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Research Article

A Longitudinal Study of Atrazine and 2,4-D Exposure and Oxidative Stress Markers Among Iowa Corn Farmers

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Reactive oxygen species, potentially formed through environmental exposures, can overwhelm an organism's antioxidant capabilities resulting in oxidative stress. Long-term oxidative stress is linked with chronic diseases. Pesticide exposures have been shown to cause oxidative stress *in vivo*. We utilized a longitudinal study of corn farmers and non-farming controls in Iowa to examine the impact of exposure to the widely used herbicides atrazine and 2,4-dichlorophenoxyacetic acid (2,4-D) on markers of oxidative stress. 225 urine samples were collected during five agricultural time periods (pre-planting, planting, growing, harvest, off-season) for 30 farmers who applied pesticides occupationally and 10 controls who did not; all were non-smoking men ages 40–60. Atrazine mercapturate (atrazine metabolite), 2,4-D, and oxidative stress markers (malondialdehyde [MDA], 8-hydroxy-2′-deoxyguanosine [8-OHdG], and 8-isoprostaglandin-$F_{2\alpha}$ [8-isoPGF]) were measured in urine. We calculated β estimates and 95% confidence intervals (95%CI) for each pesticide-oxidative stress marker combination using multivariate linear mixed-effect models for repeated measures. Farmers had higher urinary atrazine mercapturate and 2,4-D levels compared with controls. In regression models, after natural log transformation, 2,4-D was associated with elevated levels of 8-OHdG ($\beta = 0.066$, 95%CI = 0.008–0.124) and 8-isoPGF ($\beta = 0.088$, 95%CI = 0.004–0.172). 2,4-D may be associated with oxidative stress because of modest increases in 8-OHdG, a marker of oxidative DNA damage, and 8-isoPGF, a product of lipoprotein peroxidation, with recent 2,4-D exposure. Future studies should investigate the role of 2,4-D-induced oxidative stress in the pathogenesis of human diseases. Environ. Mol. Mutagen. 58:30–38, 2017. © 2016 Wiley Periodicals, Inc.

Key words: agriculture; pesticide; herbicide; oxidative damage

Additional Supporting Information may be found in the online version of this article.

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BACKGROUND

Oxidative stress occurs when free radicals, typically reactive oxygen or nitrogen species, overwhelm an organism's antioxidants and DNA repair mechanisms (Block et al. 2002; Muniz et al. 2008). These reactive species, created endogenously as a part of natural cellular processes or introduced through exogenous environmental or lifestyle exposures, may result in damage to DNA, proteins, and lipids (Block et al. 2002; Bokov et al. 2004; Aseervatham et al. 2013). Oxidative stress affects a variety of cellular processes, and may consequently be associated with the pathogenesis of many human diseases, including neurodegenerative disease, inflammatory disease, cardiovascular disease, diabetes, and cancer (Halliwell and Gutteridge 2007; Sosa et al. 2013). Specifically, markers of oxidative stress and DNA damage have been associated with many types of cancer, including lung (Erhola et al. 1997), breast (Djuric et al. 1996), bladder (Chiou et al. 2003), prostate (Chiou et al. 2003), and lymphoma (van de Wetering et al. 2008).

Oxidative stress is commonly cited as a potential mechanism of carcinogenesis for various environmental exposures, including pesticides (Alavanja et al. 2013; Guyton et al. 2015; Loomis et al. 2015). Many pathways have been posited by which pesticides may induce oxidative stress, such as futile oxidative metabolism of pesticides by cytochrome P450 enzymes, generation of redox-active pesticide metabolites, and impairment of electron transport cascades in mitochondria (Alavanja et al. 2013). Human studies have generally shown increases in biomarkers for oxidative stress and DNA damage among farmers exposed to pesticides, compared with non-farming controls (Banerjee et al. 1999; Muniz et al. 2008; Kisby et al. 2009; Da Silva et al. 2012; Wafa et al. 2013). However, these studies have been unable to examine associations with specific pesticide active ingredients, as they were either underpowered to do so or did not ask participants about specific exposures. Examination of specific pesticide active ingredients is of particular importance, as farmers may be exposed to different chemical classes of pesticides depending on the type of crops and animals farmed. Grouping all pesticide or farming exposures together may dilute the effect of specific chemicals with the potential to cause oxidative stress.

Atrazine and 2,4-dichlorophenoxyacetic acid (2,4-D) are herbicides of particular interest because of their broad agricultural use in the US; in 2001–2003, atrazine and 2,4-D were among the top five pesticide active ingredients used in the agricultural market sector (Grube et al. 2011). Atrazine is used predominantly on corn, and is monitored in US groundwater due to environmental and human health concerns (EPA 2006). Atrazine has been shown to induce oxidative stress in rats and mice (Abarikwu 2014; Jin et al. 2014; Zhao et al. 2014). In agricultural settings, farmers apply 2,4-D to a variety of crops including corn, hay, wheat, and soybeans (EPA 2005). In addition, 2,4-D is registered in the US for residential use on lawns and is the most commonly used pesticide active ingredient in the home and lawn market sector, with the potential for broad exposures in the general population (EPA 2005; Grube et al. 2011). Laboratory studies in rats have shown that 2,4-D may induce oxidative stress (Tayeb et al. 2012, 2013; Pochettino et al. 2013; Loomis et al. 2015).

We utilized data from a longitudinal biomarker study of corn farmers and non-farming controls to examine the impact of atrazine and 2,4-D on three markers of oxidative stress, malondialdehyde (MDA), 8-hydroxy-2′-deoxyguanosine (8-OHdG), and 8-isoprostaglandin-$F_{2\alpha}$ (8-isoPGF), over a growing season in Iowa. MDA is created when reactive oxygen species (ROS) react with polyunsaturated fatty acids, and is an indicator of oxidative stress when measured in blood, plasma, and urine (Muniz et al. 2008). 8-OHdG is a promutagenic lesion of DNA generated in response to ROS, and is a marker of oxidative injury (Muniz et al. 2008). 8-isoPGF is a prostaglandin-like compound produced by non-enzymatic lipoprotein peroxidation (Lee et al. 2006). These markers of oxidative stress are generally considered to be immediate or short-term responses to environmental exposures, with up to a few days lag in response to a known stressor (Song et al. 2013; Lin et al. 2015). To our knowledge, no prior studies have examined the effect of atrazine or 2,4-D on oxidative stress markers in humans, and no prior studies examining pesticide exposures and oxidative stress have utilized a longitudinal study design.

METHODS

Details on the selection of the study population, biological sample collection and exposure modeling have been described (Bakke et al. 2009).

Study Population and Data Collection

Briefly, we recruited 30 Iowa farmers (10 recruited in 2002 and 20 in 2003) from the Agricultural Health Study (AHS) cohort, and 10 non-farming and non-pesticide applying controls (five each year). Controls were male agricultural extension workers from the Iowa State University Agricultural Extension Program. Two of the 30 farmers participated in both study years. All participants were male, non-smokers, 40–60 years of age, and Iowa residents. Farmers were eligible only if they planned to plant at least 300 acres of corn in the upcoming year, and personally apply atrazine as a pre-emergent herbicide. We obtained written informed consent from all participants. The study was approved by the National Cancer Institute Institutional Review Board and the University of Iowa Human Subjects Office.

We defined five study periods: pre-planting (T1, baseline), planting (T2), growing (T3, T4), harvest (T5), and off-season (T6). At baseline, interviewers administered a questionnaire that ascertained information on farming and pesticide use history, medical history, and lifestyle factors. Participants additionally kept diaries during the planting and growing periods in which they recorded details about pesticide application, tillage, equipment repair, livestock, sun exposure and sun protection practices, and other non-pesticide occupational exposures. The study was designed to examine biomarkers associated with exposure to atrazine, thus specimen collection was timed to atrazine application. Trained study personnel collected spot urine samples at each period (twice

during the growing period) for farmers. During the first year, study personnel collected urine from controls at T1, T3, T5, and T6 only; during the second year, they collected urine from controls on the same schedule as the farmers (T1-T6). In total, 244 urine samples were collected. Ascorbic acid (100 mg/100 ml urine) was added to prevent oxidation of labile metabolites. All specimens were processed within 6 hr of collection and stored at −80°C.

Exposure Assessment

We quantified pesticide metabolites (or parent compounds) in urine (μg/L) with a standard panel for non-persistent pesticides using a modification of a high-performance liquid chromatography-tandem mass spectrometry method (Olsson et al. 2004). The target analytes were quantified using isotope dilution calibration (Bakke et al. 2009). Measurements below the instrumental limit of detection (LOD; 0.1 μg/L) were imputed twenty times using a parametric model-based estimation procedure (66% for atrazine mercapturate, 19% for 2,4-D) (Lubin et al. 2004). The intraclass correlation coefficients (ICCs) were higher than 80% for all analytes, indicating high assay reliability (Bakke et al. 2009).

Oxidative Stress Measurement

Three oxidative stress markers, MDA, 8-OHdG, and 8-isoPGF were measured in urine using methods previously described by Lee et al. (2006, 2010). MDA was measured using the reaction with thiobarbituric acid (TBA). TBA and MDA standards were purchased from Sigma-Aldrich Korea (Yongin, Kyunggi, Korea). A 10 mmol/L stock of standard MDA was prepared by dissolving 123.5 μl of 1,1,3,3-tetraethoxypropane in 50 ml of ethanol (40% ethanol by volume). TBA-MDA adducts were prepared in glass tubes with a polypropylene stopper. In each tube, 300 μl of phosphoric acid (0.5M) was mixed with 50 μl of urine and 150 μl of TBA reagent. The mixtures were incubated at 95°C for 1 hr and 500 μl methanol (HAYMAN, Witham, Essex, UK) was added to each tube. Following a 5-min centrifugation (5000*g*), the samples were analyzed using high performance liquid chromatography on a 4 × 150 mm Sunfire C18 column with UV (wavelength, 532 nm). The mobile phase was potassium phosphate (0.05 mol/L; pH 6.8) and methanol (58:42, v/v). The flow rate was 0.8 ml/min. The LOD was 0.05 μmol/L and the correlation for the linearity of the standard curve was 0.99.

The level of urinary 8-OHdG was determined using a competitive ELISA kit (Japan Institute for the Control of Aging, Fukuroi, Shizuoka, Japan). In brief, 50 μl of primary monoclonal antibody and a 50 μl sample or standard were added to microtiter plates which were pre-coated with 8-OHdG, incubated at 37°C for 1 hr and washed with 250 μl of phosphate-buffered saline. One hundred microliters of horseradish peroxidase-conjugated secondary antibody was then added to each well and incubated at 37°C for 1 hr. The reaction was terminated with 100 μl of 1 N phosphoric acid. Absorbance of each well was read at 450 nm by a microplate reader (ELx808, Bio-Tek, Winooski, VT). The LOD was 0.5 ng/ml.

Urine samples were analyzed for 8-isoPGF levels using a competitive ELISA (OxisResearch, Portland, OR) (Sasaki et al. 2002). Briefly, samples were mixed with an enhancing reagent to eliminating interference due to non-specific binding. Following substrate addition, the intensity of color measured was inversely proportional to the amount of unconjugated 8-isoPGF in the sample or standard.

Forty duplicate samples were analyzed for quality control purposes. Coefficients of variation (CV) and ICCs were calculated for MDA, 8-OHdG, and 8-isoPGF to examine the reproducibility of the assays. For MDA, 8-OHdG, and 8-isoPGF CVs (the smaller the better) were 22%, 31%, and 27%, respectively, and ICCs (the larger the better) were 58%, 81%, and 83%, respectively.

Creatinine in urine (mg/dl) was determined using a commercially available enzyme slide technology (Vitros 250 Chemistry System, Ortho-Clinical Diagnostics, Raritan, NJ) (Olsson et al. 2004).

Statistical Analysis

For the two farmers participating in both years of the study, we included only data from their first participation year. In addition, we excluded five samples with missing creatinine and pesticide metabolite information due to laboratory error. For 8-isoPGF analyses, we excluded a single outlying observation that was 100 times higher than the median and 10 times higher than the next highest observation.

We compared baseline characteristics and oxidative stress biomarkers for farmers and non-farming controls. We plotted geometric mean pesticide metabolites and oxidative stress markers by study time point separately for farmers and controls, and calculated 95% confidence limits. We utilized one-way ANOVA with Tukey's method to examine if measurements differed by time point. We utilized linear mixed-effect (LME) models with an unstructured covariance matrix to examine the effect of each pesticide metabolite on MDA, 8-OHdG, and 8-isoPGF, estimating β and 95% confidence intervals (95% CI). LME models allowed us to account for correlation of samples within subjects. MDA, 8-OHdG, 8-isoPGF, atrazine mercapturate, and 2,4-D were natural log (ln) transformed to satisfy the normality assumptions of our statistical models. We adjusted all models for age (mean centered), farmer or control, study time point, and creatinine (ln transformed) to account for the effect of urine dilution on pesticide metabolites and oxidative stress outcomes (Barr et al. 2005). Additional confounders were considered one at a time, including body mass index (<25, 25–29.9, 30+), smoking history and duration (never smoker, former smoker for <10 years, former smoker for ≥10 years), alcohol use (non-drinker, <1 drink/day, ≥1 drink/day), regular physical activity (low, medium, high), multivitamin or vitamin C supplementation (yes, no; sample collection or day prior), infection or symptoms at sample collection (yes, no), allergy symptoms at sample collection (yes, no), and history of cancer (yes, no). Potential confounders were included in the model if they were associated with the outcome ($P < 0.10$) or substantively altered the estimate for the pesticide metabolite of interest (≥20% change). We assumed that the underlying level of response could differ by participant (random intercept) and that the change in response over time could differ by participant (smoothed random slope) (Ruppert et al. 2003).

As a supplementary analysis, we examined whether ln transformed cumulative exposure to atrazine and 2,4-D during a growing season was associated with oxidative stress. Predictive exposure models were developed based on urinary metabolite measures, creatinine, and self-reported application practices collected in diaries (Bakke et al. 2009). These models were used to estimate daily exposures, which were summed to estimate cumulative exposure from T1 until each study time point (T2-T6). At baseline (pre-planting), all individuals were assigned a nominal low-level exposure. Controls were assigned the same nominal level throughout the study period.

All tests were two-sided with $\alpha = 0.05$. Results for imputed data were summarized using the MIANALYZE procedure. Model results (β) indicate the effect a one-unit change in ln pesticide (metabolite or cumulative) exposure would have on the ln oxidative stress outcome. All statistical analyses were conducted in SAS version 9.3 (SAS Institute, Cary, NC).

We planned several sensitivity analyses to better understand the effect of pesticide exposures on oxidative stress markers. We excluded samples with creatinine levels outside of the World Health Organization's normal range ($n = 19$, 8%) (WHO 1996). For the two individuals who participated in both study years, we included the second study year for these individuals instead of the first. Five individuals reported a history of cancer; we performed analyses excluding these individuals. We ran models restricted to individuals with urinary pesticide values above the LOD. We extensively adjusted for exposure to other pesticide active ingredients using urinary metabolites (atrazine, 2,4-D, chlorpyrifos,

TABLE I. Sample Characteristics Measured at Baseline (T1) for Farmers ($n = 30$) and Controls ($n = 10$)

	Total Mean (SD) or N (%)	Farmers Mean (SD) or N (%)	Controls Mean (SD) or N (%)
Sociodemographic and behavioral characteristics			
Age (years)	51.0 (6.1)	51.2 (6.2)	50.2 (5.9)
Body mass index			
<25	9 (23%)	8 (27%)	1 (10%)
25–29.9	15 (38%)	10 (33%)	5 (50%)
≥30	16 (40%)	12 (4%)	4 (40%)
Smoking history			
Never smoker	26 (65%)	22 (73%)	4 (40%)
Former smoker, <10 years	7 (18%)	4 (13%)	3 (30%)
Former smoker, ≥10 years	7 (18%)	4 (13%)	3 (30%)
Cigarettes/day	23.8 (18.9)	30.3 (24.0)	16.0 (5.5)
Alcohol use			
Non-drinker	15 (38%)	12 (40%)	3 (30%)
<1 drink/day on average	19 (48%)	15 (50%)	4 (40%)
≥1 drink/day on average	6 (15%)	3 (10%)	3 (30%)
Regular physical activity			
None	17 (43%)	14 (47%)	3 (30%)
Moderate	12 (30%)	8 (27%)	4 (40%)
Vigorous	11 (28%)	8 (27%)	3 (30%)
Used vitamin supplements ≥3 times in past 2 months	20 (50%)	14 (47%)	6 (60%)
Health characteristics			
Cold, flu, or other infection (past 2 months)	21 (53%)	15 (50%)	6 (60%)
History of cancer	5 (13%)	3 (10%)	2 (20%)
Pesticide use history			
Ever used atrazine		30 (100%)	1 (10%)
Years mixed or applied		23.9 (7.2)	4.0 (NA)
Applied pesticides to home or lawn in past year		18 (60%)	8 (80%)
Farm activities in past year			
Acres of corn planted		620.9 (257.3)	
Acres treated with atrazine		564.2 (303.4)	
Applied atrazine		30 (100%)	
Applied 2,4-D		27 (90%)	

acetochlor, malathion, parathion) and self-reported exposure information where urinary markers were unavailable (glyphosate, dicamba).

RESULTS

This analysis included 30 farmers and 10 non-farming controls with a total of 225 urine samples. In general, farmers and controls were similar in age, and reported similar rates of vitamin supplementation, alcohol use, physical activity, recent cold, flu or infection, and cancer history (Table I). Controls were more likely to be overweight or obese, and were more likely to be former smokers. However, farmers who were former smokers reported smoking more cigarettes per day compared with controls who were former smokers. At baseline, only one control reported prior use of atrazine in their lifetime; however, 80% of controls reported application of other pesticides to the home or lawn in the past year.

Urinary levels of atrazine mercapturate varied significantly by study time point among farmers (Fig. 1). Levels peaked during planting (T2) and the early growing period (T3), and were lower at enrollment (T1), late growing period (T4), harvest (T5), and off-season (T6). Atrazine mercapturate was detected in only three control urine samples (two different study participants) at low levels. Among farmers, urinary 2,4-D similarly varied significantly by study time point. 2,4-D was highest during growing (T3, T4) and planting (T2), and lower at preplanting (T1), harvest (T5), and off-season (T6). Though 2,4-D exposure appeared to peak during early growing period (T3), urinary 2,4-D metabolite levels at this time point did not differ significantly from levels during the planting or later growing period (T2, T4) (results not shown). 47% ($n = 23$) of control samples had detectable 2,4-D in their urine, though levels were generally low and did not vary by study time point.

For farmers and controls, MDA, 8-OHdG, and 8-isoPGF did not vary significantly by study time point (Fig. 2). ICCs from the unconditional variance LME models for MDA, 8-OHdG, and 8-isoPGF suggest that 35%, 16%, and 29% of the variation (respectively) in these markers was due to between-individual differences. The

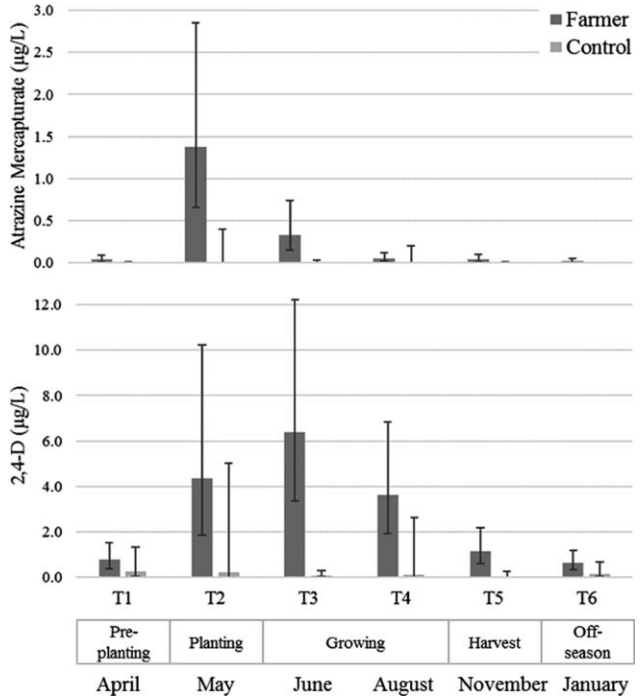

Fig. 1. Geometric mean levels and 95% confidence intervals for atrazine mercapturate and 2,4-D urinary metabolites (µg/L) measured at each study time point, for controls and farmers.

three markers were weakly to moderately positively correlated, ranging from Spearman $\rho = 0.36$ (MDA and 8-isoPGF) to 0.55 (MDA and 8-OHdG) among all study subjects (results not shown). Results for multivariate LME models showed that higher levels of 2,4-D were associated with significantly increased 8-OHdG ($\beta =$ 0.066, 95% CI = 0.008, 0.124) and 8-isoPGF ($\beta = 0.088$, 95% CI = 0.004, 0.172) (Table II). There was no association observed between 2,4-D and MDA, or atrazine mercapturate with any oxidative stress marker. We saw no association between cumulative atrazine or cumulative 2,4-D exposure with any oxidative stress outcome (Supporting Information Table SI).

Excluding observations with extreme creatinine values ($n = 19$), the associations for 2,4-D with 8-OHdG and 8-isoPGF were of the same magnitude and direction. For the two farmers who participated in both study years, when we included the second year of observations instead of the first, the associations for 2,4-D with 8-OHdG and 8-isoPGF were of similar magnitude and direction, though the confidence intervals differed slightly. The positive findings for 2,4-D with 8-OHdG and 8-isoPGF were strengthened after excluding individuals with a prior cancer diagnosis ($n = 5$). When restricting to samples with pesticide markers above the LOD, the significant association with 2,4-D and 8-OHdG remained ($n = 182$), the association with 2,4-D and 8-iso-PGF became non-significant ($n = 181$), and we additionally noted a significant association with atrazine

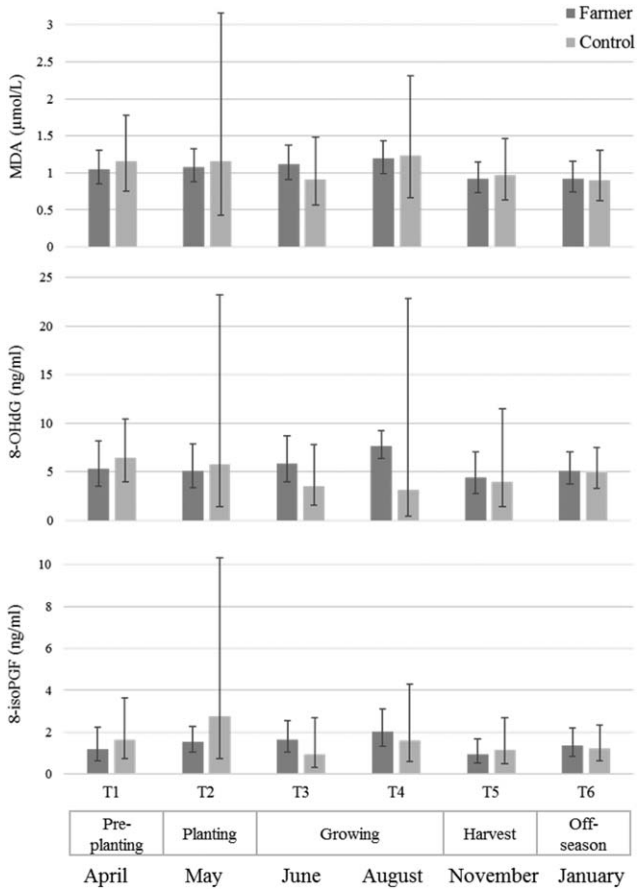

Fig. 2. Geometric mean levels and 95% confidence intervals for MDA (µmol/L), 8-OHdG (ng/ml), and 8-isoPGF (ng/ml) urinary metabolites measured at each study time point, for controls and farmers.

mercapturate and 8-OHdG ($n = 76$). Adjustment for exposure to other commonly used pesticides did not substantially impact our results.

TABLE II. Results from Multivariable Linear Mixed-Effect Models Examining the Impact of Natural Log Transformed Atrazine Mercapturate and 2,4-D in Urine on Changes in Natural Log Transformed Oxidative Stress Markers MDA, 8-OHdG, and 8-isoPGF

Outcome	Pesticide	β	95% CI
MDA[a]	Atrazine	−0.002	−0.028, 0.025
	2,4-D	0.002	−0.032, 0.035
8-OHdG[b]	Atrazine	0.032	−0.021, 0.084
	2,4-D	**0.066**	**0.008, 0.124**
8-isoPGF[c]	Atrazine	0.035	−0.033, 0.103
	2,4-D	**0.088**	**0.004, 0.172**

[a]Adjusted for age, ln(creatinine), study time point, farmer/control, vitamin use.
[b]Adjusted for age, ln(creatinine), study time point, farmer/control, vitamin use, physical activity.
[c]Adjusted for age, ln(creatinine), study time point, farmer/control, infection at blood draw.
Bold ($P < 0.05$).

DISCUSSION

To our knowledge, this is the first study to examine the association between the herbicides atrazine and 2,4-D and markers of oxidative stress in humans. Using a multivariate linear mixed-effect modeling approach, we observed associations between the herbicide 2,4-D and 8-OHdG, a biomarker of oxidative DNA damage, and 8-isoPGF, a product of non-enzymatic lipoprotein peroxidation. These associations were based on a small number of exposed individuals, with repeated samples, and were moderately robust in sensitivity analyses. There was no association for either urinary pesticide marker with MDA, a marker of lipid peroxidation. When restricted to samples with pesticide metabolite measures above the LOD, we noted an association between atrazine mercapturate and 8-OHdG; otherwise, no association with atrazine mercapturate was observed.

2,4-D is a phenoxy herbicide used for selective control of broadleaf weeds. It has been suggested that a mechanism of 2,4-D herbicidal activity may be creation of ROS and oxidative stress in target plants (Romero-Puertas et al. 2004). The toxicity of 2,4-D and creation of ROS are often attributed to the free-acid form of the pesticide (Munro et al. 1992). 2,4-D has been approved for use in the US since 1948, and is consistently ranked among the most commonly used pesticides for the agricultural, home and garden, and industry/commercial/government market sectors (EPA 2005; Grube et al. 2011). Non-occupational exposure to 2,4-D is relatively common; in a biomonitoring study of US adults and children reporting no pesticide application, almost one-quarter had detectable 2,4-D in their urine (Baker et al. 2000). The US Environmental Protection Agency (EPA) considers 2,4-D not classifiable (group D) with regard to carcinogenicity (EPA 2005). However, in 2015 the International Agency for Research on Cancer (IARC) classified 2,4-D as a possible human carcinogen (Group 2B) (IARC 2015). Though the epidemiologic literature was limited, the IARC working group found that mechanistic studies provided strong evidence that 2,4-D causes oxidative stress via a mechanism that can operate in humans (Loomis et al. 2015).

Our results demonstrated that higher levels of 2,4-D excreted in urine are associated with increased 8-OHdG, an oxidative modification of DNA. The effect of 2,4-D on oxidative stress in our study was relatively modest; a five-fold increase in urinary 2,4-D was associated with an 11% increase in 8-OHdG and a 14% increase in 8-isoPGF. This observed association in our study between 2,4-D and oxidative stress in humans is supported by findings in laboratory animals (Tayeb et al. 2012; Pochettino et al. 2013; Tayeb et al. 2013). 2,4-D treatment of rats at subacute levels has been associated with increased levels of oxidative stress at various organ sites, including the mammary gland, ovaries, ventral prostate, and liver (Tayeb et al. 2012; Pochettino et al. 2013; Tayeb et al. 2013). Total length of exposure (i.e., 45 days vs. 90 days) was not associated with higher oxidative stress (Pochettino et al. 2013). Animal studies differ from our approach in that they measured oxidative stress at specific organ sites of interest, while our study examined urinary measures.

Atrazine is a chlorotriazine herbicide used in the US against broadleaf and grassy weeds since 1958 (EPA 2006). Use of atrazine has been banned in the European Union since 2004, although it is still registered for use in the US where it is one of the most widely used agricultural herbicides (European Commission 2004; EPA 2006). Atrazine is considered not-classifiable with respect to its carcinogenicity by IARC and the US EPA (EPA 2007; IARC 2015). In our analysis, we observed no association between atrazine mercapturate and MDA, 8-OHdG, or 8-isoPGF. Laboratory studies in animals have shown associations between atrazine and oxidative stress (Blahova et al. 2013; Abarikwu 2014; Jin et al. 2014; Zhao et al. 2014); however, increased oxidative stress was seen only at very high, acutely toxic doses of atrazine, unlike the studies of 2,4-D where responses were seen also at low doses. Atrazine mercapturate was associated with 8-OHdG when we restricted our analysis to individuals with atrazine mercapturate values >LOD, but otherwise did not see any positive associations between atrazine and oxidative stress. It is possible that our imputation is misclassifying those with very low atrazine exposure; additional studies with larger sample sizes and multiple atrazine metabolites are needed to confirm this finding.

We characterized pesticide exposure using urinary metabolites, which, for the target pesticides, reflect recent exposure due to their short half-lives (CDC 2013a,b). This corresponds with the measured oxidative stress outcomes of interest, which are thought to reflect responses to recent environmental stressors. We conducted secondary analyses to examine whether cumulative pesticide exposure over the season would be an informative exposure metric. We observed no significant association between cumulative atrazine or 2,4-D exposure during the study period and any marker of oxidative stress. Compared with cumulative pesticide exposure, urinary metabolite markers provided superior model fit for these transient markers.

The longitudinal design is a major strength of our study. We collected multiple measurements for each individual both pre- and post- agricultural application, and additionally at scheduled times during the growing season when high (e.g., planting) or low (e.g., off-season) pesticide exposure was expected. Though exposure patterns varied during the study period for atrazine and 2,4-D, we do not expect that the timing of exposure would appreciably impact the associations with oxidative stress. Because our baseline (T1) urine collection occurred before planting, each individual served as his own control. As such, we have no reason to believe that unmeasured potential confounders that can vary between participants, such as diet, DNA excision

repair capacity, or other health-related factors such as cholesterol would appreciably impact our results. We might expect that some potential confounders vary between time points, most notably other pesticide exposures. To address this, we controlled for use of other pesticides commonly used in our study population, and found that adjustment for other pesticide exposures, such as glyphosate, did not impact the results. Although the total number of participants was small, collecting up to six samples per participant increased our power to detect an association. Time point was included as a smoothed continuous variable in our models to account for correlation between more closely-spaced samples. Sex and current smoking habits are important predictors of oxidative stress (Block et al. 2002); study participation here was restricted to non-smoking men thus eliminating potential confounding by these factors. The reliability of our oxidative stress assays is a limitation, as we noted CVs for blinded duplicates >20%, when ideally we would like to see values <15%. However, we calculated ICCs >80% for 8-OHdG and 8-isoPGF, indicating that although the laboratory reproducibility was lower than anticipated, much of the variability in these markers is explained by oxidative stress differences among observations. Sample collection procedures were another strength of this study; urine samples were collected by study personnel at field visits, and kept on ice packs (4°C) during transport to the University of Iowa for long-term storage (−80°C) (Bakke et al. 2009). In addition, these markers are stable even at variable storage conditions (Pratico et al. 1998; Lee and Kang 2008; Matsumoto et al. 2008). Urinary creatinine has been shown to be stable or decrease only slightly at storage temperatures below −20°C (Berg et al. 1998; Schultz et al. 2000; Garde et al. 2003), though these studies have only examined storage times under 1 year. We utilized urinary metabolites to assess exposure, which minimizes exposure misclassification due to recall or reporting bias. However, because values for 2,4-D and atrazine mercapturate were imputed for a number of samples below the LOD, we may have misclassification among these lowest exposed individuals, and additionally limited ability to detect an association, particularly for atrazine. Atrazine mercapturate, one of several urinary atrazine metabolites that may be measured to assess exposure, is widely used in epidemiologic studies; to comprehensively assess both environmental and occupational atrazine exposures, examination of multiple atrazine metabolites may provide more resolution at lower levels of exposure (Panuwet et al. 2008).

In our longitudinal biomarker study of farmers and controls, higher levels of urinary 2,4-D was associated with modest increases in 8-OHdG, a marker of oxidative DNA damage, and 8-isoPGF, a marker of non-enzymatic lipoprotein peroxidation. We only found limited evidence for an association between atrazine exposure and oxidative DNA damage (8-OHdG), when the analysis was restricted to the 76 samples with detectable levels of atrazine mercapturate. We believe this to be the first epidemiologic study to examine the association between exposure to atrazine and 2,4-D with three separate oxidative stress outcomes. Future epidemiologic studies should attempt to replicate these findings in novel study populations. Based on these results, as well as supporting evidence from laboratory animals, 2,4-D-induced oxidative stress may be important in the pathogenesis of cancer and other chronic disease outcomes.

AUTHOR CONTRIBUTIONS

Drs. Vermeulen, De Roos, Lynch, and Blair were involved in the study design and conception. Drs. Kang and Lee performed the laboratory analyses for the oxidative stress markers. Dr. Portengen consulted on the statistical methodology. Dr. Bakke provided expertise in exposure assessment. Dr. Lerro analyzed the data and prepared draft figures and tables. Dr. Lerro prepared the manuscript draft with important intellectual input from Drs. Beane Freeman and Vermeulen. All authors approved the final manuscript.

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