Exposure to Nonpersistent Insecticides and Male Reproductive Hormones

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Background: Urinary metabolites of several nonpersistent insecticides have been measured in a high percentage of men in the general population, suggesting widespread environmental exposures to these compounds. The present study explored the association of urinary concentrations of 3,5,6-trichloro-2-pyridinol (TCPY), a metabolite of chlorpyrifos and chlorpyrifos-methyl, and 1-naphthol (1N), a metabolite of carbaryl and naphthalene, with serum reproductive hormone levels in adult men.

Methods: Subjects (n = 268) were the male partners in couples presenting to a Massachusetts infertility clinic in years 2000 through 2003. TCPY and 1N were measured in a spot urine sample from each subject and adjusted for dilution using specific gravity. Reproductive hormones (follicle-stimulating hormone, leuteinizing hormone, inhibin B, testosterone, and sex hormone-binding globulin) were measured in serum collected from subjects during the same clinic visit.

Results: Multiple linear regression models showed an inverse association between TCPY and testosterone concentration. An interquartile range (IQR) increase in TCPY was associated with a decline of 25 ng/dL (95% confidence interval = −40 to −10) in testosterone concentration. The association appeared to be dose-dependent when exposure was divided into quintiles. The highest TCPY quintile was associated with a testosterone decline of 83 ng/dL (−128 to −39) compared with the lowest TCPY quintile. We also found inverse associations between TCPY and free androgen index and between 1N and testosterone, and suggestive inverse associations between TCPY and leuteinizing hormone and between 1N and free androgen index.

Conclusion: In adult men, TCPY and 1N were associated with reduced testosterone levels. On a population level, these reductions are of potential public health importance because of widespread exposure to these nonpersistent insecticides.

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Nonpersistent pesticides are widely used in agricultural, commercial, and residential settings. Due to the extensive use of these chemicals, a large proportion of the general population is exposed to low levels of nonpersistent pesticides or their environmental degradation products. The Second National Report on Human Exposure to Environmental Chemicals (carried out within NHANES 1999–2000) found that more than 90% of males in the U.S. population had urine samples with detectable levels of 3,5,6-trichloro-2-pyridinol (TCPY), the major urinary metabolite of chlorpyrifos and chlorpyrifos-methyl.[1] The report also found that over 75% of U.S. males had detectable levels of 1-naphthol (1N), a urinary metabolite of carbaryl and naphthalene.

Chlorpyrifos is a broad-spectrum organophosphate insecticide. It was one of the most commonly used insecticides in homes until restrictions were placed on its residential use by the Environmental Protection Agency (EPA) in 2000.[2] According to the most recent market estimates of pesticide sales and use, in 1999, between 13 and 19 million pounds of chlorpyrifos were applied in the United States.[3] Despite restrictions on the use of chlorpyrifos, the high rate of detection of TCPY in urine from the general population suggests ongoing environmental exposure to chlorpyrifos and TCPY. Although referred to as "nonpersistent," these insecticides applied indoors or tracked in from outdoors may persist for extended periods while protected from sunlight, rain, temperature extremes, and most microbial action.[4] For example, chlorpyrifos was found in indoor air 4 years after pest control application in a home.[5] Also, diet likely remains an important route of exposures because trace concentrations of chlorpyrifos continue to be found in many types of food.[6,7] Carbaryl, a broad-spectrum insecticide known as Sevin, is still commonly used to protect residential lawns and gardens from a variety of insects. It is estimated that between 2 and 4 million pounds of carbaryl were applied in the home and garden market sector in 1999.[3]

We recently published papers on the relationships of urinary levels of TCPY and 1N with semen quality and sperm DNA damage. Urinary 1N levels were inversely associated with sperm concentration and motility,[8] and positively associated with DNA damage in sperm cells measured by the neutral comet assay.[9] There were also suggestive associations

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between urinary TCPY levels and these male reproductive outcomes. Although our studies were not designed to explore mechanisms for the effects of these pesticides on altered semen quality and quantity, the toxicologic literature suggests that chlorpyrifos and carbaryl may be hormonally active.[10–14] One potential mechanism whereby these pesticides may alter sperm quality is through disruption of the endocrine axis, manifest as altered levels of reproductive hormones. To explore this further, the present study investigated potential relationships between biologic markers of insecticide exposure and serum reproductive hormone levels in adult men.

METHODS

Study subjects were men who were partners in subfertile couples being evaluated for infertility at the Massachusetts General Hospital Vincent Burnham Andrology Laboratory between January 2000 and April 2003. The study was approved by the Human Studies Institutional Review Boards of the Massachusetts General Hospital and the Harvard School of Public Health. After the study procedures were explained and all questions answered, subjects signed informed consent. Details of subject recruitment have been previously described.[15] Briefly, all eligible men who presented to the Andrology Laboratory were recruited to participate. Of those approached, approximately 65% consented. Most men who declined to participate cited lack of time on the day of their clinic visit as the reason for not participating.

Insecticide Metabolites in Urine

A single spot urine sample was collected from each subject on the day of the clinic visit. Although TCPY and 1N are not persistent in the body, a single urine sample may adequately predict exposure over several months.[16] Urine samples were frozen at −20°C and sent to the U.S. Centers for Disease Control and Prevention (CDC) where TCPY and 1N were measured.[17] Samples were fortified with stable isotope analogs of the target analytes and glucuronide or sulfate-bound metabolites were liberated using an enzyme hydrolysis. TCPY and 1N were isolated using liquid–liquid extraction, chemically derivatized, and measured using gas chromatography–chemical ionization–tandem mass spectrometry. The interassay coefficient of variation (CV) was 8.1% for TCPY and 10.4% for 1N. Specific gravity (SG) was measured using a handheld refractometer (National Instrument Co., Inc., Baltimore, MD). Creatinine was measured photometrically using kinetic colorimetric assay technology with a Hitachi 911 automated chemistry analyzer (Roche Diagnostics, Indianapolis, IN). Specific gravity was used as the primary method to adjust metabolite concentrations for urine dilution. We also determined volume-based (unadjusted) and creatinine-adjusted TCPY and 1N concentrations for comparison with exposure distributions from other studies. Samples with SG above 1.03 or below 1.01 (n = 54) or with creatinine concentrations above 300 or below 30 mg/dL (n = 21) were considered too concentrated or too dilute to provide valid results.[18,19] These were excluded from the primary analysis.

Reproductive Hormones

One nonfasting blood sample was drawn between the hours of 9 AM and 4 PM on the same day that the urine sample was collected. Blood samples were centrifuged and the serum was stored at −80°C until analysis. Testosterone was measured directly using the Coat-A-Count RIA kit (Diagnostics Products, Los Angeles, CA), which has interassay and intraassay CVs of 12% and 10%, respectively, with a sensitivity of 4 ng/dL (0.139 nmol/L). Sex hormone-binding globulin (SHBG) was measured using a fully automated system (Immulite; DPC, Inc.), which uses a solid-phase 2-site chemiluminescent enzyme immunometric assay and has an interassay CV of less than 8%. The free androgen index was calculated as the molar ratio of total testosterone to SHBG, both in nmol/mL.[20] Inhibin B was measured using a commercially available, double-antibody, enzyme-linked immunosorbent assay (Oxford Bioinnovation, U.K.) with interassay and intraassay CVs of 20% and 8%, respectively, limit of detection of 15.6 pg/mL, and a functional sensitivity (20% CV) of 50 pg/mL.[21] Serum luteinizing hormone (LH) and follicle-stimulating hormone (FSH) concentrations were determined by microparticle enzyme immunoassay using an automated Abbott AxSYM system (Abbott Laboratories, Chicago, IL). The Second International Reference Preparation (WHO 71/223) was used as the reference standard. The assay sensitivity for LH and FSH were 1.2 international units per liter (IU/L) and 1.1 IU/L, respectively. The intraassay CVs for LH and FSH are less than 5% and less than 3%, respectively, with interassay CVs for both hormones of less than 9%.

Statistical Analysis

For insecticide metabolite values below the limit of detection, corresponding to 0.25 μg/L for TCPY and 0.40 μg/L for 1N, we imputed a value equal to one-half the limit of detection. Reproductive hormone and insecticide metabolite levels were stratified by demographic categories to investigate the potential for confounding. We computed Spearman correlation coefficients to determine correlations among insecticide metabolites, among hormones, and between insecticide metabolite and hormone levels.

Multivariate linear regression was used to explore relationships between reproductive hormones and SG-adjusted urinary insecticide concentrations. Concentrations of testosterone and inhibin B closely approximated normality and were used in statistical models untransformed, whereas the distributions of FSH, LH, SHBG, and free androgen index concentrations were skewed left and so we transformed these hormone levels to the natural log (ln) for statistical analyses. SG-adjusted TCPY and 1N concentrations were also both ln-transformed. Inclusion of covariates was based on statistical and biologic considerations.[22] We modeled age and body mass index (BMI) as a continuous variable. Smoking status was dichotomized by current smoker versus never smoked or former smoker, and race was categorized into 4 groups. We considered for inclusion in the models the following dichotomous variables: previous examination for infertility (yes or no), prior ability to impregnate a partner (yes or no), timing of blood sample by season (winter vs spring,

summer, or fall), and time of day (9:00 AM–12:59 PM vs 1:00 PM–4:00 PM). To improve interpretability, the regression coefficients were backtransformed and expressed as a change in the dependent variable (ie, hormone levels) for an interquartile range (IQR) increase in specific gravity-adjusted metabolite levels. In secondary analyses, the multivariate models were rerun for specific gravity-adjusted metabolite levels after retaining men with specific gravity outside the acceptable range. The analyses were also tested for robustness by performing analyses using both unadjusted and creatinine-adjusted insecticide metabolite levels. Finally, we assessed nonlinear relationships between metabolite levels and hormones by regressing the hormones on tertiles and quintiles of insecticide metabolites.

RESULTS

Of the 370 men with pesticide metabolites measured in urine, 336 also had reproductive hormone levels measured in serum. We excluded an additional 14 subjects taking hormonal medications (eg, Propecia, finasteride, cabergoline, Clomid, gonadotropin-releasing hormone, testosterone, or prednisone taper). Among the remaining 322 subjects, most were white (83%) and had never smoked (72%)(Table 1). Thirty-five percent of the men had a previous infertility examination, and 40% had proven fertility (had previously made a partner pregnant). Mean age (± standard deviation [SD]) was 36 (± 5.4) years, and mean BMI was 28 (± 4.6). TCPY and 1N were detected in 95.0% and 99.7% of subjects, respectively, with SG-adjusted geometric means of 2.41 μg/L and 3.01 μg/L (Appendix Table 1, available with the online version of the article). SG-adjusted TCPY and 1N levels were moderately correlated (Spearman correlation coefficient = 0.3). Distributions of the reproductive hormones measured in serum are presented in Appendix Table 2. The mean (SD) concentrations of testosterone and inhibin B were 422 (147) ng/dL and 159 (68) pg/mL, respectively.

TABLE 1. Characteristics of Study Participants (n = 322)

Characteristic	
Age (years); mean ± SD	36.1 ± 5.4
BMI (kg/m^2); mean ± SD	28.0 ± 4.6
Race; %	
White	83
Black	5
Hispanic	5
Other	7
Smoking*; %	
Never-smoker	72
Ever-smoker	28
Current	10
Former	18
Previous examination for infertility*; %	35
Previously made a partner pregnant*; %	40
Season of blood sample; %	
Winter	26
Spring–summer–fall	74
Time of blood sample; %	
9:00 AM–12:59 PM	40
1:00 PM–4:00 PM	60

*Information on smoking status missing for 3 subjects, on previous infertility examination for 2 subjects, and on previous pregnancy for 7 subjects.

Age was associated with increased SHBG but with decreased testosterone and free androgen index. BMI was associated with increased free androgen index but decreased LH, inhibin B, SHBG, and testosterone. Current smoking was associated with increased SHBG, whereas free androgen index levels were higher among men who never smoked. Blood samples collected in the morning (between 9:00 AM and 12:59 PM) had higher inhibin B and testosterone levels compared with those collected in the afternoon, whereas blood samples collected in the winter months had lower inhibin B. For the SG-adjusted insecticide metabolites, none of the demographic variables (age, BMI, race, smoking, season) was strongly associated with urinary TCPY levels. Current smokers had higher mean (median) levels of SG-adjusted 1N (9.2 μg/mL [8.4 μg/mL]) than never-smokers (5.8 μg/mL [3.6 μg/mL]) and former smokers (3.9 μg/mL [2.8 μg/mL]). Mean and median SG-adjusted 1N concentration was also higher among men whose urine samples were collected in the winter (8.8 μg/mL [4.3 μg/mL]) versus samples collected in spring, summer or fall (4.3 μg/mL [2.8 μg/mL]).

Results from the primary multivariate regression analyses were similar to crude results and are presented in Table 2. There was an inverse association between urinary TCPY concentration and serum testosterone levels. An IQR increase in SG-adjusted TCPY was associated with a 6.2% (95% confidence interval [CI] = −10.0% to −2.5%) decrease in the median testosterone level. An IQR increase in SG-adjusted TCPY was also associated with a 7% (−11% to −2%) decrease in free androgen index. In addition, there was an inverse association between SG-adjusted 1N and testosterone. An IQR increase in 1N was associated with a 6.0% (−11% to −1%) decrease in median testosterone level.

Because both TCPY and 1N were inversely associated with testosterone, we tested for confounding and statistical interaction by running regression models that included both metabolites as independent variables. The relationships between each metabolite and each semen parameter remained essentially unchanged, although standard errors increased (data not shown). In models including an interaction term for both metabolites, there was no evidence of interaction.

Regression results for unadjusted or creatinine-adjusted TCPY and 1N concentrations were comparable with those using the SG-adjusted levels (Table 2). Results were largely unchanged when regression models were repeated after retaining 55 men with SG outside the acceptable range (data not shown). However, in addition to testosterone and FAI, SG-adjusted TCPY was also inversely associated with LH. An IQR increase in SG-adjusted TCPY was associated with a decrease in LH by a multiplicative factor of 0.94 (95% CI = 0.90 to 1.00). For the median value of LH (10.3 IU/L), this

TABLE 2. Adjusted* Coefficients (95% Confidence Intervals) for a Change in Serum Hormone Levels Associated With an IQR Increase in Insecticide Metabolite Levels†

	TCPY			1N		
	SG-Adjusted (n = 268)	Creatinine-Adjusted (n = 301)	Unadjusted (n = 322)	SG-Adjusted (n = 268)	Creatinine-Adjusted (n = 301)	Unadjusted (n = 322)
FSH‡	0.99 (0.91 to 1.06)	0.97 (0.90 to 1.04)	0.99 (0.93 to 1.05)	1.01 (0.92 to 1.10)	0.98 (0.91 to 1.06)	1.02 (0.93 to 1.11)
LH‡	0.96 (0.90 to 1.02)	0.94 (0.89 to 1.01)	0.96 (0.91 to 1.01)	0.99 (0.91 to 1.07)	0.98 (0.92 to 1.05)	1.00 (0.92 to 1.07)
Inhibin B§	2.17 (−6.65 to 11.0)	0.20 (−8.85 to 9.24)	−2.76 (−10.1 to 4.54)	−3.71 (−14.8 to 7.34)	−2.54 (−12.7 to 7.60)	−7.21 (−17.3 to 2.81)
Testosterone§	−25.2 (−40.4 to −10.1)	−25.7 (−41.1 to −10.3)	−18.4 (−30.8 to −5.8)	−24.3 (−43.3 to −5.21)	−17.5 (−35.0 to 0.04)	−21.8 (−39.1 to −4.48)
SHBG‡	1.02 (0.97 to 1.07)	1.01 (0.95 to 1.06)	1.00 (0.96 to 1.05)	1.00 (0.94 to 1.07)	1.00 (0.94 to 1.06)	1.01 (0.96 to 1.07)
Free androgen index‡	0.93 (0.89 to 0.98)	0.93 (0.89 to 0.98)	0.96 (0.91 to 0.99)	0.95 (0.90 to 1.01)	0.96 (0.92 to 1.03)	0.90 (0.90 to 1.00)

*Adjusted for age, BMI, smoking (current vs former or never), and time of day that blood was collected for hormone analysis. The testosterone models were also adjusted for (ln) SHBG.

†Ln-transformations of LH, FSH, SHBG, and free androgen index. Testosterone and inhibin B concentrations were untransformed. In all models, ln-transformations of insecticide metabolite concentrations.

‡Coefficient represents a multiplicative change in hormone level for an IQR change in insecticide metabolite concentration after backtransformation of both hormone and insecticide metabolite concentrations. For an IQR change in insecticide metabolite concentration, a coefficient equal to 1.0 indicates no change in hormone level, a coefficient <1.0 indicates a multiplicative decrease in hormone level, and a coefficient >1.0 indicates a multiplicative increase in hormone level.

§Coefficient represents the change in hormone level for an IQR change in insecticide metabolite concentration after backtransformation of the insecticide metabolite concentrations. For an IQR change in insecticide metabolite concentration, a coefficient equal to zero indicates no change in hormone level, a coefficient <0 indicates a decrease in hormone level, and a coefficient >0 indicates an increase in hormone level.

represents a 6% (0.62 IU/L) decrease in LH for an IQR increase in SG-adjusted TCPY.

To explore nonlinear relationships, we also performed analyses in which serum hormone levels were regressed on quintiles of insecticide metabolites. TCPY was associated with a monotonic decrease in testosterone (coefficients for quintiles 2, 3, 4, and 5 were −30.1 ng/dL [95% CI = −74.0 to 13.7], −54.5 ng/dL [−98.8 to −10.1], −69.2 ng/dL [−114 to −24.9], and −83.3 ng/dL [−128 to −39.0]) (Fig. 1). There was also a decreasing trend in testosterone when it was regressed on quintiles of 1N, although it was not monotonic. When (ln-transformed) LH was modeled against SG-adjusted TCPY quintiles, a dose–response pattern was not evident (Fig. 2).

FIGURE 1. Regression coefficients (diamonds) and associated 95% confidence intervals for a change in testosterone level associated with increasing quintiles of specific gravity-adjusted insecticide metabolite (A, TCPY, B, 1N), adjusted for age, BMI, ln-transformed SHBG, smoking (current vs former or never), and time of day that blood was collected for hormone analysis (n = 268).

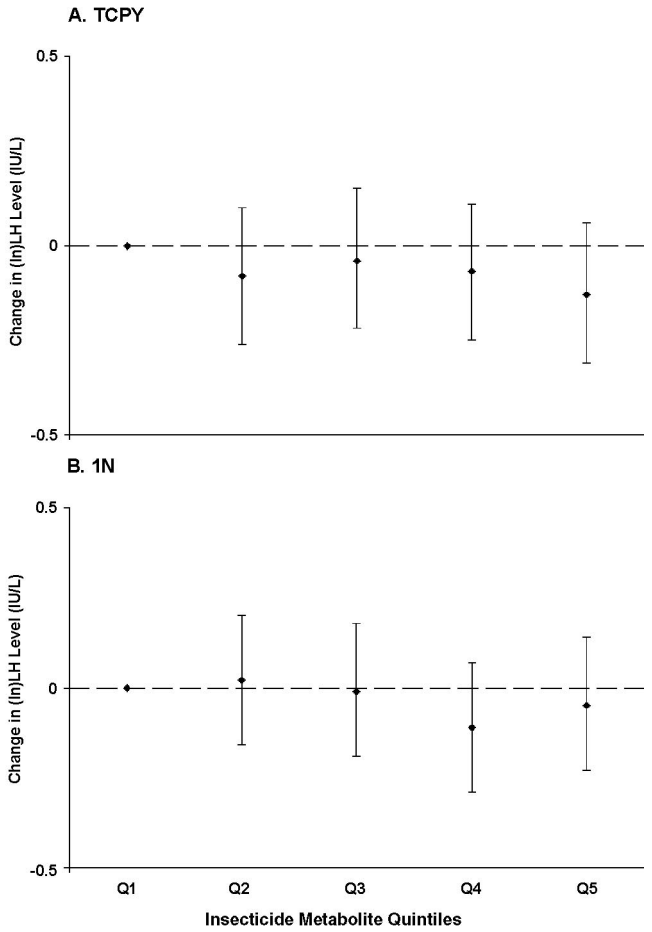

FIGURE 2. Regression coefficients (diamonds) and associated 95% confidence intervals for a change in ln-transformed LH associated with increasing quintiles of SG-adjusted insecticide metabolite (A, TCPY, B, 1N), adjusted for age, BMI, smoking (current vs former or never), and time of day that blood was collected for hormone analysis (n = 268).

DISCUSSION

We found inverse associations between urinary TCPY and testosterone levels and FAI. Increased urinary 1N concentrations were also associated with decreased testosterone levels. The concentrations of insecticide metabolites among men in our study are environmentally relevant, because the distributions of TCPY and 1N were similar to those reported in the U.S. general population.[1]

In secondary analyses, the association between TCPY and testosterone was not sensitive to the modeling approaches used. However, we found some differences between primary and secondary analyses. When men with extremely diluted or concentrated urine were retained in the analyses, there was a suggestive association between TCPY and LH (not found in the primary analyses that excluded men with dilute or concentrated urine). In addition, the dose–response relationship between testosterone and 1N became weaker when using exposure quintiles.

Although physiological mechanisms underlying the inverse association between TCPY and testosterone remain unclear, our understanding of the hypothalamic–pituitary–gonadal axis may provide some insights. Gonadotropin-releasing hormone (GnRH) from GnRH-secreting neuroendocrine cells in the hypothalamus stimulates gonadotrophs in the anterior pituitary to produce and release both LH and FSH. LH then acts on the Leydig cells to stimulate testosterone secretion. Through negative feedback, testosterone decreases the production of LH by acting on either the hypothalamus to decrease GnRH secretion or on the anterior pituitary to decrease LH secretion.[23] Thus, if the site of action of TCPY was the Leydig cells in the testes, a positive association between TCPY and LH might be expected to accompany the inverse association between TCPY and testosterone observed in the present study. However, we found a suggestive inverse association between TCPY and LH. This suggests TCPY or its parent chemicals may be acting on the hypothalamus or anterior pituitary to disrupt LH production or secretion. Chlorpyrifos is an inhibitor of cholinesterase activity and at low doses has been found to cause a dose-dependent inhibition of cholinesterase in the hypothalamus of rats.[24] Cholinesterase inhibition in the hypothalamus may in turn alter the rate of GnRH secretion.[25] In addition to effects on LH, altered GnRH secretion may also affect the production and secretion of FSH in the anterior pituitary. However, the cyclic release of FSH is governed to a lesser extent than LH by varied secretion of GnRH, and feedback control of FSH secretion is less clearly defined than that of LH.[23,26] We found no association between TCPY and FSH, which may suggest that TCPY alters GnRH recognition and/or LH secretion by the anterior pituitary, but not FSH secretion. In support of our hypothesis, the pituitary has been suggested as a site of action by phytoestrogens due to their ability to inhibit GnRH-induced LH release by gonadotrophs in female rats.[27,28]

The localization of the neuroendocrine axis, and specifically GnRH neurons, in the brain makes them anatomically and physiologically situated to mediate effects of reproductive and neurologic toxicants found in the environment.[27] However, limited studies have investigated the association between exposure to chlorpyrifos/chlorpyrifos methyl or carbaryl/naphthalene and reproductive hormone levels. Several studies suggest chlorpyrifos may be hormonally active, but specific findings have not been fully consistent. In female sheep, Rawlings et al[11] found an association between chlorpyrifos and thyroxine and cortisol, but no association between chlorpyrifos and LH or FSH. Andersen et al[13] reported weak responses to chlorpyrifos in 2 estrogenicity assays in vitro. On the other hand, chlorpyrifos-methyl, an analog of chlorpyrifos, exhibited antiandrogenic activity, but not estrogenic or estrogen-like activity, in immature rats.[14] In another study, chlorpyrifos was found to potently affect GnRH gene expression and biosynthesis in vitro.[12] In addition, chlorpyrifos and other organophosphates have been shown to inhibit steroidogenesis in adrenal cells.[29,30]

Limited studies also suggest carbaryl may be a hormonally active agent, but similar to the studies of chlorpyrifos,

specific findings lack consistency. An in vitro study of human breast and endometrial cancer cells exposed to carbamate insecticides concluded that carbaryl may act as a general endocrine modulator in mammalian cells.[31] Carbaryl was associated with decreased GnRH and gonadotropic hormone levels in fish under both laboratory and field conditions.[10] Conversely, carbaryl exposure among rats was associated with a dose-dependent increase in pituitary gonadotropic function.[32]

An alternative mechanism of hormonal effects may be directly through the phenolic metabolites (TCPY or 1N) rather than the parent compounds. These compounds may interfere with hormone signaling in the human body, although we are not aware of any studies that support or refute this hypothesized alternative mechanism.

Human studies on nonpersistent pesticide exposure and reproductive hormones are limited. A study among Danish farmers found that traditional farmers, who were presumably more highly exposed to pesticides than organic farmers, had a lower testosterone/SHBG ratio (free androgen index).[33] Among Chinese factory workers exposed to the organophosphates parathion and methamidophos, Padungtod et al[34] found exposure was associated with increased serum LH and decreased serum testosterone. Two animal studies found inverse associations between organophosphate exposure (diazinon or malathion) and testosterone levels that were also accompanied by a decline in semen quality (decreased sperm count and motility, increased percentage of morphologically abnormal sperm).[36,37] These results are largely, although not entirely, consistent with our findings, because we found TCPY and 1N to be associated with a decline in testosterone and, in previous work, semen quality.[8]

The cohort of men in the present study overlap with men from our previously published studies on semen quality and DNA damage in sperm.[8,9] We previously found evidence of an inverse association between TCPY with sperm concentration and motility and between 1N with sperm concentration. There was a strong and consistent inverse association between 1N and sperm motility. We also previously reported positive associations between TCPY and 1N and DNA damage in sperm cells, with 1N exhibiting a stronger association. In the present study, we found negative associations of TCPY and 1N with testosterone; TCPY demonstrated a stronger dose-dependent relationship.

There are limited human data on the relationship between testosterone and semen quality. Among normal couples, Uhler et al[35] found associations between FSH and inhibin B with semen quality, but reported no associations between testosterone and sperm concentration, motility, or morphology. However, in our data, we observed an association between testosterone and sperm motility when adjusting for SHBG (unpublished results). In an attempt to synthesize the results from our 3 studies, we therefore relied primarily on studies in experimental animals. In our studies, we found associations between TCPY with testosterone and associations between 1N with semen quality and sperm DNA damage. The differences in results for TCPY and 1N may suggest differing sites or mechanisms of action by chlorpyrifos and carbaryl/naphthalene on the male reproductive system. The strong association between TCPY and testosterone suggests that chlorpyrifos may be associated with altered endocrine function of the male hypothalamic–pituitary–gonad axis; in contrast, the strong associations of 1N with sperm motility and DNA damage suggests that carbaryl or naphthalene may be associated with direct damage to developing or mature sperm.

A potential limitation of the present study was the collection of only a single urine sample to measure insecticide metabolite levels and the collection of a single blood sample to measure serum hormone levels. Despite the diurnal and pulsatile fluctuations in serum hormone levels, a single blood sample can be used to provide a reliable measure of testosterone and LH over both short and long time periods in population studies.[38,39] Also, requiring multiple blood samples may limit participation rates in epidemiologic studies.[38] Measuring insecticide metabolite levels in urine provides a measure of individual internal dose. However, nonpersistent insecticides are metabolized and excreted rapidly, and levels of both TCPY and 1N measured in urine reflect insecticide exposure in the previous 24 to 48 hours.[40] Although insecticide metabolite levels in urine can vary considerably over time, suggesting that a single urine sample may not be a reliable surrogate for longer-term exposure,[41] we recently showed that a single urine sample was predictive of 3-month average urinary insecticide metabolite levels.[16] A single urine sample correctly classified men in the highest 3-month exposure tertile with a sensitivity of 0.6 and specificity of 0.9 for SG-adjusted 1N and, for SG-adjusted TCPY, a sensitivity of 0.5 and specificity of 0.8.

In our study, recruitment of subjects through an infertility clinic is not likely to introduce selection bias. A recent study among a cohort of men that overlaps with the men in the present study reported no differences when semen characteristics were compared between participants and nonparticipants, suggesting that men did not participate based on semen quality.[42] Likewise, we believe it is unlikely that men participated based on their hormone status or based on exposure to nonpersistent insecticides. In addition, the participation rate in the present study (65%) was higher than other male reproductive health study designs, which reduces the potential for selection bias and increases the internal validity of a study.

The study included both fertile and infertile men, because the female partner's infertility may be the cause of some couples' infertility and subsequent evaluation. Furthermore, pesticide metabolite levels in the present study were similar to those found in the general population, suggesting that men from the infertility clinic did not have widely different levels of exposure. For these reasons, we believe the results are generalizable to men in the general population. For generalizablity to be limited, men in the present study would need to be differentially affected by exposure (ie, more susceptible to exposure) compared with other men. There is currently no evidence suggesting that reproductive hormone levels in men visiting an infertility clinic are more sensitive to nonpersistent insecticide exposure than in other men.

In conclusion, we found an inverse association of urinary levels of metabolites of chlorpyrifos/chlorpyrifos methyl and carbaryl/naphthalene with serum testosterone. The urinary levels of these metabolites are environmentally relevant, as shown by recent NHANES data.[1] We also found a suggestive association between chlorpyrifos metabolite and decreased LH. The inverse associations with testosterone are consistent with results from our previous work, in which we found inverse associations between exposure to these insecticides and sperm concentration and motility.[8] As far as we are aware, the present study is the first to investigate these associations in humans, and additional studies are needed to substantiate our results.

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