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2,4-Dichlorophenoxyacetic acid residues in semen of Ontario farmers

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Abstract

Although paternal exposures to environmental toxicants probably play a role in adverse pregnancy outcomes, few data are available on the extent of this exposure. One semen and two 24-h urine samples were collected from 97 Ontario farmers who had recently used the phenoxy herbicides 2,4-D (2,4-dichlorophenoxyacetic acid) and/or MCPA ([4-chloro-2-methylphenoxy] acetic acid). Both samples were analyzed for 2,4-D using an immunoassay-based technique. Approximately 50% of the semen samples had detectable levels of 2,4-D (≥5.0 ppb (ng/mL)). Semen levels of 2,4-D were correlated more closely with the second of the two urine samples. Although several studies have measured 2,4-D in the urine of applicators, this study is the first to attempt to measure 2,4-D levels in semen. As these pesticides can be excreted in the semen, they could be toxic to sperm cells and be transported to the woman and developing embryo/fetus. Further research is needed to understand how pesticide handling practices can affect semen pesticide residues and the relationship between the levels observed and reproductive health.

Keywords: Semen; 2,4-Dichlorophenoxyacetic acid; Herbicides; Urine; Occupational exposure; Agriculture; Farming; Sperm; Biologic monitoring

1. Introduction

The role of paternal exposures in the etiology of adverse pregnancy outcomes has recently been highlighted in several reviews [1–3]. There is some evidence to suggest that paternal pesticide exposures may be associated with increased risks of fetal death and developmental anomalies [4,5]. A classic example of a male human reproductive toxicant is the nematocide dibromochloropropane (DBCP), which adversely affected male fertility in workers involved with its production [6]. Environmental contaminants such as pesticides may have direct effects on sperm production via genetic damage to the sperm cells or hormonal imbalances. These chemicals may also be transmitted through the seminal fluid to the woman and the fetus [3]. A major criticism of the published epidemiologic studies of pesticides and adverse reproductive outcomes has been the imprecision in the estimates of exposure. Rarely was any attempt made to quantify exposure directly by measuring these substances in body fluids or tissues. Biologic monitoring provides both a quantitative and qualitative measurement of internal dose integrated by all exposure routes. Analytic techniques exist for measuring several pesticides in urine [7,8]. However, little has been published on contaminant levels in seminal fluid, possibly due to the laboratory analytic challenges (i.e., small amounts of sample and a more complex matrix) as well as the difficulty in obtaining semen samples in occupational studies.

The Pesticide Exposure Assessment Pilot Study was designed to test newly developed protocols and analytic methods to determine the extent to which pesticide applicators and their families are exposed to pesticides during normal handling practices on the farm. In addition to the collection of urine samples, semen samples were collected to assess whether or not handling of pesticides would result in measurable levels in the semen. Based on their frequency of use

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in agricultural and residential settings, concern about possible reproductive effects [4,5] and their rapid absorption and excretion largely unchanged in urine [9,10], the phenoxy herbicides containing the active ingredients 2,4-D (2,4-dichlorophenoxyacetic acid) or MCPA ([4-chloro-2-methylphenoxy] acetic acid) were chosen as the indicator pesticides to monitor. It was hypothesized that the temporal course of exposure, distribution, and excretion of these herbicides in semen would be similar to that observed in urine. Given the small volume of sample available for semen analysis, analytical methods were developed to measure 2,4-D in urine and semen using an enzyme immunoassay. No method was available nor was one developed to analyse the structurally similar phenoxy herbicide MCPA in semen. Immunoassay methods have been used to detect 2,4-D in water, urine [11], and food [12].

Given the problems of low participation rates in occupational field studies, we also wanted to evaluate the use of plastic condoms to facilitate semen collection. Plastic condoms have been marketed as an alternative to masturbation for the collection of semen samples for clinical evaluations [13].

2. Materials and Methods

2.1. Study population

The Ontario Farm Family Health Study [14], a questionnaire-based cohort study of young farm families, was used as the sampling frame for the current study. Farm families of reproductive age living in the province of Ontario were identified in a telephone interview of farm operators enumerated by the Canadian Census of Agriculture. Farmers who had reported using phenoxy herbicides in the 1991–92 Ontario Farm Family Health Study were selected and telephoned in early 1996 to identify eligible families. To be eligible for the study, the following criteria were used: (a) they had to be planning on using the phenoxy herbicides 2,4-D or MCPA in the coming growing season; (b) they were the individuals who handled the pesticides on the farm; (c) their home was on the farm property; and (d) they were currently living with their spouse.

Geographically remote farms in northern Ontario were excluded from the study to ensure that biologic and environmental samples could be picked up by the interviewers and transported to the laboratory within a reasonable period of time.

2.2. Survey instruments

Several survey instruments were designed and given to the participants at the time they agreed to participate in the study (Table 1). The Day of Application questionnaire collected information on the pesticides and handling practices used on the first day during the year that 2,4-D or MCPA was used by the farmer, as well as the pesticides used during the previous 6 d. The Day of Application questionnaire was to be completed by the pesticide applicator during the evening after 2,4-D or MCPA was used. A male factor questionnaire identified those individuals who had a vasectomy, the time since last ejaculation, and the time interval between first handling 2,4-D or MCPA and the collection of the semen sample. In addition, the applicators were asked to keep a current diary (the Agricultural Chemical Diary) of all crop pesticides used during the study year (1996). The participants were asked to return the completed Diary by mail at the end of the season. The Day of Application Questionnaire and Agricultural Chemical Diary were used to extract information on what pesticides were used around the time of biologic sample collection. Validation against records of purchase was not attempted. Data on age, smoking status, education, per capita income, and self-rating of health were available from the Ontario Farm Family Health Study questionnaires.

Table 1
Time course of data collection, Pesticide Exposure Assessment Pilot Study

1. Keep diary of all crop pesticides used during season (Agricultural Chemical Diary)
2. Collect urine sample (single void) just before using 2,4-D or MCPA (study pesticides) for the first time during the season (pre-exposure sample)
3. Collect two consecutive 24-h urine samples, starting immediately after first use of study pesticides (Day 1 and Day 2 urine samples)
4. During the evening of first day of using study pesticides or within 24 h thereof, complete Day of Application Questionnaire
5. (optional) Within 48 h of first using study pesticides, collect semen sample and complete male factor questionnaire
6. Urine and semen samples and Day of Application and male factor questionnaires picked up by study team within 2 d of collection
7. Agricultural Chemical Diary mailed to study team at end of season

2.3. Recruitment of study population

A 2-d training session was held in late February 1996 to familiarize the interviewers with the survey instruments and to help make them comfortable with requesting and encouraging participation in all aspects of the study, including semen and urine collection. Potential participants were telephoned to arrange a visit to the farm to describe the study. If the family agreed to participate, they were asked to sign consent forms and were provided with detailed instructions, survey instruments, and biologic sample collection kits.

Participants were informed that they would receive an honorarium based on their level of participation. To fully qualify as a participant and receive the honorarium of $50, the farmer and his spouse had to agree to collect the necessary urine samples and complete all survey instruments. Semen samples were optional, with those farmers providing a semen sample were paid an additional honorarium of $100.

2.4. Biologic sampling

At the time of the farm visit, each participant who signed the informed consent form for a semen sample was given two plastic condoms (Male-Factor Pak©, Apex Medical Technologies, Inc. San Diego, CA), twist ties, a zip-lock plastic bag, a male factor questionnaire, and a large brown envelope in which to store the sample and questionnaire. As all of the farmers in this study were married, the plastic condom could be used to collect the semen during normal marital intercourse. The man was instructed to remove the condom carefully after ejaculation and tie off the open end using the twist tie provided, placing the used condom in the zip-lock plastic bag. Subsequently, he was to complete the male factor questionnaire, place the kit and questionnaire in the brown envelope, and place the envelope in the freezer until picked up by the study team. The couple was requested to collect the semen sample in the plastic condom within 48 h of first handling 2,4-D or MCPA. The couple was advised that the plastic condoms should not be used as a birth control or contraceptive device or as protection against sexually transmitted disease. If they used a condom for either of these reasons, they were instructed to use their regular condom over the one supplied, or provide a semen sample in the condom provided by some means other than intercourse.

The farmers were encouraged to follow their normal pesticide handling practices. To obtain an indication of background levels of the study pesticides, each participant was asked to collect a urine sample in the hours before handling 2,4-D or MCPA for the first time that growing season (the pre-exposure sample). Subsequently, the couple collected two consecutive 24-h urine samples (Day 1 and Day 2) immediately after starting to handle the study pesticides, in keeping with models that demonstrate that a majority of 2,4-D from any one application will be excreted in the urine over this period. The urine samples were kept cool in a sample kit with ice packs. The urine and semen samples, as well as the Day of Application and male factor questionnaires were picked up by the study team within 2 days and transported to the laboratory for pesticide analysis.

2.5. Enzyme-linked immunosorbent assay (ELISA) for 2,4-D

Chemicals and Instrumentation: Analytical standards of 2,4-D used to generate the standard curve and spike samples were obtained commercially. The [^{14}C] 2,4-D was provided by Dow AgroSciences (Indianapolis, IN). Chemical reagents were obtained from Sigma. ELISA plates were analyzed using a Model 3550-UV microplate reader (Bio-Rad Laboratories, Richmond, CA).

Preparation of 2,4-D immunogen: 2,4-D was conjugated to bovine serum albumin (BSA) as described by Fleeker [15]. Equimolar amounts of 2,4-D (42 mg), N-hydroxysuccinimide (22 mg), and N,N′-dicyclohexylcarbodiimide (39 mg) were dissolved in 2.5 mL dioxane in the same sequence as listed above. The solution was left to stand at room temperature for approximately 18 h and then filtered to remove the precipitate. The filtrate was evaporated to dryness with a rotary evaporator under vacuum at 35°C. A solution of BSA (500 mg) dissolved in 3 mL of 0.10 M borate buffer (pH 9) was added to the residue, and the mixture was agitated gently for 1 h at room temperature. The resulting solution was dialyzed against several changes of deionized water over 36 h at 4°C and lyophilized.

Antisera: New Zealand white rabbits (female) were injected subcutaneously with an emulsion consisting of 0.5 to 1.0 mg of immunogen dissolved in 0.5 mL PBS (phosphate buffered saline) and an equal volume of Freund's complete adjuvant. The injections were repeated 3, 6, and 10 d after the initial injection, substituting Freund's incomplete adjuvant for complete adjuvant. A booster injection was given 1 month after the initial injection and was repeated at monthly intervals thereafter. The rabbits were bled for antibody titer determinations 10 d after each boost. Antisera for 2,4-D immunoassay development were prepared from a single bleed in each case.

ELISA assay and standard curve: Plates were coated with 2,4-D ovalbumin (OVA) coating conjugate (1/64,000 dilution; 100 μL/well), incubated 1 h at 37°C, washed three times with a PBS–Tween solution, and blocked with 200 μL/well of 0.1% gelatin in PBS. The plates were further incubated for 20 min and again washed three times with a PBS–Tween solution. The standards, controls, and samples were made up in a 1:1 ratio with a 1/500 dilution of D1 serum. This solution was added to the plates (100 μL/well) and the plates were incubated for 30 min at room temperature. The plates were washed three times with a PBS–Tween solution. Goat antirabbit–horseradish peroxidase (GAR-HRP) at 1/5000 dilution was added to plates (100 μL/well), which were incubated for 30 min at room temperature. The plates were washed three times with a PBS–Tween solution. Substrate was added to the plates (100 μL/well). The substrate consisted of 1 mg/mL urea hydrogen peroxidase and 1 mg/mL ABTS (2,2′-azino-bis(3-ethylbenzthiazoline 6-sulfonic acid) diammonium; Sigma A9941) tablets in citrate buffer pH 5.0 at room temperature. The plates were incubated 20 min in darkness at room temperature prior to the absorbance being read at 405 nm with the BioRad 3550 UV plate reader.

A standard curve was generated by spiking semen sample from a control subject who had not been exposed to herbicides for several months using the protocol outlined above. A new standard curve was generated for each new batch of antibodies. Absorbance values of the standards and the samples (A) were normalized by dividing by the absorbance values of the negative controls (wells containing 0 ng/mL 2,4-D; A_0). The A/A_0 values for standards were plotted against the log of 2,4-D concentration to construct a standard curve. Concentrations of the samples in water and soil were determined by interpolating from a PBS standard

curve. Fleeker [15] reported the limit of detection (LOD) of an assay to be three times the standard deviation of the A_0 from its mean absorbance; whereas, Midgely et al. [16] calculated the LOD as the concentration that corresponds to 90% of the A/A_0. Though the former LOD description is used by the American Association of Official Analytical Chemists (AOAC), the latter LOD description may be a more accurate assessment of the true LOD because LOD is a function of the ability of a compound to inhibit antibody-hapten binding, rather than a function of A_0 precision. Using both criteria, the LOD of the direct ELISA was 1.0 ppb. The limit of quantitation (LOQ) of an assay has been reported as ten times the standard deviation of the A_0 from its mean absorbance [15]. The LOD and LOQ for urine (accounting for the 1:5 dilution) are 1 and 5 ppb, respectively. The semen samples were diluted 1:25 (5 times more dilute than urine), and therefore the LOD and LOQ were 5 and 25 ppb, respectively.

2.6. Laboratory analysis of biologic samples

The extraction procedure was carried out in a Level 2 containment fumehood. Semen samples were thawed, removed from condoms, and placed in Nalgene centrifuge tubes. Semen was mixed by pipetting 15 to 20 times with an Eppendorf pipette, before placing 500 μL in a borosilicate culture tube. Two mL PBS with 0.1% Triton X-100 was added to each culture tube. The tubes were capped, vortexed 2 to 3 seconds, and shaken on a platform shaker for 2 h. A 1.5-mL aliquot of this fluid was transferred to a 2.0-mL centrifuge vial and centrifuged at 16,000 *g* relative centrifugal factor (RCF) for 15 min. The supernatant was removed and analyzed for 2,4-D content using the enzyme-linked immunosorbent assay (ELISA) developed in our laboratory (JCH). The semen samples were diluted 1:25. Recoveries were determined by spiking blank semen samples with ^{14}C radiolabelled 2,4-D as described previously by Johnson and Hall [17]. Recovery of 2,4-D was 98.2% with a less than 10% cross-reactivity with MCPA. Urine values were not adjusted for creatinine levels.

2.7. Data management and statistical analysis

All data were double checked to verify accuracy. As the semen and urine levels were not normally distributed, nonparametric statistical tests were performed using the statistical package SAS [18]. The nonparametric Kruskal-Wallis test based on ranks was used to test the null hypothesis that the distribution of semen levels of 2,4-D was the same in multiple independent subgroups of the study population (for example subgroups varying by age, smoking status, reported use of index herbicides). Multiple regression analyses for predictors of log-transformed semen levels did not result in identification of significant predictors and so are not reported here.

Table 2
Participation in the Pesticide Exposure Assessment Pilot Study and the Semen Component

Recruitment stage and reasons for nonparticipation	Nonparticipants (No., % of stage)	Potential participants
Total selected from sampling frame[a] for telephone screen		773
Unable to contact	104 (13.4%)	
Refused telephone screen	45 (5.8%)	
Not eligible[b]	295 (38.2%)	
Potential Farm Visits		329
Unable to contact	2 (0.6%)	
Not eligible[b]	34 (10.3%)	
Refused visit	55 (16.7%)	
Unable to recruit[c]	2 (0.6%)	
Refused consent	21 (6.4%)	
Potential participants in study		215
Dropped out of study[d]	89 (41.4%)	
Participants in Study		126
Refused to provide semen	12 (9.5%)	
Agreed but did not provide semen sample	17 (13.5%)	
Provided semen sample		97

[a] A farm where the husband indicated that he applied 2,4-D or MCPA on the farm.

[b] No longer farming in 1996, not expecting to use 2,4-D or MCPA that season, other people handle pesticides on farm, home not on farm property, lived in geographically remote area of northern Ontario, and/or not currently living with spouse.

[c] Could not visit due to snow storm or farmer could not read.

[d] After signing consent form, did not participate for various reasons generally associated with weather conditions.

3. Results

3.1. Participation rates

A total of 773 farmers were selected from the Ontario Farm Family Health Study population as farmers who had used 2,4-D and/or MCPA at that time. After the telephone screen, 329 families were identified as potentially eligible for the biologic monitoring study (Table 2). In order to be eligible, the farmer had to be planning to use the study pesticides during the coming season. At each phase of the recruitment, the refusal rate was generally low. Approximately 6% of the farmers refused the telephone screen, 17% refused the farm recruitment visit, and once visited, 6% of the families refused to participate. We were unsuccessful in our attempts to contact 13% of the families, which is understandable given that recruitment for this study took place approximately 5 years after the sampling frame was assembled. These families probably had moved off the farm and therefore were no longer eligible for the study.

A total of 215 families signed informed consent forms. Unfortunately, the spring of 1996 was one of the coolest and wettest on record and contributed to a high drop-out rate. Approximately 40% of the farmers ($n = 89$) dropped out of the study for one of the following reasons: not being able to use the pesticides of interest ($n = 39$), time constraints due

Table 3
Comparison of characteristics of farmers[a] at each recruitment stage and final participants in semen component of Pesticide Exposure Assessment Pilot Study

Characteristic	Number of Farmers and Percentage of Sampling Stage[b]				
	Sampling frame (n = 773)	Potential farm visit (n = 329)	Potential participant (n = 215)	Potential semen provider (n = 126)	Semen providers (n = 97)
Age					
<40	261 (33.8%)	101 (30.7%)	72 (33.5%)	41 (32.5%)	35 (36.1%)
40–44	209 (27.0%)	99 (30.1%)	65 (30.2%)	44 (34.9%)	32 (33.0%)
>44	303 (39.2%)	129 (39.2%)	78 (36.3%)	41 (32.5%)	30 (30.9%)
Smoking status					
Current smoker	132 (17.1%)	48 (14.6%)	30 (14.0%)	12 (9.5%)	8 (8.2%)
Former smoker	154 (19.9%)	63 (19.1%)	43 (20.0%)	31 (24.6%)	21 (21.6%)
Never smoked	486 (63.0%)	218 (66.3%)	142 (66.0%)	83 (65.9%)	68 (70.1%)
Education					
Grade 1–11	199 (25.8%)	100 (30.4%)	53 (24.7%)	24 (19.0%)	15 (15.5%)
High school graduate	271 (35.1%)	117 (35.6%)	81 (37.7%)	51 (40.5%)	42 (43.3%)
Some post-secondary	302 (39.1%)	112 (34.0%)	81 (37.7%)	51 (40.5%)	40 (41.2%)
Per capita income					
<$15,000	530 (75.1%)	236 (78.7%)	161 (80.1%)	97 (82.2%)	75 (80.6%)
≥$15,000	176 (24.9%)	64 (21.3%)	40 (19.9%)	21 (17.8%)	18 (19.4%)
Farmer's rating of health status					
Excellent	361 (46.8%)	149 (45.3%)	99 (46.0%)	61 (48.4%)	49 (50.5%)
Good	369 (47.8%)	160 (48.6%)	103 (47.9%)	59 (46.8%)	43 (44.3%)
Fair or poor	42 (5.4%)	20 (6.1%)	13 (6.0%)	6 (4.8%)	5 (5.2%)
Average no. days/ season applying herbicides					
1–3	149 (22.5%)	47 (16.8%)	30 (16.1%)	16 (14.7%)	13 (15.3%)
4–9	208 (31.4%)	86 (30.7%)	59 (31.7%)	32 (29.4%)	24 (28.2%)
10–14	142 (21.5%)	68 (24.3%)	43 (23.1%)	29 (26.6%)	22 (25.9%)
>14	163 (24.6%)	79 (28.2%)	54 (29.0%)	32 (29.4%)	26 (30.6%)

[a] Based on data obtained in 1991/1992 Ontario Farm Family Health Study.
[b] Numbers may not add up correctly due to rounding and missing values.

to the weather conditions (n = 13), another person did the spraying (n = 23), forgot to collect urine samples (n = 7), and other problems (n = 7). The time between recruitment (and signing consent form) and sample collection varied from 2 to 4 months. After removing those families known to be ineligible (n = 329), as well as those that we could not contact during the telephone screening interview (n = 104), 37% of the remaining families (n = 126) participated in the biologic monitoring study and 28% of the husbands (n = 97) provided a semen sample.

Of the 215 families that had signed the informed consent form, 86% (n = 184) had agreed to provide a semen sample. Among the 89 families that dropped out of the study, 80% of the husbands were intending to provide a semen sample. We did not receive a semen sample from 17 men who had consented to provide one. Three of these men reported problems with the condom, three forgot or lost the kit, and the remaining men did not provide a reason.

A comparison of characteristics of the farmers that participated at each recruitment stage of the study is presented in Table 3. Compared to the men from the sampling frame who did not provide a semen sample, those who did were statistically more likely to be nonsmokers and high school graduates.

3.2. Semen analysis

Approximately 50% of the semen samples had 2,4-D levels at or above the detection limit of 5.0 ppb (ng/mL). The semen values were highly skewed, with values ranging from below the detection limit to 650 ppb, with a mean of 29.8 ppb and a median of 4.8 ppb. The Spearman correlation coefficients for the semen and urine levels were 0.002 for the pre-exposure urine sample (P = 0.99), 0.18 for the Day 1 urine (P = 0.08), and 0.33 for the Day 2 urine sample (P = 0.001). If 2,4-D was detected in the Day 2 urine, it was not necessarily a good predictor that detectable levels would be measured in the semen (Pr = 0.54); however, positive semen levels were indicative of positive Day 2 urine levels (Pr = 0.90).

Median semen levels of 2,4-D did not differ significantly by any of the personal characteristics of the applicator measured (Table 4). The percentage of samples with detectable levels of 2,4-D was not equally distributed by smoking status, with current smokers more likely to have residues of 2,4-D in their semen.

Median semen levels of 2,4-D were statistically higher in men who reported using 2,4-D only, particularly if the semen sample was collected more than 24 h after the man

Table 4
2,4-D Levels (ppb) measured by ELISA in semen samples of farmers from the Pesticide Exposure Assessment Pilot Study

Characteristic	Mean (standard deviation) (ppb)	Median (range) (ppb)	% of Semen samples with detectable levels (≥5 ppb)
All Samples (n = 97)	29.8 (84.26)	4.8 (0–650)	49.5%
Personal factors			
Vasectomy			
Yes (n = 30)	57.8 (143.22)	2.6 (0–650)	43.3%
No (n = 67)	17.2 (28.25)	5.0 (0–140)	52.2%
Time since last ejaculation			
≤48 h (n = 28)	20.3 (33.44)	6.2 (0–140)	53.6%
>48 h (n = 69)	33.6 (97.61)	4.4 (0–650)	47.8%
Per Capita Income			
<$15,000 ($n$ = 75)	35.9 (94.87)	5.0 (0–650)	50.7%
≥$15,000 ($n$ = 18)	9.6 (12.78)	4.4 (0–45)	50.0%
Education			
Grades 1–11 (n = 15)	20.1 (33.20)	12.2 (0–125)	60.0%
High School (n = 42)	29.9 (101.41)	4.9 (0–650)	50.0%
Post-secondary (n = 40)	33.2 (78.87)	4.1 (0–400)	45.0%
Age			
<40 (n = 35)	29.1 (57.63)	7.8 (0–300)	54.3%
40–44 (n = 32)	26.0 (71.12)	5.4 (0–400)	50.0%
>44 (n = 30)	34.5 (119.0)	4.2 (0–650)	43.3%
Smoking status			
Current smoker (n = 8)	19.5 (24.13)	12.1 (0–75)	75.0%[a]
Former smoker (n = 21)	28.7 (87.54)	0.8 (0–400)	23.8%
Never smoked (n = 68)	31.3 (88.37)	7.6 (0–650)	54.4%
Self-rated health status			
Excellent (n = 49)	42.3 (114.65)	7.8 (0–650)	53.1%
Good (n = 43)	17.7 (28.47)	3.8 (0–125)	46.5%
Fair to Poor (n = 5)	10.5 (14.26)	0.5 (0–27.5)	40.0%
Time between handling of 2,4-D/MCPA and semen collection			
<24 h (n = 24)	47.8 (133.68)	3.1 (0–650)	45.8%
≥24 h (n = 73)	23.8 (60.11)	5.0 (0–400)	50.7%
Reported use of 2,4-D and MCPA[b]			
No reported use of 2,4-D on pre, Day 1, or Day 2 (n = 53)	14.2 (24.83)	5.0 (0–140)	52.8%
2,4-D but no MCPA used on pre, Day 1, or Day 2 (n = 23)	73.2 (151.87)	13.8[c] (0–650)	56.5%
2,4-D & MCPA used on pre,Day 1, or Day 2 (n = 14)	25.2 (79.44)	0.7 (0–300)	28.6%
Time between first started handling herbicide and semen collection <24 h			
No reported use of 2,4-D on pre, Day 1, or Day 2 (n = 9)	21.4 (45.21)	2.5 (0–140)	44.4%
2,4-D & no MCPA used on pre, Day 1, or Day 2 (n = 8)	106.9 (223.87)	8.8 (0–650)	50.0%
2,4-D & MCPA used on pre, Day 1, or Day 2 (n = 3)	9.6 (15.47)	0.8 (0.6–27.5)	33.3%
Time between first started handling herbicide and semen collection ≥24 h			
No reported use of 2,4-D on pre, Day 1, or Day 2 (n = 44)	12.7 (18.77)	6.4 (0–75)	54.6%
2,4-D & no MCPA used on pre, Day 1, or Day 2 (n = 15)	55.3 (100.96)	25.0[d] (0.2–400)	60.0%
2,4-D & MCPA used on pre, Day 1, or Day 2 (n = 11)	29.5 (89.79)	0.5 (0–300)	27.3%
Day 2 urine level of 2,4-D	26.6 (57.02)	9.6 (0–312)	
<1.0 ppb (LOD)			29.4%
≥1.0 ppb			53.8%

[a] Chi-square P = 0.02.
[b] Missing agricultural chemical diary for 7 applicators.
[c] Kruskal-Wallis P = 0.03.
[d] Kruskal-Wallis P = 0.01.

had started handling the herbicide. However, approximately 50% of the men who indicated both in the Day of Application Questionnaire and Agricultural Chemical Diary that they had not used 2,4-D within 2 d of the semen sample being collected had detectable levels of 2,4-D in their semen. Possible explanations for this result include the assay's cross-reactivity with MCPA (believed to be in the order of 10%), misreporting, or indirect exposure to 2,4-D in the home or work environment. Time between last ejaculation and semen collection was not significantly associated with median 2,4-D levels.

4. Discussion

The data from this study indicate that those men willing to provide 24-h urine samples are generally agreeable to

collecting a semen sample in a plastic condom. Of those who actually provided urine samples, 77% provided semen samples. Although it is impossible to know how many of the families that refused to be interviewed would have been eligible for the study, assuming that all would be eligible, we estimated that our participation rate among those potentially eligible was 37% for the urine component and 28% for the semen component, using a 5-year-old sampling frame. Bearing in mind that to be included in the semen component, both the husband and wife had to agree to provide a pre-exposure and two consecutive 24-h urine samples and complete several questionnaires over a one-year period, we feel that the use of a plastic condom was effective in yielding a good participation rate among married couples.

As detectable levels of 2,4-D were measured in the semen samples, this active ingredient can be excreted by this route and thus could be toxic to sperm cells and be transported to the woman exposing her eggs at fertilization and/or the developing embryo/fetus [19]. The levels measured in the semen were of the same order of magnitude as those measured in the 24-h urine samples using the same ELISA assay and were measurable after a brief period of exposure (at most 2 d). However our results do not justify using urine analysis on a routine basis to estimate semen levels. One of the advantages of the ELISA method used was that it could measure pesticide levels in a relatively small sample volume. To our knowledge no other data on phenoxy herbicide concentrations in semen are available. In previously published research, lead [20], mercury [21], dioxins [22], organochlorine pesticides [23], several drugs [24], and tobacco smoke byproducts [25] have been detected in seminal fluid.

Dermal exposure is an important route of entry of pesticides into the body. The amount of pesticide absorbed will depend on a number of factors including the pesticide's active ingredient(s), solvent, temperature, and anatomic site. A study using radioactive labeled pesticides has demonstrated that follicle-rich areas of the body including the scalp, angle of the jaw, postauricular area, and forehead permit greater penetration of pesticides than the forearm [26]. Among several anatomic regions studied, applications to the scrotum area provided virtually no significant barrier to percutaneous penetration and resulted in the highest percentage of applied dose excreted in the urine, with a total excretion ratio 11.8 times greater than when the pesticide was applied to the forearm region.

Great variations in excretion rates of 2,4-D have been observed in volunteer studies [27]. The type of formulation (for example 2,4-D acid or 2,4-D dimethylamine salt) was also found to influence skin penetration and therefore total body burden. Volunteers excreted an average of 4% of the applied acid and 2% of the applied dimethylamine salt. When the amount of pesticide removed by hand wash 6 h after application was accounted for, it was still not possible to account for the total dose applied, indicating that the skin may have served as a reservoir for the herbicide, and other routes of excretion may have been involved. In addition, the routes of exposure likely affect absorption and excretion patterns. Another volunteer study reported that at least 88% of the ingested dose was excreted in the urine [28].

The pharmacokinetics of human absorption and urinary excretion of 2,4-D are generally well understood, with absorbed doses being bound to protein in plasma and excreted as the parent compound in urine [29]. Peak urinary excretion occurs approximately 24 h after peak plasma concentrations. The primary route of elimination is the renal organic anion (acid) secretory system [30]. When this system becomes saturated, 2,4-D accumulates in the plasma and becomes more available for glomerular filtration and distribution to other tissues. The male reproductive tract provides no barrier to many exogenous chemicals, allowing such compounds to cross into the fluids secreted by the testes and male accessory organs and ultimately pass into semen. There are several ways by which the chemical could find its way into the ejaculate including via testicular plasma, epididymal plasma, vas deferens and ampullary secretions, or the secretory fluids contributed to the whole ejaculate by the seminal vesicles, prostate, Cowper's gland, and Littré's glands, respectively [31].

The few epidemiologic studies of pesticide exposure and male fertility conducted to date have shown somewhat conflicting results. A study of occupation and semen quality for men attending a diagnostic semen laboratory in Calgary, Alberta, reported that men working in agriculture, where occupational exposure to pesticides could occur, had significantly higher semen volume, lower sperm density and motility, and a higher percentage of tapering sperm head defects [32]. In the Netherlands, exposure to herbicides was described as associated with an imprecise but elevated risk of abnormal semen parameters (OR = 1.82; 95% CI 0.4–8.25) [33]. In a Danish study, however, the authors concluded that the use of pesticides was not a likely cause of short-term effects on semen quality or concentration of reproductive hormones [34]. It is noteworthy that among the Danish farmers who sprayed herbicides, those who sprayed for more than 12 h during the season did have a significantly lower proportion of normal sperm and reduced curvilinear velocity. In previously published results of the original cohort of farmers used in this study (the Ontario Farm Family Health Study), paternal application of crop herbicides and use of phenoxy herbicides was associated with odds ratios of 1.3 (95% CI 0.9–1.9) for miscarriage, 1.4 (95% CI 0.5–3.6) for preterm delivery, 0.7 (95% CI 0.4–1.2) for small for gestational age birth [35], and had no effect on time to pregnancy (Fecundability Ratio = 1.00; 95% CI 0.86–1.16) [36]. There was some indication that preconception exposure (from 3 months before conception to the month of conception) was associated with an elevated risk of early miscarriages (<12 weeks) (OR = 2.5; 95% CI 1.0–6.4) [37].

Only one published study to date has examined sperm parameters in farmers using 2,4-D [38]; however, this study

did not attempt to measure 2,4-D in seminal fluid. Among 32 farmers using 2,4-D, the mean level measured in their urine was 9.02 mg/L (ppm), compared to no detectable concentration of 2,4-D in the unexposed group. The authors did not indicate when the urine sample was collected. Sperm density, motility, vitality, and morphology were adversely affected in the group of farmers exposed to 2,4-D. In our study, the mean urine levels of 2,4-D measured by ELISA were much lower (23.0 ppb on Day 1 and 26.6 ppb on Day 2). Unfortunately, given the logistics involved in collecting semen samples and transporting them to a laboratory within one hour of collection (as recommended by WHO [39]), and the very hectic schedule for farmers during the study period, we were not able to collect fresh sperm samples for analyses of density, motility, and morphology. As a result we were not able to determine whether the levels measured in our study had any effect on sperm quality.

Our study was designed as a rather large pilot study. As the number of samples was small and the levels of 2,4-D measured varied widely, it was difficult to identify any significant predictors of the level measured in the semen. As this study is the first, to our knowledge, to attempt to measure 2,4-D in the semen, we have little understanding of how physiologic differences among individuals, pesticide handling practices, and exposure by other routes could affect semen levels. In addition, it is not clear how exposure to other pesticides and cross-reactivity with these other pesticides in the assay may have affected our results. Although we only collected one sample of semen from each subject shortly after his exposure to 2,4-D or MCPA, we hypothesize that the excretion rate in semen is similar to that in urine. Given the importance of semen as a potential carrier of chemicals that pose reproductive hazards directly to the site of fertilization, further research is needed to understand pesticide excretion patterns in the semen and the dose-response relationship of 2,4-D in semen with sperm quality and the risk of adverse reproductive outcomes.

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