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Lack of an association between environmental exposure to polychlorinated biphenyls and *p,p′*-DDE and DNA damage in human sperm measured using the neutral comet assay

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BACKGROUND: Chlorinated organic chemicals, such as polychlorinated biphenyls (PCB), hexachlorobenzene (HCB), dichlorodiphenyl trichloroethane (DDT), and dichlorodiphenyl dichloroethene (DDE, the most stable daughter compound of DDT) are persistent lipophilic compounds found in a large portion of the general population. To explore the hypothesis that environmental exposure to these compounds is associated with altered DNA integrity in human sperm, a study of 212 male partners of a sub-fertile couple who presented to the Massachusetts General Hospital Andrology Laboratory was conducted. METHODS: The neutral single cell microgel electrophoresis assay (comet assay) was used to assess DNA integrity in sperm. VisComet image analysis software was used to measure total comet length, the proportion of DNA present in the comet tail, and tail distributed moment, an integrated measure of length and intensity. RESULTS: In the regression analyses, there were no statistically significant consistent associations between the comet assay parameters and any of the individual PCB congeners, sum of PCB, or *p,p′*-DDE. CONCLUSION: These results suggest that there are not strong relationships between adult levels of these chlorinated organic compounds and sperm DNA damage as measured by the comet assay.

Key words: comet assay/DDT/DNA damage/polychlorinated biphenyls/sperm

Introduction

Polychlorinated biphenyls (PCB), hexachlorobenzene (HCB), dichlorodiphenyl trichloroethane (DDT), and dichlorodiphenyl dichloroethene (DDE, the most stable daughter compound of DDT) are persistent lipophilic chemicals. DDT was widely used as an insecticide, while PCB were used in cutting oils, lubricants and as electrical insulators. HCB was used as a fungicide and is formed as a by-product in the manufacture of chemicals such as solvents, chlorine-containing compounds, and pesticides (Agency for Toxic Substances and Disease Registry, 1996). Although the use and manufacture of these chlorinated compounds were banned nearly 30 years ago, they are ubiquitous and persist in the environment. They are distributed worldwide as environmental pollutants and have been measured in air, water, aquatic and marine sediments, fish, and wildlife (De Voogt and Brinkman, 1989). Furthermore, they are biologically concentrated and stored in human adipose tissue. The general population continues to be exposed through ingestion of contaminated foods (fish, meat, eggs and dairy products) and water, as well as through dermal contact (soil and house dust), and inhalation (indoor air in buildings that have various sources, as well as outdoor air).

Studies have shown that PCB, HCB and *p,p′*-DDE are found in a large proportion of the general population (Murphy and Harvey, 1985; Stehr-Green 1989; Longnecker *et al*., 1997; Centers for Disease Control, 2003). It is estimated that >99% of individuals have detectable blood levels of *p,p′*-DDE (Stehr-Green, 1989; Longnecker *et al*., 1997; Centers for Disease Control, 2003). Serum levels of PCB, HCB and *p,p′*-DDE are an integrated measure of internal dose, reflecting exposure from all sources over the previous years; depending on the congener, the half-life of PCB in the blood ranges from 1 to 10 or more years, while *p,p′*-DDE has a half-life of ⩾10 years (Phillips *et al*., 1989a; Brown 1994).

In our previously published study on PCB, HCB and *p,p′*-DDE and male reproductive health, we found evidence of an inverse dose–response relationship between PCB 138 and sperm concentration, motility and morphology (Hauser *et al*., 2003). There was limited evidence of an inverse relationship between sum of PCB and those PCB classified as cytochrome

*P*450 enzyme inducers with sperm motility and sperm morphology, as well as limited evidence of an inverse association between *p,p′*-DDE and sperm motility. The lack of a consistent relationship among semen parameters and other individual PCB congeners and groupings of congeners may indicate a difference in spermatotoxicity between congeners.

The epidemiological evidence on whether PCB can damage human DNA is limited. In an occupational study on workers exposed to PCB, Kalina *et al*. (1991) found a higher frequency of chromosome aberrations in peripheral lymphocytes of workers with long-term (>10 years) PCB exposure. When mixtures of PCB and individual congeners were tested in short-term experimental tests for genotoxicity, most of the tests were negative (Silberhorn *et al*., 1990). This may be partly a result of the slow rate of metabolism of these compounds under the assay conditions (Srinivasan *et al*., 2001). PCB are metabolized by cytochrome *P*450-dependent mono-oxygenases to mono- and dihydroxylated compounds (Sipes and Schnellmann, 1987). The metabolism of the dihydroxy-metabolites, in which the hydroxyl groups are *ortho* (catechols) or *para* to each other (hydroquinones), may result in the formation of semiquinones and quinones, which are reactive electrophiles (Oakley *et al*., 1996a). These reactive electrophiles can produce reactive oxygen species (ROS), which may produce oxidative DNA damage and strand breaks (Li and Trush, 1993; Srinivasan *et al*., 2001). Srinivasan *et al*. showed that PCB metabolites, both *in vitro* and in cells in culture, produced superoxide anion radicals and other ROS which produced DNA damage in the form of strand breaks *in vitro*. Similar mechanisms have been proposed for the genotoxicities of benzene (Snyder *et al*., 1993) and *o*-phenylphenol (Hiraga and Fugii, 1981).

DNA damage in human sperm has been associated with male-mediated adverse pregnancy outcomes such as decreased IVF and ICSI fertilization (Sakkas *et al*., 1996; Lopes *et al*., 1998) and pregnancy rates (Hammadeh *et al*., 1996; Evenson *et al*., 1999; Larson *et al*., 2000). Currently, there are limited data on whether PCB metabolites may be associated with oxidative DNA damage in human cells, including sperm. In one of the few experimental studies on sperm, Green *et al*. (1975), using in-vivo tests, failed to find an increased level of chromosome aberrations in rat bone marrow and spermatogonia following exposure to Aroclor 1242 and 1254. Using human lymphocytes, Sargent *et al*. (1989) found a dose–response relationship between chromosome breakage in human lymphocytes exposed *in vitro* to PCB 77. However, Belpaeme *et al*. (1996) failed to confirm this finding and failed to find an increase in human lymphocyte DNA single strand breaks using the alkaline comet assay.

There are several laboratory techniques used to evaluate sperm DNA such as the sperm chromatin structure assay (SCSA) (Evenson *et al*., 1991). The SCSA may prove to be a useful clinical test because of its high repeatability and its ability to measure an aspect of fertility that differs from what can be offered by the traditional semen analysis (Evenson *et al*., 1999). Other DNA tests include fluorescence in-situ hybridization, used to measure aneuploidy, as well as assays used to measure DNA integrity, including the single cell microgel electrophoresis (comet assay) and the terminal deoxynucleotidyl transferase-mediated dUTP-biotin end-labelling assay (Martin, 1993; Lähdetie *et al*., 1996; Sun *et al*., 1997; World Health Organization, 1999).

In the present study, we used the neutral comet assay to investigate whether DNA integrity in human sperm was associated with serum levels of PCB, HCB and *p,p′*-DDE. We are not aware of published human studies that have examined the relation between these compounds and DNA integrity in human sperm as measured by the comet assay. Detecting even an association of small magnitude may have large public health significance because of the widespread distribution of these compounds in the general population.

Materials and methods

Subjects

The study was approved by the Harvard School of Public Health and Massachusetts General Hospital (MGH) Human Subjects Committees and all subjects signed an informed consent. Subjects were male partners of a couple who presented to the Vincent Burnham Andrology laboratory at MGH between January 2000 and April 2002 for semen analysis to evaluate the couples' inability to achieve a pregnancy. Sixty-six per cent of eligible men aged 20–54 years agreed to participate. Men presenting for post-vasectomy semen analysis and men receiving treatment for infertility, such as hormonal treatments, were excluded. Height and weight were measured and a questionnaire was used to collect information on medical history and lifestyle factors.

Since both study subjects and non-study subjects provide a semen sample as part of their clinical care at MGH Andrology Laboratory, we were able to obtain semen analysis results from non-study subjects for the year 2001. Non-study subjects include men who declined participation, as well as men not approached for recruitment because of scheduling conflicts (i.e. nurse could not approach them because of clinic appointment overlaps). We identified 142 non-study subjects fitting the same inclusion criteria as the men recruited in the present study.

Semen analysis

Each man produced a single semen sample by masturbation into a sterile plastic specimen cup. The sample was liquefied at 37°C for 20 min prior to analysis. Subjects were instructed to abstain from ejaculation for 48 h prior to producing the semen sample and to complete a questionnaire on the length of the sexual abstinence period.

Semen analyses were performed without knowledge of subjects' PCB, HCB and *p,p′*-DDE levels. All semen samples were analysed for sperm concentration and motion parameters by computer-aided semen analysis (CASA; Hamilton–Thorn Version 10HTM-IVOS, USA). Setting parameters and the definition of measured sperm motion parameters for CASA were established by Hamilton–Thorn Company [frames acquired: 30; frame rate: 60 HZ; straightness (STR) threshold: 80.0%; medium VAP threshold: 25.0 μm/s; and the duration of the tracking time: 0.50 s]. To measure both sperm concentration and motility, aliquots of semen samples (5 μl) were placed into a pre-warmed (37°C) Makler counting chamber (Sefi-Medical Instruments, Israel). A minimum of 200 sperm from at least four different fields was analysed from each specimen. Percentage motile sperm was defined as WHO grade 'a' sperm (rapidly progressive with a velocity $\geq$25 μm/s at 37°C) plus 'b' grade sperm (slow/sluggish progressive with velocity $\geq$5 but <25 μm/s).

Using the 'feathering' method (World Health Organization, 1999), at least two slides were made for each fresh semen sample. The resulting thin smear was allowed to air-dry for 1 h before staining with a Diff-Quik staining kit (Dade Behring AG, Switzerland). Morphological assessment was performed with a Nikon microscope using an oil immersion ×100 objective (Nikon Company, Japan). Sperm were assessed and scored as normal or abnormal using the strict criteria by Kruger *et al*. (1998). A minimum of 200 sperm were scored from two slides for each specimen. Results were expressed as the percentage of normal sperm.

Remaining raw semen was then frozen in 0.25 ml cryogenic straws (CryoBiosystem, I.M.V. Division, USA) by immersing the straws directly into liquid nitrogen (–196°C). Previous work in our laboratory showed that this freezing method produced results that were highly correlated with results from fresh, unfrozen samples (Duty *et al*., 2002). The straws were thawed by gently shaking in a 37°C water bath for 10 s and the semen was immediately processed for comet assay.

Comet assay

The entire comet assay procedure was conducted under low indirect incandescent light situations (60 W) to minimize light-induced damage to sperm DNA. All chemicals were purchased from VWR Scientific (USA) unless otherwise specified. After thawing, semen (with ~2×10^5 sperm) was mixed with 400 µl of 0.7% agarose (3:1 high resolution; Amresco, USA). Fifty microlitres of this semen–agarose mixture was embedded between two additional 200 µl layers of 0.7% agarose on specially designed, partially frosted, microgel electrophoresis glass slides with a clear central window (Erie Scientific, USA). Cover glasses were removed prior to submersion of slides in a cold lysing solution (4° C) of 2.5 mol/l NaCl, 100 mmol/l EDTA tetrasodium salt, 10 mmol/l Tris-base (pH 10), 1% sodium lauroyl sarcosine and 1% Triton X-100 (Boehringer Mannheim); this step mainly allows for dissolution of the cell membrane so that the sperm chromatin is accessible during the next two enzyme digestion steps. The slides were then transferred to enzyme treatment (2.5 mol/l NaCl, 5 mmol/l Tris, 0.05% sodium lauroyl sarcosine with pH adjusted to 7.4) and 10 mg/ml of RNAse (Amresco). After 4 h at 37°C, the slides were transferred into enzyme treatment plus 1 mg/ml of DNAse-free Proteinase K (Amresco) for 18 h at 37°C. These two steps are crucial for decondensing sperm chromatin and allowing migration of broken DNA out of the nucleus. Slides then equilibrated in neutral electrophoresis solution (300 mmol/l sodium acetate, 100 mmol/l Tris, pH 9) for 20 min before being electrophoresed under neutral conditions at 12 V and 130 mA for 1 h at room temperature. This was followed by precipitation and fixation of cells, first in absolute alcohol mixed with 10 mol/l ammonium acetate for 15 min, and second in 70% ethanol with 1 mg/ml spermine for 30 min. The resulting slides were air-dried and subsequently stained with YOYO dye (Molecular Probes, USA), an intensely fluorescent DNA dye. Fluorescent comet patterns were examined with a Leica fluorescence microscope model DMLB under ×400 magnification and fluoro-isothiocyanate (FITC) filter combination.

Image analysis

VisComet image analysis software, kindly donated by Impuls Bildanalyse GMBH (Gilching, Germany), was used to measure 'comet extent', 'percentage DNA in tail' (tail %) and 'tail distributed moment' (TDM) on 100 sperm in each semen sample. Comet extent is a measure of total comet length from the beginning of the head to the last visible pixel in the tail. This measurement is similar to that obtained by manual analysis utilizing an eyepiece micrometer. Percentage DNA in tail is a measurement of the proportion of the total DNA that is present in the tail. The TDM is an integrated value that takes into account both the distance and intensity of comet fragments. The formula used to calculate the TDM is:

$$M_{\text{dist}} = \sum(I \times X)/\sum I$$

where $\sum I$ = sum of all intensity values that belong to the head, body or tail, and X = x-position of intensity value. In addition to these three parameters, cells too long to measure ('long cells') with VisComet (>300 µm) were tallied and used as a third measure of DNA damage. Because of the presence of 'long cells' in most subjects, >100 cells may have been screened and scored to allow for the measurement of comet extent, tail %, and TDM on 100 cells per subject.

Serum PCB, HCB and p,p′-DDE measurements

Blood samples were collected on the same day as the semen sample and analysed by the Organic Chemistry Analytical Laboratory at the HSPH. Target analytes included 57 individual PCB congeners, HCB and *p,p′*-DDE. Details of the sampling, analytical and quality control procedures are described elsewhere (Korrick *et al*., 2000). Briefly, the blood samples were collected in red top Vacutainer™ tubes and the serum fraction was separated by centrifugation. Serum samples were stored in solvent-rinsed glass vials with Teflon-lined caps at –80°C until analysis.

The extraction followed procedures developed by the Centers for Disease Control (Needham, 1981) with modifications to conform to ultra-trace-level analyses. These modifications included additional cleaning of glassware and dry reagents used in the column chromatography clean-up and reducing the final extract volume to 100 µl.

Since PCB, HCB and *p,p′*-DDE partition according to the lipid content of tissues, and serum lipid levels vary between fasting and non-fasting states, a correction for serum lipids is needed for the valid interpretation of serum levels (Phillips *et al*., 1989b). Therefore, percentage lipid for each serum sample was measured gravimetrically, by weighing an aliquot of sample extract evaporated to dryness. The mean (SD) for the 212 samples was 0.51% (0.20).

The serum extracts were analysed by gas chromatography with electron capture detection (GC/ECD) using a Hewlett-Packard 5890 Series II GC with a fused silica capillary column (DB5, 30 meters, 0.25 mm, 0.25 µm, from J&W Scientific, USA). Confirmatory analysis was done using a Hewlett-Packard 6980 GC with a Micro-ECD (GC/uECD) and capillary column of different polarity. Quantification was based on the response factors of individual PCB congeners, HCB and *p,p′*-DDE relative to the internal standard (PCB 166 by IUPAC nomenclature). PCB concentrations were reported as individual congeners and as the sum of all congeners assayed (∑PCB). The amount of each PCB congener in samples was corrected by the amount of that analyte in the procedural blank associated with the analytical batch. Results were not adjusted for surrogate recoveries.

The PCB, HCB and *p,p′*-DDE concentrations were adjusted for total serum lipids and are expressed in units of ng/g total lipids. PCB congeners 118, 138 and 153 were especially of interest because they are prevalent in human serum and the limited human data suggest that they may be associated with altered sperm motility (Bush *et al*., 1986; Dallinga *et al*., 2002).

The laboratory follows strict quality control and quality assurance procedures. The laboratory successfully participates in various intercalibration exercises including international inter-comparison programme organized by the Institute for Quality Management and Medicine at the University of Erlangen–Nuremberg, Germany (annually) and in international Ring tests sponsored by AMEP (Arctic Monitoring and Assessment Program) and organized by

Quebec National Institute of Public Health, Canada (three times per year).

Method detection limits (MDL) were determined as 3×SD obtained from the analysis of the eight aliquots of pooled serum fortified with target analytes at 0.02 ng/g serum, as recommended by the US Environmental Protection Agency (1984). The MDL values for all PCB congeners were <0.05 ng/g, with most of the congeners <0.01 ng/g. The MDL for *p,p′*-DDE was higher, but only because unfortified serum had high *p,p′*-DDE concentrations at 6.3 ng/g.

Background contamination in 13 analytical batches was determined by procedural blanks. The mean (SD) for ∑PCB was 0.18 (0.02) ng/g. Analytical accuracy, precision, and extraction efficiency were evaluated by the analyses of two pairs (one pair in each batch) of matrix spike samples (aliquots of pooled bovine serum spiked at 0.24 ng/g of each PCB congener, 0.13 ng/g of *p,p′*-DDE and two surrogate compounds, PCB 30 and PCB 112, added to each sample at 0.67 ng/g). The mean (SD) percentage recovery for matrix spike samples was 95% (6.6) for ΣPCB and 99% (10) for DDE. The mean (SD) percentage recovery for two surrogate compounds, PCB 30 and PCB 112, were 96% (6.8) and 98% (4.1) respectively. Precision, expressed as mean relative percentage difference (RPD) between ∑PCB in matrix spike duplicates, was 5.5% (4.8).

Statistical analysis

For data analysis, Statistical Analysis Software (SAS), version 8.2 (SAS Institute Inc., USA) was used. Descriptive analyses of subject characteristics were performed. In univariate and multiple regression analyses, the mean comet extent, tail % and TDM of 100 cells per person was used as the dependent variable. Since mean comet extent and TDM were normally distributed, they were not transformed in the regression analyses. However, since tail % was not normally distributed, analyses using both untransformed and log-transformed tail % were performed. Since the results and their interpretation did not differ, we chose to present only the untransformed tail % results for ease of interpretation. The number of 'long cells' in a semen sample was not normally distributed; it was transformed using the arcsine transformation (Zar, 1984) and regressed on serum levels of PCB, HCB and *p,p′*-DDE. Spearman correlation coefficients were used to determine correlations between PCB, *p,p′*-DDE and comet parameters. Wilcoxon signed rank tests were used to compare comet parameters by smoking status and race.

Based on earlier studies (Bush *et al*., 1986; Dallinga *et al*., 2002; Richthoff *et al*., 2003), we explored the relationship between comet parameters and three individual PCB congeners (118, 138 and 153), as well as the sum of PCB. Exploratory analyses using the other individual PCB congeners were not conducted. In addition, an analysis of the relationship between comet parameters and groupings of PCB, based on structural and biological activity as proposed by Wolff *et al*, (1997), was conducted. PCB were grouped as follows: group 1: potentially estrogenic and weak phenobarbitol inducers (congeners 44, 49, 52, 101, 187, 174, 177, 157/201); group 2: potentially anti-estrogenic and dioxin-like (congeners 95/66, 74, 77/110, 105/141, 118, 156, 167, 128, 138, 170); and group 3: phenobarbital, CYP1A and CYP2B inducers (congeners 99, 153, 180, 196/203, 183).

To determine the relationship between each comet parameter and PCB, HCB and *p,p′*-DDE, a separate multivariate linear regression model was used. In the primary regression analyses, PCB, HCB and *p,p′*-DDE were modelled as continuous variables. In addition, in a secondary analysis, PCB levels were divided into tertiles and entered into the model as dummy variables to explore dose–response relationships. The use of tertile thresholds offers more flexibility for linear regression modelling and does not impose an assumption of linearity between exposure and outcome. Covariates considered for inclusion in the multivariate models included smoking status, race, age, body mass index (BMI), and abstinence time. Their inclusion in the multivariate models was based on statistical and biological considerations (Hosmer and Lemeshow, 1989). In the regression models, age was modelled as a continuous independent variable after checking for appropriateness using a quadratic term. Smoking status was used as dummy variables (current, former smokers versus never).

Results

Demographic information and the distribution of semen parameters are summarized in Table I. Of the 212 men (66% participation) in the study population, 32 men (15%) had a sperm concentration $<20\times10^6$/ml, 89 men (42%) had <50% motile sperm, and 50 men (24%) had <4% normally shaped sperm. There were 105 (50%) men with above reference values on all three semen parameters. The semen parameter categories were not mutually exclusive. A man could contribute data to any one, two or all three of the below reference value groups. Overall, the subjects were primarily Caucasian (83%), with 5% African-American, and 7% Hispanic. The mean (SD) age was 36.1 (5.2) years. Seventy-four per cent had never smoked.

Semen analysis results for non-study subjects and study subjects were similar. For instance, for the 212 study subjects in the present study, the mean (SD) sperm concentration was 108.3×10^6/ml (98.1×10^6/ml) and 15% of study subjects were below the WHO reference value of 20×10^6/ml. In comparison,

Table I. Subject demographics, semen and comet parameters (n = 212)

	Mean (SD)	*n* (%)
Age (years)	36.1 (5.2)	
Body mass index (kg/m^2)	28.4 (5.4)	
Race		
White		175 (83)
Black/African-American		11 (5.2)
Hispanic		14 (6.6)
Other		12 (5.7)
Smoking status		
Never smoker		156 (74)
Ever smoker		56 (26)
Current smoker		19 (9.0)
Ex-smoker		37 (18)
Semen parameters		
Sperm concentration ($\times10^6$/ml)	108 (98.1)	
Subjects $<20\times10^6$ sperm/ml		32 (15)
Sperm motility (% motile)	51.1 (23.9)	
Subjects <50% motile sperm		89 (42)
Sperm morphology[a] (% normal morphology)	6.9 (4.4)	
Subjects <4% normal morphology		50 (24)
Comet assay parameters[b]		
Comet extent (μm)	131 (32.0)	
% DNA in tail	24.5 (9.9)	
Tail distributed moment (μm)	59.8 (12.7)	
No. of 'long cells'	11.0 (13.4)	
Subjects without 'long cells'		26 (12)
Subjects with 'long cells'		186 (88)

[a]Kruger strict criteria used for morphology determination.
[b]VisComet image analysis software was used to measure comet extent (μm), a measure of total comet length; % DNA in tail, a measure of the proportion of total DNA present in the comet tail; and tail distributed moment (TDM), an integrated measure of length and intensity (μm); one person missing both TDM and % DNA in tail. A 'long cell' was a cell with a comet tail that extended beyond the measuring boundaries in the VisComet image analysis software (>300 μm).

among the non-study subjects, the mean sperm concentration was 98.3×10^6/ml (83.2×10^6/ml), and 17% were below the WHO reference value. For the study subjects, the mean (SD) sperm motility was 51% (24%) compared with 46% (25%) for the non-study subjects, while 42% of study subjects were below the WHO motility reference value compared with 53% of the non-study subjects. For morphology, the mean (SD) value for study subjects was 6.9% (4.4%) compared with 6.7% (4.2%) among the non-study subjects, while 24% of the study subjects were below the WHO reference value for morphology compared with 23% of the non-study subjects.

The distribution of comet parameters and serum levels of PCB and *p,p′*-DDE is shown in Table II. Fifty-seven PCB congeners, HCB and *p,p′*-DDE were measured. There was a wide distribution of both PCB congeners, HCB and *p,p′*-DDE concentrations. The median *p,p′*-DDE concentration was 220 ng/g lipid (range 72.5–7776). The median of the sum of PCB was 218 ng/g lipid (range 56.0–1590). The levels of *p,p′*-DDE in serum were higher than the levels of individual PCB congeners, which is also consistent with other studies (Bush *et al*., 1986).

The median comet extent was 130 μm (range 54.0–223). The median tail % was 21.9% (range 9.9–64.4). Median TDM was 58.6% (range 29.5–107). The number of long cells ranged from 0 to 95 cells; 26 subjects (12%) had no 'long cells' and 50% of subjects had ⩽7 cells. Figure 1A–C demonstrates the heterogeneity of comet tail lengths within an individual, while Figure 1D shows the comet cell referred to as 'long cell', a cell which was too long to measure with image analysis software.

Comet extent and TDM were highly correlated ($r = 0.86$; $P < 0.0001$); however, tail % was moderately correlated with comet extent ($r = 0.40$; $P < 0.0001$) and weakly correlated with TDM ($r = 0.10$; $P < 0.10$). Moderate correlations existed between the number of long cells and both comet extent and TDM ($r = 0.46$, and $r = 0.42$ respectively; $P < 0.0001$), but the correlation between long cells and tail % was weak ($r = 0.17$; $P = 0.01$).

Associations previously described between the sum of PCB concentrations and specific PCB congeners (Koopman-Esseboom *et al*., 1994; De Voto *et al*., 1997) were confirmed in these analyses. A strong correlation was observed between the sum of PCB concentrations and congener 153 ($r = 0.96$, $P < 0.0001$), and between congeners 153 and 138 ($r = 0.94$, $P < 0.0001$). There were weak correlations between *p,p′*-DDE and individual PCB congeners ($0.29 < r < 0.35$, $P < 0.0001$).

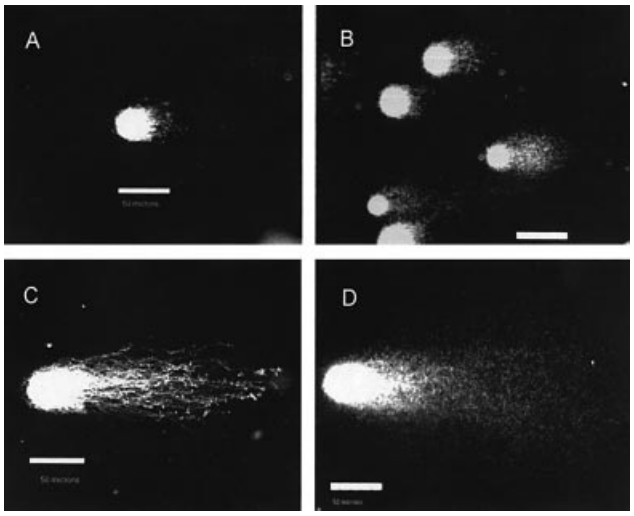

Figure 1. (**A**) Cell with a short comet tail. (**B**) Considerable heterogeneity of comet tail lengths within an individual. (**C**) Cell with a long comet tail. (**D**) Comet referred to as a long cell, a cell with highly damaged DNA that was too long to measure with the image analysis software. Scale bars = 50 μm.

Table II. Distribution of *p,p′*-DDE, HCB, PCB 118, PCB 138, PCB 153, sum of PCB, grouped PCB and comet assay parameters

	n	Percentile							Geometric mean
		Min	5th	25th	50th	75th	95th	Max	
Organochlorines[a]									
p,p′-DDE	212	72.5	96.8	153	220	334	1305	7776	254
HCB	212	6.6	8.7	11.6	15.2	20.6	38.9	68.1	16.0
PCB congener 118	212	3.0	5.3	7.6	12.0	18.4	34.0	82.9	12.3
PCB congener 138	212	7.3	14.3	23.5	32.3	44.8	91.6	260	33.6
PCB congener 153	212	9.3	19.5	30.2	42.2	59.5	125	421	44.0
Sum of PCB congeners	212	56.0	154	108	218	298	592	1590	226
Group 1 (estrogenic) PCB	212	3.9	7.4	11.4	15.9	22.7	47.8	114	16.7
Group 2 (dioxin-like) PCB	212	19.6	36.9	56.0	75.8	109	227	560	80.9
Group 3 (inducing) PCB	212	19.7	40.1	61.6	89.7	128	263	775	92.9
Comet parameters[b]									
Comet extent	212	54.0	78.1	110	130	151	185	223	127
% DNA in tail	212	9.9	13.6	18.0	21.9	27.4	46.5	64.4	22.9
Tail distributed moment	212	29.5	39.3	51.5	58.6	66.9	82.5	107	58.4
No. of long cells	212	0	0	3	7	13	34	95	8.0

[a]*p,p′*-dichlorodiphenyl dichloroethene (DDE), hexachlorobenzene (HCB) and polychlorinated biphenyls (PCB) in ng/g lipid.
[b]VisComet image analysis software was used to measure comet extent (μm), a measure of total comet length; % DNA in tail, a measure of the proportion of total DNA present in the comet tail; and tail distributed moment, an integrated measure of length and intensity (μm).
A 'long cell' was a cell with a comet tail that extended beyond the measuring boundaries in the VisComet image analysis software (>300 μm).

Table III. Adjusted regression coefficients for a change in comet assay parameters associated with an interquartile range (IQR) increase in *p,p′*-DDE, HCB and PCB levels (n = 212)

Organochlorines	IQR (ng/g lipid)	Comet assay parameter[a]		
		Comet extent[b,e]	% DNA tail[c,e]	Tail distributed moment[d,e]
p,p′-DDE	182	–0.13 (–0.96, 0.73)	–0.09 (–0.36, 0.16)	–0.11 (–0.44, 0.24)
Hexachlorobenzene	9.0	0.32 (–3.69, 4.32)	0.47 (–0.75, 1.69)	0.19 (–1.40, 1.79)
PCB congener 118	10.8	1.66 (–2.32, 5.65)	–0.10 (–1.32, 1.12)	0.70 (–0.89, 2.29)
PCB congener 138	21.3	0.89 (–2.51, 4.28)	0.58 (–0.45, 1.62)	0.28 (–1.09, 1.64)
PCB congener 153	29.3	–0.04 (–3.31, 3.22)	0.32 (–0.67, 1.32)	–0.04 (–1.35, 1.26)
Sum of PCB congeners	144	0.43 (–3.30, 4.16)	0.43 (–0.72, 1.58)	0.22 (–1.26, 1.71)
Group 1 (estrogenic) PCB	11.3	0.36 (–3.10, 3.81)	0.68 (–0.37, 1.73)	0.11 (–1.27, 1.49)
Group 2 (dioxin-like) PCB	53.3	0.96 (–2.88, 4.74)	0.43 (–0.69, 1.60)	0.31 (–1.20, 1.83)
Group 3 (inducing) PCB	66.1	–0.20 (–3.83, 3.44)	0.33 (–0.79, 1.39)	–0.01 (–1.45, 1.43)

[a]VisComet image analysis software was used to measure comet parameters.
[b]Comet extent (μm) is a measure of total comet length; coefficient units are μm/IQR (interquartile range).
[c]% DNA tail is a measure of the proportion of total DNA present in the tail; coefficient units are tail %/IQR.
[d]Tail distributed moment (μm) is an integrated measure of length and intensity; coefficient units are μm/IQR.
[e]Coefficient (95% confidence interval) adjusted for age (continuous), and smoking (current, former versus never).
DDE = *p,p′*-dichlorodiphenyl dichloroethene; HCB = hexachlorobenzene; PCB = polychlorinated biphenyls.

There were weak to moderate correlations between age with sum of PCB, groupings of PCB, PCB 138 and PCB 153 ($0.36 < r < 0.45$, $P < 0.0001$), and between *p,p′*-DDE with age (r = 0.24, P = 0.0005). There were weak to moderate correlations between BMI and PCB 118 and group 3 PCB (enzyme inducing), and a weak inverse relationship between BMI and *p,p′*-DDE (r = –0.11, P = 0.1). There were non-significant relationships between smoking status and individual PCB, sum of PCB and *p,p′*-DDE. Except for PCB 118, Hispanics had significantly lower PCB concentrations, but higher *p,p′*-DDE concentrations, than Caucasians. For Hispanics, the median concentration of the sum of PCB and *p,p′*-DDE was 163 and 958 ng/g lipid respectively, as compared with 270 and 268 ng/g lipid respectively, for Caucasians. African-Americans also had significantly higher *p,p′*-DDE, 539 ng/g lipid, than Caucasians, but sum of PCB concentrations in African-Americans (205 ng/g lipid) did not differ with those measured in Hispanics and Caucasians.

Current smoking and ex-smoking status, as compared to never smoking, were not associated with any of the four comet parameters (data not shown). Although there were no statistically significant relationships between age and comet extent, % tail and TDM, the regression coefficients were all positive, indicating an increase in these parameters with age. Comet extent increased 0.28 μm/year [95% confidence interval (CI): –0.54, 1.1], TDM increased 0.02 μm/year (95% CI: –0.31, 0.35), and tail % increased 0.18%/year (95% CI: –0.07, 0.43). The number of long cells increased marginally as age increased (<1 cell/year, P = 0.05).

Although the relationships between smoking and comet assay parameters were inconsistent, we included smoking as a potential confounder in the multiple regression models since several studies have reported increased DNA damage in smokers (Fraga *et al*., 1996; Sun *et al*., 1997; Ündeðer *et al*., 1999). Additionally, age was included in the multiple regression models since there is evidence that DNA damage increases with age (Møller *et al*., 2000; Singh *et al*., 2001). Generally, the crude and adjusted coefficients in the multiple regression models were similar, indicating that there was minimal confounding by age and smoking status.

In the adjusted multiple regression analyses, the three primary comet assay parameters were not statistically significantly associated with *p,p′*-DDE or PCB (either individual congeners, sum of congeners, or groups of congeners). Although not significant, the sum of PCB was associated with a small increase in comet extent [0.43 μm per interquartile range (IQR) increase in sum of PCB; 95% CI: –3.30, 4.16], tail % (0.43%/IQR increase in sum of PCB; 95% CI: –0.72, 1.58), and TDM (0.22 μm/IQR increase in sum of PCB; 95% CI: –1.26, 1.71).

The group 1 PCB congeners (potentially estrogenic congeners) and group 2 PCB congeners (potentially antiestrogenic and dioxin-like), as well as individual congeners 118 and 138, were generally associated with a small, non-significant increase in comet extent, tail %, and TDM. The relationships between group 3 (cytochrome P450 enzyme inducers) PCB were not consistently positive.

Hexachlorobenzene was associated, though not significantly, with a small increase in comet extent (0.32 μm/IQR increase in sum of PCB; 95% CI: –3.69, 4.32), tail % (0.47 %/IQR in sum of PCB; 95% CI: –0.75, 1.69), and TDM (0.19 μm/IQR in sum of PCB; 95% CI: –1.40, 1.79). There were no significant consistent relationships between PCB, *p,p′*-DDE and HCB and the number of long cells.

In secondary analyses, multiple regression was performed using PCB, *p,p′*-DDE and HCB categorized into tertiles. Overall, the results were similar to when PCB, *p,p′*-DDE and HCB were modelled as a continuous variable; there were not strong or consistent associations between PCB, HCB or *p,p′*-DDE and comet parameters (data not shown).

Discussion

The present study, conducted among men from an Andrology Laboratory, did not show statistically significant relationships between comet assay parameters and *p,p′*-DDE, HCB and

individual PCB congeners, sum of PCB, or PCB structure–activity groupings. Overall, these results suggest that there are not strong relationships between adult levels of these chlorinated organic compounds and DNA damage as measured by the neutral comet assay. Further study in diverse populations is needed to confirm the lack of an association between adult levels of organochlorines and sperm DNA damage.

The analysis using individual congeners and groupings of congeners based on structure–activity may represent a more appropriate approach to the analysis between PCB and health endpoints. As widely discussed in the literature, individual congeners have different toxicities and biological activities (Safe, 1990, 1993; Ahlborg *et al*., 1994; Hansen, 1999). Depending on the number and pattern of the chlorine substitutions, the biological activity of individual PCB congeners will vary. Therefore, in addition to analyses using individual PCB congeners and sum of PCB, we used congener groupings based on both structure and potential biological activity based on groupings proposed by Wolff *et al*. (1997). Although these groupings represent an improvement in exposure classification compared with earlier studies that only used sum of PCB, they still have several limitations. For instance, within groups, congeners are summed using concentration but no weighting factor is applied to account for differential activities. In addition, these groupings are based on PCB activity in animal and in-vitro systems, and not in humans. Finally, the groupings are not specifically based on potential DNA damage in human sperm, but rather on general potential biological activity. These limitations will contribute to exposure misclassification of PCB, generally biasing measures of association to the null hypothesis since we expect the misclassification to be random.

In the neutral comet assay, a cell with fragmented DNA has the appearance of a 'comet' with a brightly fluorescent head and a fluorescent tail whose intensity represents the relative amount of DNA strand breaks present (Singh *et al*., 1988; Hughes *et al*., 1997; Singh and Stephens, 1998). The comet assay for human sperm was adapted from methods used on somatic cells, which can be conducted under alkaline or neutral conditions. Neutral conditions were used for human sperm because of the abundance of alkali-sensitive sites in sperm. Alkaline test conditions can induce damage at alkali-labile sites and produce DNA strand breaks (Singh *et al*., 1989).

In previous studies using the comet assay, changes in DNA migration were detected (comet length) at low levels of radiation, 12.5 cGy (rads) of X-rays in human lymphocytes (Singh and Stephens, 1997) and 50 cGy (rads) of X-rays in human sperm (Duty *et al*., 2002). Therefore, we considered comet extent and TDM to represent sensitive quantitative measures of DNA damage. However, tail moment is purported to be a more sensitive measure of DNA damage than TDM and comet extent. This increased sensitivity results from observations that with increasing levels of DNA damage the tail length may not continue to increase but tail % may continue to increase (McKelvey-Martin *et al*., 1993). In addition to these traditional comet assay parameters, we also tallied the number of 'long cells'. We hypothesize that the parameter 'long cell' represented an independent measure of DNA damage. This was partially confirmed by the weak correlation with the traditional comet assay parameters. 'Long cells', which have diffusely dispersed fragmented DNA, represent cells with highly damaged DNA. Definitive characterizations of the comet assay parameters and the significance of the 'long cells' remain to be resolved. Although the present study was not designed to investigate this, we felt it was important to quantify 'long cells' as a separate measure since this may prove useful in future studies using the neutral comet assay.

Although PCB have been well studied, the mechanisms of toxicity are not completely understood. One potential mechanism whereby PCB may produce DNA damage is through PCB metabolites, namely the hydroxy PCB metabolites, inducing free radical-mediated oxidative DNA damage (Oakley *et al*., 1996a; McLean *et al*., 2000). Amaro *et al*. (1996) showed experimentally that lower chlorinated PCB (mono- and dichlorinated biphenyls) may be metabolized to hydroxy-metabolites which are then metabolized to hydroquinones, which can be further oxidized to reactive metabolites, particulary semiquinones, which react with O_2 to form superoxide and quinones. These ROS may produce oxidative DNA damage in the form of DNA strand breaks (Li and Trush, 1993; Srinivasan *et al*., 2001).

PCB are metabolized by cytochrome *P*450-dependent mono-oxygenases to mono- and dihydroxylated compounds (Sipes and Schnellmann, 1987). Researchers have measured hydroxy-PCB metabolites in human blood (Bergman *et al*., 1994) and have shown experimentally that PCB hydroxy-metabolites are activated to reactive intermediates that produce oxidative DNA damage (Oakley *et al*., 1996a). If PCB produce oxidative damage in human sperm, this may be one potential mechanism to support the hypothesis that PCB may contribute to male infertility and male-mediated reproductive adverse pregnancy outcomes. However, in the present study we did not find a strong association between DNA damage (as measured using the neutral comet assay) and PCB.

One potential limitation of our study was that it was a cross-sectional study in which semen and blood samples were collected on the same day. However, the long biological half-life for PCB and *p*,*p*′-DDE (Phillips, 1989a; Brown, 1994) and the relatively short time interval for spermatogenesis (3 months) makes this limitation less of a concern.

In conclusion, in adult men there were not strong relationships between PCB, *p*,*p*′-DDE and HCB and comet assay parameters as measured using the neutral comet assay. The lack of a consistent relationship between comet assay parameters and these organochlorine compounds may indicate that they are not associated with DNA damage in human sperm. Conversely, the small, though positive, non-significant associations found between comet assay parameters and PCB 118, PCB 138 and sum of PCB may have been difficult to detect due to the small sample size. The current and ongoing research on the relationship between environmental agents and semen quality emphasizes the need for a better understanding of the relationship between environmental exposures and semen quality.

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