Semen Quality in Relation to Biomarkers of Pesticide Exposure

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We previously reported reduced sperm concentration and motility in fertile men in a U.S. agrarian area (Columbia, MO) relative to men from U.S. urban centers (Minneapolis, MN; Los Angeles, CA; New York, NY). In the present study we address the hypothesis that pesticides currently used in agriculture in the Midwest contributed to these differences in semen quality. We selected men in whom all semen parameters (concentration, percentage sperm with normal morphology, and percentage motile sperm) were low (cases) and men in whom all semen parameters were within normal limits (controls) within Missouri and Minnesota (sample sizes of 50 and 36, respectively) and measured metabolites of eight current-use pesticides in urine samples provided at the time of semen collection. All pesticide analyses were conducted blind with respect to center and case–control status. Pesticide metabolite levels were elevated in Missouri cases, compared with controls, for the herbicides alachlor and atrazine and for the insecticide diazinon [2-isopropoxy-4-methylpyrimidinol (IMPY)]; for Wilcoxon rank test, p = 0.0007, 0.012, and 0.0004 for alachlor, atrazine, and IMPY, respectively. Men from Missouri with high levels of alachlor or IMPY were significantly more likely to be cases than were men with low levels [odds ratios (ORs) = 30.0 and 16.7 for alachlor and IMPY, respectively], as were men with atrazine levels higher than the limit of detection (OR = 11.3). The herbicides 2,4-D (2,4-dichlorophenoxyacetic acid) and metolachlor were also associated with poor semen quality in some analyses, whereas acetochlor levels were lower in cases than in controls (p = 0.04). No significant associations were seen for any pesticides within Minnesota, where levels of agricultural pesticides were low, or for the insect repellant DEET (*N,N*-diethyl-*m*-toluamide) or the malathion metabolite malathion dicarboxylic acid. These associations between current-use pesticides and reduced semen quality suggest that agricultural chemicals may have contributed to the reduction in semen quality in fertile men from mid-Missouri we reported previously. *Key words:* agriculture, pesticides, semen quality, sperm concentration, sperm morphology, sperm motility. *Environ Health Perspect* 111:1478–1484 (2003). doi:10.1289/ehp.6417 available via *http://dx.doi.org/* [Online 18 June 2003]

A study by Nelson and Bunge (1974), noting poor semen quality in fertile men from Iowa City, Iowa, relative to men from New York, concluded,

> Confirmation of our findings would imply that some unknown factor has caused a decrease in male fertility potential as measured by semen analysis.

Although the question of a possible decline in semen quality has been widely studied (Carlsen et al. 1992; Swan et al. 1997), before 2003 no other study included a population drawn from an agrarian environment similar to that of Iowa City to confirm or refute this conjecture. Earlier this year we reported results from the Study for Future Families (SFF), a multicenter study of semen quality in fertile men that included men from mid-Missouri, an area comparable demographically and agriculturally with Iowa City (Swan et al. 2003). Iowa City, Iowa, like Columbia, Missouri, has more than 50% of county acreage in farms, and both are located in counties in which pesticide use is high (U.S. Census Bureau 2001).

In SFF we found, as had Nelson and Bunge (1974), reduced sperm concentration and motility in men from a U.S. agrarian area (Columbia, MO) relative to men from U.S. urban centers: Los Angeles, California; Minneapolis, Minnesota; and New York, New York. Unlike earlier studies, tight quality control and standardization of all study methods made it unlikely that the variation in semen quality we observed was attributable to differences in laboratory or recruitment methods. We examined multiple potential confounders, and results were largely unchanged after statistical adjustment for these factors. Therefore, we sought to identify environmental agents associated with these between-center differences in semen quality. We hypothesized that pesticides used widely in mid-Missouri, and rarely in urban areas, might have contributed to the poor semen quality seen in men from mid-Missouri, and perhaps shed light on the finding of Nelson and Bunge (1974). In this article, we follow common use and apply the term "pesticide" not only to insecticides but also to a variety of other agricultural chemicals, including herbicides, fungicides, and various other pest control substances [U.S. Environmental Protection Agency (U.S. EPA) 1997].

It is well known that exposure to pesticides at occupational levels can adversely affect semen quality. In the late 1970s the nematocide dibromochloropropane affected more than 26,000 plantation workers in 12 countries; 64% had low sperm concentrations and 28% were involuntarily childless (Goldsmith 1997; Slutsky et al. 1999; Thrupp 1991). The chlorinated hydrocarbon pesticide chlordecone (kepone) was withdrawn in 1975 because of oligozoospermia and decreased motility resulting from occupational exposures (Faroon et al. 1995). Ethylene dibromide was an active component of approximately 100 pesticides. Its use was severely restricted in 1984 because of reduced sperm counts and semen volume in exposed workers (Schrader et al. 1988; Whorton 1981). More recently, a small study of herbicide sprayers in Argentina showed decreased sperm concentration and morphology related to high urinary levels of 2,4-D (2,4-dichlorophenoxyacetic acid) metabolites (Lerda and Rizzi 1991). Greenhouse workers in Denmark with greater/longer pesticide exposure had lower sperm counts and percentages of morphologically normal sperm (Abell et al. 2000). After a report of high sperm counts in organic farmers in Denmark, a series of studies were designed to compare reproductive health between traditional and organic farmers. Although questionnaire data showed

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no differences in fertility, sperm counts and percentage of morphologically normal sperm were reduced in traditional compared with organic farmers (Ekbom et al. 1996).

The relationship between environmental pesticide exposure and semen quality has been examined only in a single small (n = 29) study of infertile and fertile men (Hauser et al. 2002). This study suggests an association between increased serum levels of organochlorines (polychlorinated biphenyls and *p,p′*-dichlorodiphenyldichloroethylene) and decreased sperm motility, sperm concentration, and percentage of normal sperm. No study to date has examined environmental exposure to current-use, nonpersistent pesticides in relation to semen quality.

Materials and Methods

Selection of pesticides to be tested. We began our search for causes of poor semen quality in men from Missouri by examining pesticides currently used in agriculture in mid-Missouri but used less frequently in the Minneapolis area. We reviewed records of crops grown at high volume in the Midwest (primarily corn, soybeans, sorghum, and winter wheat) and agricultural products used on these crops (Pauley 2002) to identify pesticides for initial testing. We selected 15 nonpersistent pesticide metabolites included in a standard screening panel used by the Pesticide Laboratory at the Centers for Disease Control and Prevention (CDC). These include metabolites of the herbicides alachlor, atrazine, metolachlor, acetachlor, 2,4-D, and 2,4,5-trichlorophenoxacetic acid (2,4,5-T); metabolites of the insecticides carbofuran, diazinon, carbaryl, chlorpyrifos, malathion, propoxur, methyl parathion, and permethrin; and the insect repellent *N,N*-diethyl-*m*-toluamide (DEET).

Three pesticides that were found to be used more often in Minnesota than Missouri were unrelated to semen quality; comparing the proportion of cases and controls with levels above the limit of detection (LOD) by Fisher's exact test yielded p-values of 0.80, 0.57, and 0.42 for 1-naphthol, 3,5,6-trichloropyridinol, and 4-nitrophenol, respectively. These metabolites, none derived predominantly from agricultural products, were not considered further. We also eliminated four metabolites that were not detectable or measurable in either center; 2,4,5-T, 2-isopropoxyphenol (metabolite of propoxur), carbofuranphenol, and permethrin.

Selection of subjects to be tested. All subjects were participants in SFF, a 4-year multicenter study funded by the National Institute of Environmental Health Sciences. These men were partners of pregnant women who were recruited between 1999 and 2001 at prenatal clinics affiliated with university hospitals in Los Angeles, California (Harbor-UCLA and Cedars-Sinai Medical Center); Minneapolis, Minnesota (University of Minnesota Health Center); Columbia, Missouri (University Physicians); and New York, New York (Mount Sinai School of Medicine). Protocols were approved by institutional review boards at the institutions in which all clinical centers, the Data Coordinating Center, and the Andrology Coordinating Center are located, as well as at the CDC. Methods for clinical examination, data collection, and semen analysis, which were identical across centers, have been described previously (Swan et al. 2003). Briefly, all prenatal patients were approached at a prenatal visit by study staff, who asked for permission to explain the study to both partners, usually by phone. If the couple was eligible and interested in participating, both partners completed a questionnaire and gave a blood sample. The man received a physical examination and in most cases provided two semen samples (average 24 days apart). After 30 October 2000, men were asked to provide a urine sample on the day they provided their first semen samples.

In the present study, we sought to explain the differences in semen quality between men at the Missouri and Minnesota centers, which, despite large differences in semen quality, are comparable demographically and with respect to most risk factors for impaired semen quality. We selected the sample for pesticide analyses from SFF subjects in Missouri and Minnesota who had provided urine and semen samples and permitted us to store these for future analyses.

For cases, we selected men for whom the average (abstinence-time adjusted) sperm concentration was below the population median. Controls were men for whom the average (abstinence-time adjusted) sperm concentration was above the median. This resulted in a case group in which motility and morphology were also below the population median and a control group in which these parameters were above the median. Because our goal was to select cases and controls with no or few risk factors for impaired semen quality, we first selected men who were 21–40 years of age, white, nonsmokers, with no history of infertility, sexually transmitted diseases (STDs), or fever in the 3 months preceding sample collection. Because there were not enough men negative for these risk factors who satisfied the criterion for low (or high) sperm concentration, it was necessary to include 17 men (14 of whom were Missouri controls) with one or more risk factors. Because most men who were positive for one or more risk factors were controls, any bias should be in the direction of underestimating differences between cases and controls. We confirmed this in an analysis that compared results for all cases and controls with the subset of men with no risk factors.

For the initial analysis, the 30 men with the lowest sperm concentrations formed the case group and the 30 men with highest sperm concentrations served as their controls. This group included 24 men from Missouri and 36 from Minnesota.

To increase our power to examine pesticide levels in relation to semen quality within Missouri, we then selected additional Missouri subjects (4 cases and 22 controls) to achieve a total of 25 cases and 25 controls within Missouri. These men were selected according to the same criteria as the first 60 men. In all, pesticide metabolite levels were obtained on 86 men: 50 from Missouri (25 cases and 25 controls) and 36 from Minnesota (9 cases and 27 controls).

Pesticide metabolite analyses. We used a single herbicide/insecticide screen that employs a mass spectrometry–based method and quantification using the isotope dilution (ID) calibration (Hill et al. 1995). Using ID calibration, the samples are enriched with isotopically labeled analogues before preparation. The ID technique is widely regarded as the "gold standard" method for trace analysis because chemically the isotope analogue behaves identically to the native analyte but can be discriminated with a mass filter (Beeson et al. 1999). This allows complete recovery correction for each sample and improves the sensitivity, accuracy, and selectivity of the analysis. After addition of the labeled standard to urine samples, glucuronide- or sulfate-bound urinary metabolites are liberated by enzyme hydrolysis, and the analytes are isolated using solid-phase extraction. The extractants are concentrated to dryness and reconstituted in solvent for analysis by ID–high-performance liquid chromatography–atmospheric chemical ionization–tandem mass spectrometry. All results were adjusted for concentration by creatinine, and these adjusted metabolite levels were log-transformed for analysis. The LOD for all analytes was 0.1 μg/g creatinine. Measured values were used when available, and values that were too low to be quantified were assigned a value of 0.071 [the LOD × $(2)^{-1/2}$].

Semen collection and analysis. Men collected semen samples by masturbation at the clinic, and almost all samples were analyzed within 45 min of collection. Although men were requested to observe a 2–5 day abstinence period, we stressed the importance of accurately reporting the actual abstinence period, which was used in all analyses. Most men (85%) provided two samples an average of 24 days apart. All semen parameters reported here were estimated on both the first and second samples. Sperm counts were made by μ-Cell (a disposable counting chamber; Conception Technologies, San Diego, CA). Ejaculate volumes were estimated by specimen weight, assuming a semen density of 1.0 g/mL. The

percentage of motile sperm was counted in a μ-Cell chamber (Overstreet and Brazil 1997) and refers to the percentage of sperm with any flagellar movement, whether twitching or progressive. A single technician assessed sperm morphology using the strict morphology method recommended by the World Health Organization (WHO) (Guzick et al. 2001; WHO 1999), in which only sperm with absolutely no defects are classified as normal.

Statistical methods. We compared the proportion of samples with values above the LOD in Missouri and Minnesota by Fisher's exact test (Hollander and Wolfe 1999). We examined pesticide metabolite levels (singly and in combination) in relation to semen quality by conducting a series of nonparametric and parametric analyses in which the exposure variables (pesticide metabolites) and outcome variables (semen parameters) were treated alternatively as categorical or continuous variables.

The first analysis was nonparametric and compared the rank of individual metabolite levels within center by the Wilcoxon rank test (Hollander and Wolfe 1999). In the second analysis, we categorized pesticide levels, using three categories (low, medium, and high) when at least 15 men had values above the LOD. Otherwise, we divided measurements into two categories at the LOD (low and high). Therefore, values labeled "high" were those above 0.7, 3.0, and 0.3 μg/g creatinine for alachlor, 2-isopropoxy-4-methyl-pyrimidinol (IMPY), and metolachlor, respectively, and above 0.1 μg/g creatinine for atrazine and 2,4-D. We calculated odds ratios (ORs) and their 95% confidence intervals (CIs) to examine the association between pesticide level (high vs. low, or medium vs. low) and case–control status.

We also examined exposure to multiple pesticides at a "high" level by means of an ad hoc score constructed as follows. For each of the five pesticides—alachlor, IMPY, atrazine. 2,4-D, or metolachlor—if the metabolite level was high, one unit was added to the man's score, for a maximum of five units. This score does not, therefore, indicate which pesticides were high, but only the number that were high.

We then analyzed ($\log_{10}$) pesticide metabolite level in relation to case–control status within center. Finally, we used a mixed model that controls for multiple semen samples per man to examine pesticide level in relation to semen parameters (concentration, percentage of motile sperm, and percentage of normal sperm) as continuous variables. Because the number of covariates was large compared with the number of subjects in the pesticide analysis, we calculated a confounder score (Miettinen 1976) using data from all 441 men from Missouri and Minnesota. This score (the predicted value of the outcome based on the covariates, ignoring center and pesticide level) was then added as a covariate to models that also included terms for pesticide, center, and the interaction of pesticide and center.

Results

Study subjects and samples. Sample sizes were comparable for the total SFF population in Missouri and Minnesota (202 and 215, respectively) and recruitment rates were equal (27%). Missouri men were somewhat younger than Minnesota (mean age of 30.7 and 32.2 in Missouri and Minnesota, respectively), and the populations were similar with respect to ethnicity, smoking, and several variables associated with decreased semen quality (history of infertility or STDs and fever in the 3 months before semen collection).

Subject characteristics are shown in Table 1. Most cases (91%) and controls (80%) were 21–40 years of age, white, nonsmokers, with no history of infertility, STDs, or fever in the 3 months before sample collection. Occupational pesticide exposure was reported by only three men in Missouri and none in Minnesota. Self-reports of current pesticide exposure and residence within one-half mile of a farm were similar in cases and controls. Semen samples from cases and controls in both centers were similar with respect to abstinence time and analysis time, whereas all semen parameters differed significantly between cases and controls in both centers (Table 1). Therefore, although cases were selected initially on the basis of sperm concentration, they are also cases of poor semen quality by all measures examined, and controls are in the normal range for all parameters.

Pesticide analyses. **Comparison of Missouri and Minnesota pesticide metabolite levels.** The 15 urinary pesticide metabolites measured initially are shown in Table 2. Three analytes derived primarily from industrial sources were observed above the LOD more often in Minnesota than Missouri subjects, and none of these were related to semen quality. A metabolite of the organophosphate insecticide chlorpyrifos (3,5,6-trichloropyridinol) that is used to control residential pests, as well as on crops, was found above the LOD for 86% of subjects in Minnesota and 50% of those in Missouri, but the level was unrelated to case–control status ($p = 0.39$). A second analyte found more often in Minnesota samples and unrelated to semen quality ($p = 0.60$) was 1-naphthol, a carbamate metabolite and a metabolite of naphthalene. The concentration of 4-nitrophenol, a metabolite of methyl parathion and an industrial chemical used in the manufacture of drugs, fungicides, and dyes, was found above the LOD in only seven men, all from Minnesota (two cases and five controls). Carbofuranphenol, 2,4,5-T, and 2-isopropoxphenol were below the LOD for all subjects, and permethrin results were not usable because of quality control problems. None of these seven pesticides were tested further.

Five metabolites were present above the LOD more often in Missouri than Minnesota subjects, including those of the herbicides alachlor, metolachlor, atrazine, 2,4-D, and IMPY, a metabolite of the organophosphate insecticide diazinon. Levels of malathion dicarboxylic acid (MDA), acetochlor, and DEET did not differ appreciably between centers. These eight biomarkers of exposure were measured in all 86 subjects.

Pesticide exposure in relation to case–control status. We first examined pesticide

Table 1. Characteristics of subjects and semen samples for cases and controls by center.

	Missouri			Minnesota		
Characteristic	Cases	Controls	*p*-Value[a]	Cases	Controls	*p*-Value[a]
Subjects						
Mean age (years)	31.5	31.0	0.73	31.0	33.2	0.08
No. of men						
Nonwhite	0	6	0.0009	0	0	1.00
Light smokers (< 10 cigarettes/day)	0	3	0.06	0	0	1.00
Smokers (≥ 10 cigarettes/day)	0	2	0.12	0	0	1.00
Recent fever	0	1	0.31	0	0	1.00
History of STD	2	1	0.55	1	1	1.00
History of infertility	0	0	1.00	0	2	0.40
Recent pesticide exposure	8	7	0.76	0	1	0.56
Current residence within 0.5 miles of farm	7	5	0.51	0	1	0.56
Total number of men	25	25		9	27	
Semen samples						
Mean abstinence time (hr)	71.3	79.6	0.27	71.5	72.1	0.93
Mean time for semen analysis (min)	44.0	45.1	0.69	51.4	53.4	0.33
Semen parameters						
Mean sperm concentration ($\times 10^6$/mL)[b]	32.9	72.1	0.0001[c]	23.9	106.4	0.0001[c]
Percent motile sperm	43.2	48.0	0.03	46.5	56.4	0.04
Percent morphologically normal sperm	8.5	12.8	0.002	7.2	12.8	0.004
Mean total motile sperm ($\times 10^6$)[b]	65.5	110.4	0.0002[c]	57.2	220.5	0.0001[c]
No. of semen samples	47	47		18	54	

[a]Determined by by *t*-test. [b]Abstinence-time–adjusted μ-Cell concentration. [c]Based on $\log_{10}$-transformed μ-Cell concentration.

exposure in relation to semen quality by comparing pesticide metabolite levels between cases and controls, within center, using a nonparametric two-sample rank test. Results of these tests, as well as mean and median metabolite levels by case–control status and center, are shown in Table 3. In Minnesota subjects, pesticide levels in cases and controls did not differ significantly. In Missouri subjects, where levels were higher, alachlor, atrazine, and IMPY were quite different between cases and controls (p = 0.0007, 0.0004, and 0.01, respectively). Metabolite levels for two other herbicides were higher in cases, but the difference was of borderline statistical significance (p = 0.06 and 0.10 for metolachlor and 2,4-D, respectively). Acetochlor levels were lower in cases than controls (p = 0.04). We repeated this analysis excluding the 16 men (most of whom were Missouri controls) with one or more of the following risk factors that might impair semen quality: cryptorchidism, smoking, history of STD, history of infertility, recent fever, age greater than 40 years, and nonwhite race. Results among men with none of these factors were substantially unchanged from Table 3, although significance probabilities were somewhat larger because of smaller sample sizes (23 cases and 14 controls in Missouri subjects; 8 cases and 24 controls in Minnesota subjects).

We found some evidence that the month of sample collection was related to case–control status. In Missouri, men providing samples in spring or summer months (March–August) were somewhat more likely to be cases than were men who gave samples in winter months (December–February). This difference was more marked for samples given in the fall (September–November), although 95% CIs were wide (for spring–summer compared with winter, OR = 2.4; 95% CI, 0.7–8.7; and for fall compared with winter, OR = 4.7; 95% CI, 0.9–24.8). Only one case in Minnesota provided a sample in the winter, making this an unstable reference group. When fall months are compared with other months for Minnesota subjects, the OR for case–control status is 2.2, suggesting a somewhat less marked seasonal effect in that center.

Sixteen men (15 in Missouri) reported pesticide exposure at home or at work in the 3 months before sample collection. Cases who self-reported exposure were more likely to have multiple metabolites elevated. Among the eight cases in this group, on average the pesticide score was 2.5, and all had at least one elevated score. However, among the controls in this group, only two people had elevated pesticide levels, one with a score of 1 and one with a score of 2. Larger samples sizes are needed to further examine the relationship between self-reported pesticides and measured metabolite levels.

We categorized pesticides into two (< LOD and > LOD) or three (low, medium, or high) levels, as described in "Materials and Methods." We then examined the OR for poor semen quality associated with elevated pesticide levels, within center. The results (Table 4) suggest that higher levels of alachlor, which were seen only in Missouri, are associated with a particularly high risk of reduced semen quality. For high alachlor exposure compared with low exposure (> 0.7 vs. < 0.15 µg/g creatinine), the OR for poor semen quality was 30.0 (p < 0.0001). IMPY was also strongly associated with case–control status, particularly at the highest level. Few men (9) had atrazine levels > LOD. However, all but one was a case, yielding an estimate of the OR that was elevated (11.3) and statistically significant, but with a very large confidence interval. Higher levels of metolachlor were associated with case–control status, but weakly. An association of similar magnitude, but in the opposite direction, was seen for acetochlor. Metabolite levels of DEET and MDA differed little between cases and controls.

ORs within strata were usually higher in Missouri than Minnesota subjects, due at least in part to the fact that within-strata metabolite levels tended to be higher in Missouri than Minnesota subjects. For example, in the highest strata for alachlor, the maximum in Missouri subjects was 2.62 µg/g creatinine compared with 1.26 µg/g creatinine in Minnesota subjects. We included a term for the interaction of center and metabolite in the mixed models and found no significant interactions. However, the power to detect varying effects across centers was limited.

Table 3. Mean and median pesticide metabolite levels[a] in cases and controls.

	Missouri			Minnesota		
Pesticide	Cases Mean (median)	Controls Mean (median)	p-Value[b]	Cases Mean (median)	Controls Mean (median)	p-Value[b]
Alachlor	0.72 (0.67)	0.30 (0.14)	0.0007	0.31 (0.07)	0.23 (0.07)	0.60
IMPY	4.96 (1.73)	1.05 (0.07)	0.0004	1.84 (0.93)	1.56 (1.25)	0.70
Atrazine	0.17 (0.07)	0.08 (0.07)	0.01	0.07 (0.07)	0.09 (0.07)	0.40
Metolachlor	0.48 (0.25)	0.28 (0.16)	0.06	0.20 (0.07)	1.28 (0.07)	0.90
2,4-D	0.56 (0.07)	0.10 (0.07)	0.10	0.07 (0.07)	0.07 (0.07)	1.00
Malathion	0.37 (0.07)	0.37 (0.07)	0.40	0.92 (0.07)	0.58 (0.07)	0.90
DEET	0.33 (0.07)	0.09 (0.07)	0.40	0.22 (0.07)	4.52 (0.07)	0.60
Acetochlor	0.10 (0.07)	0.26 (0.11)	0.04	0.08 (0.07)	0.58 (0.07)	0.80

[a]µg/g creatinine. [b]Comparison of cases and controls within center by Wilcoxon rank test.

Table 2. Percentage of men with metabolite levels above the LOD[a] in Missouri (n = 24) and Minnesota (n = 36).[b]

	Percent > LOD[a]			
	Missouri	Minnesota	p-Value[c]	Chemical type (parent chemical; likely source)
More often > LOD in Missouri than Minnesota subjects				
Alachlor mercapturate	92	25	< 0.0001	Herbicide (alachlor; agricultural)
IMPY	96	58	0.001	Insecticide (diazinon; agricultural and residential)
Atrazine mercapturate	38	6	0.004	Herbicide (atrazine; agricultural)
Metolachlor mercapturate	96	39	< 0.0001	Herbicide (metolachlor; agricultural)
2,4-D	12	0	0.059	Herbicide (2,4-D; agricultural and residential)
More often > LOD in Minnesota than Missouri subjects				
3,5,6-Trichloropyridinol	50	86	0.004	Insecticide (chlorpyrifos/chlorpyrifos methyl; residential and agricultural)
1-Naphthol	38	64	0.065	Polycyclic aromatic hydrocarbon (naphthalene; auto exhaust) Insecticide (carbaryl; residential and agricultural)
4-Nitrophenol	0	19	0.035	Insecticide (methyl parathion; agricultural) Industrial precursors (*para*-aminophenol; unknown)
Similar no. > LOD in Missouri and Minnesota subjects[d]				
Malathion dicarboxylic acid	38	19	0.15	Insecticide (malathion; agricultural and residential)
Acetachlor mercapturate	21	8	0.25	Herbicide (acetachlor; agricultural)
DEET	12	25	0.33	Insecticide (DEET; personal repellants)

[a]Limit of detection (0.1 µg/g creatinine). [b]Initial sample of 60 men. [c]Fisher's exact test. [d]In addition, 2,4,5-T, 2-isopropoxyphenol, and carbofuranphenol were not found over the LOD in either center, and no valid permethrin values were obtained.

We examined the correlation among metabolite levels. Although IMPY was not strongly correlated with any other measured pesticide metabolite, many of the other pesticide metabolite levels were highly correlated. For example, the Pearson correlation coefficients between alachlor and acetochlor, atrazine, metolachlor, and 2,4-D were 0.89, 0.74, 0.76, and 0.92, respectively, with all $p < 0.009$. Thus, associations with semen parameters are not independent. Therefore, we examined simultaneous exposure to multiple pesticides using a score ranging from 0 to 5 (as defined in "Materials and Methods"). Within Missouri, the pesticide score was strongly associated with semen quality; the score was 0 for 15 men, 13 of whom were controls, and 3–5 for 6 men, all of whom were cases (Figure 1). We also dichotomized the score (0–1 vs. 2–5) and found that in Missouri subjects a score of 2–5 yielded an OR for poor semen quality of 3.4 (95% CI, 2.0–25.5) compared with subjects with a score of 0–1. In Minnesota subjects, this score was unrelated to case–control status; the corresponding OR was 1.3 (95% CI, 0.2–8.0).

We examined metabolite levels as continuous variables in relation to case–control status in a regression model. These results were very consistent with those in Table 3 (data not shown). We also examined pesticide level as a predictor of three (continuous) measures of semen quality ($\log_{10}$ sperm concentration, morphology, and motility). Table 5 includes results from the regression analysis for the five pesticides most associated with semen quality within Missouri subjects. None of the five pesticides were significantly associated with any semen parameter within Minnesota subjects. Within Missouri subjects, none of the pesticides were significantly associated with motility. The herbicides alachlor and metolachlor were more strongly associated with sperm morphology than with concentration, whereas IMPY was more strongly associated with concentration than with morphology. Because the distributions of these semen parameters, even after logarithmic transformation of sperm concentration, are not Gaussian, the results of these models must be viewed as exploratory.

Discussion

This study was designed to examine the hypothesis that pesticides used commonly in mid-Missouri (and rarely in urban counties) contributed to the poor semen quality in men from Columbia relative to those from Minneapolis. The data presented here support that hypothesis and identify several currently used herbicides (particularly alachlor and atrazine) and one insecticide (diazinon) that are associated with decreased semen quality in Missouri subjects. We have extended our study of partners of pregnant women (SFF) to Iowa City, but have not yet obtained pesticide levels in those subjects. However, based on the similarity of agricultural practices and pesticide use in Columbia and Iowa City (U.S. Census Bureau 2001), we hypothesize that metabolite levels will also be associated with semen quality in Iowa.

Levels of alachlor and atrazine in this sample of Missouri men were higher than expected based on a national sample of adults and children. In that sample the 95th percentiles for both herbicides were below the LOD (CDC 2003). Levels in our study were considerably higher. Because the same laboratory, methods, and LODs were used for both studies, it appears that individuals in our sample have

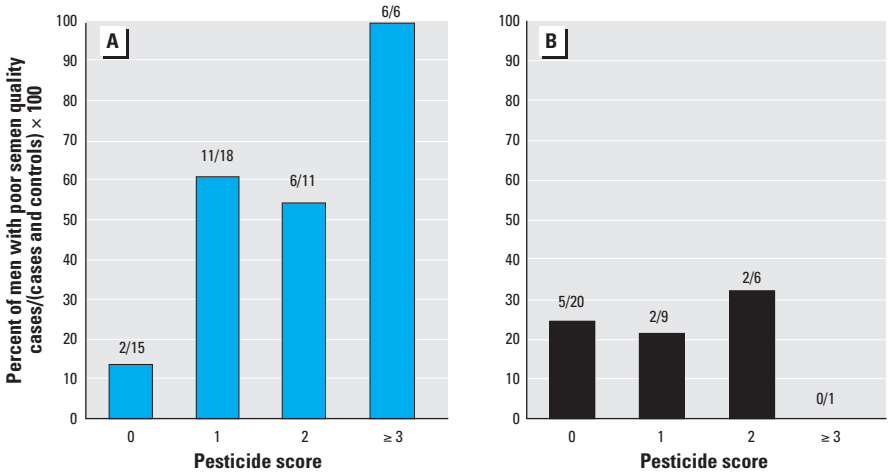

Figure 1. The proportion of men with poor semen quality in relation to pesticide score (the number of pesticides found at higher levels). (*A*) Missouri. (*B*) Minnesota.

Table 4. ORs for low semen quality for men exposed to elevated pesticide levels.

	Missouri				Minnesota			
Pesticide	Level (μg/g creatinine)	Cases	Controls	OR (95% CI)	Level (μg/g creatinine)	Cases	Controls	OR (95% CI)
Alachlor	< 0.15	3	15	Reference	< 0.15	6	21	Reference
	0.15–0.7	10	8	6.3 (1.3–29.4)	≥ 0.15	3	6	1.8 (0.3–9.2)
	> 0.7	12	2	30.0 (4.3–210)				
IMPY	< 0.1	6	20	Reference	< 0.1	3	12	Reference
	0.1–3.0	9	3	10.0 (2.0–49.2)	0.1–3.0	3	9	1.3 (0.2–8.2)
	> 3.0	10	2	16.7 (2.8–98.0)	> 3.0	3	6	2.0 (0.3–13.1)
Atrazine	< 0.1	17	24	Reference	< 0.1	9	25	
	≥ 0.1	8	1	11.3 (1.3–98.9)	≥ 0.1	0	2	
Metolachlor	< 0.15	5	11	Reference	< 0.15	5	17	Reference
	0.15–0.3	11	8	3.0 (0.7–12.2)	≥ 0.15	4	10	1.4 (0.3–6.3)
	> 0.3	9	6	3.3 (0.8–14.5)				
2,4-D	< 0.1	20	19	Reference	< 0.1	9	27	
	≥ 0.1	5	6	0.8 (0.2–3.0)	≥ 0.1	0	0	
1-Naphthol	< 1.5	9	2	Reference	< 2.0	3	8	Reference
	> 1.5	12	1	2.7 (0.2–34.2)	2.0–4.0	2	4	1.3 (0.2–11.5)
					> 4.0	4	15	0.7 (0.1–4.0)
3,5,6-Trichloropyridinol	< 0.5	5	2	Reference	< 1.25	1	3	Reference
	≥ 0.5	16	1	6.4 (0.5–86.3)	1.25–5.0	2	11	0.5 (0.04–8.3)
					> 5.0	6	13	1.4 (0.1–16.2)
4-Nitrophenol	< 0.1	20	3		< 0.1	5	14	Reference
	≥ 0.1	1	0		≥ 0.1	4	13	0.9 (0.2–3.9)

considerably higher exposures to these chemicals, particularly those in Missouri.

The sources of the pesticides that we measured are not known. Elevated metabolite levels in cases compared with controls do not reflect occupational exposure, home pesticide use, or residence near a farm, because these differed little among these groups of men. This suggests that the pesticide exposure reflected in these urinary levels is likely to be environmental. The most relevant exposure period is also uncertain, but because these pesticides are nonpersistent, with half-lives within the body of hours and days rather than years, these measured levels are likely to reflect recent exposure. Although the biologic half-life of these pesticides is short (from a few hours to a few days; International Programme on Chemical Safety 1996), they are applied over an extended period; therefore, metabolite levels measured in a man's urine sample should be relevant to the quality of both semen samples, which averaged 24 days apart.

A recent U.S. Geological Survey (2001) report on water quality noted that extensive herbicide use in agricultural areas (accounting for ~70% of total national use of pesticides) has resulted in widespread occurrence of herbicides in agricultural streams and shallow groundwater in those areas. The most frequently detected pesticides in recent surveys of ground and surface waters are the triazine herbicides (atrazine, simazine), chloroacetamides (alachlor, acetochlor, metolachlor), and 2,4-D (Thurman et al. 1996), as well as the insecticides carbofuran and diazinon (Kolpin et al. 1998). Thus, water appears to be a plausible source of the exposures measured in these subjects.

Published data on the reproductive toxicity of the pesticides associated with semen quality in this study are limited. Alachlor, which is found in a variety of commercial herbicides, is an aniline herbicide used to control annual grasses and certain broadleaf weeds in field corn, soybeans, and peanuts. It has been classified by the U.S. EPA as a probable human carcinogen, but to date, no reproductive effects have been demonstrated in human or animal studies.

Atrazine and other triazine herbicides have demonstrated antiestrogenic activity at high doses in several laboratory studies. In one, atrazine increased secretion of leutinizing hormone and prolactin (Tennant et al. 1994). In another study, atrazine was shown to inhibit the enzymatic conversion of testosterone to 5α-dihydrotestosterone in the rat prostate (Kniewald et al. 1995). Estrogenic action is also suggested by some studies. For example, triazines have been shown to induce aromatase *in vitro* (Sanderson et al. 2001). Recently, Hayes et al. (2002) reported that exposure to low, environmentally relevant, doses of atrazine demasculinized the male African clawed frog (*Xenopus laevis*), and adult exposure to 25 ppb induced a 10-fold decrease in testosterone. As far as we know, ours is the first study to examine human reproductive risk associated with atrazine exposure.

Diazinon is, according to the U.S. EPA, one of the most commonly found pesticides in air, rain, and drinking and surface water. On 5 December 2000, the U.S. EPA announced an agreement to phase out diazinon in the United States beginning in March 2001. This phaseout was based on the insecticide's acute toxicity, being "one of the leading causes of acute toxicity poisoning for humans and wildlife." To date, however, there has been little evidence of its reproductive toxicity (U.S. EPA 2000).

Our study has many strengths but also some limitations. Although we have sufficient power to demonstrate significant associations between pesticide metabolite levels and several semen parameters, these results are based on small numbers. In particular, the number of cases in Minnesota was small and thus not adequate to rule out associations within that center between semen quality and the (predominantly home and industrial) pesticides detected in those subjects. Also, because few men reported pesticide exposure at home or work (and all but one of these was in Missouri), we were limited in our ability to examine the relationships between self-reported and measured exposures, but the higher metabolite levels in cases self-reporting exposure (but not controls) is intriguing. Moreover, the regression analyses of metabolite level as a function of a (continuous) semen parameter must be viewed as exploratory. In those analyses, the number of parameters was large relative to the number of subjects; the distribution of semen parameter was not normal (because of the method of selecting subjects for the case–control analysis); and for several pesticides (notably atrazine and 2,4-D) there were very few values > LOD.

The participation rate was low in both Missouri and Minnesota (27%), but comparable with rates in other population-based studies of semen quality (Jorgensen et al. 2001). Although participation bias is a potential concern, in order for the associations of semen quality and pesticide exposure to be the result of selection bias, men with low exposure and high semen quality would have to participate at a higher rate than men with high exposure and poor semen quality. Because men had no knowledge of either their pesticide levels or their semen quality, it is very unlikely that these factors could have influenced men's decision to participate.

Because several of these pesticides are highly correlated, the associations presented here may reflect multiple exposures, not only to measured metabolites but also to exposures to compounds that were not measured but are correlated with those measured.

We attempted to address the problem of multiple correlated exposures using the pesticide score, but the number of results above the LOD was too small for most pesticides to allow us to examine the covariance structure of these variables or to assess the degree of synergy between them. Nonetheless, the score does demonstrate, more precisely than data from any single pesticide, a strong association between exposure and case–control status within Missouri and the absence of any association in Minnesota, where few men were exposed > LOD to the agricultural pesticides summarized by the pesticide score.

We have analyzed these data using a range of methods; the simplest models (the nonparametric test of dichotomized pesticide level and outcome) make no assumptions and are clearly appropriate but do not make full use of the available data. The more complex models make increasingly restrictive assumptions that are more difficult to justify. We felt that this range of analyses provided valuable alternative interpretations of the data. By and large, the results are consistent across analyses. None found any appreciable associations between metabolite level and semen quality within Minnesota subjects. In the case of both alachlor and IMPY, for which there were substantial numbers of subjects above the LOD, all methods found strong and significant associations. Within Missouri subjects, most methods also found significant associations with atrazine, although numbers of men with values above the LOD were too small for the mixed model to provide useful information. Results for metolachlor and 2,4-D should be

Table 5. Slopes (*p*-values) within center from regression of semen parameters on pesticide metabolite level.[a]

	Missouri			Minnesota		
Metabolite	Concentration[b]	Percent normal morphology	Percent motile	Concentration[b]	Percent normal morphology	Percent motile
Alachlor	–0.15 (0.07)	–3.26 (0.01)	–2.40 (0.32)	–0.008 (0.94)	–0.39 (0.82)	–1.31 (0.69)
IMPY	–0.09 (0.05)	–1.33 (0.12)	–1.81 (0.20)	–0.001 (0.99)	0.79 (0.45)	2.31 (0.22)
Atrazine	–0.17 (0.29)	–2.41 (0.35)	–4.47 (0.34)	0.09 (0.76)	–1.84 (0.71)	8.21 (0.35)
2,4-D[c]	–0.12 (0.19)	–2.36 (0.11)	–0.06 (0.98)	–0.12 (0.19)	–2.36 (0.11)	–0.06 (0.98)
Metolachlor	–0.16 (0.14)	–3.42 (0.05)	–3.31 (0.31)	–0.38 (0.66)	–2.19 (0.11)	1.39 (0.58)

[a]Within-center pesticide coefficient from the regression of semen parameter on risk score, $\log_{10}$ metabolite level, center, and interaction of metabolite and center. [b]$\log_{10}$ μ-Cell concentration. [c]Interaction of metabolite and center could not be estimated, so estimates are equal in both centers.

considered "borderline," with small and somewhat inconsistent associations.

The differences in results for the three chloroacetanilide herbicides were unexpected. We saw a strong inverse association in Missouri between alachlor and semen quality in all analyses, a weak inverse association for metolachlor, and, in some analyses, a positive association for acetochlor. Within Missouri subjects ($\log_{10}$), alachlor and metolachlor levels were strongly correlated (Pearson correlation = 0.82, p = 0.0001), but neither was correlated with acetochlor in either center.

The available data on season, although limited, are consistent with an association between pesticide use and semen quality. Most of the pesticides studied are applied primarily in May and June (Pauley 2002). Although varying with soil and other local conditions, the environmental half-lives of these pesticides can be several months (Konda and Pasztor 2001). Because semen samples reflect exposures that occurred during the 3 months before sample collection, the finding that semen quality is poorer in late summer and fall, compared with winter, is plausibly linked to agricultural exposures. However, studies in urban populations have also found lower sperm concentration in summer compared with winter months (Jorgenson 2001), which may be related to heat or hours of daylight.

The mixed model allowed us to examine individual semen parameters as a function of pesticide metabolite level. The available data, although limited, suggest that sperm concentration and morphology, rather than motility, are affected. This is consistent with previous studies that found sperm concentration and the percentage of morphologically normal sperm decreased among sprayers (Lerda and Rizzi 1991), greenhouse workers (Abell et al. 2000), and traditional compared with organic farmers (Ekbom et al. 1996). Effects of these pesticides on specific semen parameters should be explored further in larger data sets.

It is unlikely that observer bias can explain the associations presented here. All pesticide analyses were blinded with respect to semen quality and center, and semen quality was measured without knowledge of pesticide status. We eliminated known confounders in our sample selection and controlled for remaining confounding in regression analyses. Although unmeasured confounders may remain, it is difficult to postulate a scenario that would create the associations seen here. With relatively small sample size and many metabolites found at very low levels, it is possible, although not likely, that these results are due to chance.

This is the first population-based study to demonstrate links between specific biomarkers of environmental exposures and biomarkers of male reproduction in humans. Given the current widespread use of these pesticides, if further study confirms these findings, the implications for public health and agricultural practice could be considerable.

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