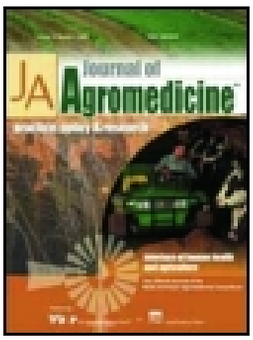

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Oxidative Stress and DNA Damage in Agricultural Workers

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ABSTRACT. Oxidative stress and DNA damage have been proposed as mechanisms linking pesticide exposure to health effects such as cancer and neurological diseases. A pilot study of pesticide applicators and farm workers working in the fruit orchards of Oregon (i.e., apples, pears) was conducted to examine the relationship between organophosphate (OP) pesticide exposure and oxidative stress and DNA damage. Urine samples were analyzed for OP metabolites and 8-hydroxy-2′-deoxyguanosine (8-OH-dG). Lymphocytes were analyzed for oxidative DNA repair activity and DNA damage (Comet assay) and serum analyzed for lipid peroxides (i.e., malondialdehyde [MDA]). Cellular DNA damage in agricultural workers was validated using lymphocyte cell cultures. Urinary OP metabolites were significantly higher in farm workers and applicators ($p < .001$) when compared to controls. 8-OH-dG levels were 8.5 times and 2.3 times higher in farm workers and applicators, respectively, than in controls. Serum MDA levels were 4.9 times and 24 times higher in farm workers and applicators, respectively, than in controls. DNA damage and oxidative DNA repair were significantly greater in lymphocytes from applicators and farm workers when compared with controls. A separate field study showed that DNA damage was also significantly greater ($p < .001$) in buccal cells (i.e., leukocytes) collected from migrant farm workers working with fungicides in the berry crops in Oregon. Markers of oxidative stress (i.e., reactive oxygen species, reduced levels of glutathione) and oxidative DNA damage were also observed in lymphocyte cell cultures treated with an OP. The findings from these in vivo and in vitro studies indicate that pesticides induce oxidative stress and DNA damage in agricultural workers. These biomarkers may be useful for increasing our understanding of the link between pesticides and cancer.

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KEYWORDS. Apurinic/apyrimidinic endonuclease (APE), comet assay, fungicides, glutathione, malondialdehyde (MDA), organophosphate pesticides, oral leukocytes, reactive oxygen species (ROS), 8-hydroxydeoxyguanosine (8-OH-dG)

INTRODUCTION

Multiple studies have reported associations between exposure to agricultural chemicals and various health outcomes including cancer, Parkinson's disease, and other neurological diseases. However, these investigations are often limited by self-reported exposures and do little to advance our understanding of the biological mechanisms linking exposure to pesticides and adverse health outcomes. Oxidative stress is one mechanism that could link pesticide exposures to a number of the health outcomes observed in epidemiological studies.

Oxidative stress is a condition in which there are elevated levels of reactive oxygen species (ROS) caused by overproduction of cellular oxidants. These highly reactive metabolites disrupt cellular metabolism, reduce cellular antioxidant defenses, damage macromolecules (e.g., protein, RNA, DNA) and disrupt the function of DNA repair proteins.[1] A reduction in cellular antioxidants and DNA repair function can lead to the accumulation of oxidative DNA lesions (e.g., 8-hydroxy-2′-deoxyguanosine [8-OH-dG]), which are reportedly reliable biomarkers of oxidative stress.

There is compelling evidence from human and animal studies that pesticides, especially organophosphate pesticides, induce oxidative stress.[2] Increased serum and urinary levels of lipid peroxides and altered blood levels of glutathione (GSH) and antioxidant enzymes have been detected in several cases of pesticide poisoning. Moreover, DNA damage comparable to that seen after oxidative stress has been detected after exposure to pesticides,[3] and may explain, in part, the link between pesticide exposure and cancer.

This paper describes the results of studies designed to assess the relationship between pesticide exposure in agricultural workers and measures of oxidative stress and DNA damage. Results were validated by examining similar biomarkers in lymphocyte cultures treated with a common organophosphate, azinphos methyl (AZM). The overall goal of these studies was to demonstrate that exposure to pesticides induces oxidative stress and DNA damage in agricultural workers.

METHODS

Target Populations

We conducted a pilot study in 2000 of 20 certified pesticide applicators, farm workers, and controls as part of a research study taking place in Oregon agricultural communities.[4–6] Ten nonagricultural adults were recruited from an urban Oregon community. We followed this pilot work with a larger study in 2004 in which we recruited 133 farm workers harvesting Oregon small fruit and berry crops and 56 nonagricultural controls.

Procedures

Questionnaires were administered to characterize work history and job activities. Spot urine samples were collected at the end of the workday. In the 2000 pilot study, we obtained venous blood samples for analysis and in our larger 2004 investigation, we obtained saliva samples for analysis of DNA damage. Methods for isolating lymphocytes are described in Muniz et al.[7] Methods for saliva collection and the extraction of leukocytes have been described in McCauley et al.[8]

Analysis of Urine for OP Metabolites and 8-OH-Deoxyguanosine

Urine samples were analyzed for organophosphate (OP) pesticide metabolites by gas chromatography with pulsed flame photometric detector (GC-PFPD)[4] or concentrated over a C_{18} SFE SepPak cartridge (Waters Associates) as described by Muniz and colleagues,[7] and analyzed for 8-OH-dG by high-performance

liquid chromatography (HPLC) with amperometric detection.

Analysis of Serum for Malondialdehyde (MDA)

An aliquot of serum (200 μl) was examined for MDA levels using a microtiter plate assay (LPO-586 kit; OXIS, Portland, OR) and the lipid peroxide analyzed by ultraviolet-visible (UV-Vis) absorbance according to the manufacturer's instructions. Serum samples were run in duplicate and the averaged values reported as nmol MDA/ml of serum.

Oxidative DNA Repair Activity

We developed a unique oligonucleotide probe for assessing apurinic/apyrimidinic endonuclease (APE) activity in lymphocytes of agricultural workers[7] that examines only APE activity and not other competing enzymes (e.g., exonucleases).

Lymphocyte Cell Culture

Lymphocytes from a healthy male volunteer were isolated using the Ficoll gradient centrifugation method and treated with azinphos methyl (AZM) for 24 h. ROS and glutathione levels were determined by incubating the cells with 10 μM 5-(and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCF-DA) or 40 μM monochlorobimane and the fluorescence read on a microplate plate reader according to procedures described by Muniz and colleagues.[7]

Comet Assay

Lymphocytes and oral leukocytes were analyzed by the comet assay according to the methods described by Muniz[7] and McCauley[8] and their colleagues. DNA damage was determined by measuring the tail length and tail moment using a fluorescence microscope equipped with an automated digital imaging system running COMET Assay III software (Perceptive Instruments, UK). Lymphocyte cultures were also examined for oxidative DNA damage by pretreating the cells with 0.4 units of *Esherichia coli* formamidopyramidine DNA glycosylase (Fpg) according to manufacturer's instructions (Trevigen, Inc).

Statistical Analysis

Levels of oxidative stress and urinary dialkylphosphate metabolites below the limit of detection (LOD) were treated as 0.5 LOD in all analyses. Measures of oxidative stress and urinary dialkylphosphate (DAP) metabolites were log-transformed to improve symmetry. General linear models were used to examine group differences in OP metabolites and measures of oxidative stress and DNA damage adjusting for age, smoking status, dietary intake of antioxidants, and creatinine concentration.

RESULTS

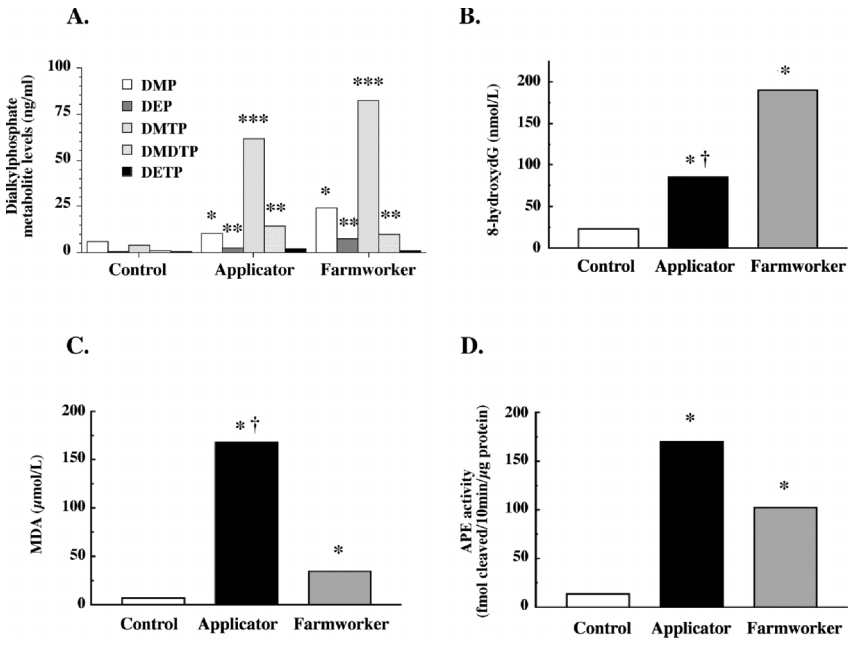
Biomarkers of Exposure and Oxidative Stress in Pilot Study of Agricultural Workers

Figure 1A shows the estimated median concentration of OP metabolites in the applicator, farm worker, and control samples. Farm workers had a 8.2-fold increase in the sum of methyl DAP when compared to controls, whereas applicators had a 6.1-fold increase compared to controls. Figure 1B shows the levels of 8-OH-dG in urine samples, with controls having the lowest levels compared to median values of 85 nmol/L for applicators and 190 nmol/L for farm workers. Figure 1C shows that the serum MDA level among the three groups was lowest in the control group and higher in the farm workers and applicators. A similar pattern is observed for lymphocyte APE activity (Figure 1D) in both farm workers and applicators. Adjusting the comparisons for age and dietary score did not affect the observed differences for APE activity or MDA levels.

DNA Damage in Lymphocytes and Oral Leukocytes

Figure 2 shows the extent of DNA damage in lymphocytes (from our pilot study) and oral leukocytes from agricultural workers. In our pilot study (Figure 2A), applicator and farm worker groups combined had a mean tail length 3.2-fold greater than controls. There is a significant partial correlation of the combined methyl

FIGURE 1. Biomarkers of exposure and oxidative stress are elevated in the urine, serum and lymphocytes of agricultural workers. (**A**) Median urinary dialkylphosphate metabolite levels (ng/ml) in each of the comparison groups, adjusted for age and levels of creatinine. (**B**) Median urinary levels of 8-oxodeoxyguanosine in the three groups, adjusted for creatinine, age, and dietary intake of antioxidants. (**C**) Median serum levels of malondialdehyde (MDA) in the three groups. (**D**) Median lymphocyte apurinic/apyrimidinic endonuclease (APE) activity in the three groups. Bars marked with an asterisk indicate groups that differ from the control at the .05 (*), .01 (**), or .001 (***) level; cross symbol indicates applicators differ from farm workers at the .05 level.



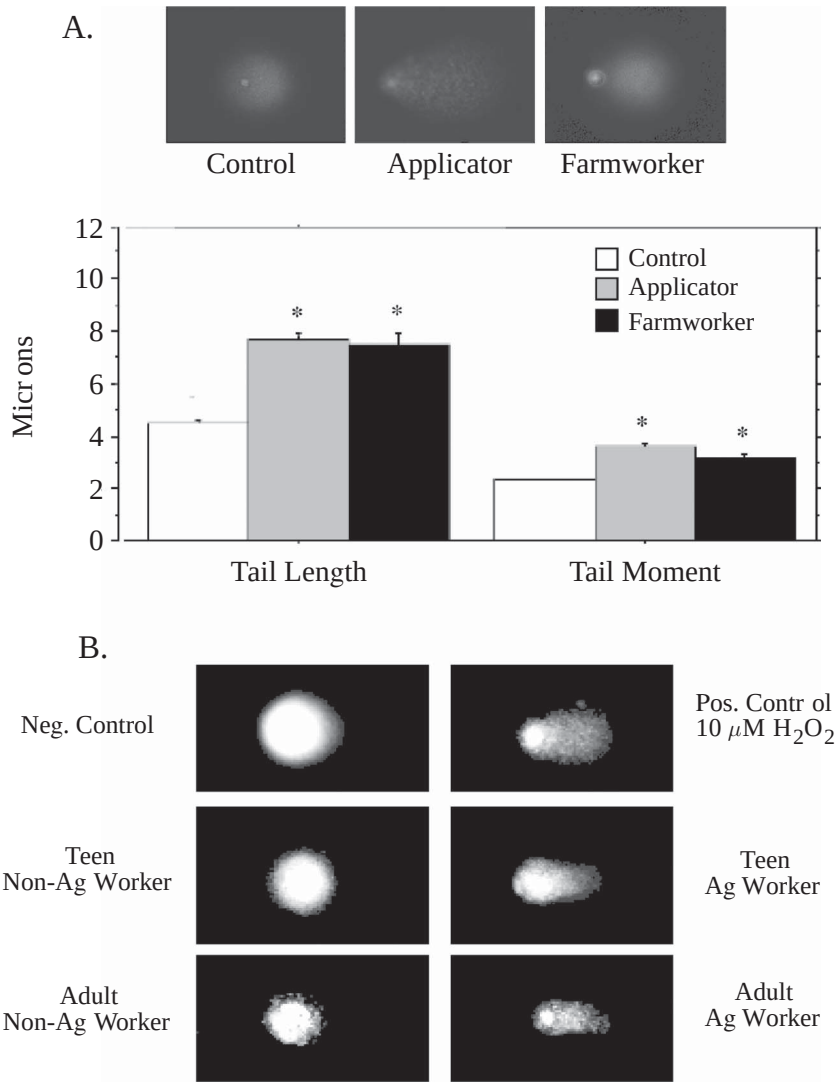
sum of the OP metabolites (partial correlation = .56, $p < .01$) and the tail length of the lymphocytes from all subjects tested.

Similarly, we found in our larger study (2004) of agricultural workers that buccal mucosa cells from farm workers (Figure 2B) had a mean tail intensity that was significantly greater than controls (1-sided p value < .001). Tail moment was also significantly greater in oral leukocytes from agricultural workers than nonagricultural workers (1-sided p value < .001). No comet parameter was significantly associated with years spent working in agriculture, age, sex, body mass index, diet, or alcohol or tobacco use.

In Vitro Oxidative Stress and DNA Damage

Cultures of human lymphocytes were treated with AZM to determine if the pesticide used in Oregon orchards[5] induces oxidative stress and DNA damage (Figure 3). AZM induced a concentration-dependent increase in ROS and a reduction in GSH levels (Figure 3A). Lymphocytes treated with AZM were also examined for the exent of DNA damage by the comet assay (Figure 3B). Figure 3B shows that 10 μM AZM induced a significant increase in both tail length (239%) and tail moment (149%) compared to the negative control. The extent of DNA damage in AZM-treated lymphocytes was comparable to that observed in lymphocytes from agricultural workers (see Figure 2A) and in lymphocyte cultures treated with 10 μM H_2O_2. Conversely, vitamin E pretreatment reduced (~31%) both tail length and moment in AZM-treated lymphocytes (Figure 3B). When AZM-treated lymphocytes were incubated with Fpg (the enzyme that removes 8-OH-dG), there was a 7.2% increase in tail length and a 16.6% increase in tail moment. These results indicate that AZM induces oxidative DNA damage.

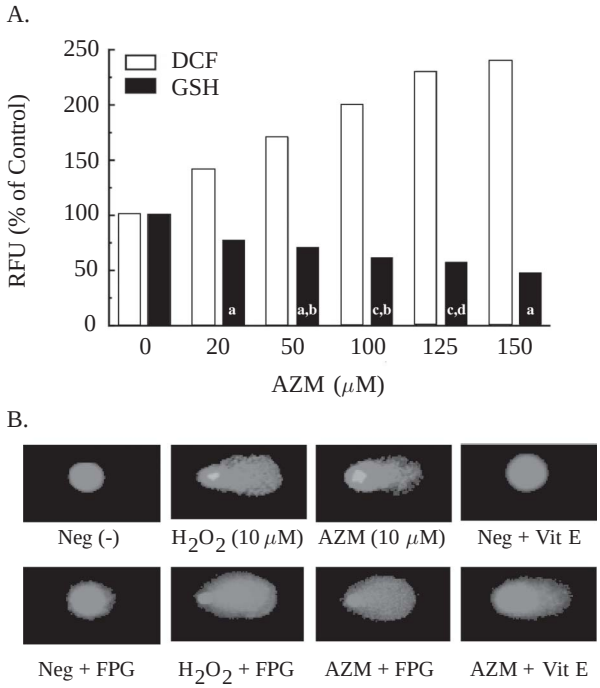
FIGURE 2. Analysis of lymphocytes and buccal cells from agricultural workers for DNA damage. Lymphocytes from fruit orchard workers and buccal cells from berry crop workers were examined by the Comet assay for DNA damage. (**A**) Photomicrographs of lymphocytes from controls, applicators, and farm workers. Lower graph shows mean values of the tail length and tail moment for lymphocytes from controls (n = 9), applicators (n = 10), and farm workers (n = 9). Bars marked with an asterisk indicate groups that differ from the control at the .05 level. (**B**) Photomicrographs of leukocytes from the buccal cavity of teenage (Teen) or adult berry crop workers. For comparison, leukocytes from the buccal cavity of a non-Ag control were treated with 10 μM H_2O_2 for 10 min.



DISCUSSION

Most studies of DNA damage and pesticide exposure have focused on cytogenetic end points (e.g., chromosomal aberrations, sister-chromatid exchanges, and micronuclei) and with conflicting results.[9] More recent studies have documented increased levels of DNA damage in lymphocytes using the Comet assay,[10,11] but they did not examine specific biomarkers of organophosphate pesticide exposure. We have shown in this paper that pesticide-exposed workers have increased levels of biomarkers of oxidative stress and

FIGURE 3. Biomarkers of oxidative stress and DNA damage in human lymphocyte cell cultures treated with an OP pesticide. (**A**) Intracellular levels of glutathione (GSH) and reactive oxygen species (ROS) in lymphocyte cultures treated with various concentrations (20 to 150 μM) of azinphos methyl (AZM) for 24 h. The values represent the mean relative fluorescence intensity units (RFU) (n = 8) of two separate experiments. All nonzero concentrations of AZM significantly differ from control; bars of the same color that share a common letter do not significantly differ at the .05 level (Turkey's HSD). (**B**) Comet images. *Top*: Untreated lymphocytes (negative control), lymphocytes treated with 10 μM H_2O_2 (positive control), lymphocytes treated with 10 μM azinphos methyl (AZM) for 24 h, and lymphocytes treated with 20 μM vitamin E (negative control). *Bottom*: Comet image of negative control + Fpg, lymphocytes treated with 10 μM H_2O_2 for 5 min (positive control) + Fpg, lymphocyte culture treated with 10 μM AZM for 24 h + Fpg, and lymphocyte culture pretreated with 20 μM vitamin E for 24 h prior to treatment with 10 μM AZM.



DNA damage, and that the amount of DNA damage correlated with the extent of pesticide exposure. This was corroborated by our in vitro studies that showed the extent of oxidative stress and DNA damage induced by AZM in human lymphocytes was concentration dependent and blocked by antioxidants. The in vitro studies also showed that AZM induced a significant amount of oxidative DNA damage (i.e., 8-OH dG), which is elevated in many cancers.[12] Similar results were also observed in human neuroblastoma cells treated with AZM,[13] suggesting that oxidative stress and DNA damage are common mechanisms by which pesticides disrupt the function of human cells.

Oxidative DNA damage reportedly plays an important role in a number of pathological conditions, including carcinogenesis, but few epidemiological studies have reported the usefulness of measuring biomarkers of oxidative stress and DNA damage. Lagorio and colleagues[14] reported a dose-response effect between personal exposure to benzene and urinary levels of 8-OH-dG and the influence of gender, smoking, and body mass index on this biomarker. All three of the biomarkers that we used indicate higher levels of oxidative stress among farm workers and applicators than that observed in a control population. We did not observe a strong association between occasional

smoking among three of our study participants and measures of oxidative stress or DNA damage. Leanness is reported to be associated with an increased excretion of 8-OH-dG,[15] possibly due to the influence of a higher metabolic rate on these factors. We did not obtain body mass index in this study, but it is possible that the high exertion associated with farm work could have contributed to higher urinary levels of 8-OH-dG among agricultural workers. For example, vigorous exercise (~10 h/day) for 30 days increased urinary excretion of 8-oxo-7,8-dihydro-2′-deoxyguanosine by 33% in men.[16] However, urinary 8-OH-dG levels have been reported to be significantly lower one day after moderate exercise for 1 h.[17] Other factors that could have contributed to the variable urinary levels of 8-OH-dG include diet and/or the consumption of alcohol. We did not find an association between self-reported alcohol consumption and diet among the individuals participating in this study; however, self-reported consumption may not be a reliable indicator of exposure.

The 8-OH-dG levels were significantly different for each group ($p \leq .01$ for all comparisons). Both applicators and farm workers had higher levels than controls. Previous studies that used 8-OH-dG as a marker of oxidative damage used a 24-h urine collection, but in our study, only spot urines were feasible. Spot urines contain adequate levels of 8-OH-dG for measurement among healthy men and women.[18] However, recent studies indicate that urinary levels of 8-OH-dG can vary over a 24-h period when measured for consecutive days.[19] Therefore, single values of 8-OH-dG should be considered with caution, in particular for small study groups and when spot urines are used. In addition, oxidative DNA lesions generally appear in the urine within hours after exposure to mutagenic chemicals and their persistence may be brief as indicated by the diminished mutagenicity within 24-h or less after exposure.[20] It may be that urinary levels of 8-OH-dG as well as the urinary levels of DAP metabolites reflect short-term exposures compared to the other markers examined in the serum (MDA) and lymphocytes (APE activity, DNA damage) of agricultural workers. Given that not all of our applicators had recently used pesticides, their urinary 8-OH-dG levels may reflect more current exposures (similar to the general farm worker group), whereas the MDA levels, APE activity, and DNA damage (Comet assay) may reflect more cumulative or long-term exposure.

The comet assay has been widely used to determine the extent of DNA damage in lymphocytes from farmers with occupational exposure to a variety of pesticides.[10,11] Our study, showing that DNA damage was greater in lymphocytes and oral leukocytes of farm workers and applicators than nonagricultural workers, is consistent with this previous work. Our findings build significantly upon this previous work in that we have demonstrated a dose-dependent association with oxidative stress and DNA damage by providing correlations with urinary biomarkers of organophosphate pesticides. Moreover, the comet studies with oral leukocytes demonstrate the ability to detect subtle differences in DNA damage among working populations exposed to agricultural chemicals compared to control populations. Such noninvasive methods could be particularly useful in larger studies to examine further the relationship between pesticide exposure and genotoxocity and the potential health risks (e.g., cancer).

One of the main objectives of these studies was to examine the effects of a specific organophosphate pesticide on Hispanic farm workers and applicators in Oregon. Since previous studies by McCauley and colleagues[5] indicate that azinphos methyl (AZM) was the most prevalent OP pesticide detected in the homes of agricultural workers in Hood River County, Oregon, we compared the extent of DNA damage in lymphocytes treated in vitro with AZM with that of lymphocytes from the agricultural workers. At AZM concentrations detected in the homes of field workers, we observed comparable DNA damage as in lymphocytes from agricultural workers. The addition of oxidative DNA repair enzymes (i.e., Fpg) in the comet assay demonstrated that a significant amount of the DNA damage induced by AZM was due to oxidative stress, and that the DNA damage observed in lymphocytes from agricultural workers was also due to OP pesticide induced

oxidative stress. The ability of AZM to induce ROS and reduce GSH levels in human lymphocytes (Figure 3A) and neuroblastoma cell cultures,[13] and for antioxidants (e.g., vitamin E), spin-trapping agents, and glutathione precursors (e.g., *N*-acetylcysteine) to significantly reduce the effects of AZM in human neuroblastoma cell cultures, is consistent with this hypothesis. These results suggest that pesticide exposure in agricultural workers induces oxidative stress by depleting intracellular GSH and increasing ROS production. GSH is known to play a key role in regulating intracellular levels of ROS by scavenger action and maintaining the intracellular redox status. Organophosphate pesticides appear to disturb this cellular pathway by an unknown mechanism, or perhaps by disrupting mitochondrial metabolism as suggested by Kaur and his colleagues.[21]

The implications of these markers of oxidative stress and DNA damage are undetermined with respect to permanent DNA damage and their relationship to cancer. However, the use of simple noninvasive measures of oxidative stress and DNA damage provides a unique opportunity for occupational and environmental scientists who have cohorts to study prospectively the association between marker levels and subsequent health effects. Currently the only biomonitoring occurring among agricultural workers is the measurement of acetylcholinesterase levels in pesticide applicators and handlers. These results indicate that other mechanisms of cell disruption may play a role in the association of pesticide exposure and chronic diseases, including cancer, and will surely lead to additional areas of investigation to explore a cause-and-effect relationship. Although we focused on organophosphate and fungicide exposures in these investigations, the capacity of other classes of pesticides to induce similar patterns of oxidative stress and DNA damage should be investigated. We also demonstrated that biomarkers of damage can be examined in oral leukocytes of agricultural workers. Because oral leukocytes can be obtained noninvasively, they may be particularly important for assessing pesticide exposures and DNA damage in young agricultural workers. Although many of these biomarkers are currently under development and lack the link with specific health outcomes,[22] they provide an important foundation for increasing our understanding of the biological mechanisms associated with pesticide exposures in multiple epidemiological investigations.

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