

Human semen quality and sperm DNA damage in relation to urinary metabolites of pyrethroid insecticides

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BACKGROUND: Exposure to synthetic pyrethroid insecticides is widespread, and is expected to increase among the general population due to the need to replace other common insecticides following regulatory use restrictions. On the basis of limited studies, there is animal and human evidence for altered reproductive or endocrine function following pyrethroid exposure. **METHODS:** The present study measured urinary pyrethroid metabolites [3-phenoxybenzoic acid (3PBA) and *cis*- and *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (CDCCA and TDCCA)], semen quality, sperm motion parameters and sperm DNA damage with the neutral comet assay in 207 men recruited from an infertility clinic. **RESULTS:** In multivariate analysis, the highest 3PBA quartile was associated with a suggestive 20.2 million sperm/ml reduction (95% confidence interval –37.1 to +2.6) in sperm concentration compared with men below the 3PBA median. There were significant inverse associations between TDCCA and sperm motility and sperm motion parameters when adjusting for CDCCA and other covariates. The highest TDCCA quartile was associated with a 15.5% decline (95% confidence interval –26.2 to –4.8) in sperm motility compared with men below the median. In multiple logistic analyses, there were dose-dependent increased odds for below reference sperm concentration, motility and morphology in relation to TDCCA. Among the comet assay measures, 3PBA and CDCCA were associated with increased sperm DNA damage, measured as percent DNA in the comet tail. **CONCLUSIONS:** We found evidence for reduced semen quality and increased sperm DNA damage in relation to urinary metabolites of pyrethroid insecticides. These findings may be of concern due to increased pyrethroid use and prevalent human exposure.

Keywords: biomarkers; epidemiology; male; pesticides; permethrin

Introduction

Synthetic pyrethroid insecticides are among the most commonly available to consumers today. Pyrethroid usage has increased in recent years due to the need to replace common organophosphate insecticides following use restrictions in the USA and other countries. A consequence of the increased availability, use and broad-spectrum applicability of pyrethroids is widespread exposure among the general population. Urinary metabolites of pyrethroid insecticides have been measured in a substantial proportion of the general population in the USA and in Germany (CDC, 2005; Heudorf *et al.*, 2006), and it is reasonable to expect these proportions to increase as organophosphorus insecticides continue to be phased out. Diet is a primary route of exposure to pyrethroids among non-occupationally exposed individuals (ATSDR, 2003), but permethrin and other pyrethroids have also been measured in a high proportion of household dust samples suggesting that

the home environment may also comprise a major exposure source (Rudel *et al.*, 2003; Colt *et al.*, 2004; Tulse *et al.*, 2006; Julien *et al.*, 2007). Thus, exposure to pyrethroids is likely to be multi-media and multi-route, making the use of exposure biomarkers to estimate internal dose advantageous in human epidemiological studies.

Data on altered reproductive or endocrine function resulting from pyrethroid exposure are limited, but animal and *in vitro* studies suggest that some pyrethroids or their metabolites may possess endocrine disrupting properties (Tyler *et al.*, 2000; Mani *et al.*, 2002; ATSDR, 2003; Zhang *et al.*, 2007) and adversely affect semen quality (Salem *et al.*, 1988; Elbetieha *et al.*, 2001; Yousef *et al.*, 2003; el-Demerdash *et al.*, 2004; Zhang *et al.*, 2007). In humans, several studies of occupational exposure to pyrethroids, namely fenvalerate, suggest reductions in semen quality and increased DNA damage and chromosomal aberrations in sperm among

exposed workers (Bian *et al.*, 2004; Kamijima *et al.*, 2004; Xia *et al.*, 2004; Lifeng *et al.*, 2006). Two recent studies among Chinese men have also assessed non-occupational exposure to pyrethroids by measuring metabolite concentrations in urine, and have reported suggestive associations with reduced sperm concentration (Perry *et al.*, 2007; Xia *et al.*, 2007).

The present study adds to the previous human studies of non-occupational pyrethroid exposure and altered semen quality by offering more statistical power (Perry *et al.*, 2007), by including additional pyrethroid metabolites in the analysis (Xia *et al.*, 2007) and by expanding outcome measures to include the assessment of sperm DNA damage measured by neutral comet assay. Investigation of environmental impacts on sperm DNA damage is important since growing and consistent evidence shows that sperm DNA damage adversely affects male fertility, contributing to poorer embryo development and lower pregnancy rates among partners of men undergoing assisted reproductive treatments (Duran *et al.*, 2002; Morris *et al.*, 2002; Agarwal and Allamaneni, 2004; Lewis and Aitken, 2005; Borini *et al.*, 2006).

Methods

Subject recruitment

Between January 2000 and April 2003, men between 18 and 54 years of age were recruited from the Vincent Memorial Andrology lab at Massachusetts General Hospital (MGH) and invited to participate in the study. Approximately 65% of eligible men agreed to participate. The primary reason cited by non-participants was lack of time. Exclusionary criteria included prior vasectomy, self-reported medical risk factors for infertility (e.g. varicocele or orchidopexy) or current use of exogenous hormones. A retrospective review of anonymized clinic records of non-participants, who met the same eligibility criteria as the study subjects, found that there were no differences between participants and non-participants in regards to age or semen parameters (Hauser *et al.*, 2005). Height and weight were measured, and all men completed a brief nurse administered questionnaire at the time of recruitment, and provided health information. The Harvard School of Public Health (HSPH), MGH and University of Michigan Human Subjects Committees approved the study and all subjects signed an informed consent.

Semen sample collection

Semen was collected on site at MGH in a sterile plastic specimen cup after a recommended period of abstinence of 48 h. After liquefaction at 37°C for 30 min, semen quality parameters and motion characteristics were measured at the clinic. The remaining unprocessed semen was frozen in 0.25 ml cryogenic straws (CryoBiosystem, I.M.V. Division, San Diego, CA, USA) by immersing the straws directly into liquid nitrogen (−196°C). Previous work in our laboratory showed that this freezing method produced comet assay results that were highly correlated with results from fresh, unfrozen samples (Duty *et al.*, 2002). Semen samples were later analyzed in batches, where straws were thawed by gently shaking in a 37°C water bath for 10 s and the semen was immediately processed for the comet assay.

Semen quality

Concentration, motility and motion parameters

Semen samples were analyzed for sperm concentration and motion parameters by computer-aided semen analyzer (CASA, HTM-IVOS Version 10HTM-IVOS, Beverly, MA, USA). Setting parameters and the definition of measured sperm motion parameters for CASA were established by the Hamilton-Thorn Company. To measure both sperm concentration and motility, 5 µl of semen from each sample was placed into a pre-warmed (37°C) Makler counting chamber (Sefi—Medical instruments, Haifa, Israel). A minimum of 200 sperm cells from at least four different fields were analyzed from each specimen. Motile sperm was defined as World Health Organization (WHO) grade 'a' sperm (rapidly progressive with a velocity $\geq 25 \mu\text{m/s}$ at 37°C) plus 'b' grade sperm (slow/sluggish progressive with a velocity $\geq 5 \mu\text{m/s}$ but $< 25 \mu\text{m/s}$) (WHO, 1999). Measurement of CASA motion characteristics has been previously described (Duty *et al.*, 2004; Meeker *et al.*, 2004). Of seven CASA variables that were measured, only three were chosen [straight-line velocity (VSL), curvilinear velocity (VCL) and linearity (LIN=VSL/VCL \times 100)] for inclusion in the present analysis due to a high degree of dependence between several of the measures.

Morphology

At least two slides were made for each fresh semen sample. The resulting thin smear was allowed to air dry for an hour before staining with a Diff-Quik staining kit (Dade Behring AG, Duding, Switzerland). Morphological assessment was performed with a Nikon microscope using an oil immersion 100 \times objective (Nikon Company, Tokyo, Japan). A minimum of 200 sperm cells were counted from two slides for each specimen. Strict scoring criteria were used to classify men as having normal or below normal morphology (Kruger *et al.*, 1988).

Neutral comet assay

The comet assay procedure used in the present study to assess sperm DNA damage has been previously described (Singh and Stephens, 1998; Duty *et al.*, 2003). Briefly, 20 µl of a semen/agarose mixture (0.7% 3:1 high resolution agarose; Amresco, Solon, OH, USA) was embedded between two additional layers of agarose on microgel electrophoresis glass slides (Erie Scientific, Portsmouth, NH, USA). Slides were then immersed in a cold lysing solution to dissolve the cell membrane and make chromatin accessible for the enzyme digestion steps. After 1 h cold lysis, slides were transferred to a solution for enzyme treatment with 10 µg/ml of RNase (Amresco) and incubated at 37°C for 4 h. Slides were then transferred to a second enzyme treatment with 1 mg/ml proteinase K (Amresco) and incubated at 37°C for 18 h. The slides were placed on a horizontal slab in an electrophoretic unit, equilibrated for 20 min and underwent electrophoresis for 1 h. DNA in the gel was then precipitated, fixed in ethanol and dried. Slides were stained and observed with fluorescence microscope. Comet extent, tail distributed moment (TDM) and percent DNA located in the tail (Tail%) were measured on 100 sperm in each semen sample using VisComet software (Impuls Computergestutzte Bildanalyse GmbH, Gilching, Germany). Comet extent is a measure of total comet length from the beginning of the head to the last visible pixel in the tail. Tail% is a measurement of the proportion of total DNA that is present in the tail. TDM is an integrated value that takes into account both the distance and intensity of comet fragments:

$$\text{TDM} = \frac{\sum(I \times X)}{\sum I}$$

where ΣI is the sum of all intensity values that belong to the head, body or tail, and X is the x -position of the intensity value.

Urine sample collection and analysis

A single spot urine sample was collected from each subject. Urine samples were frozen at -20°C and sent to the US Centers for Disease Control and Prevention (CDC) where the pyrethroid metabolites 3-phenoxybenzoic acid (3PBA), *cis*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (CDCCA) and *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (TDCCA) were measured. 3PBA is a common metabolite of several pyrethroids including cyhalothrin, cypermethrin, deltamethrin, fenvalerate and permethrin. CDCCA and TDCCA are metabolites of *cis*-permethrin and *trans*-permethrin, respectively, in addition to other pyrethroids such as cyfluthrin and cypermethrin. Analytical chemistry procedures used for measurement of pyrethroid metabolites in urine have been previously described (Baker *et al.*, 2004). Briefly, samples were fortified with stable isotope analogs of the target analytes, underwent solid-phase extraction and were analyzed using high-performance liquid chromatography coupled with tandem mass spectrometry using turbo ion-spray atmospheric pressure ionization. The limit of detection (LOD) was $0.1\ \mu\text{g/l}$ for all three metabolites. 4-Fluoro-3PBA (4F3PBA) and *cis*-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane carboxylic acid (DBCA), specific metabolites of cyfluthrin and deltamethrin, respectively, were also measured, but due to low detection rates were excluded from further analysis. Specific gravity (SG) was used to adjust urine samples for dilution. SG was measured using a handheld refractometer (National Instrument Company, Inc., Baltimore, MD, USA).

Statistical analysis

Data analysis was performed using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). Descriptive statistics on subject demographics were calculated, along with the distributions of urinary pyrethroid metabolite concentrations, semen quality and motion parameters, and sperm DNA damage measures. Bivariate analysis was conducted between all semen quality, sperm DNA damage, pyrethroid metabolite and demographic variables to investigate differences between distributions or categories and the potential for confounding. Differences were tested statistically using parametric or non-parametric methods where appropriate.

Owing to the high proportion of samples below the LOD for 3PBA (46%), CDCCA (47%) and TDCCA (49%), SG-adjusted pyrethroid metabolites were categorized into low, medium or high groups. The low group consisted of values below the median for each metabolite. The medium group was comprised of values greater than or equal to the median but <75 th percentile value, whereas the high groups consisted of values ≥ 75 th percentile. Categories of the sum of CDCCA and TDCCA, as well as the sum of all three pyrethroid metabolites, were also modeled as exposure variables. For values below the LOD, zero was used in the calculation of these summed variables prior to categorizing into low, medium or high groups.

Multiple linear regression was used to assess associations between urinary pyrethroid metabolite categories and continuous measures of semen quality, sperm motion and sperm DNA damage. Sperm concentration was transformed using the natural logarithm, whereas all other semen quality, sperm motion and DNA damage measures were modeled untransformed. Age, body mass index (BMI), race, abstinence period and smoking were considered as covariates, and were included or excluded from models based on biologic and statistical considerations (Kleinbaum *et al.*, 1998). The association between urinary pyrethroid metabolite categories and sperm concentration, motility and morphology was also assessed by multiple logistic

regression, where subjects were dichotomized as either above or below WHO (1999) reference levels for sperm concentration (20 million sperm/ml) and motility (50% motile sperm). The strict criteria (4% normal) were used as a cut-off for sperm morphology (Kruger *et al.*, 1988). Reference subjects were those that were above the reference level for all three parameters. Azoospermic men were not included in the analyses for CASA motion and sperm DNA damage parameters since they were not measurable.

Results

Among the 207 men for whom data on urinary pyrethroid metabolite concentration and semen quality parameters were available, the majority was white and had never smoked (Table I). The mean (SD) age and BMI were 36 (5.6) years and 28 (4.7), respectively. Distributions of semen quality, sperm motion and sperm DNA damage measures, and of SG-adjusted urinary pyrethroid metabolite concentrations, are presented in Tables II and III, respectively. In preliminary bivariate analyses, the proportion of subjects in the high metabolite concentration groups did not differ by season (P -values > 0.05), but increasing exposure groups for 3PBA, CDCCA and TDCCA were associated with greater age (P -values for trend < 0.05). Semen quality parameters were associated with abstinence period categories as previously reported (Meeker *et al.*, 2007). Pyrethroid metabolite groups for CDCCA and TDCCA were moderately correlated with each other (Spearman coefficient = 0.66) and with 3PBA (Spearman coefficients = 0.54 and 0.68, respectively).

In multivariate linear regression, semen quality parameters, sperm motion parameters and sperm DNA damage measures were regressed on categories (<50 th, 50th–75th and >75 th percentile) of SG-adjusted pyrethroid metabolite concentrations (Table IV). Compared with men with values below the median, men in the highest 3PBA category had suggestive declines in sperm concentration and sperm motility. For sperm concentration, which was transformed by the natural logarithm, the regression coefficient of -0.37 represents a 21.2 [95% confidence intervals (CI) -37.1 to $+2.6$] million sperm/ml decrease in adjusted geometric mean sperm concentration compared with men with 3PBA below the median. TDCCA concentration above the 75th percentile was also associated with a suggestive 20.3 (95% CI -35.3 to $+2.6$) million sperm/ml decline in sperm concentration and a statistically significant 10% (95% CI -19% to -1%) reduction in sperm motility.

Since the regression estimates for CDCCA and TDCCA differed and those for TDCCA were noticeably stronger, further analysis was conducted by including both metabolites in the same multivariate model to account for the shared parent compounds between the two metabolites (e.g. cypermethrin). When CDCCA concentration above or below the median was also included in the TDCCA models, the associations of TDCCA with sperm motility and all three sperm motion parameters were stronger and the P -values for trend were statistically significant (data not shown). For example, for sperm motility, the regression estimates for the three TDCCA groups were: 0 (reference), 0.54 (95% CI -8.9 to $+9.0$) and -15.5 (-26.2 to -4.8), with a P -value for trend of 0.01. Trend P -values

Table I. Demographic categories by semen parameters^a (*n* = 207).

	Comparison subjects (<i>n</i> = 90) ^b	Semen parameters		
		Sperm concentration <20 million/ml (<i>n</i> = 42)	Sperm motility <50% motile (<i>n</i> = 106)	Sperm morphology <4% normal (<i>n</i> = 51)
Age, mean (SD)	35.7 (5.3)	36.5 (6.5)	36.6 (5.6)	36.5 (5.9)
BMI, mean (SD)	28.5 (4.8)	28.5 (4.2)	27.5 (4.3)	28.7 (4.3)
Abstinence time, <i>n</i> (%)				
≤2 days	24 (27)	14 (33)	27 (25)	9 (18)
3 days	26 (29)	10 (24)	31 (29)	16 (31)
4 days	17 (19)	6 (14)	20 (19)	11 (22)
5 days	10 (11)	6 (14)	12 (11)	5 (10)
6 or more days	13 (14)	6 (14)	16 (15)	10 (20)
Race, <i>n</i> (%)				
White	82 (91)	33 (79)	85 (80)	42 (82)
Black/Afr-Amer	2 (2)	2 (5)	6 (6)	3 (6)
Hispanic	1 (1)	2 (5)	5 (5)	1 (2)
Other	5 (6)	4 (10)	8 (8)	4 (8)
Smoking status, <i>n</i> (%)				
Never smoker	67 (74)	26 (62)	74 (70)	36 (71)
Ever smoker	22 (24)	15 (36)	30 (28)	14 (27)
Former	15 (17)	10 (24)	24 (23)	9 (18)
Current	7 (8)	5 (12)	6 (6)	5 (10)

^aInformation on race and smoking missing for four men.

^bComparison group consists of men with all three semen parameters above the reference level.

Table II. Distribution of semen quality parameters, sperm motion parameters and sperm DNA damage measures.

	Mean	Selected percentiles					
		10th	25th	50th	75th	90th	95th
Semen quality (<i>n</i> = 207)							
Concentration (10 ⁶ /ml)	107	5.2	26.8	76.8	157	236	263
Motility (%)	47.1	11	25	49	70	77	82
Morphology (% normal)	7.1	1	4	7	10	14	15
Sperm motion (<i>n</i> = 193)							
Straight-line velocity (μm/s)	45.2	32.4	38.4	46.7	52.4	57.7	63.6
Curvilinear velocity (μm/s)	79.0	57.3	67.2	79.0	91.0	104	107
Linearity (%)	57.8	50	54	58	62	67	69
DNA damage (<i>n</i> = 143)							
Comet extent (μm)	116	78.1	92.5	112	134	162	177
Tail distributed moment (μm)	51.1	34.6	40.9	50.2	58.4	71.0	74.5
Percent DNA in tail (%)	33.8	17.1	22.8	32.3	44.8	51.9	57.9

Table III. Distribution of SG-adjusted pyrethroid metabolites in urine (ng/ml), *n* = 207.

Pyrethroid metabolite	Selected percentiles						
	10th	25th	50th	75th	90th	95th	Max
3PBA	<0.10	<0.10	0.14	0.45	1.31	2.48	61.3
<i>cis</i> -DCCA	<0.10	<0.10	0.14	0.29	0.58	1.55	23.2
<i>trans</i> -DCCA	<0.10	<0.10	0.12	0.39	1.25	4.07	36.8
<i>cis</i> + <i>trans</i> -DCCA	<0.10	0.11	0.27	0.65	1.85	6.01	59.9
Sum pyrethroid	<0.10	0.16	0.46	1.02	3.12	8.13	121

for VSL, VCL and LIN were 0.02, 0.04 and 0.008, respectively. The association between TDCCA and sperm concentration also became stronger after adjusting for CDCCA. TDCCA concentration above the 75th percentile was associated with a significant 34.2 (95% CI -51.1 to -5.7) million sperm/ml decline in sperm concentration (*P*-value for trend across the three TDCCA groups = 0.06).

In multiple logistic regression analyses in which sperm concentration, motility and morphology were categorized as above or below reference value (Table V), the odds of having sperm concentration below 20 million/ml were increased 2.7-fold (95% CI 1.1–6.9) for men with TDCCA above the 75th percentile compared with those with TDCCA below the median (*P*-value for trend = 0.06). As with the linear regression

Table IV. Adjusted regression coefficients for change in semen quality and sperm motion parameters associated with urinary pyrethroid metabolite groups.

Metabolite percentiles	Semen quality parameters ^a			CASA motion parameters ^b		
	Concentration ^c	Motility	Morphology	VSL	VCL	LIN
3PBA						
<50th	0	0	0	0	0	0
50th–75th	0.13 (–0.26, 0.53)	1.37 (–7.23, 9.97)	1.17 (–0.44, 2.78)	–1.31 (–5.08, 2.46)	–3.59 (–10.1, 2.88)	1.08 (–1.82, 3.98)
>75th	–0.37 (–0.78, 0.04)	–6.73 (–15.5, 2.07)	–0.22 (–1.87, 1.43)	–1.62 (–5.52, 2.28)	–3.91 (–10.6, 2.79)	–0.71 (–3.71, 2.29)
<i>P</i> for trend	0.14	0.19	0.98	0.38	0.2	0.78
cis-DCCA						
<50th	0	0	0	0	0	0
50th–75th	0.38 (–0.02, 0.78)	5.66 (–2.92, 14.2)	1.17 (–0.44, 2.78)	–2.66 (–6.43, 1.12)	–4.21 (–10.7, 2.29)	–0.49 (–3.41, 2.43)
>75th	–0.29 (–0.69, 0.11)	–3.34 (–12.1, 5.37)	0.31 (–1.32, 1.95)	–1.66 (–5.50, 2.19)	–3.97 (–10.6, 2.65)	–0.70 (–3.67, 2.27)
<i>P</i> for trend	0.36	0.65	0.54	0.29	0.18	0.62
trans-DCCA						
<50th	0	0	0	0	0	0
50th–75th	0.24 (–0.15, 0.64)	2.66 (–5.89, 11.2)	0.42 (–1.21, 2.04)	–0.11 (–3.87, 3.64)	–0.99 (–7.47, 5.50)	0.88 (–2.00, 3.76)
>75th	–0.38 (–0.80, 0.04)	–9.96 (–18.8, –1.06)	–0.26 (–1.95, 1.43)	–2.96 (–6.99, 1.06)	–4.02 (–11.0, 2.92)	–2.04 (–5.13, 1.05)
<i>P</i> for trend	0.19	0.06 ^d	0.86	0.19 ^d	0.27 ^d	0.30 ^d
cis-+trans-DCCA						
<50th	0	0	0	0	0	0
50th–75th	0.20 (–0.20, 0.59)	2.72 (–5.80, 11.2)	0.43 (–1.19, 2.04)	2.51 (–1.22, 6.23)	2.61 (–3.82, 9.05)	1.27 (–1.61, 4.14)
>75th	–0.33 (–0.75, 0.08)	–9.84 (–18.5, –1.14)	–0.12 (–1.77, 1.54)	–2.32 (–6.23, 1.59)	–3.70 (–10.5, 3.07)	–1.59 (–4.61, 1.44)
<i>P</i> for trend	0.23	0.06	0.98	0.46	0.43	0.47
Sum pyrethroids						
<50th	0	0	0	0	0	0
50th–75th	–0.01 (–0.40, 0.38)	–0.31 (–8.90, 8.28)	0.48 (–1.14, 2.10)	–2.23 (–5.96, 1.51)	–4.50 (–10.9, 1.91)	0.59 (–2.29, 3.46)
>75th	–0.36 (–0.77, 0.05)	–7.64 (–16.4, 1.14)	0.08 (–1.57, 1.74)	–1.97 (–5.89, 1.94)	–3.90 (–10.6, 2.82)	–1.14 (–4.15, 1.88)
<i>P</i> for trend	0.11	0.11	0.84	0.25	0.18	0.56

^aAdjusted for age and abstinence period, *n* = 207.^bAdjusted for age and abstinence period, *n* = 193.^cIn-transformed.^dWhen additionally adjusting for *cis*-DCCA, became statistically significant (results not shown).

analysis, the associations between the high TDCCA group and semen quality became larger and stronger after adjustment for CDCCA. For below reference sperm concentration, the odds ratios for the three TDCCA groups were 1.0 (reference), 1.32 (95% CI 0.5–3.7) and 6.3 (1.8–21.9) with a *P*-value for trend of 0.006. For below reference sperm motility and morphology, the odds ratio estimates for the highest TDCCA group compared with the lowest was 2.7 (1.1–6.6) and 3.0 (1.0–9.0), respectively, with trend *P*-values of 0.06 and 0.07.

Because the comet assay was introduced after the study began, sperm DNA damage measures were available for only 143 of the men in the present analysis (Table VI). 3PBA and CDCCA were associated with increased sperm DNA damage measured as Tail%, but not comet extent or TDM. The association between 3PBA categories and Tail% was monotonic, suggesting a dose–response trend (*P*-value for trend = 0.02). Compared with the low 3PBA group, the regression coefficients for medium and high 3PBA groups were equivalent to increases in Tail% of 7.5% (95% CI –10% to 25%) and 20% (3–37%), respectively, relative to the study population median (median = 32.3% of DNA in comet tail).

Discussion

In the present study, we found associations between urinary metabolites of pyrethroid insecticides and decreased semen quality, as well as evidence for a relationship between pyrethroid metabolites and sperm DNA damage. We cannot rule

out the possibility that some of our statistically significant or suggestive results were due to chance since multiple comparisons were made. Nevertheless, these findings may be of concern due to the increased use of pyrethroid pesticides resulting in widespread exposure among the general population. In addition, the associations we report are among men with urinary pyrethroid metabolite concentrations similar to or slightly lower than those measured among the US general population in the most recent (Third) National Report on Human Exposure to Environmental Chemicals (CDC, 2005). Median, 75th and 95th percentile concentrations for unadjusted 3PBA among males in the Third National Report were 0.29, 0.68 and 3.23 µg/l compared with 0.12, 0.42 and 2.27 µg/l in the present study. For TDCCA, these percentiles were <LOD, 0.40 and 2.37 µg/l among males in the Third National Report compared with 0.10, 0.36 and 3.34 in the present study. Pyrethroid metabolite concentrations in the present study were also similar to those found in urine samples from adults and children in the German general population (Heudorf and Angerer, 2001; Schettgen *et al.*, 2002). A recent cross-validation study showed that comparable pyrethroid metabolite data were obtained among unknown urine samples between the US and German laboratories using different extraction and analytical techniques (Barr *et al.*, 2007).

Exposure biomarkers are the preferred method for estimating individual exposures in environmental epidemiological studies of pesticides since they can account for all exposure sources and exposure routes, and urinary metabolites are

Table V. Adjusted^a odds ratios for below reference semen quality parameters associated with urinary pyrethroid metabolite groups.

Metabolite percentiles	<i>n_a</i>	Concentration		Motility		Morphology	
		<i>n_b</i>	OR (95% CI)	<i>n_b</i>	OR (95% CI)	<i>n_b</i>	OR (95% CI)
3PBA							
<50th	47	20	1	51	1	25	1
50th–75th	24	6	0.59 (0.21, 1.68)	25	0.93 (0.46, 1.85)	11	0.80 (0.33, 1.91)
>75th	19	16	1.95 (0.82, 4.67)	30	1.36 (0.66, 2.78)	15	1.35 (0.58, 3.17)
<i>P</i> for trend			0.2		0.46		0.58
<i>cis</i>-DCCA							
<50th	41	22	1	55	1	28	1
50th–75th	29	6	0.38 (0.14, 1.06)	21	0.53 (0.26, 1.05)	10	0.49 (0.20, 1.18)
>75th	20	14	1.28 (0.54, 3.06)	30	1.05 (0.52, 2.13)	13	0.90 (0.38, 2.11)
<i>P</i> for trend			0.84		0.83		0.58
<i>trans</i>-DCCA							
<50th	46	17	1	52	1	23	1
50th–75th	27	9	0.92 (0.36, 2.35)	21	0.66 (0.33, 1.34)	13	0.91 (0.39, 2.11)
>75th	17	16	2.72 (1.07, 6.92)	33	1.61 (0.77, 3.36)	15	1.59 (0.66, 3.83)
<i>P</i> for trend			0.06 ^b		0.35 ^c		0.37 ^d
<i>cis</i>+<i>trans</i>-DCCA							
<50th	44	16	1	52	1	25	1
50th–75th	29	10	0.94 (0.37, 2.36)	21	0.60 (0.30, 1.20)	11	0.67 (0.29, 1.58)
>75th	17	16	2.66 (1.07, 6.63)	33	1.57 (0.76, 3.25)	15	1.47 (0.62, 3.47)
<i>P</i> for trend			0.05		0.41		0.54
Sum pyrethroids							
<50th	48	19	1	49	1	26	1
50th–75th	23	9	0.99 (0.39, 2.54)	26	1.08 (0.54, 2.16)	11	0.86 (0.36, 2.06)
>75th	19	14	1.86 (0.76, 4.54)	31	1.51 (0.74, 3.08)	14	1.26 (0.54, 2.96)
<i>P</i> for trend			0.21		0.28		0.67

n_a, number of men above reference level for all three semen parameters by pyrethroid metabolite category.

n_b, number of men below reference level for that semen parameter by pyrethroid metabolite category.

^aAdjusted for age and abstinence period.

^bWhen also adjusted for *cis*-DCCA, odds ratio (OR) estimates become 1.0, 1.32 (0.48, 3.67) and 6.29 (1.80, 21.9); *P* for trend = 0.006.

^cWhen also adjusted for *cis*-DCCA, OR estimates become 1.0, 0.85 (0.40, 1.81) and 2.66 (1.08, 6.59); *P* for trend = 0.06.

^dWhen also adjusted for *cis*-DCCA, OR estimates become 1.0, 1.18 (0.49, 2.91) and 2.98 (0.99, 8.98); *P* for trend = 0.07.

Table VI. Adjusted^a regression coefficients for change in DNA damage associated with urinary pyrethroid metabolite groups.

Metabolite percentiles	Sperm DNA damage measures		
	Comet extent	TDM	Tail %
3PBA			
<50th	0	0	0
50th–75th	−14.4 (−27.9, −0.92)	−5.58 (−11.3, 0.16)	2.42 (−3.11, 7.94)
>75th	4.59 (−9.05, 18.2)	−1.29 (−7.09, 4.52)	6.45 (0.86, 12.0)
<i>P</i> for trend	0.81	0.46	0.02
<i>cis</i>-DCCA			
<50th	0	0	0
50th–75th	−2.19 (−16.2, 11.9)	−2.44 (−8.36, 3.47)	4.46 (−1.20, 10.1)
>75th	3.93 (−10.1, 17.9)	−0.17 (−6.07, 5.73)	4.97 (−0.67, 10.6)
<i>P</i> for trend	0.64	0.85	0.06
<i>trans</i>-DCCA			
<50th	0	0	0
50th–75th	−5.11 (−19.0, 8.77)	−3.64 (−9.48, 2.20)	4.09 (−1.54, 9.73)
>75th	4.53 (−9.58, 18.6)	0.97 (−4.96, 6.90)	−0.47 (−6.20, 5.25)
<i>P</i> for trend	0.65	0.94	0.92
<i>cis</i>+<i>trans</i>-DCCA			
<50th	0	0	0
50th–75th	−9.05 (−22.9, 4.82)	−4.47 (−10.3, 1.37)	5.57 (−0.07, 11.2)
>75th	4.82 (−9.11, 18.7)	1.15 (−4.72, 7.02)	1.42 (−4.24, 7.08)
<i>P</i> for trend	0.69	0.93	0.43
Sum pyrethroids			
<50th	0	0	0
50th–75th	−16.2 (−29.7, −2.62)	−7.63 (−13.3, −1.93)	4.68 (−0.92, 10.3)
>75th	2.61 (−11.1, 16.3)	−0.42 (−6.18, 5.35)	2.51 (−3.16, 8.18)
<i>P</i> for trend	0.94	0.55	0.27

^aAdjusted for age and smoking, *n* = 143.

advantageous due to ease of urine sample collection and large sample volumes (low limits of detection). However, a limitation of relying solely on their use is that they usually do not allow for the determination of primary exposure sources or the differentiation between exposure to the parent compound or the breakdown products in the environment. In addition, pyrethroids and other non-persistent pesticides are rapidly metabolized and excreted, so metabolite concentrations in urine reflect exposure over hours or days preceding sample collection. The ability of a single urine sample to predict metabolite concentrations over longer periods of interest is not yet known, but consistent individual time-activity patterns from day to day and month to month coupled with stable microenvironmental pyrethroid concentrations (or pyrethroid residues in food) may lead to 'pseudo-steady state' metabolite concentrations over long periods of time (NRC, 2006). Likewise, the temporal reliability for non-persistent insecticide metabolites, such as those from carbaryl and chlorpyrifos, may be adequate when categorizing individuals into broad exposure groups (Meeker *et al.*, 2005), as was done in the present analysis. Despite these potential limitations, the use of exposure biomarkers of pyrethroid insecticides is a strength of the present study.

Our suggestive findings of an association between 3PBA and sperm concentration are consistent with those recently reported by Xia *et al.* (2007). Similar in design to the present study, Xia and colleagues recruited 376 men (mean age ~30 years) who had no specific occupational exposure to pyrethroids through a Chinese infertility clinic from March 2004 to March 2006. Urinary 3PBA concentrations were measured, as were semen quality and sperm motion parameters. The men were divided into 3PBA quartiles, and men in the highest quartile had significantly increased odds (OR = 2.0, 95% CI 1.0–4.1) of having <20 million sperm/ml compared with men in the lowest 3PBA quartile. This is similar in magnitude to the odds ratio we report among the highest 3PBA quartile (OR = 2.0, 95% CI 0.8–4.7), though the comparison group in the present study is men below the 50th percentile for 3PBA concentration (i.e. combined first and second quartiles). The wider confidence intervals in the present study likely reflect the smaller study size and thus lower statistical power. In addition, 3PBA concentrations among the Chinese men were higher, where the 50th and 75th percentile values (lower bound concentrations for third and fourth quartiles) for unadjusted 3PBA were 1.25 and 1.90 $\mu\text{g}/\text{l}$ compared with 0.12 and 0.42 $\mu\text{g}/\text{l}$ in the present study. However, the highest unadjusted 3PBA concentration was greater in the present study (53.6 $\mu\text{g}/\text{l}$ compared with 30.9 $\mu\text{g}/\text{l}$ in the Chinese study). We also reported an increased odds of having below reference sperm concentration for men in the highest TDCCA quartile, but CDCCA and TDCCA were not measured in the Chinese study which limits our ability to further compare results.

Our findings of a suggestive inverse association between sperm concentration and both 3PBA and TDCCA are consistent with preliminary results from another recent Chinese study (Perry *et al.*, 2007). In that study, Perry *et al.* recruited 202 young and newly married men from an agricultural area in rural China. Urine samples from 18 of the men were randomly chosen to be analyzed for pesticide metabolite

concentrations, including 3PBA, CDCCA and TDCCA, at the same CDC laboratory that analyzed the urine samples from the present study. Sperm concentrations were compared among men above or below the median pesticide metabolite concentration. CDCCA was only detected in two of the samples and was thus excluded from further analysis. The crude geometric mean sperm concentration among men above the median for 3PBA was 12 million sperm/ml lower than among the men below the 3PBA median. For TDCCA, men above the median concentration had a crude geometric mean sperm concentration 34 million sperm/ml less than men with TDCCA below the median. Although the study was too small to perform detailed or powerful statistical analyses, the results were suggestive of an association and were consistent with our findings among a larger study population. Unadjusted 3PBA concentrations were higher than those found in the present study (median 1.1 $\mu\text{g}/\text{l}$ compared with 0.12 $\mu\text{g}/\text{l}$), whereas median and range TDCCA concentrations were very similar between the two studies.

Our results do not support additive effects on semen and sperm quality among the pyrethroids represented by the various metabolites assessed, as summed metabolite values generally showed weaker associations with semen quality and sperm DNA damage than individual metabolites. The differences in our findings between CDCCA and TDCCA suggest an association between environmental exposures to *trans*-permethrin or TDCCA, but not *cis*-permethrin or CDCCA, and reduced sperm concentration, motility and sperm motion parameters. Conversely, CDCCA and 3PBA, but not TDCCA, were associated with increased sperm DNA damage, potentially suggesting differential biological mechanisms on the male reproductive system between pyrethroid insecticides. Studies of pyrethroid exposure and sperm DNA damage or genotoxicity are limited to men exposed occupationally to fenvalerate (Bian *et al.*, 2004; Xia *et al.*, 2004), and exposure information on fenvalerate among the general population is scarce. According to estimates from 1997, only 30 000 pounds of fenvalerate was applied in the USA, compared with over 1 million pounds of permethrin that same year (ATSDR, 2003). More studies on pyrethroid exposure and sperm DNA damage/fragmentation are needed, as sperm DNA integrity is becoming more clinically relevant as increased evidence surfaces on its ability to predict male fertility and birth outcomes (Erenpreiss *et al.*, 2006).

Data on endocrine or reproductive effects specific to *trans*-permethrin or TDCCA are also lacking, as *cis*-permethrin is associated with higher acute toxicity than *trans*-permethrin (ATSDR, 2003). A recent study of mice orally administered *cis*-permethrin at doses of 0, 35 or 70 mg/kg-day for 6 weeks reported significant dose-dependent declines in epididymal sperm count and sperm motility, and testicular and circulating levels of testosterone, along with a dose-dependent increase in circulating LH (Zhang *et al.*, 2007). Further, testicular residue concentrations of *cis*-permethrin from the individual animals were strongly inversely correlated with testicular testosterone levels. Exposure-related reductions in mRNA and protein expression levels of peripheral benzodiazepine receptor, steroidogenic acute regulatory protein, and cytochrome P450

side-chain cleavage were also observed, as well as structural changes in Leydig cell mitochondria, suggesting these disruptions resulted in a reduction of cholesterol transport and conversion essential for testicular steroidogenesis. A previous study in rats also found reduced sperm counts, testosterone, FSH and LH following ingestion of tap water containing cypermethrin (Elbetieha *et al.*, 2001). On the basis of these findings and the results of the present human study, similarly detailed work on *trans*-permethrin and other pyrethroids is needed to provide a clearer understanding on the specific exposures and mechanisms potentially involved in the associations reported here.

In conclusion, we found evidence for reduced semen quality and increased sperm DNA damage in relation to urinary metabolites of pyrethroid insecticides, though the specific pesticide(s) and biological mechanisms potentially responsible are not yet clear. Some of our findings are consistent with other human studies among populations with higher levels of pyrethroid exposure, which suggests future research should be concerned with male reproductive effects from both occupational and environmental (non-occupational) pyrethroid exposure. Future work in our group includes the assessment of reproductive hormone levels associated with pyrethroid exposure among these men, and investigation of additive or synergistic effects between pyrethroids and other insecticides (organophosphates and carbamates; see Hodgson and Rose, 2007) on intermediate measures of male reproductive health among a larger, more statistically powerful, cohort of men.

Conflict of interest: The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the NIEHS or CDC.

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