




Human Semen Quality, Sperm DNA Damage, and the Level of Urinary Concentrations of IN and TCPY, the Biomarkers of Nonpersistent Insecticides

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Abstract

The aim of the study was to evaluate the association between environmental exposure to nonpersistent insecticides and semen quality (concentration, motility, morphology, computer-aided semen analysis [CASA] parameters, and sperm DNA damage). Urine samples ($n = 315$) collected from men who attended the infertility clinic with normal semen concentration of 15 to 300 mln/ml and age under 45 years were analyzed for two metabolites (1-naphthol [IN] and 3,5,6-trichloro-2-pyridinol [TCPY]) of nonpersistent insecticides. Participants provided semen, blood, and saliva samples; additionally, men filled a detailed questionnaire. The results identified that urinary TCPY concentration was significantly associated with a decrease in motility; also there was a positive association between TCPY and DNA fragmentation index (DFI). IN concentration was negatively associated with a percentage of sperm with normal morphology and positively with one of the CASA parameters (curvilinear velocity [VCL]). The results suggest that environmental exposure to nonpersistent insecticides may have an impact on semen quality parameters and sperm DNA damage.

Keywords

semen quality, DNA damage, carbaryl, chlorpyrifos

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Several studies have reported a meaningful decline in sperm quality over the past decades (Carlsen, Giwercman, Keiding, & Skakkebaek, 1992; Jorgensen et al., 2011; Kumar & Singh, 2015; Levine et al., 2017; Mendiola et al., 2013; Swan, Elkin, & Fenster, 1997). It has been hypothesized that the widespread exposure to the group of human-made chemicals, namely, endocrine disruptors, may be an important contributing factor to that trend. The general population is known to be exposed to a diverse group of endocrine disruptor chemicals like bisphenol A, phthalates, parabens, triclosan, or pesticides (Melgarejo et al., 2015).

Pesticides are a large and heterogeneous group of chemicals used to control and repel pests in different fields (Mostafalou & Abdollahi, 2017). The literature reports that the development of new and strong organic chemical targets has brought pesticides into widespread use during the past century (Mostafalou & Abdollahi,

2017). Pesticides typically occur in mixtures with other pesticides (Hernandez et al., 2013) and these substances are widely used in agricultural, commercial, and residential settings (Meeker, Ryan, Barr, & Hauser, 2006). Considering the extensive use of these chemicals, a

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large proportion of the general population is exposed to low levels of contemporary-use nonpersistent pesticides like carbaryl, chlorpyrifos, or chlorpyrifos-methyl (Meeker et al., 2006). Carbaryl (1-naphthyl-*N*-methylcarbamate) widely known as Sevin[®] is an ester of carbamic acid (Fujino et al., 2016; Meeker, Barr, Serdar, Rappaport, & Hauser, 2007). It is a broad-spectrum insecticide commonly applied to residential lawns and gardens to protect from a variety of insects. Carbaryl is considered as the most effective pesticide for crops as well as agricultural animals and pets (Fujino et al., 2016). While chlorpyrifos and chlorpyrifos-methyl are organophosphorus insecticides (Centers for Disease Control and Prevention, 2009). Chlorpyrifos is a broad-spectrum insecticide that has been extensively used to control insects on food crops. It has been applied directly on animals to kill mites and sprayed to kill mosquitoes. In 2000, the Environmental Protection Agency (EPA) placed a restriction on chlorpyrifos residential use (U.S. EPA, 2002). Chlorpyrifos-methyl is an organophosphorus insecticide used in agriculture but not registered for residential use (Meeker et al., 2006). Human exposure to nonpersistent insecticides can occur through a variety of routes. Potential exposure routes include inhalation of air or dust, ingestion of insecticide residues on food, or dermal contact from insecticide-treated areas (Meeker, Singh, et al., 2004). In the general population, the main exposure pathway is through dietary ingestion, especially via the consumption of fruit and vegetables (Chiu et al., 2016). Despite restrictions on the use of carbaryl, chlorpyrifos, and chlorpyrifos-methyl, the high rate of detection of 1N and TCPY in urine of the general population demonstrates ongoing environmental exposure to the insecticides.

Scientists have become more aware that human-made chemicals may have an impact on reproductive function in both animals and humans (Moline et al., 2000). The results of studies suggest a potential association between exposure to some commonly used insecticides and human reproduction. A study of workers who packaged carbaryl reported an increased oligozoospermia (<20 mln sperm/ml) and tetraspermia (>60% abnormal sperm morphology) in men compared with the reference group of chemical workers (Wyrobek et al., 1981). More information about carbaryl's testicular toxicity comes from studies on laboratory rats. Luca and Balan (1987) identified an association between carbaryl and sperm shape abnormalities. Dose-response relationships between carbaryl and a decline in epididymal sperm count and motility were reported by Pant, Srivastava, Prasad, Shankar, and Srivastava (1995). Chlorpyrifos is less studied than carbaryl is for its testicular toxicity because of the ban on its residential use since 2000 (U.S. EPA, 2002). It has been found that exposure to chlorpyrifos can disrupt endocrine regulation in ewes (Rawlings, Cook, & Waldbilling, 1998). The results of these studies

have indicated that insecticides may have an impact on reproductive functions.

During the past decades a possible degradation in human semen quality has been debated intensively and has become an important public health issue. A controversial review article of 61 studies analyzing sperm concentrations in fertile men and in men of unknown fertility published between 1938 and 1990 by Carlsen et al. (1992) identified a significant decrease in sperm concentrations and in semen volume. Further evidence for declining sperm quality was provided by Swan, Elkin, and Fenster (2000), who performed multivariate analysis of 101 studies from 1934 to 1996, taking into account many of the confounding factors, and reported an even greater reduction in sperm concentration, indicating an annual decline of 1.5% in the United States compared with the 1% previously determined by Carlsen et al. (1992). There are many hypotheses regarding the increase of male infertility. One of them is the exposure to environmental factors like pesticides, which may affect reproductive functions. To our knowledge, relatively little information is available considering human reproductive health and environmental exposure to insecticides like carbaryl and chlorpyrifos/chlorpyrifos-methyl. The hypothesis of the study is that widespread environmental exposure to insecticides may affect male fertility. Therefore, the aim of this study was to evaluate the association between environmental exposure to nonpersistent insecticides, measured through urinary concentrations of two main metabolites (1N, TCPY), and semen quality and sperm DNA damage.

Methods

Participant Recruitment and Biological Samples

The participants were men who attended an infertility clinic in Lodz, Poland, for diagnostic purposes between 2008 and 2011 from the study "Environmental Factors and Male Infertility." Criteria for inclusion to the study were age under 45 years and normal semen concentration of at least 15 mln/ml (WHO, 2010). The study was performed according to the Declaration of Helsinki guidelines and the procedures employed were approved by the Nofer Institute of Occupational Medicine. The Bioethical Committee Board approved the study (Resolution No 9/2007 [June 4, 2007]). Written informed consent was obtained from all subjects before their participation. This was a cross-sectional study, where 315 men were included. Participants completed a detailed questionnaire and provided semen, urine, saliva, and blood samples during their clinic visit on the same day. The cotinine level in saliva was measured to verify the smoking status by using high-performance liquid chromatography coupled with tandem mass spectrometry/positive

electrospray ionization (LC-ESI+MS/MS) and isotope dilution (Jurewicz et al., 2017).

Main Semen Quality Parameters and Sperm Chromatin Structure

Participants provided a semen sample for diagnostic analysis as part of a fertility investigation, which was used for the study. Semen samples were collected following masturbation into a sterile plastic container and were liquefied at 37°C for 30 min. Sperm concentration, motility, morphology, and motion parameters were determined. Motion parameters were measured using a computer-aided semen analysis (CASA; Hamilton-Thorne Version 10HTM-IVOS, Hamilton-Thorne research, Danvers, MA). Out of seven CASA motion variables measured, three main parameters for the vigor and pattern of sperm motion were analyzed (straight-line velocity [VSL], curvilinear velocity [VCL], and linearity [LIN]). Three CASA parameters, VSL, VCL, and LIN, were used as measures of sperm progression, sperm vigor, and swimming pattern, respectively (Duty et al., 2004). In addition, sperm morphology was quantified using strict Kruger criteria to rank men as having normal or below normal morphology. More details of analysis for semen parameters have been previously described (Jurewicz et al., 2014).

To assess sperm with DNA damage, sperm chromatin structure assay (SCSA) was conducted using flow cytometry (DAKO Galaxy DAKO, Glostrup, Denmark; ASRM Practice Committee, 2006). The DNA fragmentation index (DFI) was calculated based on the formula: $DFI = (\text{cells with a shift of the alpha-t parameter/all cells}) \times 100$ each analyzed sperm cell and was shown on a histogram. The full description of the method used for sperm chromatin structure assessment is presented elsewhere (Jurewicz et al., 2014).

Urinary IN and TCPY Analysis

A single spot urine sample was collected from each subject. Urine samples were frozen at -20°C and sent to the laboratory in the Department of Toxicology, Medical University of Gdańsk, for analysis, where the nonpersistent insecticide metabolites were measured.

Standards and Materials

Analytical standards were purchased from Riedel de Haën: 1-naphthol (1N; purity 99.9%) and 3,5,6-trichloro-2-pyridinol (TCPY; purity 98.8%). Labeled analog of 3,5,6-trichloro-2-pyridinol (4,5,6-¹³C, 99%, 100 µg/ml in acetonitrile, CP97%) was obtained from Cambridge Isotope Laboratories (CIL) and was used as the internal standard.

Hexane (HEX, ≥99%), *tert*-butyl methyl ether (MTBE, ≥99%), and a mixture of *N,O*-bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (BSTFA:TMCS, 99:1, BSTFA 99.5%, TMCS 99.2%) were purchased from Sigma-Aldrich. Formic acid (80%), magnesium sulfate anhydrous (98.5%), sodium acetate (99%), and primary secondary amine sorbent (PSA) were obtained from POCH (Gliwice, Poland) and Scharlab (Barcelona, Spain), respectively. β-Glucuronidase (Type HP-2 from *Helix pomatia*, activity 184973 U/ml) was obtained from Supelco.

Standard stock solutions were prepared in acetonitrile at a concentration of 10 mg/ml and then were used to prepare working solutions of 100 and 10 µg/ml. Working solutions were applied to prepare sequential dilutions for method calibration. Mixed internal standard solutions were prepared in acetonitrile at a final concentration level of 1 µg/ml. All solutions were stored at -20°C. Acetate buffer solution (1 M, pH 5.0) containing 230 U of β-glucuronidase per 750 µl was prepared freshly for each analytical batch.

Sample Preparation

Three milliliters of urine were placed in 10-ml screw-cap glass tube followed by 50 µl of mixed internal standard solution and 750 µl of freshly prepared acetate buffer (1 M, pH 5.0) containing 230 U of β-glucuronidase. Overnight incubation (at least 12 hr) at 37°C was performed. Then the sample was acidified with 450 µl of 80% formic acid, and 3 ml of HEX:MTBE mixture (3:1, v:v) was added to the sample and the tube was shaken for 10 min. After centrifugation, the organic layer was transferred into an open glass tube and the extraction was repeated. Combined extracts were cleaned up with 200 mg of MgSO₄ and 10 mg of PSA by shaking in hand for 1 min. Then 5 ml of cleaned extract was transferred into a new open glass tube and evaporated to dryness under a stream of nitrogen at 35°C. The residue was dissolved with 50 µl of BSTFA:TMCS (99:1) and derivatized for 30 min at 40°C. One microliter of final extract was analyzed by gas chromatography (GC)-MS/MS.

GC-MS/MS Conditions

Analyses were performed using GC (Varian GC-450) coupled with tandem MS (Varian 220-MS, ion-trap mass spectrometer). Separation was achieved on a VF-5ms (Varian, Palo Alto, USA) low bleed capillary column (30 m × 0.25 mm inner diameter, 0.25-µm film thickness with integrated 10-m guard column) using the following temperature oven program: 60°C for 3 min, 60–140°C (120°C/min), 140–290°C (17°C/min), 280°C held for 13 min. The flow rate of the carrier gas, helium, was 1.0 ml/min. Temperature of the manifold, trap, and transfer line were 45°C, 200°C, and 290°C, respectively.

One microliter of the sample extract was injected in splitless mode into a 1,177 split/splitless injector (injector temperature 280°C).

The limit of detection (LOD) was 0.5 µg/L.

Statistical Analysis

R statistical software (ver.3, R Foundation for Statistical Computing, Vienna, Austria) was used to perform statistical analysis (R Core Team, 2016). Descriptive statistics on subject demographics were calculated, along with the distributions of urinary 1N, TCPY concentrations, and main semen parameters and sperm DNA damage. Bivariate analysis was performed among all sperm measures, urine insecticide metabolites, and demographic variables to investigate differences between distributions or categories and the potential confounders. The frequency of samples below LOD was as follows: 1NA was 5.08% for INA and 0% for TCPY. Creatinine-adjusted urinary 1N and TCPY were categorized into four groups: The first consisted of values below LOD to 25th percentile value, second greater than 25th percentile value to the median, third greater than the median to 75th percentile value, and the last group consisted of values greater than the 75th percentile. Additionally, 1N and TCPY levels in urine samples were presented as continuous variables and in categories below and above the median. Multiple linear regression models were used to evaluate the associations of urinary insecticide metabolites with selected semen parameters and sperm DNA damage. Some dependent variables (motility, percentage of sperm with abnormal morphology, DFI, and high DNA stainability [HDS]) were log transformed to obtain approximate more systematic quasi-normal distribution. Inclusion of covariates in the multivariable regression models was based on biological and statistical considerations. The following covariates were evaluated as potential confounders: sexual abstinence (days), age (years), smoking (yes/no based on cotinine level in saliva), alcohol consumption (none or less than 1 drink per week, 1 to 3 drinks per week, and every day), past diseases (yes/no; past diseases that may have impact on semen quality: mumps, cryptorchidism, testes surgery, testes trauma), creatinine. Missing values in abstinence variables were imputed with an auxiliary regression model using sperm volume as a predictor.

Results

Demographic and Clinical Participant Characteristics

Demographic characteristics of participants are presented in Table 1.

The study population consisted of 315 men who attended infertility clinics. The mean ($\pm SD$) age and

Table 1. Characteristics of the Study Population ($N = 315$).

Characteristics	<i>n</i> (%)
Education <i>n</i> (%)	
Primary and vocational	65 (20.63)
Secondary	119 (37.78)
Higher	131 (41.59)
Smoking determined by cotinine level <i>n</i> (%)	
No	224 (71.11)
Yes	91 (28.89)
BMI (kg/m²) <i>n</i> (%)	
<25	106 (33.65)
≥25	209 (66.35)
Mean (SD)	26.8 ± 3.4
Median (min–max)	27.6 (18.3–39.5)
Duration of couple's infertility (years) <i>n</i> (%)	
1–2	120 (38.10)
2–3	104 (33.01)
3–5	45 (14.29)
>5	46 (14.60)
Past diseases that may have an impact on semen quality <i>n</i> (%)	
No	276 (87.62)
Yes	39 (12.38)
Abstinence (days) <i>n</i> (%)	
<3	11 (3.49)
3–7	242 (76.82)
>7	16 (5.08)
Missing data	46 (14.60)
Mean (SD)	5.0 ± 2.3
Median (min–max)	5.0 (0.0–20.0)
Age (years)	
Mean (SD)	32.14 (4.23)
Median (min–max)	31.60 (22.01–44.26)
Alcohol use <i>n</i> (%)	
None or <1 drink/week	104 (33.01)
1–3 drinks/week	163 (51.75)
Every day	48 (15.24)

Note. Past diseases that may have impact on semen quality—mumps, cryptorchidism, testes surgery, testes trauma. BMI = body mass index.

BMI of the subjects were 32.14 ± 4.2 years and 26.8 ± 3.4 kg/m². Most of the men participating in the study had higher (41.6%) or secondary (37.8%) education. Most of them were nonsmokers (71.1%) and drank one to three drinks per week (51.8%). Twelve percent of the study population reported past diseases that may have an impact on semen parameters.

Table 2 presents semen quality parameters, distribution of CASA parameters, DFI, and HDS of the study population.

The mean ($\pm SD$) sperm concentration was 50.6 mln/ml (52.4). The percentages of motility and sperm with normal morphology were (mean $\pm SD$) 55.7 ± 19.9 and 53.7 ± 23.9 , whereas the mean values for sperm DNA

Table 2. Semen Quality, Distribution of CASA Parameters, and DNA Fragmentation Index of the Study Population.

Semen quality	Statistical variables						Geometric mean \pm SD	N	Frequency of detection
	25%	50%	75%	95%	Max	Mean \pm SD			
Main semen parameters									
Concentration (mln/ml)	22.5	32.6	85.77	125	360.0	50.6 \pm 52.4	–	334	–
Motility (%)	46	55.0	66	83	99.0	55.7 \pm 19.9	–	315	–
Sperm with normal morphology (%)	31.22	50.0	69.51	75.25	96.0	53.7 \pm 23.9	–	315	–
CASA parameters									
VSL (μ m/s)	37	43.1	50.7	61.41	77.1	43.6 \pm 10.3	–	315	–
VCL (μ m/s)	37.5	79	92.0	108	146	78.1 \pm 16.8	–	324	–
LIN (%)	51	56	61	66.1	74	56 \pm 6.4	–	315	–
DNA fragmentation index									
DFI	8.34	14.0	20.43	41.18	68.7	16.5 \pm 11.4	–	262	–
HDS	5.22	7.16	9.31	13.46	30.70	7.7 \pm 3.9	–	262	–

Note. CASA = computer-aided semen analysis; FSH = follicle-stimulating hormone; HDS = high DNA stainability; LIN = linearity; VCL = curvilinear velocity; VSL = straight-line velocity.

Table 3. Summary Statistics for the Unadjusted and Adjusted Urinary IN and TCPY Concentrations.

IN and TCPY concentration in urine									
	25%	50%	75%	95%	Max	Mean \pm SD	Geometric mean \pm SD	N	Percentage of detection
IN unadjusted (μ g/L)	0.68	1.60	4.64	11.38	26.34	3.36 \pm 4.10	1.70 \pm 3.40	315	94.92%
IN creatinine adjusted (μ g/g creatinine)	0.53	1.34	4.31	11.12	26.14	3.25 \pm 7.12	1.55 \pm 3.44	315	94.92%
TCPY unadjusted (μ g/L)	0.58	1.14	2.20	7.99	76.91	2.57 \pm 6.00	1.18 \pm 3.10	315	100%
TCPY creatinine adjusted (μ g/g creatinine)	0.48	0.98	2.10	7.55	76.42	2.42 \pm 7.11	1.05 \pm 4.11	315	100%

Note. IN = 1-naphthol; TCPY = 3,5,6-trichloro-2-pyridinol.

damage and HDS were (mean \pm SD) 16.5 \pm 11.4 and 7.7 \pm 3.9, respectively.

IN and TCPY Levels in Urine Samples

Summary statistics for the unadjusted and adjusted urinary insecticides metabolites concentrations are presented in Table 3. The geometric mean concentrations of unadjusted urinary IN and TCPY were (mean \pm SD) 1.70 μ g/L \pm 3.40 and 1.18 μ g/L \pm 3.10, respectively. The median values of IN and TCPY were 1.60 μ g/L and 1.14 μ g/L. The geometric mean concentrations of creatinine-adjusted IN and TCPY were (mean \pm SD) 1.55 μ g/L \pm 3.44 and 1.05 μ g/L \pm 4.11, respectively.

Urinary IN and TCPY Concentrations and Semen Parameters and Sperm DNA Damage

Multivariate linear regression models were performed to evaluate the association between two major metabolites of carbaryl, chlorpyrifos and chlorpyrifos-methyl, and human semen parameters and sperm DNA damage. A

negative association was observed between urinary concentration of TCPY \geq 50th percentile and sperm motility ($p = .024$). Urinary TCPY also was associated with one of the CASA parameters. TCPY concentration greater than 25th percentile value to the median was positively associated with VSL ($p = .02$). In addition, TCPY urinary concentrations greater than the median to 75th percentile increase the DNA damage (DFI; $p = .04$). When the urinary concentration of IN was treated as a continuous variable and above median variable, a negative association with percentile of sperm with normal morphology ($p = .03$ and $p = .01$, respectively) was found. One of the three assessed CASA parameters (VSL) was related to the urinary IN, both categories of urinary concentrations greater than the median to 75th percentile, and continuous concentrations of IN ($p = .03$ and $p = .01$, respectively). Also, VSL was related to the urinary concentration of IN in the category presented as above the median ($p = .006$). There was no association between urinary IN and other semen motion parameters (VCL and LIN). Neither category of urinary concentrations of IN and TCPY was associated with HDS (Tables 4 and 5).

Table 4. IN and TCPY Concentration in Urine and Semen Quality Categories of Urinary Concentrations.

Semen parameters	Exposure	IN			TCPY		
	Percentile of exposure	Coef	95% CI	<i>p</i>	Coef	95% CI	<i>p</i>
Conc	≤25th		Ref			Ref	
	25th–50th	–0.21	[–0.55, 0.13]	.22	–0.1	[–0.43, 0.24]	.56
	50th–75th	–0.07	[–0.41, 0.28]	.71	–0.29	[–0.63, 0.04]	.08
	>75th	0.02	[–0.35, 0.38]	.93	–0.27	[–0.60, 0.06]	.11
	Cont	0.05	[–0.12, 0.22]	.55	–0.13	[–0.31, 0.04]	.14
Motility	≤25th		Ref			Ref	
	25th–50th	–2.64	[–9.05, 3.78]	.42	3.55	[–2.78, 9.89]	.27
	50th–75th	2.11	[–4.45, 8.68]	.53	–2.46	[–8.81, 3.89]	.45
	>75th	3.81	[–3.07, 10.68]	.28	–4.26	[–10.6, 2.08]	.19
	Cont	2.71	[–0.53, 5.95]	.1	–3.31	[–6.64, 0.02]	.051
Morph	≤25th		Ref			Ref	
	25th–50th	0.01	[–7.66, 7.68]	.1	–0.3	[–7.98, 7.38]	.94
	50th–75th	–7.22	[–15.03, 0.6]	.07	–3.71	[–11.38, 3.95]	.34
	>75th	–7.27	[–15.49, 0.95]	.08	4.07	[–3.56, 11.7]	.29
	Cont	–4.21	[–8.09, –0.32]	.03	1.79	[–2.22, 5.81]	.38
VSL	≤25th		Ref			Ref	
	25th–50th	0.32	[–2.99, 3.64]	.85	3.83	[0.54, 7.13]	.02
	50th–75th	3.85	[0.48, 7.22]	.03	1.02	[–2.28, 4.32]	.55
	>75th	3.34	[–0.16, 6.91]	.06	3.22	[–0.06, 6.49]	.054
	Cont	2.2	[0.52, 3.87]	.01	0.58	[–1.15, 2.32]	.51
VCL	≤25th		Ref			Ref	
	25th–50th	–1.68	[–4.49, 1.14]	.24	1.83	[–0.94, 4.61]	.2
	50th–75th	1.39	[–1.47, 4.24]	.34	–1.2	[–3.98, 1.59]	.4
	>75th	0.03	[–2.97, 3.02]	.99	2.05	[–0.7, 4.81]	.14
	Cont	0.57	[–0.87, 1.98]	.44	0.1	[–0.46, 2.46]	.18
LIN	≤25th		Ref			Ref	
	25th–50th	1.14	[–0.93, 3.21]	.28	1.08	[–0.97, 3.14]	.3
	50th–75th	0.93	[–1.17, 3.03]	.38	1.14	[–0.92, 3.2]	.28
	>75th	2.05	[–0.15, 4.26]	.07	0.43	[–1.62, 2.47]	.68
	Cont	0.91	[–0.13, 1.95]	.09	–0.33	[–1.4, 0.75]	.55
DFI	≤25th		Ref			Ref	
	25th–50th	–0.09	[–0.39, 0.2]	.54	0.23	[–0.04, 0.49]	.09
	50th–75th	0.01	[–0.29, 0.32]	.94	0.29	[0.008, 0.56]	.04
	>75th	–0.1	[–0.4, 0.19]	.49	0.1	[–0.2, 0.4]	.53
	Cont	–0.02	[–0.15, 0.12]	.81	0.06	[–0.1, 0.21]	.47
HDS	≤25th		Ref			Ref	
	25th–50th	–0.01	[–1.91, 1.89]	.99	–0.4	[–2.15, 1.36]	.66
	50th–75th	–1.49	[–3.47, 0.48]	.14	–0.58	[–2.42, 1.27]	.54
	>75th	–0.97	[–2.9, 0.95]	.32	–0.24	[–2.23, 1.75]	.81
	Cont	–0.7	[–1.59, 0.18]	.12	0.07	[–0.93, 1.06]	.89

Statistically significant at the level .05. Multivariate model adjusted for sexual abstinence, age, smoking, alcohol consumption, past diseases, and creatinine. IN = 1-naphthol; Coef = β coefficient; Conc = sperm concentration; Cont = continuous variable; DFI = DNA fragmentation index; HDS = high DNA stainability; LIN = linearity; Morph = percentage of sperm with normal morphology; Ref = reference; TCPY = 3,5,6-trichloro-2-pyridinol; VCL = curvilinear velocity; VSL = straight-line velocity.

Bold values represents statistically significant variable (level of significance <0.05).

Discussion

In the present study, a relationship between urinary metabolites of nonpersistent insecticides (carbaryl, chlorpyrifos,

and chlorpyrifos-methyl) and semen quality has been found. Specifically, the evidence that urinary concentration of TCPY is significantly associated with a decrease in the sperm motility has been reported. TCPY concentration

Table 5. 1N and TCPY Concentration in Urine and Semen Quality Categories of Urinary Concentrations.

Semen quality	Percentile of exposure	1N			TCPY		
		Coef	95% CI	<i>p</i>	Coef	95% CI	<i>p</i>
Conc	<50th	Ref			Ref		
	≥50th	0.08	[-0.17, 0.33]	.53	-0.23	[-0.47, 0.003]	.053
Motility	<50th	Ref			Ref		
	≥50th	4.26	[-0.56, 9.08]	.08	-5.14	[-9.62, 0.67]	.024
Morph	<50th	Ref			Ref		
	≥50th	-7.25	[-13.01, -1.49]	.01	0.35	[-5.08, 5.78]	.9
VSL	<50th	Ref			Ref		
	≥50th	3.47	[0.99, 5.95]	.006	0.25	[-2.1, 2.6]	.84
VCL	<50th	Ref			Ref		
	≥50th	1.63	[-0.48, 3.74]	.13	-0.45	[-2.43, 1.53]	.65
LIN	<50th	Ref			Ref		
	≥50th	0.85	[-0.7, 2.4]	.28	0.25	[-1.21, 1.7]	.74
DFI	<50th	Ref			Ref		
	≥50th	0	[-0.21, 0.21]	.1	0.009	[-0.12, 0.29]	.4
HDS	<50th	Ref			Ref		
	≥50th	-1.22	[-2.57, 0.14]	.08	-0.23	[-1.56, 1.11]	.74

Statistically significant at the level .05. Multivariate model adjusted for sexual abstinence, age, smoking, alcohol consumption, past diseases, and creatinine. 1N = 1-naphthol; Coef = β coefficient; Conc = sperm concentration; DFI = DNA fragmentation index; HDS = high DNA stainability; LIN = linearity; Morph = percentage of sperm with normal morphology; Ref = reference; TCPY = 3,5,6-trichloro-2-pyridinol; VCL = curvilinear velocity; VSL = straight-line velocity.

in urine greater than the median to 75th percentile was positively associated with DFI. Exposure to 1N was also negatively related to percentage of sperm with normal morphology. Additionally, 1N and TCPY were positively associated with one of the CASA parameters (VSL). On the other hand, there was no association between the metabolites of nonpersistent insecticides and HDS.

To our knowledge, only three (Meeker, Ryan, et al., 2004; Meeker, Singh, et al., 2004; Perry, Venners, Barr, &, 2007) studies have investigated the association between metabolites of nonpersistent insecticides (1N, TCPY) and human semen parameters and sperm DNA damage. Meeker and coauthors performed two studies. In the first study, increased urinary 1N concentration was significantly associated with percentage DNA in comet tail (tail%), one of the parameters of comet assay. Urinary TCPY concentration was also associated with increased tail% but with decreased tail distributed moment (TDM), another comet assay parameter. Moreover, in the second study Meeker and coauthors found associations between urinary metabolites of nonpersistent insecticides and decreased sperm concentration and sperm motility. On the other hand, Perry and coauthors conducted a pilot biomonitoring study to assess whether men were exposed to pesticides environmentally. The results identified the high prevalence of environmental exposure to organophosphorus insecticides and pyrethroids. Additionally, the preliminary analyses indicated that the higher exposure group had lower sperm concentration. However, the small sample size ($n = 18$) limited statistical power.

The inconsistency between the study results may be associated with the fact that a smaller sample size was examined in the U.S. study. Additionally, lower levels of 1N and TCPY in urine among participants from the study performed by Meeker, Singh, et al. (2004) may affect the study results. Different confounding factors used in the studies, creatinine adjustment in the present study, and specific gravity adjustment in the study by Meeker et al. may also impact on the final results.

Limited animal data have identified an association between exposure to carbaryl and decreased semen quality. During a 90-day study of rats, Pant and coauthors (Pant et al., 1995; Pant, Shankar, & Srivastava, 1996) found a statistically significant dose-related decline in epididymal sperm count and percent motile sperm, as well as increased sperm with abnormal morphology. In the study performed to explore relationships between chlorpyrifos exposure and semen quality, Everret and coworkers (Everret, 1982) observed decreased sperm production and sperm motility in Holstein bulls 6 months after dermal lice treatment with an unknown amount of chlorpyrifos. On the contrary, another animal study did not identify associations between chlorpyrifos and altered male reproductive health (Breslin, 1996).

Nonpersistent pesticides may operate through hormonal or genotoxic pathways to affect male reproduction (Perry, 2008). Impacted semen quality parameters included concentration, morphology, and motility. The mechanism of action of carbaryl and chlorpyrifos on

male reproductive function is based on animal toxicology studies and remains unclear. One hypothesized mechanism suggested that pesticides may penetrate the blood–testis barrier to potentially interrupt spermatogenesis, by affecting either genetic integrity or hormone production (Rodríguez & Bustos-Obregon, 2000; Toppari et al., 1996). In addition, changes in sperm motility could be due to the disturbance of tail structural protein components and/or ATP synthesis (Contreras & Bustos-Obregon, 1999).

The present study has several limitations. A single urine sample has been used to assess nonpersistent insecticide exposure, and a single semen sample to describe the semen quality. The main biomarker used to estimate the exposure to carbaryl is 1N and it accounts for more than 85% of all carbaryl metabolites in urine (Maroni, Colosio, Ferioli, & Fait, 2000). It should be mentioned that 1N is a primary metabolite of both carbaryl and naphthalene; urinary concentrations of 1N can arise from exposure to either of these compounds. Unlike carbaryl, naphthalene is metabolized to both 1N and 2N (Meeker et al., 2007). Thus, if levels of 1N and 2N are highly correlated in urine samples, it would be reasonable to deduce that naphthalene and not carbaryl is the source of 1N, whereas if levels of 1N and 2N are poorly correlated, then carbaryl is likely to be a major source of urinary 1N (Meeker et al., 2007). The major urinary metabolite of chlorpyrifos and chlorpyrifos-methyl is TCPY. Carbaryl, chlorpyrifos, and chlorpyrifos-methyl are metabolized and excreted rapidly; for example, TCPY has an estimated half-life of 27H in humans (Nolan, Rick, Freshour, & Saunders, 1984). Levels of both TCPY and 1N measured in urine samples reflect insecticide exposure in the previous 24–48 hr (Maroni et al., 2000). Additionally, the 1N levels in urine may reflect the exposure to naphthalene or carbaryl. To check the major sources of 1N, the correlation with 2N was assessed. The result of Spearman correlation indicates that 1N and 2N were poorly correlated ($p > .05$).

On the other hand, spermatogenesis is a cyclical process that takes approximately 3 months. Insecticide metabolites in urine may vary considerably over time, suggesting that a single urine sample cannot reflect long-term exposure. Meeker and coauthors reported that a single urine sample was predictive of 3-month average urinary metabolite concentrations (Meeker et al., 2005). By contrast, Stokes-Riner et al. (2007) provide evidence that one semen sample may be representative of semen quality over several weeks in epidemiological studies. In the present study, all subjects were recruited in the same center and all semen samples were collected and analyzed using a standardized protocol. The next limitation of the study was that participants were recruited from an infertility clinic; this may limit the ability to generalize the results to the general population.

Nevertheless, men with normal semen parameters according to the World Health Organization (WHO) classification (WHO, 2010) were included in the study. Consequently, the results may apply to general population samples as well. Participants were heterogeneous in their semen profiles and had normal semen parameters. Because a large number of analyses were performed in the present study, some of the observations could be chance findings due to multiple testing. On the contrary, these findings may be of concern because of the widespread use of insecticides and because pesticides almost always are found in mixtures with other pesticides.

This study had several methodological strengths. Smoking status of participants was verified using the level of cotinine in saliva. Also, detailed information on demographics and medical and lifestyle risk factors received from the men allowed for control of confounding in the statistical analyses. Additionally, strength arises from the fact that this is one of the few studies concerning environmental exposure to nonpersistent insecticides and male reproductive health.

In conclusion, human environmental exposure to nonpersistent insecticide like carbaryl and chlorpyrifos may have an impact on semen quality parameters. In the present study we have observed that intermediate levels of nonpersistent insecticide exposures are associated with the greatest negative effects on the semen parameters. Further studies are needed to confirm these findings.



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