigen (PSA) and vesical fructose are released into seminal fluid. These excretion products are...
traditionally used as markers for the functional activity of post-testicular sex organs.

In Sweden, consumption of fatty fish from the Baltic Sea is a major exposure source for POPs (Asplund et al., 1994; Svensson et al., 1995). Fishermen from the Swedish east coast, by the Baltic Sea, and fishermen from the Swedish west coast, have approximately the same consumption of locally caught fish and are socio-economically comparable. However, the fatty fish caught in the Baltic Sea is much more polluted with POPs compared to fish caught in the sea off the Swedish west coast. This is reflected in higher serum levels of POPs among east coast than west coast fishermen (Svensson et al., 1995; Rignell-Hydbom et al., 2004). The Swedish fishermen population is an appropriate and feasible study base for epidemiological studies assessing exposure to POPs and male reproductive function. To ensure a broad range of POP exposure levels, fishermen from both coastal stretches formed the study base.

In the present study we used 2,2',4,4',5,5'-hexachlorobiphenyl (CB-153) and the major metabolite of DDT, 1,1-dichloro-2,2-bis (p-chlorophenyl)-ethylene (p,p'-DDE) in serum as biomarkers for POP exposure. Previous studies indicate that CB-153 is a good marker for total PCBs ($r = 0.9$) (Grimvall et al., 1997; Glynn et al., 2000) hydroxylated PCBs (OH-PCBs) (Åke Bergman, personal communication) and dioxin-like compounds (Gladen et al., 1999). The anti-androgenic compound p,p'-DDE, is another relevant biomarker which is present in relatively high serum concentrations among men consuming fatty fish from the Baltic Sea (Sjödin et al., 2000).

The aim of this study was to investigate whether there were any associations between serum levels of CB-153 or p,p'-DDE, and excretion of biochemical markers of epididymal or accessory sex gland function.

Materials and methods

Study population

Cohorts of fishermen from the Swedish west ($n = 3505$) and east ($n = 1678$) coasts have been established previously (Svensson et al., 1995). In the year 2000, these fishermen, born 1935 or later, received a questionnaire regarding fracture incidence. In connection to this the men were asked if they wanted information about a male post-testicular sex organs. The non-participants from the original fishermen’s cohort had a similar age distribution (median 52 years, range 29–67) as the 155 participants in our study (median 47, range 27–67). In a previous study by Rylander et al. (1995) fishermen’s wives on average gave birth to 2.0 children, and this was also the average number of children the participants in our study had fathered.

There were no statistical differences with respect to exposure levels of CB-153 and p,p'-DDE, age, sperm concentration and percentage of motile sperms, between the 155 men included in the present study and the 38 men excluded due to limited amount of semen.

Semen samples and blood samples

Information on how to collect the semen sample was given to the participants during a telephone interview as well as in written form. Seventy-four percent of the men had an abstinence time of 2–4 days (median 3 days, range 1–15) before the semen samples were obtained by masturbation. Information on fever, or medical treatments during the past 3 months, and spillage during collection was obtained from the participants. Venous blood samples were collected at the participant’s homes in connection with the delivery of the semen samples. The blood samples were centrifuged and sera were frozen at $–80^\circ$C. The study was approved by the Ethical Committee at Lund University.

Preparation of semen for biochemical analyses

Four-hundred and fifty microlitres of the ejaculate was mixed with 50 $\mu$l of 0.1 M benzamidine after 20 min of liquefaction. The mixture was centrifuged for 20 min at 4500 g, and thereafter the supernatant was poured off into tubes which were kept in $–80^\circ$C until analysis.

Prostate specific antigen

The concentration of PSA (mg/l) in seminal plasma was determined with Prostatus™ kit (Wallac Oy, Finland). This is a Delfia™ method, using three monoclonal antibodies against PSA. The coefficient for the total variation was 9% (mean concentration 979 $\mu$g/ml).

Zinc

The concentration of zinc (mmol/l) in seminal plasma was determined with a colometric method (Makino et al., 1982). The proteins in the sample were precipitated with trichloroacetic acid, the supernatant mixed with a water soluble pyridylazo dye and the absorbance measured at 560 nm. The coefficient of variation (CV) was 7% for control samples with a mean zinc concentration of 2.0 mmol/l.

Neutral $\alpha$-glucosidase

Seminal plasma NAG (mU/ml) was measured using a commercially available kit (Episcreen™; Fertipro, Beerem, Belgium) according to the manufacturer’s instructions. The test is based on the measurement of the intensity of a colour change evoked by the reaction between $\alpha$-glucosidase and 125 $\mu$l of reagent 1 (0.09 % Na-azide) which was added to 125 $\mu$l of thawed seminal plasma. The mixture was well mixed by pipetting, one diagnostic tablet ($p$-nitophenyl-$\alpha$-D-glucopyranoside) was added, and thereafter the mixture was vortexed for 60 s and then incubated for 4 h at 37°C. After incubation, 3 ml of reagent was centrifuged for 6 min at 3000 g. The absorbance was well mixed by pipetting, one diagnostic tablet ($p$-nitophenyl-$\alpha$-D-glucopyranoside) was added, and thereafter the mixture was vortexed for 60 s and then incubated for 4 h at 37°C. After incubation, 3 ml of reagent was centrifuged for 6 min at 3000 g. The absorbance was
value, obtained by reading the supernatant against reagent 2 (0.02 mol/l NaOH) as a blank, was measured spectrophotometrically at 450 nm. This value was plotted on a standard curve and the corresponding total α-glucosidase activity was read on the abscissa. The NAG concentration was estimated by use of the corresponding table provided by the manufacturer.

Fructose

The concentrations of fructose (mmol/l) in seminal plasma was determined with a spectrophotometric method, essentially described by Wetterauer and Heite (1976), run on Beckman Synchron LX20 instrument. Proteins in the sample were precipitated with perchloric acid and the absorbance of the supernatant measured. After addition of phosphoglucose isomerase, resulting in conversion of fructose to glucose, the absorbance was measured again. The absorbance difference corresponds to the concentration of fructose in the sample. The CV was 5% at a mean fructose concentration of 12.7 mmol/l.

Hormone analyses

Serum testosterone and sex hormone-binding globulin (SHBG) were measured by commercially available immunoassays. The total assay variation coefficients were 5.5 and 4.6%, respectively. Free androgen index (FAI) was defined by total testosterone/SHBG ratio.

CB-153 and p,p’-DDE

The levels of CB-153 and p,p’-DDE in serum were determined as previously described (Rignell-Hydbom et al., 2004). Briefly, CB-153 and p,p’-DDE were extracted from serum by solid phase extraction (Isolute ENV+; IST, Hengoed, UK) using on-column degradation of the lipids and analysis by gas chromatography mass spectrometry. 13C12-labeled CB-153 and 13C12-labeled p,p’-DDE were used as internal standards. The selected ion monitoring of p,p’-DDE was performed at m/z 318 while m/z 330 was used for the internal standard. The CV, calculated from samples analyzed in duplicate at different days, was 7% at 0.6 ng/ml (n = 76) and 5% at 1.5 ng/ml (n = 37) for CB-153 and 12% at 0.6 ng/ml (n = 56) and 7% at 2.4 ng/ml (n = 50) for p,p’-DDE. The detection limits were 0.05 ng/ml for CB-153 and 0.1 ng/ml for p,p’-DDE. The analyses of CB-153 and p,p’-DDE were part of the Round Robin inter-comparison program (Hans Drexler, Institute and Out-Patient Clinic for Occupational, Social and Environmental Medicine, University of Erlangen-Nuremberg, Germany) with analysis results within the tolerance limits.

Determination of lipids by enzymatic methods

Serum concentrations of triglycerides, cholesterol and phospholipids were determined by enzymatic methods using reagents from Boehringer–Mannheim (triglycerides and cholesterol; Mannheim, Germany) and Waco Chemicals (phospholipids; Neuss, Germany). The total lipid concentration in serum was calculated by summation of the amounts of triglycerides, cholesterol and phospholipids. In these calculations, the average molecular weights of triglycerides and phospholipids were assumed to be 807 and 714. For cholesterol we used an average molecular weight of 571, assuming that the proportion of free and esterified cholesterol in serum was 1:2.

Statistical analysis

Bivariate associations between lipid-adjusted CB-153 and p,p’-DDE and the outcome variables (total PSA, zinc, NAG, and fructose, respectively, per ejaculate; Table I) were evaluated using Pearson’s correlation coefficient (r). Scatter plots were used for all bivariate comparisons to ensure that assumptions of linear associations were reasonable and accordingly the use of Pearson’s r. The effect of the exposure variables on the outcome variables were evaluated by linear regression models. p,p’-DDE and CB-153 correlated strongly (r = 0.73) and were therefore not included in the model at the same time. The exposure variables were treated as continuous variables (untransformed and log transformed) as well as categorized into five equally sized groups. In the latter case, dummy variables were used. Due to skewed distributions of the outcome variables PSA and zinc, log transformed data were compared with untransformed data by means of residual analysis. Neither PSA nor zinc better fulfilled the model assumptions after log transformation. Thus, we therefore used untransformed data in the analysis. There was a strong correlation between PSA and zinc levels (r = 0.83), which is in accordance with a previous published study (Elzanaty et al., 2002). Thus, it could not be expected that independent effects of POP on PSA and zinc could be assessed in the present data set.

As potential confounders we considered age (continuous), current smoking status (yes versus no), abstinence time (as continuous or categorized into 0–2, >2–4, >4–6, >6 days) and FAI. We used the method suggested by Greenland (1989) for deciding which of the potential confounders should be included in the final multivariate linear regression models. Potential confounders were entered into bivariate and multivariate models if they changed the effect estimates by 10% or more, and excluded if their exclusion changed the effect estimates by <5%. Since age and FAI most likely are part of the same causal chain-of-event, we chose to evaluate three different models with respect to confounding selection: One model assessed age as a potential confounder, but not FAI, another model assessed FAI but not age, and the third model assessed both these variables.

Results

CB-153

There was a significant negative association between CB-153 and total PSA (slope [β] = −2.5, 95% confidence interval [CI] −4.0, −0.9, P = 0.02; Figure 1). When assessing age as a confounder, irrespective of whether FAI also was assessed, the final models included age, smoking and abstinence time (β = −1.4, 95% CI −3.0, 0.1, P = 0.07). In the model that assessed FAI but not age, inclusion of potential

<table>
<thead>
<tr>
<th>Exposure variables (serum)</th>
<th>n</th>
<th>Mean (SD)</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB-153 (ng/g lipid)</td>
<td>155</td>
<td>200 (193)</td>
<td>188</td>
<td>40–1460</td>
</tr>
<tr>
<td>p,p’-DDE (ng/g lipid)</td>
<td>155</td>
<td>322 (306)</td>
<td>229</td>
<td>40–2251</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Outcome variables (semen)</th>
<th>n</th>
<th>Mean (SD)</th>
<th>Median</th>
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<tbody>
<tr>
<td>PSA (µg/ejaculate)</td>
<td>155</td>
<td>3075 (1930)</td>
<td>2741</td>
<td>376–11062</td>
</tr>
<tr>
<td>Zinc (µM/ejaculate)</td>
<td>155</td>
<td>6.8 (4.9)</td>
<td>5.96</td>
<td>0.3–30.0</td>
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<tr>
<td>Neutral α-glucosidase (mU/ejaculate)</td>
<td>154</td>
<td>29 (19)</td>
<td>24</td>
<td>3–115</td>
</tr>
<tr>
<td>Fructose (µM/ejaculate)</td>
<td>155</td>
<td>47 (37)</td>
<td>39</td>
<td>0.6–263</td>
</tr>
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</table>

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<th>Potential confounding variables</th>
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<th>Mean (SD)</th>
<th>Median</th>
<th>Range</th>
</tr>
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<tbody>
<tr>
<td>Age</td>
<td>155</td>
<td>47 (9)</td>
<td>47</td>
<td>27–67</td>
</tr>
<tr>
<td>Abstinence time (days)</td>
<td>155</td>
<td>3.8 (2.3)</td>
<td>3.0</td>
<td>0.5–15</td>
</tr>
<tr>
<td>FAI (total testosterone/SHBG) in serum</td>
<td>155</td>
<td>0.44 (0.18)</td>
<td>0.42</td>
<td>0.17–1.54</td>
</tr>
</tbody>
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| Current smokers | 33 |

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confounders did not change the effect estimate >10%. When CB-153 was categorized into equally sized quintiles, the highest exposed quintile had 1352 μg/l (95% CI 407, 2298, $P = 0.005$) lower PSA concentration compared to the lowest exposed quintile. However, after adjustment for confounders the significant difference between the two extreme groups did not remain (556 μg/l; 95% CI–421, 1532, $P = 0.26$; Table II). There were no significant univariate associations between CB-153, irrespectively of whether the exposure variable was untransformed or categorized, and total seminal concentrations of zinc, NAG and fructose (all $P$-values ≥0.10, data not shown).

**Table II.** The effect of serum levels of 2,2',4,4',5,5'-hexachlorobiphenyl (CB-153) and 1,1-dichloro-2,2-bis-(chlorophenyl)-ethylene (p,p'-DDE), respectively, on total amount of seminal prostate specific antigen (PSA). The differences (β) between highest exposed quintile and the four less exposed quintiles, respectively, were estimated using linear regression. β denotes the regression coefficients and is presented with a 95% CI.

<table>
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<tr>
<th></th>
<th>Crude estimates</th>
<th>Adjusted for confounders*</th>
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<tr>
<td></td>
<td>β</td>
<td>95% CI</td>
</tr>
<tr>
<td>CB-153 (ng/g lipid)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;113 (n = 30)</td>
<td>1352</td>
<td>407, 2298</td>
</tr>
<tr>
<td>113–168 (32)</td>
<td>865</td>
<td>–65, 1794</td>
</tr>
<tr>
<td>169–232 (33)</td>
<td>866</td>
<td>–57, 1789</td>
</tr>
<tr>
<td>233–328 (25)</td>
<td>758</td>
<td>–247, 1762</td>
</tr>
<tr>
<td>329–460 (33)</td>
<td>ref.</td>
<td>ref.</td>
</tr>
<tr>
<td>p,p'-DDE (ng/g lipid)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;136 (n = 33)</td>
<td>707</td>
<td>–254, 1667</td>
</tr>
<tr>
<td>136–192 (31)</td>
<td>629</td>
<td>–346, 1603</td>
</tr>
<tr>
<td>193–273 (28)</td>
<td>856</td>
<td>–144, 1856</td>
</tr>
<tr>
<td>274–472 (30)</td>
<td>257</td>
<td>–726, 1240</td>
</tr>
<tr>
<td>473–2252 (30)</td>
<td>ref.</td>
<td>ref.</td>
</tr>
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</table>

*Age, smoking and abstinence time (days).

**Figure 1.** The correlation between serum levels of 2,2',4,4',5,5'-hexachlorobiphenyl (CB-153) and the total amount of prostate specific antigen (PSA) per ejaculate, among 155 Swedish fishermen ($r = –0.25; P = 0.002$).

**Discussion**

The present study gave only some very minor support that exposure to POP, assessed by serum levels of CB-153, was negatively associated with total seminal PSA. The results were clearly negative for the other outcome markers zinc, NAG and fructose. Moreover, the alternative POP marker, p,p'-DDE, was not related to any of the seminal markers.

As for most human semen studies the participation rate was low and selection bias could be of concern. A study by Larsen et al. (1998) showed that age and fertility status could be sources of bias in fertility studies. Another recently published study did not, however, find any differences among participants and non-participants regarding age, fertility status and socioeconomic status (Eustache et al., 2004). In the present study the age distribution and fertility status among the participants compared to the non-participants were similar and therefore we do not suspect that selection bias was of major concern. In addition, the study base was socioeconomically homogeneous. Furthermore, according to World Health Organization guidelines, 83% of the participants were normozoospermic which corresponds to another study of men from the general population (Bonde et al., 1998).

In order to be able to assess other sperm parameters, biochemical degradation of seminal products was stopped, by adding benzamidine, after 20 min post ejaculation. Intracellular variation in the rate of degradation of biochemical markers during the period of liquefaction, cannot be excluded. Such variation might limit the statistical power of the present study design, which should be taken into consideration when evaluating our statistically non-significant findings.

The function of epididymis and accessory sex glands are under strong androgenic control, and the AR mediated action is one of the most important regulators of PSA secretion. Therefore, a negative effect of POPs on seminal PSA might be due to anti-androgenic effects of these environmental toxicants. However, ER α and β as well as AhR were also found to be expressed in the prostate (Enmark et al., 1997). In vitro studies have shown that stimulation of these receptors leads to a suppression of prostate cell function and growth (Jana et al., 1999; Endo et al., 2003). Adult male rodents, which were exposed to estrogens showed structural and functional changes in both epithelial and stromal compartments in the accessory sex glands (Jarred et al., 2000; Prins et al., 2001). Experimental data indicate that postnatal as well as prenatal PCB exposure may lead to reduced weight of prostate and seminal vesicles (Sager, 1983; Faqi et al., 1998). Since
CB-153 correlates very well with both total PCB concentration and the dioxin-like activity in serum and plasma (Grimvall et al., 1997; Gladen et al., 1999; Glynn et al., 2000), any of the above mentioned receptor mediated mechanisms might be involved in changes in prostate function. In conclusion, the present study gave only some very minor support for CB-153 in serum being negatively associated with total seminal PSA. The indicated, but non-significant association, can be a random finding and a firm conclusion on whether POP exposure might effect seminal markers epididymal and accessory sex gland functions in man, has to await further studies.

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