Human Reproduction Vol.19, No.11 pp. 2573–2580, 2004
Advance Access publication August 27, 2004
doi:10.1093/humrep/deh444

Urinary levels of insecticide metabolites and DNA damage in human sperm

John D.Meeker[1], Narendra P.Singh[2], Louise Ryan[3], Susan M.Duty[4], Dana B.Barr[5], Robert F.Herrick[1], Deborah H.Bennett[1] and Russ Hauser[1,6,7]

[1]Department of Environmental Health and [3]Department of Biostatistics, Harvard School of Public Health, [4]Nursing Program, School for Health Studies, Simmons College, [6]Vincent Memorial Obstetrics and Gynecology Service, Andrology Laboratory and In Vitro Fertilization Unit, Massachusetts General Hospital, Boston, MA, [2]Department of Bioengineering, University of Washington, Seattle, WA and [5]Centers for Disease Control and Prevention, Atlanta, GA, USA

[7]To whom correspondence should be addressed at: Department of Environmental Health, Occupational Health Program, Harvard School of Public Health, Building 1 Room 1405, 665 Huntington Avenue, Boston, MA 02115, USA.
E-mail: rhauser@hohp.harvard.edu

BACKGROUND: Members of the general population are exposed to non-persistent insecticides at low levels. The present study explored whether environmental exposures to carbaryl and chlorpyrifos are associated with DNA damage in human sperm. METHODS: Subjects ($n = 260$) were recruited through a Massachusetts infertility clinic. Individual exposures were measured as spot urinary metabolite concentrations of chlorpyrifos [3,5,6-trichloro-2-pyridinol (TCPY)] and carbaryl [1-naphthol (1N)], adjusted using specific gravity. Sperm DNA integrity was assessed by neutral comet assay and reported as comet extent, percentage DNA in comet tail (Tail%) and tail distributed moment (TDM). RESULTS: A statistically significant increase in Tail% was found for an interquartile range (IQR) increase in both 1N [coefficient = 4.1; 95% confidence interval (CI) 1.9–6.3] and TCPY (2.8; 0.9–4.6), while a decrease in TDM was associated with IQR changes in 1N (−2.2; −4.9 to 0.5) and TCPY (−2.5; −4.7 to −0.2). A negative correlation between Tail% and TDM was present only when stratified by comet extent, suggesting that Tail% and TDM may measure different types of DNA damage within comet extent strata. CONCLUSIONS: Environmental exposure to carbaryl and chlorpyrifos may be associated with increased DNA damage in human sperm, as indicated by a change in comet assay parameters.

Key words: comet assay/DNA damage/exposure/insecticides

Introduction

Human exposure to environmental levels of non-persistent, contemporary use insecticides occurs through a variety of pathways. Potential exposure routes include ingestion of insecticide residue on food and inhalation or dermal exposure from insecticide-treated areas, such as indoor air and dust that may contain insecticides or their environmental breakdown intermediates. The Second US National Report on Human Exposure to Environmental Chemicals, National Health and Nutrition Examination Survey (NHANES 1999–2000) reported measurable levels of urinary 3,5,6-trichloro-2-pyridinol (TCPY), a metabolite of chlorpyrifos and chlorpyrifos-methyl, and 1-naphthol (1N), a metabolite of carbaryl and naphthalene, in >90% and 75% of males in the USA, respectively (CDC, 2003).

We recently found an association between urinary metabolites of carbaryl (1N) and chlorpyrifos (TCPY) and altered human semen quality (Meeker *et al.*, 2004a). Specifically, we found statistically significant associations between 1N and decreased sperm concentration and motility, along with suggestive associations between TCPY and sperm concentration and motility. The observed associations between 1N and sperm concentration and motility were consistent with previous studies of carbaryl exposure in humans (Whorton *et al.*, 1979; Wyrobek *et al.*, 1981) and laboratory rats (Rybakova, 1966; Shtenberg and Rybakova, 1968; Pant *et al.*, 1995, 1996). Few human and animal studies exist for comparison with our suggestive results for TCPY.

In addition to semen parameters, laboratory measures of sperm DNA damage are increasingly used to evaluate sperm function. The comet assay has been used to monitor DNA damage in a number of different cell types (Singh *et al.*, 1988; Olive and Banath, 1996), including human sperm (Singh and Stephens, 1998; Duty *et al.*, 2003). The comet assay relies on the electrophoretic movement of DNA out of single cells, in which low molecular fragments migrate away from the cell towards the positive electrode in a pattern resembling a comet tail. It is useful as an epidemiological

end-point in environmental and occupational studies since it is a measure of DNA damage, which is predictive of fertilization and embryo cleavage rates (Sun *et al.*, 1997).

To our knowledge, the relationship between DNA damage in human sperm and exposure specifically to carbaryl and chlorpyrifos has not been investigated. The pesticide dibromochloropropane (DBCP), a well-documented testicular toxicant (ATSDR, 1992), was found to be associated with increased DNA single strand breaks (SSBs) in rat sperm cells (Bjorge *et al.*, 1995). Exposure to a complex mixture of pesticides was found to be associated with increased DNA damage in blood leukocytes and lymphocytes of pesticide production workers (Garaj-Vrhovac and Zeljezic, 2000; Grover *et al.*, 2003), but it was not possible to discern which pesticides were most strongly associated with DNA damage due to the non-specific nature of the exposure assessments. Numerous animal and *in vitro* studies have been conducted to investigate genotoxic properties of specific pesticides, and provided evidence that both carbaryl and chlorpyrifos may be associated with genotoxic effects in various cell types (Ahmed *et al.*, 1977a,b; Woodruff *et al.*, 1983; Onfelt, 1983, 1987; Lieberman *et al.*, 1998; Rahman *et al.*, 2002). The objective of the present study was to explore the association between environmentally relevant levels of carbaryl and chlorpyrifos metabolites in urine and DNA damage to human sperm cells.

Methods

Study subjects were men who were partners in subfertile couples seeking infertility diagnosis from the Vincent Burnham Andrology Laboratory at Massachusetts General Hospital (Boston, USA) between January 2000 and April 2003. Details of subject recruitment and sample collection have been described previously (Hauser *et al.*, 2003). Briefly, consecutive eligible men were recruited to participate. Of those approached, 65% consented. A single spot urine sample was collected from each subject on the same day as the semen sample. Urine samples were frozen at −20°C and sent to the US Centers for Disease Control and Prevention (CDC) where TCPY and 1N were measured (Hill *et al.*, 1995). Samples were fortified with stable isotope analogues of the target analytes, and glucuronide or sulphate-bound metabolites were liberated using an enzyme hydrolysis. TCPY and 1N were isolated using liquid–liquid extraction, chemically derivatized, and measured using gas chromatography–chemical ionization–tandem mass spectrometry.

Creatinine (CRE) concentrations are frequently used to adjust for variable urine dilution in spot samples when measuring pesticide metabolites. However, CRE adjustment may not be appropriate for compounds that undergo active tubular secretion, which includes organic compounds such as TCPY and 1N that can be conjugated by the liver in the form of glucuronides or sulphates (Boeniger *et al.*, 1993). In addition, CRE levels may vary by gender, age, muscle mass, race, diet, activity and time of day. Therefore, adjusting urine insecticide metabolite concentrations using specific gravity (SG) may be more appropriate than CRE. Thus, in the present study, SG-adjusted metabolite concentrations were used as the primary method for dilution adjustment. However, in addition to SG-adjusted results, volume-based (unadjusted) and CRE-adjusted TCPY and 1N concentrations were also determined for comparison with exposure distributions from other studies. Samples with CRE concentrations >300 or <30 mg/dl, or with SG $>$ 1.03 or <1.01 were considered too concentrated or too dilute, respectively, to provide valid results and were excluded from the primary analysis (Teass *et al.*, 1998). CRE was measured photometrically using kinetic colorimetric assay technology with a Hitachi 911 automated chemistry analyser (Roche Diagnostics, Indianapolis, IN). SG was measured using a hand-held refractometer (National Instrument Company, Inc., Baltimore, MD).

Semen samples were preserved in liquid nitrogen (−196°C) within 1 h of collection and later analysed at the Harvard School of Public Health for DNA damage by the neutral comet assay. The comet assay is used widely for somatic cell types, and has been modified to de-condense chromatin in sperm cells. Spermatazoa DNA is tightly bound by protamine molecules (Haines *et al.*, 1998), and a more extensive lysis step allows the liberation of DNA from proteins (Singh and Stephens, 1998). Neutral conditions were used 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). The comet assay procedure used in the present study has been described previously (Duty *et al.*, 2003). Briefly, 50 μl of a semen–agarose mixture was embedded between two additional layers of agarose on microgel electrophoresis glass slides. 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 lyses, slides were transferred to a solution for enzyme treatment with 10 mg/ml of RNase (Amresco, Solon, OH) 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 under a fluorescence microscope. Comet extent, tail distributed moment (TDM) and percentage 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:

$$\mathrm{TDM} = \Sigma(I \times X)/\Sigma I$$

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. Cells with high DNA damage (CHD), which are cells too long to measure with VisComet (>300 μm), were counted for each subject and used as an additional measure of DNA damage.

Statistical analysis

Data analysis was performed using SAS version 8.0 (SAS Institute Inc., Cary, NC). Descriptive statistics on subject demographics were tabulated, along with the distributions of insecticide metabolite levels and comet parameters. For insecticide metabolite values below the limit of detection (LOD), corresponding to 0.25 μg/l for TCPY and 0.40 μg/l for 1N, an imputed value equal to one-half the LOD was used. Values for comet extent, TDM and Tail% represent the arithmetic mean of the 100 cells measured per subject. Comet parameters and insecticide metabolite levels were stratified by demographic categories to investigate the potential for confounding. Spearman correlation coefficients were used to determine correlations among insecticide metabolites and between comet assay parameters. The normality of the comet parameters and metabolite

concentrations was assessed and appropriate transformations were performed. Comet extent and TDM were normally distributed. Tail% was not normally distributed, but because results and interpretation from analyses using both unadjusted and log-transformed Tail% were consistent, results using unadjusted Tail% are presented for ease of interpretation. The number of CHD in each subject's semen sample was not normally distributed and an arcsine transformation was used (Zar, 1984). SG-adjusted TCPY and 1N concentrations were both log-transformed.

Univariate and multivariate linear regression were used to explore relationships between comet assay parameters and SG-adjusted urinary insecticide concentrations. Inclusion of covariates was based on statistical and biological considerations (Hosmer and Lemeshow, 1989). Age was modelled as a continuous variable and abstinence time modelled as an ordinal five-category variable ($\leq$2 days, 3, 4, 5 or $>$6 days). Smoking status was dichotomized by current smoker versus never smoked or former smoker, and race was categorized into four groups: white, African-American, Hispanic and other. Regression coefficients were transformed into coefficients representing an interquartile range (IQR) increase in SG-adjusted metabolite level to improve the interpretability of results. A test for interaction between smoking status and urine insecticide metabolites was assessed by including an interaction term in the multivariate linear regression models.

Results

Comet assay parameters and urinary insecticide metabolite concentrations were available from 260 of 368 men recruited into the study. The comet assay was not performed on 74 men: comet assay parameters were not available for the first 46 men recruited into the study because the study initially did not archive semen samples for comet assay analysis; 19 men were azoospermic (had no sperm cells available for the comet assay); and nine samples were lost in processing. Insecticide metabolite levels were not available for 34 of the remaining 294 men. Demographic information is provided in Table I. The mean ($\pm$SD) age in years and body mass index were 36.1 $\pm$ 5.6 and 28.3 $\pm$ 4.7, respectively. Approximately 82% of subjects were white, and no other race/ethnicity contributed $>$7% of subjects. Most subjects had never smoked (74%), and only 9% were current smokers.

Distributions of unadjusted, CRE-adjusted and SG-adjusted TCPY and 1N levels are presented in Table II. Forty-six (18%) men were excluded from the primary data analysis because SG values were too concentrated (SG $>$ 1.030) or too dilute (SG $<$ 1.010). Of the 214 men with SG in the acceptable range, SG-adjusted TCPY and 1N levels were moderately correlated (Spearman correlation coefficient = 0.3; $P < 0.001$). SG-adjusted TCPY concentrations ranged from below detection to 40.7 μg/l, with a geometric mean of 1.71 μg/l, and SG-adjusted 1N ranged from below detection to 160 μg/l, with a geometric mean of 2.12 μg/l.

Table I. Subject demographics, $n = 260$

Age, mean (SD) years	36.1 (5.6)
Body mass index, mean (SD)	28.3 (4.7)
Race, *n* (%)	
White	214 (82)
African-American	14 (5)
Hispanic	16 (6)
Other	16 (6)
Smoking status, *n* (%)	
Never smoker	192 (74)
Ever smoker	68 (26)
Ex-smoker	46 (18)
Current smoker	22 (9)

Percentages are rounded and may not total 100.

Distributions of comet parameters are presented in Table III. Comet extent ranged from 47.6 to 223 μm, with a mean of 126 μm. TDM had a range of 25.7 to 107 μm, with a mean of 57.1 μm, and Tail% ranged from 9.9 to 64.4 μm, with a mean of 26.5 μm. The number of long cells per subject ranged from 0 to 95. Comet extent and TDM were highly correlated among the 214 men with SG in the acceptable range (Spearman coefficient = 0.9; $P < 0.001$). However, comet extent and Tail% were only slightly correlated (Spearman coefficient = 0.2; $P = 0.001$), and TDM and Tail% were not correlated (Spearman coefficient = -0.08; $P = 0.2$).

In univariate linear regression, none of the demographic variables were significant predictors of comet extent, TDM or Tail%. Increased age was a suggestive predictor of CHD. Although the relationships between demographic characteristics and comet parameters were not significant, current smoking and age were included in the multivariate models since several studies have reported increased DNA damage in smokers (Fraga *et al.*, 1996; Sun *et al.*, 1997; Undeger *et al.*, 1999) and an association between DNA damage and age (Moller *et al.*, 2000; Singh *et al.*, 2001, 2003). Crude and adjusted regression coefficients were similar, indicating that there was minimal confounding by age and smoking status.

The final multiple regression models are summarized in Table IV. After adjusting for age and current smoking, the strongest and most stable association was found between SG-adjusted 1N and Tail%. For an IQR increase in 1N, Tail% significantly increased by 4.13% [95% confidence interval (CI), 1.92–6.32]. An IQR increase in 1N resulted in a non-significant but suggestive reduction in TDM (-2.18 μm; 95% CI -4.88 to 0.50) and a non-significant decrease in comet extent (-0.92 μm; 95% CI -7.39 to 5.55). A significant increase in Tail% of 2.76% (95% CI 0.89 to 4.62) was also found for an IQR increase in SG-adjusted TCPY. Conversely, an IQR increase in TCPY was associated with a significant decline in TDM (coefficient = -2.47 μm; 95% CI -4.71 to -0.22) and a non-significant decline in comet extent (-2.72 μm; 95% CI -8.13 to 2.68). Coefficients for the relationships between the number of arcsine-transformed CHD and 1N (-0.03; 95% CI -0.18 to 0.15) and TCPY (-0.02; 95% CI -0.24 to 0.17) were near zero and non-significant.

In models with an interaction term between smoking status (current smokers versus current non-smokers) and urinary metabolite levels, for TDM there was a borderline significant interaction between smoking status and SG-adjusted ln(TCPY) ($P = 0.06$). In the strata of current smokers, there was a strong statistically significant inverse relationship between SG-adjusted TCPY and TDM (coefficient = -8.42 μm for an IQR increase in TCPY; $P = 0.01$). Among current

Table II. Distribution of TCPY and 1N levels in urine of adult men

Insecticide metabolite[a]	n[b]	Geometric mean	Selected percentiles						
			10th	25th	50th	75th	90th	95th	Max
Unadjusted (μg/l)[c]									
TCPY	260	2.03	0.37	1.24	2.49	4.15	7.28	9.76	32.2
1N	260	2.55	0.76	1.34	2.59	4.40	7.36	12.7	140
SG-adjusted[d]									
TCPY	214	2.21	0.47	1.28	2.82	4.74	7.65	9.76	40.7
1N	214	2.72	0.83	1.38	2.75	4.70	8.38	12.1	160
CRE-adjusted (μg/g)[e]									
TCPY	243	1.71	0.47	0.94	1.94	3.30	5.45	7.24	35.1
1N	243	2.12	0.58	1.10	2.04	4.26	7.19	10.4	151

[a]TCPY = 3,5,6-trichloro-2-pyridinol, primary metabolite of chlorpyrifos; 1N = 1-naphthol, a metabolite of carbaryl.
[b]n = number of subjects.
[c]Limit of detection (LOD) for TCPY = 0.25 μg/l; LOD for 1N = 0.40 μg/l. 92.7% of TCPY samples above LOD; 99.6% of 1N samples above LOD.
[d]Forty-six samples excluded with specific gravity >1.03 or <1.01.
[e]Seventeen samples excluded with creatinine >300 or <30 mg/dl.

Table III. Distribution of comet assay parameters among 260 men

Comet parameter	Mean	Selected percentiles						
		10th	25th	50th	75th	90th	95th	Max
Percentage tail DNA (%)	26.5	14.8	18.2	22.5	32.2	45.3	49.6	64.4
Comet extent (μm)	126	81.1	106	124	149	167	182	223
Tail distributed moment (μm)	57.1	39.2	47.6	57.0	65.7	74.5	82.2	107
CHD (count)[a]	10.1	0	2	7	12	24	35	95

[a]CHD = cells with a high level of damage (>300 μm).

Table IV. Adjusted regression coefficients[a,b] for comet parameters associated with an interquartile range (IQR)[c] increase in SG-adjusted urinary insecticide metabolite concentrations, $n = 214$[d]

	Percentage DNA tail (%)		Comet extent (μm)		Tail distributed moment (μm)	
	Estimate (95% CI)	*P*-value	Estimate (95% CI)	*P*-value	Estimate (95%CI)	*P*-value
TCPY	2.76 (0.89 to 4.62)	0.004	−2.72 (−8.13 to 2.68)	0.3	−2.47 (−4.71 to −0.22)	0.03
1N	4.13 (1.92 to 6.32)	0.0003	−0.92 (−7.39 to 5.55)	0.8	−2.18 (−4.88 to 0.50)	0.1

[a]Adjusted for age and smoking status.
[b]Regression coefficients represent the change in comet parameter for an IQR change in insecticide metabolite concentration (0 indicates no change in semen parameter for an IQR change in insecticide metabolite concentration, while a coefficient <0 indicates a decrease in comet parameter for an IQR change in insecticide metabolite concentration and a coefficient >0 indicates an increase in comet parameter for an IQR change in insecticide metabolite concentration).
[c]TCPY IQR = 1.28–4.74 μg/l; 1N IQR = 1.38–4.70 μg/l.
[d]Forty-six samples excluded with specific gravity >1.03 or <1.01.

non-smokers, the relationship was weaker and not statistically significant (coefficient = −1.64 μm for an IQR increase in TCPY; $P = 0.18$). For comet extent and Tail%, interaction terms between current smoking status and TCPY did not approach statistical significance ($P > 0.2$). There were also no interactions between smoking and 1N for any of the three comet parameters ($P > 0.4$).

In a sensitivity analysis, the data were reanalysed after retaining the 46 men that were excluded from the primary analysis because their urine SG was outside the acceptable range. For TDM among the 260 men, adjusted coefficients for an IQR change in both SG-adjusted TCPY (−2.65; 95% CI −4.61 to −0.68) and 1N (−2.39; 95% CI −4.67 to −0.13) increased. A similar increase was observed when retaining the 46 men for the relationships between comet extent and TCPY (−3.44; 95% CI −8.16 to 1.26) and 1N (−2.17; 95% CI −7.60 to 3.25). Conversely, adjusted coefficients for Tail% were slightly attenuated for both TCPY (2.44; 95% CI 0.80–4.07) and 1N (3.82; 95% CI 1.98–5.67), but remained statistically significant. Similar results were also obtained when analyses were repeated using unadjusted (n = 260) as well as CRE-adjusted (n = 260 and n = 243, when 17 men with CRE outside the acceptable range were excluded) urinary insecticide metabolite levels (data not shown).

To better understand the inconsistent associations among the three comet parameters and insecticide metabolite levels (i.e. metabolite levels exhibited positive associations

Table V. Correlation coefficients for percentage DNA tail (Tail%) and tail distributed moment (TDM) or comet extent, stratified by comet extent or TDM, respectively

Comet extent strata (μm)	*n*	Tail% versus TDM	TDM strata	*n*	Tail% versus comet extent
All	214	−0.08	All	214	0.2**
<90	31	−0.5*	<40	26	0.5*
90–110	33	−0.6**	40–48	27	0.5*
110–130	46	−0.6**	48–56	45	0.8**
130–150	47	−0.5**	56–64	47	0.6**
150–170	38	−0.5*	64–72	34	0.7**
>170	19	−0.4	>72	35	0.7**

* $P < 0.05$; ** $P < 0.001$.

with Tail% versus negative associations with comet extent and TDM), correlation coefficients were calculated for Tail% and either comet extent or TDM while stratifying by TDM or comet extent, respectively. Table V shows that even though there was no overall correlation between Tail% and TDM, when the data were stratified by comet extent, a strong negative association was found between Tail% and TDM. Conversely, when the data were stratified by TDM, the weak correlation between Tail% and comet extent became stronger. These findings suggest that some previously unrecognized interrelationships may exist between comet parameters.

Discussion

The present study found that increased urinary 1N concentrations were significantly associated with increased Tail%, but associated with a suggestive decrease in TDM. Increased urinary TCPY was also associated with increased Tail%, but decreased TDM. Previous studies have found increased DNA damage among smokers, so interactions between smoking and urinary metabolites of chlorpyrifos and carbaryl were explored. Results from the tests for interaction suggest that the inverse association between TDM and SG-adjusted TCPY concentration was stronger in current smokers than in current non-smokers.

Associations in the opposite direction for the relationships between insecticide metabolite levels and Tail% as compared with comet extent and TDM were unexpected, but may reflect the type of DNA damage associated with these compounds. Although comet extent and TDM were highly correlated, there was no association between TDM and Tail%. However, when stratified by comet extent, a strong inverse relationship between TDM and Tail% was found (Table V). A potential explanation for the TDM and Tail% association when stratified by comet extent may relate to differing types of DNA damage. Double strand breaks (DSBs) may lead to small DNA fragments that migrate large distances from the comet head but are of low intensity (contain a small amount of DNA). This in turn will have a larger influence on TDM, which is an integrative measure of distance multiplied by intensity, as compared with Tail% which is the proportion of tail to head intensity. For example, comet tails shown in Figure 1a, c, e and g consist of many small DNA fragments (probably produced by DSBs) and possess relatively high TDM values and relatively low Tail%. In contrast, SSBs may contribute to a large proportion of DNA intensity in the tail (increased Tail%), whereas TDM (dependent on both distance and intensity) may increase less. Since there are few small fragments of DNA that are far away from the comet head, TDM does not increase at the same rate as Tail%. For example, comet tails in Figure 1b, d, f and h have relatively high Tail% and low TDM. They also appear to have DNA stretching from the comet head in the proximal portion of the comet, the length of which can depend on the number of SSBs (Singh, 2000). Thus, depending on whether SSBs or DSBs are more prevalent, for a given comet extent, the cell can have either a relatively high Tail% but low TDM (SSBs predominate) or a relatively high TDM but low Tail% (DSBs predominate), hence the negative correlation within comet extent strata. As an example, comparisons of Figure 1a and b, c and d, e and f, and g and h show an inverse relationship between Tail% and TDM within comet extent strata. Based on this hypothesis, results from the present study would suggest that urinary levels of 1N and TCPY are associated with increased DNA SSBs measured as Tail%. These compounds also showed inverse associations with DSBs, suggesting competition between the two types of DNA damage. In addition, in our assay, DSBs may be more susceptible to the loss of small DNA fragments in the gel or may create fragments too small to be measured depending on the sensitivity of the imaging technique (N.P.Singh, personal communication).

A number of *in vitro*, animal and human studies have shown that insecticides may alter DNA integrity of different cell types. Carbaryl has been shown to induce unscheduled DNA synthesis, and presumably DNA damage, in cultured human fibroblasts (Ahmed *et al.*, 1977a). Carbaryl also acted as a weak mutagen and spindle-disturbing agent in Chinese hamster cells (Ahmed *et al.*, 1977b; Onfelt, 1987). In leukocytes of mice, significant dose-dependent associations were found between chlorpyrifos and increased DNA damage measured by comet assay (Rahman *et al.*, 2002). In humans, a case study of eight patients exposed to chlorpyrifos following residential application found increased DNA damage (measured as chromosome alterations) compared with reference ranges, suggesting that chlorpyrifos may be associated with human genotoxicity (Lieberman *et al.*, 1998).

If, as hypothesized, associations between non-persistent insecticide metabolites and DNA damage are related to SSBs, it may provide information on the possible mechanisms involved. DNA SSBs can be caused by ionizing

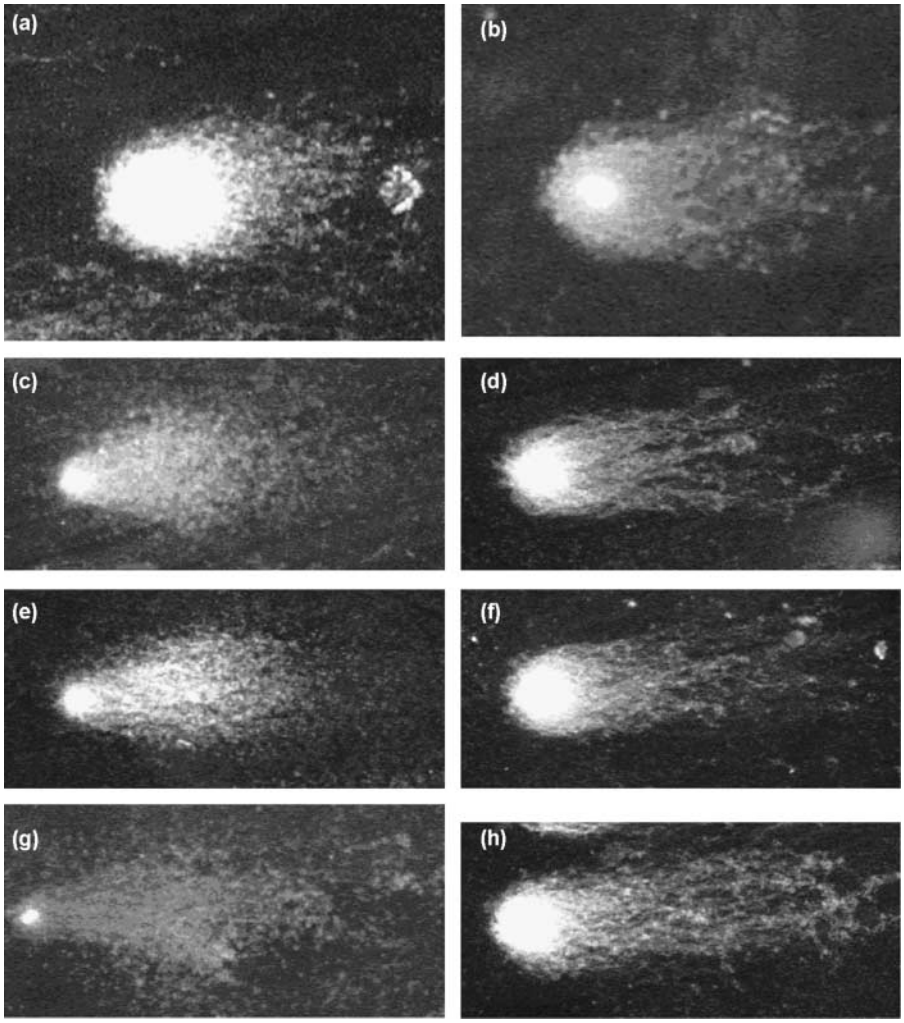

Figure 1. Images of comet pairs (a-b, c-d, e-f, g-h) within increasing comet extent strata that have inversely related Tail% and TDM. (**a**) Comet extent = 101, Tail% = 31, TDM = 59; (**b**) comet extent = 102, Tail% = 62, TDM = 43; (**c**) comet extent = 170, Tail% = 34, TDM = 83; (**d**) comet extent = 167, Tail% = 57, TDM = 66; (**e**) comet extent = 198, Tail% = 38, TDM = 90; (**f**) comet extent = 198, Tail% = 56, TDM = 65; (**g**) comet extent = 231, Tail% = 32, TDM = 138; (**h**) comet extent = 238, Tail% = 66, TDM = 88.

radiation, oxidizing agents and alkylating agents (Memisoglu and Samson, 2000). Carbamates and organophosphates, including carbaryl and chlorpyrifos or their metabolic intermediates, have been suspected to act as alkylating agents on DNA bases, directly or indirectly through protein alkylation (Wild, 1975; Ahmed *et al.*, 1977a; Rahman *et al.*, 2002). Bifunctional alkylating agents used as cancer drugs (e.g. busulfan and cyclophosphamide) are known to adversely affect spermatogenesis and cause sperm DNA damage in mammals (Bucci and Meistrich, 1987; Qiu *et al.*, 1995). Some alkylating agents affect spermatids and sperm without morphological damage (Jackson and Craig, 1969). This is consistent with our previous findings that 1N was significantly associated with decreased sperm concentration and motility, but not with sperm morphology (Meeker *et al.*, 2004a). There may also be other mechanisms contributing to DNA damage. Carbaryl efficiently reduces intracellular levels of glutathione (Soderpalm-Berndes and Onfelt, 1988), which could lead to an increase of reactive oxygen species that contribute to the formation of DNA SSBs and/or DSBs (Saleha Banu *et al.*, 2001; Rahman *et al.*, 2002). It is also possible that carbaryl may be reacting with nitrite in the environment or diet to create nitrosocarbaryl, which is then split into aromatic and aliphatic fragments. The aliphatic fragments then bind irreversibly to DNA, forming many alkali-labile bonds and SSBs (Regan *et al.*, 1976). For chlorpyrifos, damage could also be initiated by phosphorylation of the DNA, as the phosphorus moiety in organophosphates may be a good substrate for nucleophilic attack (Wild, 1975; Rahman *et al.*, 2002).

Biomonitoring for insecticide metabolite concentrations in urine is a commonly used indicator of internal dose integrating the various pathways of exposure (Barr *et al.*, 1999). However, non-persistent insecticides are metabolized and excreted rapidly, so metabolite levels measured in urine reflect insecticide exposure in the previous 24–48 h. Urinary insecticide metabolite levels can vary considerably over time, and it has been suggested that a single sample may not be a reliable surrogate for long-term exposure (MacIntosh *et al.*, 1999). An unreliable measure would lead to increased

non-differential exposure misclassification, which can attenuate associations between exposure and outcome. However, we recently found that a single urine sample may adequately predict 3 month average exposure (Meeker *et al.*, 2004b).

1N is a major metabolite of both carbaryl and naphthalene, and in the present study it was not possible to attribute 1N levels to either compound. Animal studies have found no associations between naphthalene and male reproductive health (ATSDR, 2003), while carbaryl has exhibited potential testicular toxicity (Rybakova, 1966; Shtenberg and Rybakova, 1968; Whorton *et al.*, 1979; Wyrobek *et al.*, 1981; Pant *et al.*, 1995, 1996). Thus, one potential limitation in using 1N as a biomarker of carbaryl is that the unknown contribution of naphthalene exposure to 1N levels may introduce random exposure misclassification and attenuate the observed associations between 1N and comet assay parameters.

Distributions of unadjusted and CRE-adjusted TCPY and 1N levels in the present study were compared with those recently reported for males in CDC's Second National Report on Human Exposure to Environmental Chemicals which was derived from samples collected as a part of the National Health and Nutrition Examination Survey (NHANES) (CDC, 2003). Median and 95th percentile unadjusted and CRE-adjusted TCPY concentrations in the present study were nearly identical to NHANES concentrations, while the present study had slightly higher median and 95th percentile concentrations for unadjusted and CRE-adjusted 1N. The similarities of insecticide metabolite concentrations to those of NHANES 1999–2000 suggest that exposure levels in the present study are representative of exposures to chlorpyrifos/chlorpyrifos methyl, carbaryl/naphthalene and their environmental degradates among men in the general population.

Although it is unclear currently which comet assay parameter is the most relevant measure of DNA damage in sperm and whether they reflect different mechanisms and types of DNA damage as we hypothesize, the percentage of DNA in the comet tail (Tail%) has been shown to be proportional to the frequency of DNA strand breaks (Olive *et al.*, 1990). Also, Tail% may represent a more sensitive measure of DNA damage than both TDM and comet extent, because Tail% continues to increase with increased DNA damage while comet extent may not (McKelvey-Martin *et al.*, 1993). Significant associations between urinary metabolites of non-persistent insecticides and Tail% found in the present study suggest that environmental exposure to carbaryl and chlorpyrifos may be related to increased DNA damage in human sperm. However, future studies are needed to elucidate the inconsistent results between the different measures of DNA damage obtained by the neutral comet assay. Furthermore, future studies should seek to better differentiate carbaryl exposures from naphthalene exposures so that associations can be attributed unequivocally to one pesticide.

Acknowledgements

We thank Ms Ana Trisini, research assistant, for performing a portion of the comet assays, Roberto Bravo, Centers for Disease Control and Prevention, Linda Godfrey-Bailey, research nurse, Janna Frelich, data manager, and Ramace Dadd, research assistant, Harvard School of Public Health. Supported by grants ES09718 and ES00002 from the National Institute of Environmental Health Sciences, NIH. Contents are solely the responsibility of the authors and do not necessarily represent the official views of NIEHS, NIH.

References

Ahmed FE, Hart RW and Lewis NJ (1977a) Pesticide induced DNA damage and its repair in cultured human cells. Mutat Res 42,161–174.

Ahmed FE, Lewis NJ and Hart RW (1977b) Pesticide induced ouabain resistant mutants in Chinese hamster V79 cells. Chem Biol Interact 19, 369–374.

ATSDR (1992) Toxicological Profile for Dibromochloropropane. Agency for Toxic Substances and Disease Registry, Atlanta, GA.

ATSDR (2003) Toxicological Profile for Naphthalene, 1-Methylnapthalene, and 2-Methylnapthalene. Agency for Toxic Substances and Disease Registry, Atlanta.

Barr DB, Barr JR, Driskell WJ, Hill RH Jr, Ashley DL, Needham LL, Head SL and Sampson EJ (1999) Strategies for biological monitoring of exposure for contemporary-use pesticides. Toxicol Indust Health 15, 168–179.

Bjorge C, Wiger R, Holme JA, Brunborg G, Andersen R, Dybing E and Soderlund EJ (1995) In vitro toxicity of 1,2-dibromo-3-chloropropane (DBCP) in different testicular cell types from rats. Reprod Toxicol 9, 461–473.

Boeniger MF, Lowry LK and Rosenberg J (1993) Interpretation of urine results used to assess chemical exposure with emphasis on creatinine adjustments: a review. Am Indust Hyg Assoc J 54,615–627.

Bucci LR and Meistrich ML (1987) Effects of busulfan on murine spermatogenesis: cytotoxicity, sterility, sperm abnormalities, and dominant lethal mutations. Mutat Res 176,259–268.

CDC (2003). Second National Report on Human Exposure to Environmental Chemicals. Atlanta, GA. Centers for Disease Control and Prevention, 2003 (Accessed February 2, 2004, at http://www.cdc.gov/exposurereport/reportresults.htm.)

Duty SM, Singh NP, Silva MJ, Barr DB, Brock JW, Ryan L, Herrick RF, Christiani DC and Hauser R (2003) The relationship between environmental exposures to phthalates and DNA damage in human sperm using the neutral comet assay. Environ Health Perspect 111,1164–1169.

Fraga CG, Motchnik PA, Wyrobek AJ, Rempel DM and Ames BN (1996) Smoking and low antioxidant levels increase oxidative damage to sperm DNA. Mutat Res 351,199–203.

Garaj-Vrhovac V and Zeljezic D (2000) Evaluation of DNA damage in workers occupationally exposed to pesticides using single-cell gel electrophoresis (SCGE) assay. Pesticide genotoxicity revealed by comet assay. Mutat Res 469,279–285.

Grover P, Danadevi K, Mahboob M, Rozati R, Banu BS and Rahman MF (2003) Evaluation of genetic damage in workers employed in pesticide production utilizing the Comet assay. Mutagenesis 18,201–205.

Haines G, Marples B, Daniel P and Morris I (1998) DNA damage in human and mouse spermatozoa after in vitro-irradiation assessed by the comet assay. Adv Exp Med Biol 444,79–91.

Hauser R, Chen Z, Pothier L, Ryan L and Altshul L (2003) The relationship between human semen parameters and environmental exposure to polychlorinated biphenyls and p,p′-DDE. Environ Health Perspect 111, 1505–1511.

Hill RH Jr, Head SL, Baker S, Gregg M, Shealy DB, Bailey SL, Williams CC, Sampson EJ and Needham LL (1995) Pesticide residues in urine of adults living in the United States: reference range concentrations. Environ Res 71,99–108.

Hosmer DW Jr and Lemeshow S (1989) Model building strategies and methods for logistic regression. In Applied Logistic Regression. John Wiley & Sons, New York, pp. 82–134.

Jackson H and Craig AW (1969) Effects of alkylating chemicals on reproductive cells. Ann NY Acad Sci 160,215–227.

Lieberman AD, Craven MR, Lewis HA and Nemenzo JH (1998) Genotoxicity from domestic use of organophosphate pesticides. J Occup Environ Med 40,954–957.

MacIntosh DL, Needham LL, Hammerstrom KA and Ryan PB (1999) A longitudinal investigation of selected pesticide metabolites in urine. J Expo Anal Environ Epidemiol 9,494–501.

McKelvey-Martin VJ, Green MH, Schmezer P, Pool-Zobel BL, De Meo MP and Collins A (1993) The single cell gel electrophoresis assay (comet assay): a European review. Mutat Res 288,47–63.

Meeker JD, Ryan L, Barr DB, Herrick RF, Bennett DH, Bravo R and Hauser R (2004a) The relationship of urinary metabolites of carbaryl and chlorpyrifos with human semen quality. Environ Health Perspect in press.

Meeker JD, Barr DB, Ryan L, Herrick RF, Bennett DH, Bravo R and Hauser R (2004b) Temporal variability of urinary levels of non-persistent insecticides in adult men. J Expo Anal Environ Epidemiol in press.

Memisoglu A and Samson L (2000) DNA Repair by Reversal of Damage. In Nature Encyclopedia of Life Sciences. Nature Publishing Group, London.

Moller P, Knudsen LE, Loft S and Wallin H (2000) The comet assay as a rapid test in biomonitoring occupational exposure to DNA-damaging agents and effect of confounding factors. Cancer Epidemiol Biomarkers Prev 9,1005–1015.

Olive PL and Banath JP (1996) Sizing highly fragmented DNA in individual apoptotic cells using the comet assay and a DNA crosslinking agent. Exp Cell Res 221,19–26.

Olive PL, Banath JP and Durand RE (1990) Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the 'comet' assay. Radiat Res 122,86–94.

Onfelt A (1983) Spindle disturbances in mammalian cells. I. Changes in the quantity of free sulfhydryl groups in relation to survival and C-mitosis in V79 Chinese hamster cells after treatment with colcemid, diamide, carbaryl and methyl mercury. Chem Biol Interact 46,201–217.

Onfelt A (1987) Spindle disturbances in mammalian cells. III. Toxicity, c-mitosis and aneuploidy with 22 different compounds. Specific and unspecific mechanisms. Mutat Res 182,135–154.

Pant N, Srivastava SC, Prasad AK, Shankar R and Srivastava SP (1995) Effects of carbaryl on the rat's male reproductive system. Vet Hum Toxicol 37,421–425.

Pant N, Shankar R and Srivastava SP (1996) Spermatotoxic effects of carbaryl in rats. Hum Exp Toxicol 15,736–738.

Qiu J, Hales BF and Robaire B (1995) Damage to rat spermatozoal DNA after chronic cyclophosphamide exposure. Biol Reprod 53,1465–1473.

Rahman MF, Mahboob M, Danadevi K, Saleha Banu B and Grover P (2002) Assessment of genotoxic effects of chloropyriphos and acephate by the comet assay in mice leucocytes. Mutat Res 516,139–147.

Regan JD, Setlow RB, Francis AA and Lijinsky W (1976) Nitrosocarbaryl: its effect on human DNA. Mutat Res 38,293–302.

Rybakova MN (1966) Toxic effect of sevin on animals. Hyg Sanit (USSR) 31,402–407.

Saleha Banu B, Danadevi K, Rahman MF, Ahuja YR and Kaiser J (2001) Genotoxic effect of monocrotophos to sentinel species using comet assay. Food Chem Toxicol 39,361–366.

Shtenberg AI and Rybakova MN (1968) Effect of carbaryl on the neuroendocrine system of rats. Food Cosmet Toxicol 6,461–467.

Singh NP (2000) Microgels for estimation of DNA strand breaks, DNA protein crosslinks and apoptosis. Mutat Res 455,111–127.

Singh NP and Stephens RE (1998) X-ray-induced DNA double-strand breaks in human sperm. Mutagenesis 13,75–79.

Singh NP, McCoy MT, Tice RR and Schneider EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 175,184–191.

Singh NP, Danner DB, Tice RR, McCoy MT, Collins GD and Schneider EL (1989) Abundant alkali-sensitive sites in DNA of human and mouse sperm. Exp Cell Res 184,461–470.

Singh NP, Ogburn CE, Wolf NS, van Belle G and Martin GM (2001) DNA double-strand breaks in mouse kidney cells with age. Biogerontology 2, 261–270.

Singh NP, Muller CH and Berger RE (2003) Effects of age on DNA double-strand breaks and apoptosis in human sperm. Fertil Steril 80,1420–1430.

Soderpalm-Berndes C and Onfelt A (1988) The action of carbaryl and its metabolite alpha-naphthol on mitosis in V79 Chinese hamster fibroblasts. Indications of the involvement of some cholinester in cell division. Mutat Res 201,349–363.

Sun J-G, Jurisicova A and Casper RF (1997) Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro. Biol Reprod 56,602–607.

Teass AW, Biagini RE, DeBord DG and Hull RD (1998) Application of Biological Monitoring Methods. In NIOSH Manual of Analytical Methods. National Institute for Occupational Safety and Health, Cincinnati, OH, pp. 52–62.

Undeger U, Zorlu AF and Basaran N (1999) Use of the alkaline comet assay to monitor DNA damage in technicians exposed to low-dose radiation. J Occup Environ Med 41,693–698.

Whorton MD, Milby TH, Stubbs HA, Avashia BH and Hull EQ (1979) Testicular function among carbaryl-exposed exployees. J Toxicol Environ Health 5,929–941.

Wild D (1975) Mutagenicity studies on organophosphorus insecticides. Mutat Res 32,133–150.

Woodruff RC, Phillips JP and Irwin D (1983) Pesticide-induced complete and partial chromosome loss in screens with repair-defective females of Drosophila melanogaster. Environ Mutagen 5,835–846.

Wyrobek AJ, Watchmaker G, Gordon L, Wong K, Moore D, 2nd and Whorton D (1981) Sperm shape abnormalities in carbaryl-exposed employees. Environ Health Perspect 40,255–265.

Zar J (1984) The Arcsine Transformation. In Biostatistical Analysis. Prentice-Hall, Inc., Englewood Cliffs, NJ, pp. 239–324.

Submitted on May 6, 2004; accepted on July 9, 2004