ORIGINAL RESEARCH

2,4-D exposure and urinary markers of oxidative DNA damage and lipid peroxidation: a longitudinal study

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ABSTRACT

Objective 2,4-Dichlorophenoxyacetic acid (2,4-D) is a herbicide that is commonly used commercially, agriculturally and residentially worldwide. There is concern about its potential for carcinogenicity based on studies in laboratory animals demonstrating the potential for induction of oxidative stress. We conducted a longitudinal biomarker study of 31 pesticide applicators in Kansas who heavily applied 2,4-D and 34 non-applicator controls.

Methods We used multivariable generalised linear mixed-effect models to evaluate the association between urinary 2,4-D and natural log-transformed 8-iso prostaglandin $F_{2\alpha}$ (8-isoprostane) and 8-hydroxy-2′-deoxyguanosine (8-OHdG), adjusting for urinary creatinine, age, tobacco use and concomitant use of the herbicide picloram.

Results Compared with non-applicator controls, urinary 2,4-D in the third quartile of exposure was associated with elevated 8-isoprostane (e^{β}=1.38, 95% CI 1.03 to 1.84). There was no association among the highest exposed and no exposure-response trend. 2,4-D exposure was not associated with 8-OHdG. Results were unchanged when restricted to participants who only applied 2,4-D (no picloram use).

Conclusions We did not find evidence that increasing 2,4-D exposure was associated with 8-isoprostane or 8-OHdG. Future work should carefully evaluate potential confounders of this association, such as diet and physical activity, as well as additional biological markers of oxidative stress and damage.

Key messages

What is already known?

► 2,4-Dichlorophenoxyacetic acid (2,4-D) is among the most commonly used herbicides in USA in both agricultural and residential settings; there has been concern about its carcinogenicity based on induction of oxidative stress in laboratory animals and some epidemiologic evidence.

What are the new findings?

► In a longitudinal biomarker study, we did not observe an association between increasing 2,4-D exposure and urinary 8-iso prostaglandin $F_{2\alpha}$ (a marker of lipid peroxidation) or 8-hydroxy-2'-deoxyguanosine (a marker of oxidative DNA damage).

How might this impact policy or clinical practice in the foreseeable future?

► There is limited evidence from this study to suggest an association between 2,4-D and oxidative stress, however, given the ubiquity of 2,4-D exposure, careful monitoring of exposure and effects is warranted.

BACKGROUND

2,4-Dichlorophenoxyacetic acid (2,4-D) is a phenoxy herbicide used for selective control of broadleaf weeds. It has been approved for use in USA since 1948, and is still one of the most commonly used pesticides.[1 2] In 2012, an estimated 13.6–18.1 million kilograms of 2,4-D were used in US agriculture.[1] In addition to agricultural applications, 2,4-D is licensed for residential use in USA; thus, there is potential for broad exposure in the general population. Among a sample of children and adults from the National Health and Nutrition Examination Survey, nearly a quarter had 2,4-D metabolites in their urine.[3] 2,4-D may be applied alone, or as a product mixture in combination with other pesticides.

Epidemiologic studies have examined the association between 2,4-D exposure and various cancer outcomes. Early studies found increased risk of non-Hodgkin's lymphoma among persons exposed to 2,4-D;[4–7] however, more recent analyses have observed elevated but non-significant associations.[8 9] A large case-control study in Canada found that 2,4-D was associated with increased risk of prostate cancer,[10] but this finding was not supported by data from two prospective cohort studies.[8 11]

The International Agency for Research on Cancer recently classified 2,4-D as a possible human carcinogen (2B), based on limited epidemiologic evidence and animal models that demonstrated consistent and strong associations between 2,4-D and increased oxidative stress.[12] A recent longitudinal study of pesticide applicators and non-applicator controls noted modest elevations in urinary markers of oxidative stress shortly after 2,4-D exposure.[13] Oxidative stress occurs when the presence of free radicals overwhelm an organism's antioxidants and DNA repair mechanisms.[14] These reactive oxygen or nitrogen species, created endogenously as a part of natural cellular processes or introduced through environmental or lifestyle factors, may cause oxidative stress, resulting in damage to DNA, proteins and lipids.[14] Oxidative stress potentially affects a

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variety of cellular processes, and consequently may be associated with the pathogenesis of many human diseases, including cancer.[15]

To better understand whether 2,4-D may induce oxidative stress in humans, we conducted a longitudinal biomarker study of pesticide applicators in Kansas. We evaluated two non-specific urinary markers of oxidative stress that are widely used in epidemiologic studies. 8-iso prostaglandin $F_{2\alpha}$ (8-isoprostane), a prostaglandin-like compound, is produced by non-enzymatic peroxidation of arachidonic acid in phospholipid membranes.[16] 8-hydroxy-2'-deoxyguanosine (8-OHdG) is a promutagenic lesion of DNA and marker of oxidative injury generated in response to reactive oxygen species.[17]

METHODS

Study design

The Kansas County Noxious Weed Applicator Study is a longitudinal biomarker study conducted during 1994–1995, and designed specifically to evaluate the biological effects of 2,4-D exposure.[18 19] The banked urine specimens from this highly exposed study population provide a unique resource to evaluate new hypotheses regarding 2.4-D and oxidative stress and damage. Pesticide applicators (n=31) were recruited from Kansas county noxious weed offices, charged with controlling troublesome agricultural weeds on public and private land. To be included, applicators had to be cancer-free at enrolment. Applicators applied only herbicides (predominantly 2,4-D), and during the application season typically sprayed daily. Each participant completed a baseline in-person interview regarding prior pesticide exposure and various lifestyle and other environmental factors. They were followed between April and August for approximately 12 weeks, or until 2,4-D use was discontinued for the application season. Five applicators participated in both study years (1994 and 1995). Six applicators participated in 1994 and gave an additional baseline interview in 1995. During the application season, each applicator kept a diary to record his or her daily pesticide exposure.

Non-applicator controls were recruited using newspaper advertisements and word of mouth in the same counties from which applicators were recruited. Prospective participants with a history of cancer or other disease that might interfere with laboratory assays were excluded, as were those taking prescribed medications. Of the eligible controls, n=34 were matched to applicators by gender, 5-year age group, alcohol and tobacco use, and geography. Non-applicator control baseline interviews were conducted throughout the applicator study period.

Spot urine samples were collected at baseline for both applicators and controls, and overnight (12 hours) urine samples were collected every 2 weeks after a typical day of 2,4-D use for applicators (136 samples total). Each applicator gave three to six samples (median = five) during a single application season. Urine collection and storage procedures have been described in detail.[18] Samples were processed the morning of collection and shipped in glass vials on dry ice to the University of Kansas Medical Center. All urine samples were stored at −80 °C.

Exposure

Urinary 2,4-D was analysed using high performance liquid chromatography and tandem mass spectrometry with isotope dilution quantification.[20] At baseline, 2,4-D was measured for a random sample of applicators and controls and not detected in any sample evaluated.[18 21] In our analyses, 2,4-D was classified as baseline controls (referent), baseline applicators and quartiles of urinary 2,4-D among applicators (Q1: 0.47–17.31 µg/L, Q2: 17.32–81.98 µg/L, Q3: 81.99–283.87 µg/L, Q4: >283.87 µg/L).

Oxidative stress

8-Isoprostane and 8-OHdG were quantified in urine using acetylcholinesterase competitive ELISA kits from Cayman Chemical (Ann Arbor, Michigan, item numbers 516351 and 501130, respectively). For 8-isoprostane, samples were first purified by C-18 solid phase extraction. For both assays, if necessary, samples were diluted to fall within the linear range of the standard curve. Samples were run in triplicate on 96-well plates, with blinded duplicates interspersed for quality control. Plates were incubated for 90 min and read using a Spectramax 340PC384 at 414 nm. The concentration of each sample was calculated from a logistic four-parameter fit of the standard concentrations versus % bound/maximum bound.

Coefficients of variation (CV) and intraclass correlation coefficients (ICC) were calculated for 30 blinded duplicate samples to analyse assay reproducibility. For 8-isoprostane and 8-OHdG, CVs were 11.0% and 9.1%, respectively, and ICCs were 74.2% and 92.8%, respectively.

Creatinine

Creatinine was quantified in urine using a colorimetric assay kit from Cayman Chemical (Ann Arbor, Michigan, item number 500701). This assay relies on the Jaffe reaction, where a yellow/orange colour forms when creatinine reacts with alkaline picrate. Plates were incubated for 90 min and read using a Spectramax 340PC384 at 490 nm.

Detailed laboratory methods for 8-isoprostane, 8-OHdG and creatinine are described in the online supplementary material.

Statistical analyses

We included in our analysis 31 pesticide applicators and 34 controls with a total of 214 urine samples. We used generalised linear mixed-effect models to evaluate the association between 2,4-D with natural log-transformed 8-isoprostane and 8-OHdG. Effect estimates were expressed as the exponentiated model parameter estimates (β), indicating that exposure in a given category is the expected geometric mean of that category divided by that of controls (referent). Models were adjusted for tobacco use (ever/never, including cigarettes, cigars and oral tobacco), age at study enrolment, urinary creatinine,[22] and recent picloram use (last 5 days). In our study, picloram was the next most frequently applied herbicide during the study period and was typically applied as a mixture with 2,4-D. We considered adjustment for additional covariates including physical activity, detailed tobacco smoking history (pack-years), time in days since enrolment, alcohol use and education; however, these covariates did not alter the associations for 2,4-D use and were not included in the final models. Models included a random intercept with urinary creatinine included as a random effect, and an unstructured covariance matrix. As a sensitivity analysis, if applicators participated in both study years we included only the first year. For the main analysis, exposure was classified as controls (referent), baseline applicators and quartiles of urinary 2,4-D among applicators. We also considered different referent categories, such as the baseline sample from applicators and the lowest quartile of urinary 2,4-D levels among applicators. In analyses restricted to applicators (single baseline category), we conducted tests for trend by including the median of each 2,4-D exposure category as a continuous variable. We performed analyses excluding samples with extreme creatinine values, excluding observations

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with recent picloram use (last 5 days), and restricting to non-Hispanic white men. Analyses were performed in SAS V.9.4 (Cary, North Carolina, USA). Figures were created using ggplot2 in R (V.3.4.3). All tests were two-sided with $\alpha=0.05$.

RESULTS

Pesticide applicators and controls were similar with respect to important demographic and behavioural characteristics including age, sex, race/ethnicity, tobacco current/former use and physical activity (table 1). Pesticide applicators were slightly more likely to be oral tobacco users, reported greater pack-years of cigarettes and were more likely to drink at least one alcoholic beverage per day, but these differences were not statistically significant. Self-reported herbicide and insecticide use prior to study enrolment was more common among pesticide applicators than non-applicator controls. During the study period, on average applicators applied 2,4-D for 23 days (range: 7–46) and picloram for 7 days (range: 0–45).

In general, once 2,4-D application began, urinary exposure fluctuated somewhat within an individual with no obvious trends over time (figure 1). The range of measured urinary 2,4-D was wide (online supplementary figure 1), with an IQR of 17.31–283.87 µg/L. 8-Isoprostane and 8-OHdG at baseline were higher among controls compared with applicators (figure 2). Compared with applicator baseline, oxidative stress markers were higher at the study time point with highest 2,4-D exposure.

We found no clear association between increasing 2,4-D use and 8-isoprostane or 8-OHdG (table 2). Compared with non-applicator controls, 2,4-D exposure in the third quartile was associated with elevated 8-isoprostane ($e^{\beta}=1.38$, 95% CI 1.03 to 1.84); however, there was no significant association for the fourth quartile of exposure ($e^{\beta}=1.14$, 95% CI 0.85 to 1.55). 2,4-D exposure was not associated with 8-OHdG compared with controls. Models were adjusted for potential confounders including creatinine, age, year of study enrolment, tobacco use and use of the herbicide picloram. Urinary creatinine was positively associated with both 8-isoprostane and 8-OHdG. Ever tobacco use was positively associated with 8-isoprostane and inversely associated with 8-OHdG. There was little evidence for an association with age, study year or picloram with either oxidative stress marker. Model results were robust to sensitivity analyses, including specifying baseline applicators as the referent category, excluding samples with creatinine values outside of the normal range, excluding samples with recent self-reported picloram use, and restricting to non-Hispanic white men (online supplementary table 1). Among applicators with measured 2,4-D exposure (excluding all baseline samples), 8-isoprostane was significantly elevated at all levels compared with the lowest exposure quartile (Q2: $e^{\beta}=1.48$, Q3: $e^{\beta}=1.82$, Q4: $e^{\beta}=1.55$) with no evidence of an exposure-response trend ($p=0.51$).

Table 1 Descriptive characteristics stratified by pesticide applicators and non-applicator controls

	Controls	Applicators	
	n=34	n=31	
	Mean (SD)	Mean (SD)	
Age at enrolment/baseline sample	33.6 (13.5)	32.8 (13.8)	
2,4-D (days)*	--	23.0 (9.1)	
Picloram (days)*	--	7.4 (12.9)	
	N (%)	N (%)	P value†
Sex			
Male	32 (94.1)	29 (93.5)	0.92
Female	2 (5.9)	2 (6.5)	
Race/ethnicity			
White	33 (97.1)	31 (100)	0.34
Hispanic	1 (2.9)	0 (0)	
Enrolment year			
1994	9 (26.47)	12 (38.71)	0.43
1995	25 (73.53)	19 (61.29)	
Tobacco use			
Never	19 (55.9)	16 (51.6)	0.88
Former	8 (23.5)	7 (22.6)	
Current	7 (20.6)	8 (25.8)	
Pack-years smoked			
Never smoker	23 (67.6)	21 (67.7)	0.30
≤15 pack-years	7 (20.6)	3 (9.7)	
>15 pack-years	4 (11.8)	7 (22.6)	
Oral tobacco user			
No	26 (76.5)	21 (67.7)	0.43
Yes	8 (23.5)	10 (32.3)	
Alcoholic drinks			
Never drinker	9 (26.5)	8 (25.8)	0.36
≤1 drink/day	16 (47.1)	10 (32.3)	
>1 drink/day	9 (26.5)	13 (41.9)	
Physical activity			
None/low	4 (11.8)	4 (13.3)	0.85
Moderate/vigorous	30 (88.2)	26 (86.7)	
Pesticide use more than 6 months prior to study enrolment‡			
Any pesticides	9 (26.5)	27 (87.1)	<0.01
Herbicides			
Atrazine	0 (0)	9 (29)	<0.01
Dicamba	0 (0)	14 (45.2)	<0.01
Glyphosate	3 (8.8)	24 (77.4)	<0.01
2,4-D	1 (2.9)	24 (77.4)	<0.01
Kerosene/oil	4 (11.8)	5 (16.1)	0.61
Insecticides			
Carbofuran	0 (0)	5 (16.1)	0.02
Malathion	0 (0)	6 (19.4)	<0.01
Carbaryl	3 (8.8)	6 (19.4)	0.22

*Average days applied pesticide during the study period. If applicator participated in both study years, included year 1 only.
†χ^2 test for homogeneity.
‡Self-reported personal use of pesticides 6 months prior to study enrolment or earlier (applicators) or ever (non-applicators).
2,4-D, 2,4-dichlorophenoxyacetic acid.

DISCUSSION

In a longitudinal study of Kansas pesticide applicators regularly applying 2,4-D over a growing season and non-applicator controls, we did not observe a clear association between 2,4-D exposure and oxidative stress. The potentially toxic effects of 2,4-D, including induction of oxidative stress, is attributed to the free-acid form.[23] 2,4-D is not considered to be genotoxic;[23] however, in epidemiologic studies it has been associated with telomere shortening, micronucleus formation and lymphocyte replicative proliferation.[18 21 24 25] 2,4-D exposure has been demonstrated to cause oxidative tissue damage in vivo.[12] Additionally, a longitudinal epidemiologic study of 30 Iowa corn farmers and 10 non-farming controls found that urinary 2,4-D (modelled continuously) was associated with modest elevations in 8-OHdG and 8-isoprostane.[13] Here in KS applicators, we observed increased 8-isoprostane (third quartile of 2,4-D exposure), but not in the fourth quartile and no evidence of a monotonic trend. Our findings did not replicate the earlier study,

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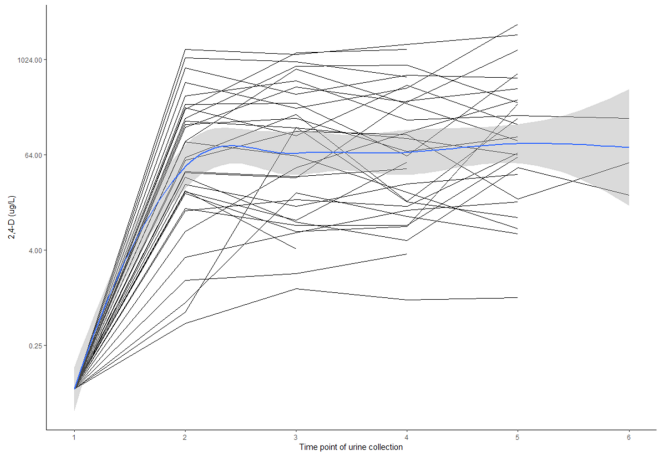


1 Categories of exposure include controls at baseline, applicators at baseline, applicators at the time of lowest measured urinary 2,4-D, and applicators at the time of highest measured urinary 2,4-D

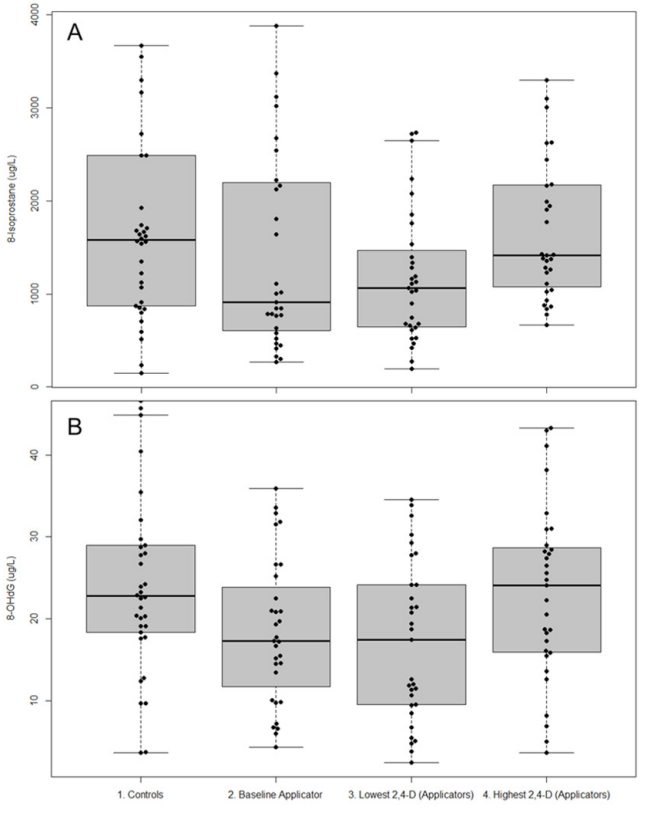
Figure 1 Spaghetti plot displaying applicator urinary 2,4-dichlorophenoxyacetic acid (2,4-D, ug/L, on the log scale) over the study period; three to six time points for each pesticide applicator[1]

despite evaluating the same urinary oxidative stress markers in applicators with measured urinary levels of 2,4-D much higher than those reported in Iowa corn farmers.[13] For example, the lowest quartile of exposure in the KS weed applicators encompassed the entire range of 2,4-D exposures reported in the Iowa corn farmers. One reason for the difference in the results could be the age difference in the two study populations; the mean age in the Iowa study is 51 years, almost 20 years older than this analysis.[13] In the present study, 2,4-D was not measured at baseline for controls or applicators. Baseline exposures were assumed to be very low based on study eligibility criteria, and the exposure contrast was assumed to be large due to the frequent, intensive 2,4-D use reported during the study period. Based on measured urinary 2,4-D levels in the general population, it is possible that baseline applicators and controls had some 2,4-D exposure, but the levels were likely very low.[12] A unique feature of this study was that many applicators applied 2,4-D exclusively (n=20), eliminating potential confounding due to concomitant exposure to other pesticides. Controlling for and excluding concurrent users of picloram, a herbicide occasionally applied as a mixture with 2,4-D, did not impact the effect estimates for 2,4-D.

Table 2 Multivariable generalised linear mixed model results evaluating the association between 2,4-D exposure and natural log-transformed markers of oxidative stress

		8-Isoprostane		8-OHdG	
	N	$e^{\beta 1}$	95% CI*	$e^{\beta 1}$	95% CI*
Urinary 2,4-D					
Non-applicator controls	34	1.00	referent	1.00	referent
Baseline applicators	42	1.07	0.82 to 1.41	1.03	0.90 to 1.17
≤17.31 µg/L	35	0.96	0.71 to 1.31	0.86	0.74 to 1.00
17.32–81.98 µg/L	34	1.25	0.93 to 1.67	0.92	0.79 to 1.06
81.99–283.87 µg/L	35	1.38	1.03 to 1.84	1.09	0.95 to 1.25
>283.87 µg/L	34	1.14	0.85 to 1.55	1.05	0.91 to 1.21
10 mg/dL urinary creatinine	214	1.06	1.05 to 1.07	1.06	1.05 to 1.06
Age (5 years)	214	1.01	0.96 to 1.06	1.02	1.00 to 1.04
Ever tobacco use					
No	111	1.00	referent	1.00	referent
Yes	113	1.22	0.95 to 1.57	0.86	0.78 to 0.96
Study year enrolled					
1994	70	0.89	0.75 to 1.06	0.92	0.83 to 1.02
1995	144	1.00	referent	1.00	referent
Picloram use last 5 days					
No	182	1.00	referent	1.00	referent
Yes	32	0.93	0.74 to 1.17	1.08	0.95 to 1.23

*Exponentiated model parameter estimate (β) represents the ratio of marker geometric mean for each category compared with the referent.
2,4-D, 2,4-dichlorophenoxyacetic acid; 8-isoprostane, 8-iso prostaglandin F2α; 8-OHdG, 8-hydroxy-2'-deoxyguanosine.



1 2,4-D was not measured at enrollment, applicators and controls were assumed to have the same nominal level.

Figure 2 Beeswarm box plot of urinary 8-iso prostaglandin $F_{2\alpha}$ (8-isoprostane) (A) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) (B) by categories of 2,4-dichlorophenoxyacetic acid (2,4-D) exposure[1]

Urine sample collection and storage procedures were an important strength of this study. Efforts were made to ensure participants were trained in self-collection procedures. Study personnel transported and processed samples quickly while maintaining cool temperatures to ensure low potential for bacterial contamination. The age of the samples is a potential limitation, as studies have not evaluated whether lengthy storage times may impact the stability of urinary markers of oxidative stress. However, literature suggests that 8-OHdG and 8-isoprostane are stable even at variable storage conditions.[26 27] Urinary creatinine is stable or may decrease in samples stored below −20°C,[28–30] though these studies have examined storage times up to 1 year. Of note, we are evaluating relative effect estimates in our analysis and do not believe that spurious formation of oxidation products would differentially impact the samples of exposed versus unexposed applicators. However, spurious formation of oxidation products would result in random measurement error and may impact our power to detect an association. A limitation of our study is lack of detailed information on a few potentially

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important confounders throughout the study period. We were able to control for certain baseline characteristics such as self-reported tobacco use, age, usual physical activity and body mass index. However, we were unable to control for certain potential confounders at each time point that may vary throughout the study period, such as recent tobacco use, physical activity and diet including vitamin use. For example, regular physical activity has been associated with increased endogenous antioxidant activity and lower oxidative stress.[31] If applicators with the highest exposure were also the most physically active, this could explain the absence of elevated oxidative stress among the highest exposed, despite significant elevation in the third quartile of exposure. Though we had a relatively small sample size, repeated measures provided additional power to evaluate associations between 2,4-D and these urinary markers of oxidative stress. An additional limitation of our study is that the urine collection protocol differed slightly at baseline and during pesticide application. At baseline, applicators provided a spot urine sample, while during application they provided overnight samples. We modelled our results including only overnight samples collected during pesticide application, comparing each quartile of urinary 2,4-D to the lowest; trends were similar to the main analysis, though the effect estimates were larger (online supplementary table 1).

CONCLUSIONS

Comparing pesticide applicators with very high occupational 2,4-D exposure to unexposed controls, we did not find evidence that increasing 2,4-D was associated with markers of urinary oxidative stress. Future work in this area should carefully consider diet and supplement use, physical activity and other behavioural factors as potential confounders, as well as additional biological markers for evaluation of oxidative stress and damage.

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Contributors AB and NR conceived and designed the original study. CCL performed the data analysis and prepared draft figures and tables. CCL prepared the manuscript draft with important intellectual input from LEBF. All authors were involved in writing and editing the manuscript and approved the submitted version. CCL is the guarantor for this work. The corresponding author attests that all listed authors meet authorship criteria and that no others meeting the criteria have been omitted.

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