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Dialkyl Phosphate Urinary Metabolites and Chromosomal Abnormalities in Human Sperm

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Abstract

Background—The past decade has seen numerous human health studies seeking to characterize the impacts of environmental exposures, such as organophosphate (OP) insecticides, on male reproduction. Despite an extensive literature on OP toxicology, many hormone-mediated effects on the testes are not well understood.

Objectives—This study investigated environmental exposures to OPs and their association with the frequency of sperm chromosomal abnormalities (i.e., disomy) among adult men.

Methods—Men (n=159) from a study assessing the impact of environmental exposures on male reproductive health were included in this investigation. Multi-probe fluorescence *in situ* hybridization (FISH) for chromosomes X, Y, and 18 was used to determine XX18, YY18, XY18 and total disomy in sperm nuclei. Urine was analyzed using gas chromatography coupled with mass spectrometry for concentrations of dialkyl phosphate (DAP) metabolites of OPs [dimethylphosphate (DMP); dimethylthiophosphate (DMTP); dimethyldithiophosphate (DMDTP); diethylphosphate (DEP); diethylthiophosphate (DETP); and diethyldithiophosphate (DEDTP)].

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Poisson regression was used to model the association between OP exposures and disomy measures. Incidence rate ratios (IRRs) were calculated for each disomy type by exposure quartiles for most metabolites, controlling for age, race, BMI, smoking, specific gravity, total sperm concentration, motility, and morphology.

Results—A significant positive trend was seen for increasing IRRs by exposure quartiles of DMTP, DMDTP, DEP and DETP in XX18, YY18, XY18 and total disomy. A significant inverse association was observed between DMP and total disomy. Findings for total sum of DAP metabolites concealed individual associations as those results differed from the patterns observed for each individual metabolite. Dose-response relationships appeared nonmonotonic, with most of the increase in disomy rates occurring between the second and third exposure quartiles and without additional increases between the third and fourth exposure quartiles.

Conclusions—This is the first epidemiologic study of this size to examine the relationship between environmental OP exposures and human sperm disomy outcomes. Our findings suggest that increased disomy rates were associated with specific DAP metabolites, suggesting that the impacts of OPs on testis function need further characterization in epidemiologic studies.

Keywords

aneuploidy; *In situ* hybridization; organophosphate pesticides; reproduction; endocrine disruptors

Introduction

The impacts of environmental endocrine disruptors on male reproductive health has received heightened research attention in recent years (Diamanti-Kandarakis et al., 2009; Woodruff, 2011; Zoeller et al., 2012; WHO, 2013). Each year more than 2 million couples in the US who want to have children are infertile, and over 2 million conceptions are lost before the twentieth week of gestation (ACOG, 2002; CDC, 2013). Most aneuploid conceptuses perish in utero; up to 50% of all spontaneous abortions are thought to be related to pre-existing chromosomal abnormalities (Jacobs, 1992; Lebedev et al., 2004). Because many chromosomal abnormalities come from the father's sperm, particularly for the sex chromosomes (X and Y), researchers have attempted to understand the paternal role in sex chromosome aneuploidy (Hassold and Hunt, 2001; Martin et al., 1991). Aneuploidy occurs when chromosome pairs fail to separate properly during cell division. In germ cells, errors in chromosome segregation during meiosis (I or II) result in imbalances in chromosome numbers; however, the exact causes of non-disjunction are unknown. Disomy is the most frequent aneuploidy observed in human sperm.

Children with sex chromosomal abnormalities (e.g., characterized in Klinefelter and Turner syndrome), may have reproductive disorders, behavioral and/or intellectual difficulties when compared to their siblings (Martin, 2006; Boyd et al., 2011). Evidence from European birth defect registries suggests that the prevalence of chromosomal abnormalities in infants (during the first 28 days after birth) increased between 1967–1988 (Morris et al., 2008). Because there was no observed increase in maternally-derived chromosomal abnormalities, underlying environmental causes affecting spermatogenesis are suspected (Morris et al., 2008). Comparable birth defect data for the US are not available.

Much concern has been raised about pesticides being potential endocrine disrupting chemicals (EDCs). EDCs can modulate the endocrine system and potentially cause adverse effects (Sharpe, 2009; Woodruff, 2011; Zoeller et al., 2012; WHO, 2013; NAS, 2014). Humans are exposed to EDCs through multiple routes of exposure (oral, dermal and inhalation) and pathways, including their diet (direct, indirect), environment (water, soil, air), and occupation (Tyler et al., 2000; Jørgensen et al., 2006; McKinlay et al., 2008a, 2008b; Mnif et al., 2011). Because organophosphate (OPs) insecticides accounted for a large share of all US insecticide use, they were the first group of pesticides to be reviewed under the Food Quality Protection Act (FQPA) of 1996. In 1999, the US EPA determined a common mechanism of action based on their ability to bind to and phosphorylate the enzyme acetylcholinesterase in both the central (brain) and peripheral nervous systems (US EPA, 1999). OPs are used in agriculture, recreational and commercial areas, and public pest control programs, accounting for 35% of the total US insecticide usage (US EPA, 2011). Urinary metabolites of OPs, such as dialkyl phosphates (DAPs), have been measured in a substantial proportion of the general population (Barr et al., 2004; CDC, 2009). OPs have been associated with effects on thyroid hormone levels (Lacasaña et al., 2010), decreased semen volume and sperm count (Yucra et al., 2008; Recio-Vega et al., 2008), lower sperm concentration (Perry et al., 2007b), abnormal morphology and decreased sperm motility (Hossain et al., 2010), DNA damage/fragmentation in sperm (Meeker et al., 2004; Muñiz et al., 2008; Atherton et al., 2009), and sperm chromatin structure alteration (Sanchez-Pena et al., 2004). However, limited information has been published about associations between OPs and sperm abnormalities (Padungton et al., 1999; Recio et al., 2001).

Toxicants may adversely affect germ cell DNA integrity (Mruk and Cheng, 2011); however, the exact causes of aneuploidy and the specific windows in which exposures impact the spermatogenic cycle are not well known (Herrera et al., 2008; Axelsson et al., 2010; Ashton Acton, 2013). This study investigated environmental exposures to OPs and their association with altered frequency of disomy among adult men.

Materials and Methods

Study Subjects

Study subjects were men from a parent study assessing the impact of environmental exposures on semen quality. The parent study has been described elsewhere (Hauser et al., 2003). Briefly, eligible participants were men aged 20–54 from couples seeking infertility evaluation at Massachusetts General Hospital (MGH) Fertility Center between January 2000 and May 2003. Sixty-five percent of eligible men agreed to participate; those declining participation cited lack of time during their clinic visit. Exclusion criteria included men who were at the center for post-vasectomy semen analysis and/or receiving treatment for infertility. None of the men reported occupational exposure to pesticides or other agents. All men completed a self-administered questionnaire that collected demographic, lifestyle factors, medical and fertility history information, and provided urine and semen samples. Eligibility for this analysis was based on the availability of both a urine and semen sample from the biorepository. Of the men enrolled in the parent study (n=341), a semen sample was available for 159 men (47%). Informed consent forms were signed by all subjects prior

to participation. The parent study was approved by the Harvard School of Public Health, the Massachusetts General Hospital Human Subjects Committees, and by the Office of Human Research at the George Washington University.

Semen Analysis

Measurement of the semen parameters have been previously described (Hauser et al., 2003). Researchers asked the participants to abstain from ejaculation for 48 hours prior to providing a semen sample at the clinic via masturbation. Samples were liquefied at 37°C for 20 minutes before analysis. Analysis of the samples took place at the MGH Andrology Laboratory. Andrologists were blind as to exposure status. The volume, pH, color, and viscosity were also determined for each semen sample. Sperm counts and percent motility were determined manually and then measured by computer-aided sperm analysis (CASA) using the Hamilton-Thorn Motility Analyzer (10HTM-IVO). A minimum of 200 sperm from 4 different fields were analyzed. A Nikon microscope with an oil immersion 100x objective was used for this analysis (Nikon Company, Tokyo, Japan). Sperm were scored normal or abnormal using the strict criteria reported by Kruger et al., 1988.

Disomy Analysis

Semen samples were stored in -80°C without cryoprotectant until FISH analysis was performed. The procedures for the detection of sex chromosome disomy have been described elsewhere (McAuliffe et al., 2012). A single investigator, blinded to exposure status, performed Fluorescence *in situ* hybridization (FISH) analysis for the detection of sex chromosome disomy, as the primary outcome of interest. Sex chromosome disomy is the most frequent form of sperm aneuploidy, occurring twice as frequently as disomy in the autosomes and resulting in viable offspring (i.e., disomic sperm for X or Y are capable of fertilization). The FISH procedure was carried out for three chromosomes of interest: X, Y and 18 (autosomal control) to determine XX18, YY18, XY18 and total sex chromosome disomy in sperm nuclei. A series of non-overlapping field images were taken for each FISH slide using a fluorescence microscope and scored using custom MATLAB (Mathworks Inc., Natick, MA) software. The software was designed to utilize scoring algorithms based on criteria for size and shape as reported by Baumgartner et al. (1999). Details of the sperm FISH control procedures and validation of the semi-automated scoring method have been previously reported (Perry et al., 2007a, 2011a).

Urine Analysis

Urinary dialkylphosphate (DAP) metabolites were used to estimate human exposure to OP insecticides. Concentrations of six DAP metabolites (i.e., dimethylphosphate (DMP); dimethylthiophosphate (DMTP); dimethyldithiophosphate (DMDTP); diethylphosphate (DEP); diethylthiophosphate (DETP); and diethyldithiophosphate (DEDTP)) were analyzed in urine specimens, using gas chromatography (GC) coupled with mass spectrometry (MS) with isotopic dilution quantification. This method is reported elsewhere (Prapamontol et al., 2014). Briefly, 2.5 mL aliquot of urine was added to 2 g NaCl. The sample was spiked with isotopically labeled internal standards, acidified using 6N HCl, and mixed. The sample was extracted three times with acetonitrile:diethylether (1:1 v/v) and the extract was dried over

anhydrous sodium sulfate. The extract was concentrated to dryness and redissolved in 1.5 mL acetonitrile. Analytes were subjected to a phase-transfer catalyzed derivatization using pentafluorobenzyl bromide and potassium carbonate. The pentafluoro-phosphate esters were extracted with hexane, concentrated to dryness and reconstituted in 50 μ L toluene for analysis by GC-MS.

The DAP metabolites are divided in two main groups: the dimethyl alkylphosphates (or DMAPs), and the diethyl alkylphosphates (or DEAPs). Measuring Σ DAP, Σ DEAP and Σ DMAP is a common approach to quantifying total exposure to organophosphates (Barr et al., 2004). The methyl-containing metabolites (or DMAPs) are derived from O,O-dimethyl-substituted OP insecticide such as azinphos-methyl, dimethoate, malathion, phosmet, among others. During metabolism, the phosphoric group of the parent OP undergoes hydrolysis to become DMP, DMTP, and/or DMDTP. The ethyl-containing metabolites (or DEAPs; including DEP, DETP, and/or DEDTP) are derived from O,O-ethyl-substituted OP pesticides such as chlorpyrifos, diazinon, parathion, among others. Most OP-ethyl parent pesticides are metabolized into DEP and DETP metabolites.

The limit of detection (LOD) for each analyte was estimated by spiking pooled urine sample with standard solutions (from serial dilution of the lowest standard calibration solution) to obtain a concentration providing a signal-to-noise ratio (S/N) of 3. The LODs were 0.6 ng/mL (DMP), 0.2 ng/mL (DMTP), 0.2 ng/mL (DEP), and 0.1 ng/mL (DETP, DMDTP, DEDTP). Total organophosphate metabolite concentration (Σ DAP), total diethyl alkylphosphate (Σ DEAP or sum of DEP, DETP and DEDTP metabolites), and total dimethyl alkylphosphate (Σ DMAP or sum of DMP, DMTP and DMDTP metabolites) were calculated by first dividing each metabolite concentration by its molecular weight (154, 170, 186, 126, 132, 158 for DEP, DETP, DEDTP, DMP, DMTP, and DMDTP, respectively). These transformed metabolite concentrations were then summed and multiplied by 1,000 to obtain units of nmol/mL. Urine samples specific gravity was measured using a handheld refractometer (National Instrument Company, Inc., Baltimore, MD, USA). Creatinine was measured photometrically using kinetic colorimetric assay technology with a Hitachi 911 automated chemistry analyzer (Roche Diagnostics, Indianapolis, IN, USA).

Statistical Analysis

Descriptive statistics were generated for demographic and semen parameter variables. Semen parameters were dichotomized using the most recent World Health Organization (WHO) reference values for sperm concentration (<15 million sperm/mL) and motility (<32% motile sperm), and the Tygerberg Strict Criteria for morphology (<4% normal morphology) (Kruger et al., 1988; World Health Organization, 2010). Urinary metabolite concentrations were used as both continuous and categorical measures. For metabolite values below the LOD, an imputed value equal to one-half the LOD was used. Descriptive statistics for pesticide metabolite levels (μ g/L) in urine were summarized. Creatinine and specific gravity adjusted concentrations as well as volume-based (unadjusted) values were calculated. Although creatinine concentrations are commonly used to adjust for urine dilution, studies have reported that creatinine levels vary by individual factors such as sex (Bjornsson et al., 1979; Turner et al., 1975); age (Edwards et al., 1959; Fuller et al., 1982);

diet (Lykken et al., 1980); decreasing muscle mass (Alessio et al., 1985; Driver et al., 1980); and time of day or seasonality (Freeman et al., 1995; O'Rourke et al., 2000). Therefore, creatinine concentrations may not be suitable to adjust for urine dilution. Specific gravity is considered to be more appropriate and was used to adjust urine metabolite concentrations (Barr et al., 2005). Because the urinary concentrations were not normally distributed, these data were log transformed. Pearson correlations were examined to explore the nature of the association between individual urinary DAP metabolites as well as with the total sum of specific metabolite products (i.e., Σ DEAP, Σ DMAP, and Σ DAP).

Due to the large number of sperm being scored and the relatively low frequency of disomy, the association between each specific volume-based urinary metabolite concentration and the disomy measures (i.e., XX18, YY18, XY18, and total sex chromosome disomy) were modeled using Poisson regression (SAS GENMOD procedure) in unadjusted and adjusted models. All outcome variables were examined, and all displayed a classic Poisson distribution shape. For each subject, the number of sperm scored and the number of disomic nuclei were summed. The individual subject was treated as the unit of analysis. The number of sperm counted was standardized across subjects using the offset variable of the natural logarithm of the number of sperm scored.

Poisson models were fitted using a disomy measure as the outcome variable (i.e., as a count of disomic cells for XX18, YY18, XY18, and total sex chromosome disomy) and the metabolite of interest as the independent variable. Age, body-mass index (BMI), motility, morphology, log of sperm concentration and specific gravity were included as continuous covariates. Smoking (never, former, and current) and race (white, black, or other) were included as categorical covariates. Because the distribution of sperm concentration is often non-normal and positively skewed (Berman et al., 1996), a log transformation of sperm concentration was used.

Metabolites were categorized as quartiles for most metabolites. Incidence rate ratios (IRRs) and 95% confidence intervals were calculated for each model. In addition, the exposure variable was included in the model as an ordinal variable to test for trend. Because the urine samples may have been too concentrated or too diluted to provide valid results, a sensitivity analysis was performed excluding individuals with creatinine concentrations >300 mg/dL or <30 mg/dL or specific gravity >1.03 or <1.01 . A sensitivity analysis was also performed excluding those individuals with fewer than 1,000 nuclei scored, as too few nuclei scored could impact the disomy estimates. Because individual DAP metabolites are correlated among themselves and constitute a portion of Σ DAPs, models were adjusted for Σ DAPs to account for exposure interrelationships. Equivalent results were obtained and Σ DAPs was removed from the final adjusted models. Statistical analysis was performed using SAS version 9.3 (SAS Institute Inc., Cary, NC).

Results

Table 1 shows the demographic and semen parameter characteristics of the study subjects ($n=159$). The men had an average age of 35 years and a mean BMI of 28 kg/m^2 . The majority of the men were white (86%) and non-Hispanic (94%). Most men (74%) had never

smoked with only 7% current smokers. Of the 159 men, 10% ($n = 16$) had sperm concentrations <15 million/mL, 21% ($n = 33$) had $<32\%$ motile sperm, and 18% ($n = 28$) had $<4\%$ normally shaped sperm based on the most recent WHO reference values for sperm concentration and the Tygerberg Strict Criteria for morphology. A median of 6848 sperm nuclei were scored per subject (Table 2). The observed median percentages of XX18, YY18, XY18, and total disomy were 0.4, 0.4, 1.1 and 1.9, respectively. The %XY18 median was 3 times higher than %XX18 and %YY18 medians.

Table 3 summarizes the unadjusted urinary OP metabolites as well as the specific gravity and creatinine adjusted concentrations. About 57% of the urinary samples were above the LOD for most metabolites. DMTP was the most frequently detected metabolite ($> 80\%$ of the samples). DMP metabolite was detected at the highest concentration level. Although the percentage above the LOD was higher for DMTP, the median levels were slightly higher for DMP and DMDTP. The specific gravity and creatinine adjusted results were similar. All three DMAP metabolites ($r = 0.48\text{--}0.57$) and DEAP metabolites ($r = 0.20\text{--}0.54$) were weakly to moderately correlated. Total DAPs and DMAPs were strongly correlated ($r = 0.97$), and there were moderate correlations between Total DAPs and DEAPs ($r = 0.73$) and between DEAPs and DMAPs ($r = 0.58$).

Table 4 provides the mean percentage of each of the evaluated disomies as well as total disomy stratified by detectable levels of OP metabolites. Men in the highest quartile had consistently higher values for %XX18 and total disomy. Similar disomy values were observed in other exposure quartiles.

Table 5 shows the results of the Poisson regression models for each outcome adjusted for specific gravity, age, race, BMI, smoking, total concentration, motility, and morphology when categorizing metabolites into exposure quartiles. Urinary DMTP levels above the lowest quartile were significantly associated with increased rates of XX18, YY18, XY18, and total sex chromosome disomy. The highest significant association was observed between the third exposure quartile of DMTP (2.21–6.47 ng/mL) and XX18, with a 52% increase in the incidence rate ratio ($IRR_{Q3} = 1.52$; 95% CI: 1.36, 1.69). Significantly increased disomy rates were also observed for men in different exposure quartiles of individual DMDTP, DEP and DETP metabolites. Specifically, increased rates were observed for DMDTP and XX18 ($IRR_{Q3}=1.24$; 95% CI: 1.14, 1.36), YY18 ($IRR_{Q2}=1.26$; 95% CI: 1.15, 1.38), XY18 ($IRR_{Q3}=1.28$; 95% CI: 1.22, 1.35) and total sex chromosome disomy ($IRR_{Q3}=1.23$; 95% CI: 1.18, 1.28). Increased rates were observed between DEP and XX18 ($IRR_{Q2}=1.23$; 95% CI: 1.12, 1.35), YY18 ($IRR_{Q3}=1.45$; 95% CI: 1.33, 1.59), XY18 ($IRR_{Q3}=1.09$; 95% CI: 1.04, 1.15) and total sex chromosome disomy ($IRR_{Q3}=1.16$; 95% CI: 1.11, 1.21). Results also showed that DETP was associated with increased rates of XX18, YY18 and total disomy but was inversely associated with XY18. Inverse associations were observed for DMP and XX18, XY18 and total sex chromosome disomy. For DEDTP, only 16 men were above the LOD; inverse associations were observed for YY18, XY18, and total sex chromosome disomy. Adjusted IRRs for XX18, YY18, XY18, and total sex chromosome disomy by Σ DAPs showed inverse associations when compared to the reference group. Additionally, adjusted IRRs for each disomy outcome by Σ DMAPs and Σ DEAPs were also calculated and were similar to those observed for Σ DAPs (data not

shown). Because individual DAP metabolites are subsets of the total DAPs and are therefore highly correlated, adjusted models that controlled for total DAPs were also run. Similar IRRs were obtained.

Linear tests for trend were examined in adjusted models (Table 5). Dose-response curves appeared nonmonotonic, with increase in disomy rates mainly occurring between the second and third exposure quartiles. For example, an increase in the magnitude of total disomy rates between the second and third exposure quartiles of DMDTP was observed; however, after the third DMTP exposure quartile, the magnitude of the XX18 disomy rate decreased (p-value for trend=0.0001). Even though significant p-values for trend were achieved, nonmonotonic patterns were widely observed across disomy types. Sensitivity analyses, excluding 9 individuals with creatinine concentrations >300 mg/dL or <30 mg/dL, and 29 individuals with specific gravity concentrations >1.03 or <1.01, showed similar results (data not shown). A sensitivity analysis with total nuclei scored < 1,000 was also conducted, as disomy estimates can be impacted by too few nuclei scored. In the reanalysis, eight men were excluded and the results remained essentially unchanged.

Discussion

This is the first epidemiologic study of this size to examine the relationship between environmental OP exposures and human sperm disomy outcomes. Results showed significant increased rates of XX18, YY18, XY18, and total sex chromosome disomy (7–52%) by increasing levels of DMTP, DMDTP, DEP and DETP metabolites after adjusting for potential confounders (i.e., age, race, BMI, smoking, specific gravity, total sperm concentration, motility, and morphology). Even though other lifestyle factors may affect chromosomal abnormalities, only risk factors with known associations with pesticide exposure and sperm disomy outcomes were included in the adjusted models. A significant inverse association was observed in adjusted models between DMP and XX18, XY18 and total disomy. The relationship between the disomy outcomes and the individual DAP metabolites as well as the relationship with Σ DAPs were examined to identify their underlying differences and distinctive associations. Adjusted IRRs for XX18, YY18, XY18, and total sex-chromosome disomy by Σ DAPs showed inverse associations when compared to the reference group. These results suggest that aggregating all six DAP metabolites into Σ DAPs may conceal the independent effects of each metabolite. Σ DAPs showed mostly inverse associations across disomy types.

Nonmonotonic dose-response trends were observed between the outcome and exposure categories, with most of the increase in disomy rates occurring between the second and third exposure quartiles and without substantial additional increases between the third and fourth exposure quartile. Because men in the reference group were not significantly different from men in the highest exposure quartile in terms of their demographic and lifestyle characteristics or semen parameters, it is unlikely that differences across exposure groups explain these nonmonotonic curves.

Unadjusted DAP urinary concentrations (Males 95th percentile) were slightly higher for the current study (0.39–39 ng/mL) than the levels reported in the U.S. general population for

2001–2002 (0.78–31 ng/mL) and 2007–2008 (<LOD–36 ng/mL), as reported in the Fourth National Report on Human Exposure to Environmental Chemicals (CDC, 2009 - Updated Tables 2015). In our study, the metabolites detected at highest concentrations were DMP, DMTP and DEP. Similarly studies have reported DMTP as the metabolite detected at highest concentration, followed by DMDTP and DEP (Recio et al., 2001), however this study consisted of a farmworker population where dimethoate was frequently applied. Dimethoate devolves to all three dimethyl metabolites; however, in general populations, DMDTP is infrequently detected (Barr et al., 2004).

Limited epidemiological information has been published about the effects of OP insecticides and their association with sex chromosome disomy (Padungtod et al., 1999; Recio et al., 2001). Increased frequency of total sperm aneuploidies (0.30%) were observed among Chinese pesticide workers manufacturing methyl parathion, ethyl parathion, and metamidophos, compared with the controls (0.19%) (Padungtod et al., 1999). Also, an increased prevalence of sperm aneuploidies in chromosomes X, Y, and 18 was also observed in these workers. A cross-sectional study among agricultural workers also found similar significant associations between frequencies of sperm aneuploidy and organophosphate urinary metabolites (Recio et al., 2001). The most frequent aneuploidy observed was the lack of a sex chromosome or sex null (0.19%), followed by XY18 (0.15%). Increased relative risks were observed between DEP (2.59, 95% CI: 1.59, 2.71) and DETP (1.68, 95%: 1.13, 2.81) and the sex null during the spraying season. Total aneuploidies were significantly higher (72%) during the pesticide spraying season when compared to before the spraying season (59%).

Several studies have reported that OP pesticide exposures were significantly associated with decreased semen volume and decreased sperm count (Yucra et al., 2008; Recio-Vega et al., 2008), lower sperm concentration (Padungtod et al., 2000; Perry et al., 2007b), sperm chromatin alteration (Sánchez-Peña et al., 2004), increased luteinizing hormone and decreased testosterone (Padungtod et al., 1998), higher abnormal morphology and decreased sperm motility (Hossain et al., 2010). Some OPs such as parathion and methylparathion are structurally similar to various hormones, including estrogens and may interact with hormone receptors and/or gene transcription. Perry et al. (2011b) reported that high levels of urinary DMP metabolites were associated with sperm concentrations and total motility below the median population levels among Chinese men. A cross-sectional study conducted among Mexican farmers evaluated the effects of OP toxicity on semen quality and DNA integrity. Two OP exposure indexes were created: at the month of sampling (representing the exposure to spermatids-spermatozoa) and during 3 months before sampling (representing cells at one spermatogenic cycle). In this study, dose-response relationships were observed between OP exposure and sperm quality parameters for both exposure indexes. This study suggested that cells at all stages of spermatogenesis are affected by OP chemicals (Perez-Herrera et al., 2008).

The commonly known mechanism of action of OP toxicity involves the inhibition of the enzyme acetylcholinesterase (AChE) which leads to neurotoxicity in the central and/or peripheral nervous system (US EPA, 1999). It is likely that OP insecticides also act through noncholinergic mechanisms (i.e., without inhibition of acetylcholinesterase) at low

environmental doses (Dam et al., 2003). Various pesticides have been found to bind and alter the function of hormone receptors, alter the synthesis or clearance of endogenous hormones, interact with various neurotransmitter systems, and cause adverse effects by other mechanisms (Stoker et al., 2000). With respect to the OPs, less is known about OP effects on other target organs such as the testis. Because spermatogenesis is a highly complex process dependent upon optimal conditions to occur correctly (Cheng and Mruk, 2009, 2010), OP secondary mechanisms that are not associated with the cholinergic system, including inhibitory mechanisms of androgenic activity mediated by hormones, may lead to endocrine disrupting effects (Elersek and Filipic, 2011; Kang et al., 2004).

OPs prevent thyroid hormone-receptor binding and increases the expression of estrogen responsive genes (McKinlay et al., 2008b). Toxicology studies have linked OPs with effects on steroid and thyroid hormones in rats (Jeong et al., 2006), anti-androgenic activity in rats (Kang et al., 2004), and increased spermatids apoptosis during spermatogonial proliferation in mice (Masoud et al., 2003). Severe spermatogenesis disruption (specifically affecting the pituitary gonadotrophins) was shown with increasing doses of quinalphos in male rats (Sarkar et al., 2000). Furthermore, increased follicle stimulating hormone (FSH) levels have been observed in rats exposed to chlorpyrifos suggesting adverse effects on their reproductive system (Sai et al., 2014). A study in adult male mice measured FSH and luteinizing hormone (LH) concentrations and reported diazinon effects on testis structure and sex hormones levels (Fattahi et al., 2009). Significant reductions were also observed in the diameter and weight of testes, Leydig and Sertoli cells, sperm count, and testosterone concentration. FSH and LH triggered testosterone production potentially altering spermatogenesis mechanisms. Similarly, Ngoula et al. (2007) observed spermatogenesis inhibition and Leydig cells density reduction after male rats were treated with pirimiphos-methyl. It appears that OP insecticides disrupt male hormones; however, it is still uncertain the relationship between OP exposure, hormone signaling and cell division.

Epidemiological studies among non-occupationally exposed adult males have found associations between OP exposure and hormones serum levels (i.e., thyrotropin (TSH) and triiodothyronine (T4)) (Meeker et al., 2006). Recio et al. (2005) found inverse associations between FSH levels and DMTP and DMDTP, and between DMTP levels and LH among Mexican agricultural workers. Another investigation among Peruvian pesticide applicators reported no significant associations between DAPs and hormone levels (Yucra et al., 2008). An inverse association between DAP and total thyroxine (T3) was also reported among floricultural workers living in Mexico (Lacasaña et al., 2010). Nonetheless, an increased association between total DAPs and TSH and T4 levels was also demonstrated among the same group of men. Both inverse and increased associations were observed between hormones (i.e., inhibin B, FSH, LH) and DAP metabolites (Blanco-Muñoz et al., 2010). These epidemiological findings are consistent with discussions of potential low-dose effect mechanisms responsible for generating human disorders (Vandenberg et al., 2012).

The endocrine system is likely to respond to very low hormone concentrations allowing a vast number of hormonally active molecules to co-occur (Welshons et al., 2003). Low doses of a chemical could affect expression of a hormone receptor in the hypothalamus, an endpoint not examined in high-dose toxicology testing (Vandenberg et al., 2012). It is

uncertain how increased exposure to OPs might be protective for sex-chromosome disomy. Our results exhibited nonmonotonic dose-response relationships between exposure and effect. When nonmonotonic dose-responses occur, the effects observed at low doses cannot be predicted by the effects observed at high doses (Vandenberg et al., 2012). Scientists have previously stated that as the dose decreases and the effect size decreases, the number of animals or individuals needed to achieve the power to detect a significant effect would have to increase substantially (vom Saal et al., 1998). Even though active compounds may induce significant biological effects at extremely low concentrations and researchers may be unable to detect small magnitudes of effect (Vandenberg et al., 2012), this study was able to detect significant associations between OP exposures and disomy.

Detailed exposure assessments that include time-specific exposure are needed to determine when the most critical exposures windows in the spermatogenic process are likely to occur. Spot urine samples are unlikely to reflect cumulative pesticide exposures, particularly when the parent compounds are rapidly metabolized (Martenies and Perry, 2013). OP metabolites measured in a single urine sample may not accurately reflect cumulative exposure over longer periods due to urine volume variability and the concentrations of endogenous and exogenous chemicals from void to void (Barr et al., 2005). Nonetheless, the reliability of non-persistent pesticide metabolites may be adequate when individuals are categorized into broad exposure groups due to their consistent individual time-activity patterns combined with constant microenvironmental concentrations that may lead to 'steady state' metabolite concentrations over longer time periods (Meeker et al., 2005). The ability of a single urine sample to predict metabolite concentrations over longer periods of interest and implications for detecting health outcomes in epidemiological studies need continued investigation.

DAP metabolites are limited as biomarkers of exposure, as they do not retain the structure from which they were derived, and cannot be attributed to a specific original parent compound (Bravo et al., 2004). Over the years, the use of DAP metabolites as biomarkers of exposure for OP insecticides has been scrutinized. DAPs may also occur in the environment as a result of degradation of organophosphorus insecticides (Lu et al., 2005). The DAP metabolites can be present in urine after low level exposures to OPs that do not cause clinical symptoms or inhibition of cholinesterase activity (Davies and Peterson, 1997; Franklin et al., 1981). Moreover, the inability to distinguish exposures to parent compounds from exposures to pre-formed DAPs has been a concern (Krieger et al., 2012; Sudakin and Stone, 2011). The presence of DAPs in a person's urine may reflect exposure the parent OP pesticide or to the metabolites themselves. Therefore, there is the need to evaluate the effects and impact on spermatogenesis caused by the exposure to preformed degradation products versus exposure to parent compounds. Despite these limitations, biological measurements of urinary DAP metabolites are the most practical and widely used method to estimate the internal dose of most OP pesticides used worldwide (Angerer et al., 2007). These analyses continue to be extensively used in biomonitoring studies due to their relatively low costs and their utility for interpreting health outcomes of interest.

The men in this study population were recruited from a fertility clinic as opposed to the general population; however, there is no evidence that our participants would differ in their response to OP exposures. More than half of the men in our study population had normal

semen motility, concentration and morphology, and extensive information on potential confounders was collected and adjusted for in the final models. A validated semi-automated method for disomy frequency determination was used in this study. The semiautomated method allowed for reliable processing of a large number of samples (Perry et al., 2007a, 2011a). The use of a semiautomated method is considered a strength in this study because it allowed for objective counting of disomic sperm in a large number of samples.

Conclusion

This is the first epidemiologic study of this size to examine the relationship between environmental OP exposures and human sperm disomy outcomes. This study assessed environmental OP exposures and human sperm disomy outcomes by conducting multiple analyses adjusted for potential confounders. Our findings suggest that increased disomy rates were associated with specific DAP metabolites. Conversely, significant inverse associations were also observed between DMP exposure levels and most disomy outcomes. Total sum of DAP metabolites concealed individual associations observed for each individual metabolite. Dose-response relationships appeared nonmonotonic, with most of the increase in disomy rates occurring between the second and third exposure quartiles and without additional increases between the third and fourth exposure quartiles. These complex relationships present a challenge when documenting the associations between OP exposures and sperm chromosomal abnormalities. Future research need to ascertain the mechanisms by which EDCs affect health reproductive outcomes. Detailed exposure assessments are also required to further our understanding of sperm health effects associated with environmental exposures.

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Highlights

First epidemiologic study of this size to examine OP exposures and sperm disomy.

Increased disomy rates were associated with specific DAP urinary metabolites.

Total sum of DAP metabolites concealed individual associations.

OP impacts on testis function need further characterization in epidemiologic studies.

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Table 1

Characteristics of study participants (n=159).

Variable	Mean ± SD
Age	35 ± 5
BMI (kg/m ²)	28 ± 5
Race	N (%)
White	137 (86)
Black	5 (3)
Other	17 (11)
Hispanic ethnicity	
No	149 (94)
Yes	10 (6)
Semen Concentration	
<15 million/mL	16 (10)
Semen Morphology	
<4% normal	28 (18)
Semen Motility	
<32% motile	33 (21)
Abstinence time	
≤2 days	35 (22)
3–4 days	74 (47)
>5 days	50 (31)
Smoking (n=2 missing)	
No	116 (74)
Current smoker	11 (7)
Former smoker	30 (19)

Table 2

Number of sperm nuclei scored and percent disomy (n=159).

Variable	Mean $\pm$ SD	Median	25 th	75 th
Nuclei (n)	6848 $\pm$ 4815	5503	2939	9976
%X18	38 $\pm$ 9	40	33	45
%Y18	37 $\pm$ 9	39	33	43
%XX18	0.4 $\pm$ 0.4	0.3	0.2	0.5
%YY18	0.4 $\pm$ 0.3	0.3	0.2	0.5
%XY18	1.1 $\pm$ 0.8	0.9	0.6	1.5
Total Disomy %	1.9 $\pm$ 1.3	1.6	1.1	2.5

Table 3

Distribution of pesticide metabolite levels in urine (n=159).

Metabolite ^a (ng/mL)	Mean ± SD	Percentile				Range	
		25th	50th	75th	90th		95th
Unadjusted							
DMP	11±25	<LOD	4	12	29	39	<LOD-271
DMTP	9±17	1	3	7	21	39	<LOD-148
DMDTP	1±2	<LOD	0.4	1	4	6	<LOD-10
DEP	4±8	0.1	1	4	9	15	<LOD-64
DETP	2±3	<LOD	1	1	4	9	<LOD-20
DEDTP	0.1±0.1	<LOD	<LOD	<LOD	0.3	0.4	<LOD-1
ΣDAP ^b	188±318	20	98	194	408	790	3–2,340
SG-adjusted							
DMP	11±24	<LOD	5	15	28	43	<LOD-259
DMTP	10±18	1	4	9	29	44	<LOD-122
DMDTP	1±3	<LOD	0.5	1	4	8	<LOD-15
DEP	4±7	0.2	1	4	9	14	<LOD-54
DETP	2±3	0.2	1	2	5	7	<LOD-17
DEDTP	0.1±0.1	<LOD	<LOD	0.1	0.3	0.4	<LOD-1
ΣDAP ^b	202±303	34	113	236	469	728	3–2,094
CR-adjusted (missing=2)							
DMP	8±16	<LOD	3	7	20	28	<LOD-148
DMTP	7±16	1	3	6	16	25	<LOD-122
DMDTP	1±2	<LOD	0.3	1	3	5	<LOD-19
DEP	2±5	0.2	1	2	7	11	<LOD-34
DETP	1±2	0.1	1	1	4	6	<LOD-16
DEDTP	0.1±0.1	<LOD	<LOD	0.1	0.2	0.5	<LOD-1
ΣDAP ^b	143±242	23	67	155	314	471	2–1,800

^a LOD= 0.6 ng/mL (DMP), 0.2 ng/mL (DMTP), 0.2 ng/mL (DEP), and 0.1 ng/mL (DETP, DMDTP, DEDTP). Percent of samples above the LOD: DMP=57% (n = 91); DMTP=87% (n = 139); DMDTP=57% (n = 90); DEP=64% (n = 101); DETP=72% (n = 114); and DEDTP=10% (n = 16).

^b ΣDAPs urinary concentration units are nmol/mL.

Table 4Aneuploidy Mean $\pm$ SD by detection of individual urinary organophosphate metabolite levels (n=159).

Metabolite ^a	%XX18	%YY18	%XY18	%Total Disomy
DMP				
Q1	0.49 $\pm$ 0.48	0.39 $\pm$ 0.37	1.27 $\pm$ 0.98	2.15 $\pm$ 1.53
Q2	0.45 $\pm$ 0.59	0.31 $\pm$ 0.20	1.00 $\pm$ 0.61	1.76 $\pm$ 1.09
Q3	0.36 $\pm$ 0.29	0.39 $\pm$ 0.26	0.92 $\pm$ 0.74	1.67 $\pm$ 1.06
Q4	0.38 $\pm$ 0.32	0.31 $\pm$ 0.17	1.13 $\pm$ 0.68	1.81 $\pm$ 0.95
DMTP				
Q1	0.36 $\pm$ 0.29	0.37 $\pm$ 0.21	1.15 $\pm$ 0.68	1.88 $\pm$ 0.89
Q2	0.52 $\pm$ 0.59	0.40 $\pm$ 0.43	1.21 $\pm$ 1.18	2.12 $\pm$ 1.86
Q3	0.42 $\pm$ 0.40	0.37 $\pm$ 0.27	1.01 $\pm$ 0.60	1.81 $\pm$ 1.01
Q4	0.41 $\pm$ 0.41	0.31 $\pm$ 0.18	1.13 $\pm$ 0.69	1.85 $\pm$ 1.01
DMDTP				
Q1	0.46 $\pm$ 0.49	0.38 $\pm$ 0.36	1.20 $\pm$ 0.97	2.04 $\pm$ 1.52
Q2	0.50 $\pm$ 0.49	0.30 $\pm$ 0.19	1.21 $\pm$ 0.75	2.01 $\pm$ 1.19
Q3	0.41 $\pm$ 0.44	0.38 $\pm$ 0.28	1.00 $\pm$ 0.80	1.79 $\pm$ 1.14
Q4	0.33 $\pm$ 0.23	0.36 $\pm$ 0.20	0.98 $\pm$ 0.53	1.67 $\pm$ 0.78
DEP				
Q1	0.37 $\pm$ 0.26	0.35 $\pm$ 0.25	1.11 $\pm$ 0.69	1.83 $\pm$ 0.97
Q2	0.58 $\pm$ 0.64	0.47 $\pm$ 0.45	1.35 $\pm$ 1.26	2.40 $\pm$ 1.98
Q3	0.36 $\pm$ 0.43	0.32 $\pm$ 0.23	0.86 $\pm$ 0.56	1.53 $\pm$ 0.93
Q4	0.48 $\pm$ 0.44	0.30 $\pm$ 0.15	1.18 $\pm$ 0.65	1.95 $\pm$ 1.00
DETP				
Q1	0.40 $\pm$ 0.36	0.33 $\pm$ 0.20	1.24 $\pm$ 0.81	1.96 $\pm$ 1.08
Q2	0.44 $\pm$ 0.48	0.36 $\pm$ 0.29	0.93 $\pm$ 0.52	1.74 $\pm$ 0.98
Q3	0.46 $\pm$ 0.44	0.44 $\pm$ 0.41	1.34 $\pm$ 1.17	2.25 $\pm$ 1.88
Q4	0.44 $\pm$ 0.51	0.32 $\pm$ 0.23	0.97 $\pm$ 0.62	1.73 $\pm$ 0.97
DEDTP				
G1	0.44 $\pm$ 0.46	0.37 $\pm$ 0.30	1.15 $\pm$ 0.86	1.95 $\pm$ 1.31
G2	0.43 $\pm$ 0.33	0.28 $\pm$ 0.16	0.93 $\pm$ 0.51	1.64 $\pm$ 0.85
ΣDAPs				
T1	0.50 $\pm$ 0.53	0.37 $\pm$ 0.38	1.27 $\pm$ 1.07	2.14 $\pm$ 1.65
T2	0.46 $\pm$ 0.48	0.40 $\pm$ 0.29	1.08 $\pm$ 0.74	1.94 $\pm$ 1.17
T3	0.35 $\pm$ 0.28	0.31 $\pm$ 0.18	1.02 $\pm$ 0.61	1.68 $\pm$ 0.86

^aDMP Exposure Quartiles: Q1=X LOD (n=68), Q2=0.60<X 7.95 ng/mL (n=30), Q3=7.95<X 13.39 ng/mL (n=30), Q4=X>13.39 ng/mL (n=31).
 DMTP Exposure Quartiles: Q1=X LOD (n=30), Q2=0.20<X 2.21 ng/mL (n=43), Q3=2.21<X 6.47 ng/mL (n=42), Q4=X>6.47 ng/mL (n=44).
 DMDTP Exposure Quartiles: Q1=X LOD (n=69), Q2=0.10<X 0.73 ng/mL (n=30), Q3=0.73<X 1.86 ng/mL (n=30), Q4=X>1.86 ng/mL (n=30).
 DEP Exposure Quartiles: Q1=X LOD (n=58), Q2=0.20<X 1.46 ng/mL (n=34), Q3=1.46<X 3.96 ng/mL (n=33), Q4=X>3.96 ng/mL (n=34).
 DETP Exposure Quartiles: Q1=X LOD (n=45), Q2=0.10<X 0.62 ng/mL (n=39), Q3=0.62<X 1.51 ng/mL (n=37), Q4=X>1.51 ng/mL (n=38).
 DEDTP Exposure Group: G1=X LOD (n=143); G2=X>0.10 ng/mL (n=16). Σ DAPs Exposure Tertiles: T1=2.76 $\times$ 35.00 nmol/mL (n=53), T2=35.00<X 155.00 nmol/mL (n=52), T3=X>155.01 nmol/mL (n=54).

Table 5

Adjusted IRRs (95% CI) for XX18, YY18, XY18, and total sex-chromosome disomy by organophosphate metabolite (n=159).

Metabolite ^a	XX18	YY18	XY18	Total Disomy
DMP				
Q1	1.00	1.00	1.00	1.00
Q2	0.69 (0.63, 0.76)	1.00 (0.92, 1.09)	0.79 (0.74, 0.84)	0.81 (0.78, 0.84)
Q3	0.81 (0.74, 0.89)	0.89 (0.80, 0.99)	0.92 (0.87, 0.98)	0.89 (0.86, 0.93)
Q4	0.77 (0.70, 0.86)	1.07 (0.97, 1.18)	0.80 (0.75, 0.85)	0.84 (0.80, 0.88)
p-Value for Trend	<0.0001	0.2440	<0.0001	<0.0001
DMTP				
Q1	1.00	1.00	1.00	1.00
Q2	1.33 (1.18, 1.50)	1.09 (0.97, 1.23)	1.09 (1.02, 1.16)	1.13 (1.08, 1.19)
Q3	1.52 (1.36, 1.69)	1.03 (0.93, 1.15)	1.06 (1.00, 1.12)	1.14 (1.08, 1.20)
Q4	1.24 (1.10, 1.38)	1.21 (1.09, 1.35)	0.91 (0.86, 0.97)	1.03 (0.98, 1.08)
p-Value for Trend	0.1214	0.0142	0.4193	0.0236
DMDTP				
Q1	1.00	1.00	1.00	1.00
Q2	1.22 (1.12, 1.34)	1.26 (1.15, 1.38)	0.98 (0.92, 1.03)	1.08 (1.04, 1.13)
Q3	1.24 (1.14, 1.36)	1.01 (0.92, 1.12)	1.28 (1.22, 1.35)	1.23 (1.18, 1.28)
Q4	1.01 (0.91, 1.13)	1.16 (1.05, 1.29)	1.10 (1.04, 1.17)	1.09 (1.04, 1.15)
p-Value for Trend	0.0521	<0.0001	0.0982	<0.0001
DEP				
Q1	1.00	1.00	1.00	1.00
Q2	1.23 (1.12, 1.35)	1.04 (0.94, 1.15)	1.07 (1.01, 1.13)	1.09 (1.05, 1.14)
Q3	1.13 (1.03, 1.24)	1.45 (1.33, 1.59)	1.09 (1.04, 1.15)	1.16 (1.11, 1.21)
Q4	0.92 (0.83, 1.02)	0.98 (0.88, 1.09)	0.84 (0.79, 0.89)	0.88 (0.84, 0.92)
p-Value for Trend	0.0010	0.5790	0.7969	0.1784
DETP				
Q1	1.00	1.00	1.00	1.00
Q2	1.07 (0.98, 1.18)	1.39 (1.26, 1.53)	0.99 (0.94, 1.05)	1.07 (1.02, 1.11)

Metabolite ^a	Adjusted IRRs ^b			Total Disomy
	XX18	YY18	XY18	
Q3	1.13 (1.02, 1.24)	1.31 (1.18, 1.45)	0.89 (0.89, 0.95)	1.00 (0.96, 1.05)
Q4	0.83 (0.75, 0.92)	1.22 (1.09, 1.36)	0.73 (0.69, 0.78)	0.83 (0.79, 0.87)
p-Value for Trend	0.0007	<0.0001	<0.0001	<0.0001
DEDTP^c				
G1	1.00	1.00	1.00	1.00
G2	1.10 (0.99, 1.22)	0.85 (0.76, 0.96)	0.90 (0.84, 0.96)	0.93 (0.88, 0.98)
ΣDAPs				
T1	1.00	1.00	1.00	1.00
T2	1.04 (0.96, 1.13)	1.36 (1.25, 1.48)	0.94 (0.90, 0.99)	1.03 (0.99, 1.07)
T3	0.85 (0.77, 0.93)	1.08 (0.98, 1.20)	0.86 (0.82, 0.91)	0.89 (0.86, 0.93)
p-Value for Trend	0.0002	0.1753	<0.0001	<0.0001

^aDMTP Exposure Quartiles: Q1=X LOD (n=68), Q2=0.60<X 7.95 ng/mL (n=30), Q3=7.95<X 13.39 ng/mL (n=31), DMTP Exposure Quartiles: Q1=X LOD (n=30), Q2=0.20<X 2.21 ng/mL (n=43), Q3=2.21<X 6.47 ng/mL (n=42), Q4=X>6.47 ng/mL (n=44), DMTP Exposure Quartiles: Q1=X LOD (n=69), Q2=0.10<X 0.73 ng/mL (n=30), Q3=0.73<X 1.86 ng/mL (n=30), Q4=X>1.86 ng/mL (n=30), DEP Exposure Quartiles: Q1=X LOD (n=58), Q2=0.20<X 1.46 ng/mL (n=34), Q3=1.46<X 3.96 ng/mL (n=33), Q4=X>3.96 ng/mL (n=34), DETP Exposure Quartiles: Q1=X LOD (n=45), Q2=0.10<X 0.62 ng/mL (n=39), Q3=0.62<X 1.51 ng/mL (n=37), Q4=X>1.51 ng/mL (n=38), DEDTP Exposure Group: G1=X LOD (n=143), G2=X>0.10 ng/mL (n=16), ΣDAPs Exposure Tertiles: T1=2.76 × 35.00 nmol/mL (n=53), T2=35.00 <X 155.01 nmol/mL (n=52), T3=X>155.01 nmol/mL (n=54).

^bIRR_s were adjusted for specific gravity, age, race, BMI, smoking, total concentration, motility, and morphology. ΣDAP_s is the sum of all six individual metabolites.

^cNo p-value for trend was calculated for adjusted disomy IRRs and DEDTP.