SEMEN QUALITY IN RELATION TO BIOMARKERS OF PESTICIDE EXPOSURE

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ABBREVIATIONS:

CA	California
CDC	Centers for Disease Control and Prevention
CI	Confidence interval
IA	Iowa
ID	Isotope dilution
IMPY	2-isopropoxy-4-methyl-pyrimidinol
LOD	Limit of detection
MDA	Malathion dicarboxylic acid
MN	Minnesota
MO	Missouri
MS	Mass spectrometric
OR	Odds ratio
NY	New York
SFF	Study for Future Families
STD	Sexually transmitted disease
US	United States
USEPA	United States Environmental Protection Agency
WHO	World Health Organization

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ABSTRACT

We previously reported reduced sperm concentration and motility in fertile men in an agrarian area (Columbia, MO) relative to men from urban centers in Minneapolis, MN, Los Angeles, CA and New York, NY. The present study addresses 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, % normal morphology and % motile) were low (cases) and men in whom all semen parameters were within normal limits (controls) within MO and MN (sample sizes 50 and 36 in MO and MN, respectively) and measured metabolites of eight non-persistent, 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 MO cases compared to controls for the herbicides alachlor and atrazine, and for the insecticide diazinon (2-isopropoxy-4-methyl-pyrimidinol, or IMPY) (P-values for Wilcoxon rank test = 0.0007, 0.012, and 0.0004, for alachlor, atrazine and IMPY, respectively). MO men with high levels of alachlor or IMPY were significantly more likely to be cases than men with low levels (OR=30.0, 16.7 for alachlor and IMPY, respectively), as were men with atrazine over the LOD (OR=11.3). The herbicides 2,4-D and metolachlor were also associated with poor semen quality in some analyses, while acetochlor levels were lower in cases than controls (P=0.04). No significant associations were seen for any pesticides within MN, where levels of agricultural pesticides were low, or for the insect repellant DEET or the malathion metabolite MDA. 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.

SEMEN QUALITY IN RELATION TO BIOMARKERS OF PESTICIDE EXPOSURE

INTRODUCTION

In 1974, a study by Nelson and Bunge, noting poor semen quality in fertile men from Iowa City, IA, relative to men from NY, concluded, "Confirmation of our findings would imply that some unknown factor has caused a decrease in male fertility potential as measured by semen analysis" (Nelson and Bunge 1974). While the question of a possible decline in semen quality has been widely studied (Carlsen et al. 1992; Swan et al. 1997), prior to 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 multi-center study of semen quality in fertile men that included men from mid-Missouri, an area comparable demographically and agriculturally to Iowa City (Swan et al. 2003). Iowa City, IA, like Columbia, MO, has over 50% of county acreage in farms and both are located in counties in which use of pesticides is high (U.S. Census Bureau 2001).

In SFF we found, as had Nelson and Bunge, reduced sperm concentration and motility in men from an agrarian area (Columbia, MO) relative to men from urban centers (Los Angeles, CA, Minneapolis, MN, and New York, NY). 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. In this discussion we follow common usage and apply the term “pesticide” to a variety of agricultural chemicals; “[pesticides] ... though often misunderstood to refer only to insecticides…also applies to herbicides, fungicides, and various other substances used to control pests” (U.S. Environmental Protection Agency 1997).

It is well known that exposure to pesticides at occupational levels can adversely affect semen quality. In the late 1970’s the nematocide dibromochloropropane affected over 26,000 plantation workers in 12 countries; 64% had low sperm concentration and 28% were involuntarily childless (Thrupp 1991; Goldsmith 1997; Slutsky et al. 1999). 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 due to reduced sperm counts and semen volume in exposed workers (Whorton 1981; Schrader SM et al. 1988). More recently, a small study of herbicide sprayers in Argentina found decreased sperm concentration and morphology related to high urinary levels of 2,4-D metabolites (Lerda and Rizzi 1991). Greenhouse workers in Denmark with greater/longer pesticide exposure had lower sperm counts and percentage of morphologically normal sperm (Abell et al. 2000). Following 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 no differences in fertility, sperm count and percent 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 only been examined 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 (PCBs and p, p'-DDE) and decreased sperm motility, sperm concentration and percent normal morphology. No study to date has examined environmental exposure to current-use, non-persistent pesticides in relation to semen quality.

METHODS

Selection of pesticides to be tested

We began our search for causes of poor semen quality in men from MO 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 Mid west (primarily corn, soybeans, sorghum and winter wheat) and agricultural products used on these crops (Danekas and Schlegel 2002) to identify pesticides for initial testing. We selected 15 non-persistent 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-T; metabolites of the insecticides carbofuran, diazinon, carbaryl, chlorpyrifos, malathion, propoxur, methyl parathion and permethrin; and the insect repellent DEET.

Three pesticides that were found more often in MN than MO 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-napthol, 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 four-year multi-center 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, CA (Harbor-UCLA and Cedars-Sinai Medical Center), Minneapolis, MN (University of Minnesota Health Center), Columbia, MO (University Physicians) and New York, NY (Mt. 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 October 30, 2000, men were asked to provide a urine sample on the day they provided their first semen samples.

In the current study we sought to explain the differences in semen quality between MO and MN, centers that, 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 MO and MN 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. Since our goal was to select cases and controls with no or few, risk factors for impaired semen quality we first selected men who were aged 21-40, Caucasian, non-smokers, with no history of infertility, sexually transmitted diseases (STDs) or fever in the three months preceding sample collection. Because there were not enough men negative for these risk factors that satisfied the criterion for low (or high) sperm concentration, it was necessary to include 17 men (14 of whom were MO controls) with one or more risk factors. Since 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 to the subset of men with no risk factors.

For the initial analysis the 30 men with the lowest sperm concentration formed the case group and the 30 men with highest sperm concentration served as their controls. This group included 24 men from MO and 36 from MN.

In order to increase our power to examine pesticide levels in relation to semen quality within MO we then selected additional MO subjects (4 cases and 22 controls) to achieve a total of 25 cases and 25 controls within MO. 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 MO (25 cases and 25 controls) and 36 from MN (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 (Beeson et al. 1999). Using ID calibration, the samples are enriched with isotopically labeled analogues prior to 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 (Hill, Jr. et al. 1995). 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 spectrometric (MS/MS). All results were adjusted for concentration by creatinine and these adjusted metabolite levels were log-transformed for analysis. The limit of detection (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 x $(2)^{-½}$].

Semen collection and analysis

Men collected semen samples by masturbation at the clinic and almost all samples were analyzed within 45 minutes 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 sample. 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 percent 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 WHO (World Health Organization 1999; Guzick et al. 2001), 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 MO and MN 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, "high" values were those above 0.7, 3.0 and 0.3 μg/g creatinine, for alachlor, 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" (as defined above) 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 ($logarithm_{10}$) 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, percent motile sperm and percent normal sperm) as continuous variables. Because the number of covariates was large compared to the number of subjects in the pesticide analysis, we calculated a confounder score (Miettinen 1976), using data from all 441 men from MO and MN. 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 MO and MN (202 and 215, respectively) and recruitment rates were equal (27%). MO men were somewhat younger than MN (mean age 30.7 and 32.2 in MO and MN, 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 three months prior to semen collection).

Subject characteristics are contained in Table 1. Most cases (91%) and controls (80%) were 21-40 years of age, Caucasian, non-smokers, with no history of infertility, STDs or fever in the three months prior to sample collection. Occupational pesticide exposure was reported by only three men in MO and none in MN. 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, while all semen parameters differed significantly between cases and controls in both centers (Table 1). Therefore, though 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 MO and MN pesticide metabolite levels

The 15 urinary pesticide metabolites measured initially are shown in Table 2. Three analytes derived primarily from industrial sources were seen above the LOD more often in MN than MO, and none of these were related to semen quality. A metabolite of the organophosphate insecticide chlorpyrifos (3, 5, 6-trichloropyridinol) which is used to control residential pests, as well as on crops, was found above the LOD for 86% of subjects in MN and 50% in MO, but level was unrelated to case-control status (P=0.39). A second analyte found more often in MN 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 7 men, all from MN (2 cases and 5 controls). Carbofuranphenol, 2,4,5-T, and 2-isopropoxyphenol were below the LOD for all subjects, and permethrin results were not usable due to quality control problems. None of these seven pesticides were tested further.

Five metabolites were present above the LOD more often in MO than MN, including those of the herbicides alachlor, metolachlor, atrazine and 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 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 MN, pesticide levels in cases and controls did not differ significantly. In MO, where levels were higher, alachlor, atrazine and IMPY were quite different between cases and controls (P-values = 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-values = 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 MO 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 over 40, non-white race. Results among men with none of these factors were substantially unchanged from Table 3, although significance probabilities were somewhat larger due to smaller sample sizes (23 cases and 14 controls in MO; 8 cases and 24 controls) in MN.

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

There were 16 men (15 in MO) who reported pesticide exposure at home or at work in the three months before sample collection. Cases who self-reported exposure were more likely to have multiple metabolites elevated. Among the 8 cases in this group, on average the pesticide score was 2.5, and all had at least one elevated. 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 (below and above the LOD) or three (low, medium or high) levels, as described above (see Statistical 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 MO, are associated with a particularly high risk of reduced semen quality. For high alachlor exposure compared to 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. There were few men (9) with atrazine over the LOD. However all but one was a case, yielding an estimate of the OR which 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.

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

We examined the correlation among metabolite levels. While 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, metalochlor and 2,4-D were 0.89, 0.74, 0.76 and 0.92, respectively, with all P-values <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 (defined above under Statistical Methods). Within MO, 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 MO a score of 2-5 yielded an OR for poor semen quality of 3.4 (95% CI 2.0-25.5) compared to 0-1. In MN 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, which were very consistent with those in Table 3, are not presented. We also examined pesticide level as a predictor of three (continuous) measures of semen quality (logarithm$_{10}$ sperm concentration, morphology and motility). Table 5 includes results from the regression analysis for the five pesticides most associated with semen quality within MO. None of the five pesticides were significantly associated with any semen parameter within MN. Within MO, none of the pesticides were significantly associated with motility. The herbicides alachlor and metolachlor were more strongly associated with sperm morphology than concentration while IMPY was more strongly associated with concentration than morphology. Since 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, MO, relative to those from Minneapolis, MN. 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 MO. We have extended our study of partners of pregnant women (SFF) to Iowa City, IA, but have not yet obtained pesticide levels in those subjects. However, based on the similarity of agricultural practices and pesticide use in Columbia, MO and Iowa City (U.S. Census Bureau 2001), we hypothesize that metabolite levels will also be associated with semen quality in IA.

Levels of alachlor and atrazine in this population of MO 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 (Centers for Disease Control and Prevention 2003). Levels in our study were considerably higher. Since the same laboratory, methods and LODs were used for both studies, it appears that individuals in our sample have considerably higher exposures to these chemicals, particularly in MO.

The sources of the pesticides that we measured are not known. Elevated metabolite levels in cases compared to controls do not reflect occupational exposure, home pesticide use, or residence near a farm, since these differed little between 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 since these pesticides are non-persistent, with half-lives within the body of hours and days rather than years, these measured levels are likely to reflect recent exposure. While the biological half-life of these pesticides is short (from a few hours to a few days)(International Programme on Chemical Safety, World Health Organization 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 US Geologic Survey report on water quality noted that extensive herbicide use in agricultural areas (accounting for about 70 percent of total national use of pesticides) has resulted in widespread occurrence of herbicides in agricultural streams and shallow ground water in those areas (U.S. Geological Survey 2001). The most frequently detected pesticides in recent surveys of ground and surface waters are the triazine herbicides (atrazine, simazine), chloroacetamides (alachlor, acetochlor, metalochlor) 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 United States Environmental Protection Agency (USEPA) 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 anti-estrogenic activity at high doses in several laboratory studies. In one, atrazine increased secretion of LH 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. 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 tenfold decrease in testosterone (Hayes et al. 2002). As far as we know, ours is the first study to examine human reproductive risk associated with atrazine exposure.

Diazinon is, according to the USEPA, one of the most commonly found pesticides in air, rain, and drinking and surface water. On December 5, 2000, the USEPA announced an agreement to phase-out diazinon in the US beginning in March 2001. This phase-out 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. Environmental Protection Agency 2000).

Our study has many strengths, but also some limitations. While 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 MN 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 MO), 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 (due to method of selecting subjects for the case-control

analysis); and for several pesticides (notably atrazine and 2,4-D) there were very few values above the LOD.

The participation rate was low in both MO and MN (27%), but comparable to rates in other population-based studies of semen quality (Jorgensen 2001). While 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. Since 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 MO, and the absence of any association in MN, where few men were exposed above the 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 MN. 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 MO, 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 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 MO between alachlor and semen quality in all analyses, a weak inverse association for metolachlor and, in some analyses, a positive association for acetochlor. Within MO (logarithm_{10}) alachlor and metolachlor levels are strongly correlated (Pearson correlation = 0.82, P=0.0001), but neither is correlated with acetochlor in either center.

The available data on season, though limited, are consistent with an association between pesticide use and semen quality. Most of the pesticides studied are applied primarily in May and June (Danekas and Schlegel 2002). While varying with soil and other local conditions, the environmental half-lives of these pesticides can be several months (Konda and Pasztor 2001). Since semen samples reflect exposures that

occurred during the three months prior to sample collection, the finding that semen quality is poorer in late summer and fall, compared to winter, is plausibly linked to agricultural exposures. However, studies in urban populations have also found lower sperm concentration in summer, compared to 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, while limited, suggest that it is sperm concentration and morphology, rather than motility that are affected. This is consistent with previous studies that found sperm concentration and the percent of morphologically normal sperm decreased among sprayers (Lerda and Rizzi 1991), greenhouse workers (Abell et al. 2000), and traditional compared to 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. While 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, though 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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Table 1: Characteristics of subjects and semen samples for cases and controls by center

	MO			MN		
	Cases	**Controls**	**P-value [a]**	**Cases**	**Controls**	**P-value [a]**
Subjects characteristics						
Mean age	31.5	31.0	0.73	31.0	33.2	0.08
Number of men:						
Non-Caucasian	0	6	0.0009	0	0	1.00
Light smokers (<10 cig/day)	0	3	0.06	0	0	1.00
Smokers (>=10 cig/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 ½ mile of farm	7	5	0.51	0	1	0.56
Total number of men	**25**	**25**		**9**	**27**	
Semen sample characteristics						
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 ($x10^6$/ml) [b]	32.9	72.1	0.0001 [c]	23.9	106.4	0.0001 [c]
% Motile sperm	43.2	48.0	0.03	46.5	56.4	0.04
% Morphologically normal sperm	8.5	12.8	0.002	7.2	12.8	0.004
Mean total motile sperm ($x10^6$) [b]	65.5	110.4	0.0002 [c]	57.2	220.5	0.0001 [c]
Number of semen samples	47	47		18	54	

[a] P-value by t-test; [b] Abstinence-time-adjusted µ-Cell concentration; [c] Based on $logarithm_{10}$ transformed µ-Cell concentration

Table 2: Percent of men with metabolite levels above the LOD [a] in MO and MN [b]

	Percent >LOD [a]			
	MO	**MN**	**P-value [c]**	**Chemical type (parent chemical; likely source)**
Found more often >LOD in MO than MN				
Alachlor mercapturate	92%	25%	<0.0001	Herbicide (alachlor; agricultural)
IMPY [d]	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)
Found more often >LOD in MN than MO				
3,5,6-trichloropyridinol [e]	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 [g]	0	19%	0.035	Insecticide (methyl parathion; agricultural) Industrial precursors (para-aminophenol; unknown)
Number >LOD similar in MO and MN [e]				
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)
Number of subjects	24	36		

[a] Limit of detection (0.1 µg/g creatinine); [b] Initial sample of 60 men; [c] Fisher's exact test; [d] 2-isopropyl-4-methyl-6-hydroxypyrimidine; [e] 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.

Table 3: Mean and median pesticide metabolite levels in cases and controls

	MO					MN				
	Cases		Controls			Cases		Controls		
Pesticide [a]	Mean	(median)	Mean	(median)	P-value [b]	Mean	(median)	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] For comparison of cases and controls by Wilcoxon rank test within center.

Table 4: Odds ratios for low semen quality for men exposed to elevated pesticide levels

	MO				MN			
Pesticide	**Level [a]**	**Cases**	**Controls**	**Odds Ratio (95% CI)**	**Level [a]**	**Cases**	**Controls**	**Odds Ratio (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-Trichloro	< 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)

[a] µg/g creatinine

Table 5: Slopes within center from regression of semen parameters on pesticide metabolite level [a]

Metabolite	MO			MN		
	Concentration [b]	% Normal Morphology	% Motile	Concentration [b]	% Normal Morphology	% 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. P-values in parentheses; [b] Log_{10} μ-cell concentration; [c] Interaction of metabolite and center could not be estimated, so estimates are equal in both centers.