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ORIGINAL ARTICLE

DNA damage in horticultural farmers: a pilot study showing an association with organophosphate pesticide exposure

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Abstract

A study of horticultural farmers exposed to organophosphate pesticides (OPs) and controls investigated the relationships between OP exposure, DNA damage and oxidative stress. Blood acetylcholinesterase (AChE) and urinary dialkylphosphate (DAP) levels determined exposure and 8-hydroxy-2′-deoxyguanosine (8OHdG) indicated oxidative stress status. The farmers had approximately 30% lower AChE activity and increased DAP levels compared with the controls, reflecting moderate OP exposure. They had higher DNA damage than the controls and there was a significant positive relationship between DAP and DNA damage with greater than 95% power. The farmers also had a significant positive relationship between urinary DAP and 8OHdG levels.

Keywords: *Acetylcholinesterase; 8-hydroxy-2′-deoxyguanosine; organophosphate pesticides*

Introduction

Man is exposed to insecticide, fungicide, herbicide and biocide mixtures (collectively 'pesticides') at low levels from environmental sources, including food and water, and, at higher levels, from occupational use. Although the acute toxicity of many of these chemicals is well recognised, being derived from studies of experimental animals and *in vitro* studies, extrapolation to man following chronic low-level environmental exposure is problematic. For example, it is well known that the parent forms of organophosphate pesticides (OPs) are activated by the cytochromes P450 (CYPs) to oxon metabolites (Mutch & Williams 2006) that have the ability to inhibit acetylcholinesterase (AChE) in the nervous system and at neuromuscular junctions to cause acute toxic effects. However, the effect of low-level exposure to OPs below the threshold to cause acute symptoms is far less well understood.

The widespread and increasing use of pesticides with poorly characterised long-term health risks has led to concern that some may be human toxins. Reports from the Agricultural Health Study, a large prospective cohort study of pesticide applicators in Iowa and North Carolina (USA), has added to the concern since it has shown positive associations between chronic exposure to several pesticides, including OPs, and various cancers (Alavanja et al. 2007). However, there has been criticism that these studies are of restricted value as the associations were made in the absence of pesticide exposure measurements. For example, although a recent study showed increased lymphocyte

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DNA damage in association with decreased plasma butyrylcholinesterase activity (a non-specific marker of OP exposure) in Brazilian farm workers exposed to pesticide mixtures, definitive exposure to OPs was not characterised (Remor et al. 2008).

In this study, DNA damage was determined in parallel with two measures of OP exposure, urinary dialkylphosphate (DAP) metabolites and blood AChE activity, to investigate whether a relationship between OP exposure and DNA damage does exist. The alkaline Comet assay determined 'background' DNA damage for male volunteers living in the UK with no occupational exposure to pesticides. DNA damage was then determined for male Spanish horticultural farmers in relation to both OP exposure and oxidative stress status, as indicated by urinary 8-hydroxy-2′-deoxyguanosine (8OHdG) levels. In parallel, OP exposure, oxidative stress and DNA damage was determined for a group of male volunteers living in the same region of Spain with no occupational exposure to pesticides.

Materials and methods

Dimethylphosphate (DMP) and dimethylthiophosphate (DMTP) were obtained from Aldrich (Milwaukee, WI, USA); diethylphosphate (DEP) and diethylthiophosphate (DETP) were purchased from Supelco (Bellefonte, USA). Lymphoprep was purchased from Axis-Shield (Oslo, Norway) and SYBR Gold from Molecular Probes (Leidon, Netherlands). The Comet Assay IV software was bought from Perceptive Instruments (Haverhill, Suffolk, UK).

DNA damage by the alkaline Comet method

Immediately upon arrival of the blood samples from Spain, lymphocytes were isolated by centrifugation through lymphoprep and DNA damage was measured using the alkaline single-cell gel electrophoresis (Comet) method of Collins and Dusinska (2002). Each assay was carried out with a positive control (in duplicate) consisting of lymphocytes treated with hydrogen peroxide (50 µM) for 5 min at 37°C in humidified 5% CO_2/95% air. Six blood samples (F2, F6, F7, F8, F9, F10) either had overcrowded lymphocyte preparations or were lost during lymphocyte isolation making it impossible to determine DNA damage in these samples.

Prior to visualization of the DNA damage (Comets), dried down slides were rehydrated in the dark for 30 min in ice-cold PBS. Each slide was flooded with SYBR Gold diluted 1 in 10000 (v/v) in Tris/EDTA buffer (pH 7.4) for 10 min before the excess was removed. Slides were left to dry overnight in the dark at room temperature. Comets were visualised using a fluorescent microscope (x200 magnification) and DNA damage was quantified using an automated image analysis system (Comet Assay IV software). Fifty cells per parameter (25 cells per duplicate slide) were randomly selected and expressed as Olive Tail Moment (OTM) by the automated software. Tail moment, the chosen measure of DNA damage, is defined as the product of the percentage of DNA in the tail and the displacement between the head and tail mean centres. Highly damaged cells, with the visual appearance of hedgehogs, were assumed to be apoptotic/necrotic and were excluded from analysis. In order to minimise assay variability, one experienced scientist (KMA) scored all the Comets. The Comets were assigned one of five predefined classes (0, 1, 2, 3, 4) on the basis of DNA damage expressed as OTM. Thus, class 0 (undamaged cells, 1–2 OTM), 1 (2–3 OTM), 2 (3–5 OTM), 3 (5–10 OTM) and 4 (very damaged cells, >10 OTM). The total damage score for 100 comets could therefore range from 0 (all undamaged) to 400 (all damaged) (Moretti et al. 1999). There was a significant positive relationship (p <0.001) between OTM and DNA damage score for the 53 subjects (Figure 1), as also reported by others (Lebailly et al. 1998).

Assay precision for measurement of DNA damage by the Comet method

To determine the intra-assay coefficient of variation (CV) of the Comet method, the assay was performed four times on different dates using freshly harvested TK6 cells, a lymphoblastoid, non-adherent cell line that are a homogeneous population. The four mean OTM values (50 cells/assay) from the four assays were averaged and expressed as 0.564 ± 0.041 (mean ± SD). The intra-assay CV of the Comet method was therefore 7.3%.

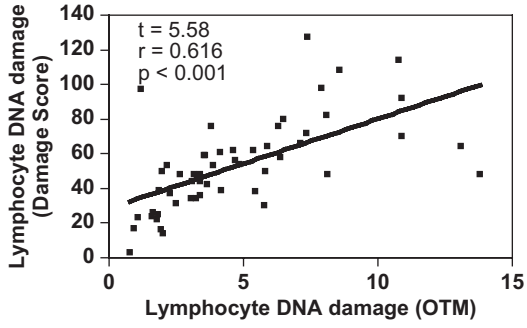

Figure 1. The correlation between lymphocyte DNA damage evaluated as Olive tail moment (OTM) and damage score (arbitrary units). Individual values are the mean of 50 cells evaluated for DNA damage by the Comet method. There was a significant (p <0.001) positive relationship between OTM and DNA damage score for the 53 subjects comprising farmers (n = 14), Spanish controls (n = 7) and UK controls (n = 32).

Subjects

Thirty-two young (33.5 ± 9.0 years, mean ± SD), non-smoking, men were recruited with local ethical committee approval (REC06/Q0905/152). The volunteers were staff or students working at Newcastle University, UK and had no occupational exposure OPs. This group established a range for 'background' DNA damage that was likely due to low-level exposure to environmental genotoxins, variable health status and antioxidant intake, among others. To evaluate intraindividual variation for DNA damage, five 'non-exposed' women working at Newcastle University volunteered to provide blood samples on three separate occasions between April and September for alkaline Comet analysis. The female subjects were non-smokers and were aged 41.8 ± 14.4 years (mean ± SD).

Seventeen young (35.5 ± 8.2 years, mean ± SD), healthy, male horticultural farmers who had worked with pesticide mixtures for at least 2 years were recruited in Almeria, Spain. A questionnaire and consent form was presented to the farmers by a Spanish-speaking clinician who ascertained the age, medication intake, smoking status, level of alcohol consumption and general health of the individuals. None of the farmers reported poor health or any symptom associated with acute pesticide toxicity. Six were smokers and eight non-smokers. Seven young (26.0 ± 4.8 years, mean ± SD), healthy, men working at the University of Almeria and with no occupational exposure to OPs served as the Spanish control population. There were three smokers and four non-smokers. The controls, therefore, were matched as far as possible for age and smoking status as these parameters have been associated with increased DNA damage.

Twenty millilitres of venous blood and 50 ml spot urine samples were obtained from the farmers and Spanish controls. The samples from both groups were sent to the UK and Spain by freight at 4°C and arrived within 48 h. Preliminary experiments had shown that DNA damage and enzyme activities were not affected by this treatment (data not shown).

Analysis of urine for dialkylphosphate metabolites (DAP)

LAB S.L, Department of Analytical Chemistry, University of Almeria (Spain), carried out analysis of the urine samples for the four major dialkylphosphate metabolites (DAPs): (DMP, DMTP, DEP and DETP). A Waters Acquity Ultraperformance Liquid Chromatograph (Waters, Milford, MA, USA) was interfaced to a Quatro Premier XE 3Q Mass spectrometer (Micromass, Manchester, UK). Separation was carried out on an Acquity UPLC column (C18, 1.7 μm 100 × 2. 1 mm, Waters) using a linear gradient of acetonitrile and water in ammonium formate (2mM). The liquid chromatography (LC)-mass spectrometry (MS)-MS conditions (Multiple Reaction Monitoring) are given in Table 1. A capillary voltage of 3.0 kV was used in negative ionisation mode. The interface temperature was set at 350°C and the source at 120°C.

The extraction procedure was based on a previously published method (Dularent et al. 2006). Briefly, 5 ml of urine was treated with 4 g of sodium chloride and 1 ml of hydrochloric acid (6 M). The mixture was extracted with 5 ml of diethyl ether and centrifuged at 3000 rpm for 5 min. The organic phase was collected and the extraction repeated with another 5 ml of ethyl acetate. Both extracts were combined and evaporated to dryness under nitrogen. The residue was reconstituted in 80 μl of 2 mM ammonium formate/methanol (50:50, v/v) and a 5 μl volume was injected onto the LC-MS-MS. The DAPs were extracted from urine with an efficiency of between 89% and 97% and a relative standard deviation of less than 9%. The analytical limit of detection of the DAPs was in the range 0.6–1.1 ng ml^{-1}. The urine samples were also analysed for creatinine concentration and these values were used to normalise the urinary DAP levels between samples.

Measurement of blood acetylcholinesterase and butyrylcholinesterase activities

Prior to transport from Spain to the UK, a 1 in 25 dilution was made by adding 40 μl of whole blood collected

Table 1. LC-MS-MS conditions for measurement of the four dialkylphosphate (DAP) metabolites: dimethylphosphate (DMP), dimethylthiophosphate (DMTP), diethylthiophosphate (DETP) and diethylphosphate (DEP).

DAP	Retention Time (min)	Precursor ion (m/z)	Declustering potential (V)	Type of transition	Collision energy (V)	Product ion (m/z)	Q/C ratio
DMP	4.07	124.9	−51	Quantification	−28	79.0	2.10
				Confirmatory	−22	63.1	
DMTP	4.23	140.9	−36	Quantification	−20	125.8	1.86
				Confirmatory	−26	96.0	
DETP	4.44	168.9	−41	Quantification	−24	95	1.22
				Confirmatory	−14	141	
DEP	4.75	152.9	−31	Quantification	−22	79.1	2.28
				Confirmatory	−16	124.8	

in lithium heparin to 1 ml buffered saponin solution (100 mg saponin in 100 ml 0.1 M phosphate buffer, pH 8). For AChE and butyrylcholinesterase (BuChE) analyses, 10 µl of the diluted blood was incubated with 190 µl phosphate buffer (pH 8) and 110 µl DTNB solution (5′5-dithio-bis-2-nitrobenzoic acid, pH 7/acetyl (β-methyl) thiocholine iodide or 5′5-dithio-bis-2-nitrobenzoic acid, pH 7/butyrylthiolcholine). The final concentrations of DTNB, acetyl (β-methyl) thiocholine and butyrylthiocholine were 0.5 mM, 5 mM and 7 mM, respectively. The change in absorbance units was read every 30 s up to 4 min at 412 nm and 37°C using a Multiskan plate reader, enabling the generation of a kinetic plot (positive slope). Reduced glutathione standards (0–5.0 nmol per well) were prepared in water and treated in the same manner as the samples. A reduced glutathione standard curve was used to quantify enzyme activity, i.e. liberation of thiocholine from the substrates by AChE or BuChE. The enzyme activities were expressed as µmol ml^{-1} min^{-1}.

Analysis of urine for 8-hydroxy-2′-deoxyguanosine

One millilitre of urine was diluted 1:1 with 0.01 M HCl and internal standard, 8-bromo-2′-deoxyguanosine was added. This was applied to a HCX, mixed mode cation exchange SPE cartridge, previously conditioned with 1 ml of methanol and 1 ml of 0.01 M HCl. After washing with 1 ml of 0.01 M HCl, analytes were eluted with 2 ml of 95:5 methanol:ammonia which was then evaporated to dryness under a stream of dry nitrogen. This was reconstituted with 200 µl of mobile phase, 80:20, 0.1% aqueous acetic acid:methanol, for LC-MS-MS analysis. Five microlitre volumes were injected onto the LC-MS-MS.

LC-MS-MS analysis was performed on a Quantum Discovery, Triple Quadrupole LC-MS-MS system, using positive electrospray ionisation and selected reactant monitoring mode of detection. LC separation was achieved on a Sunfire C_{18}, 100 mm × 2.1 mm × 3.5 µm column using a linear gradient of methanol in formic acid (0.1%, v/v) and water in formic acid (0.1%, v/v). The LC-MS-MS conditions are given in Table 2. Positive ionisation electrospray mode was used. The analytical limit of detection of 8OHdG was 10 pg ml^{-1}. The urine samples were also analysed for creatinine concentration and this was used to normalise the urinary 8OHdG levels between samples.

Statistical analysis

Statistical comparison between DNA damage score, DAP concentration, 8OHdG concentration, AChE activity and BuChE activity for the farmers and the two control populations was determined by the non-parametric Mann–Whitney *U* test (two-tailed). Correlation analysis was by the Spearman test (two-tailed). Values of $p < 0.05$ were considered to be significant.

Table 2. Selected reaction monitoring conditions for measurement of urinary 8-hydroxy-2′-deoxyguanosine (8OHdG) by LC-MS-MS.

Compound	Precursor m/z	Product m/z	Collision energy (V)	Dwell time ms^{-1}
8OHdG	284.092	168.054	13	10
		140.048	29	10
		112.032	35	10
2′-5N^{15}-deoxyguanosine	273.082	156.977	12	10
Internal standard		138.955	33	10
		112.956	33	10

Results

DNA damage in the study populations

Intraindividual variations in DNA damage

The extent of variability in Comet DNA damage within an individual with time was determined for five control female subjects on three separate occasions to assess intraindividual variations. The three DNA damage (OTM) values were averaged for each of the five female subjects and expressed as mean ± SD which gave values of 1.02 ± 0.741, 0.977 ± 0.311, 1.55 ± 0.067, 0.866 ± 0.079 and 1.69 ± 1.02 with intraindividual coefficient of variations (CVs) of 73%, 32%, 4%, 9% and 63%, respectively. For comparison, the DNA damage was converted to damage score (mean ± SD), giving values of 33 ± 23, 16 ± 3.5, 20 ± 4.0, 8.0 ± 0.1 and 51 ± 43, respectively. Although the DNA damage was low for all five subjects, the mean ± SEM intraindividual CV was 36.2% ± 13.9%, in keeping with the values reported by others (Holz et al. 1995, Pilger et al. 2001, Moller et al. 2000). The intraindividual variation in DNA damage would have been only minimally influenced by the precision of the method that had a CV of 7.3%.

Horticultural farmers: comparison with UK and Spanish controls

The horticultural farmers mostly worked inside plastic greenhouses and on the two occasions sampling took place (July and November, 2006) many of them had been using pesticides daily for several weeks. On both occasions, sampling took place during mid-morning on a Tuesday when it was known that pesticides had been used the previous day, and the same day in most cases, following the weekend break when pesticides were not used. The farmers did not wear protective clothing whether working inside or outside the greenhouses although gloves and respirators were available at some sites.

Table 3. Characteristics of the farmers (F1–F20)[a] and control Spanish males (C1–C7), including age, pesticide use, smoking status, DNA damage score, urinary 8-hydroxy-2′-deoxyguanosine (8OHdG) concentration, urinary dialkylphosphate concentrations (DMTP + DMP and DETP+DEP), blood acetylcholinesterase activity (AChE) and blood butyrylcholinesterase activity (BuChE).

I.D.	Age (years)	Pesticide use (years)	Last used pesticides (days ago)	Smoke	DNA damage score	8OH-dG (pmol mmol^{-1} creatinine)	DMTP+DMP (µmol mmol^{-1} creatinine)	DETP+DEP (µmol mmol^{-1} creatinine)	AChE (µmol ml^{-1} min^{-1})	BuChE (µmol ml^{-1} min^{-1})
F1	41	16	7	NS	108	0.63	0.063	0.170	2.90	3.77
F2	32	8	?	S	n/a	0.49	0.042	0.070	2.62	2.27
F3	30	6	?	S	98	1.24	0.082	0.179	2.87	2.86
F4	46	20	?	NS	92	1.18	0.113	0.138	2.87	2.60
F5	20	8	>50	S	97	1.67	0.055	0.196	2.60	3.25
F6	47	4	7	NS	n/a	0.83	0.089	0.141	2.92	2.32
F7	38	1–2	4	S	n/a	3.03	0.081	0.184	2.94	1.76
F8	33	16	Today	NS	n/a	2.08	0.025	0.091	2.65	2.30
F9	34	16	21	NS	n/a	1.45	0.034	0.520	3.17	1.98
F10	37	20	21	NS	n/a	1.31	0.013	0.630	3.13	2.16
F11	34	17	Today	NS	70	1.14	0.008	0.013	3.10	2.78
F12	25	1–2	Today	NS	44	0.55	0.007	0.041	3.15	2.48
F13	38	2	Today	S	22	n/a	0.030	0.006	3.78	3.78
F14	46	20	Today	NS	58	1.50	0.089	0.072	3.90	4.01
F15	47	1–2	30	NS	80	n/a	0.052	0.070	3.92	2.34
F16	29	1–2	>50	S	76	n/a	0.060	0.057	3.02	3.00
F17	21	6	Today	S	114	0.48	0.081	0.184	3.19	3.18
F18	30	1–2	>50	NS	62	n/a	0.038	0.041	3.35	3.02
F19	43	16	Today	NS	66	0.63	0.055	0.120	3.35	3.69
F20	43	4	Today	NS	82	0.45	0.071	0.079	2.78	2.82
C1	30	0	–	NS	30	0.31	–	–	4.85	3.32
C2	28	0	–	NS	64	0.40	–	–	3.80	3.90
C3	33	0	–	NS	54	0.44	–	–	4.26	2.69
C4	19	0	–	S	34	n/a	–	–	3.76	3.10
C5	22	0	–	S	72	1.07	–	–	3.77	3.58
C6	25	0	–	NS	48	0.36	–	–	4.48	3.42
C7	28	0	–	S	48	n/a	–	–	4.50	3.00

[a]Three farmers provided blood samples at both the July (F4, F7 and F9) and November (F14, F13, F11) visits, respectively.
n/a, Not available; –, values were at the analytical limit of detection of the method; ?, couldn't remember when last used pesticides; DMTP, dimethylthiophosphate; DMP, dimethylphosphate; DETP, diethylthiophosphate; DEP, diethylphosphate; blood AChE, acetylcholinesterase; BuChE, butyrylcholinesterase.

Table 3 gives the age, smoking status, DNA damage scores, urinary DAP and 8OHdG levels, and the AChE and BuChE activities of the farmers and control Spanish male subjects. As a group, the farmers had used pesticides for 8.9 ± 6.9 years (mean ± SD). Three farmers provided blood samples at both the July (F4, F7 and F9) and November (F14, F13, F11) visits, respectively (Table 3). The DNA damage score (mean ± SD and range, median) for the farmers (n = 14), Spanish controls (n = 7) and UK controls (n = 32) was 79.2 ± 26.7 (22–114, 81), 50.0 ± 15.1 (30–72, 48) and 43.5 ± 23.4 (3–127, 40.5), respectively. The farmers had significantly higher DNA damage than both the Spanish ($p < 0.05$) and UK controls ($p < 0.001$) (Figure 2).

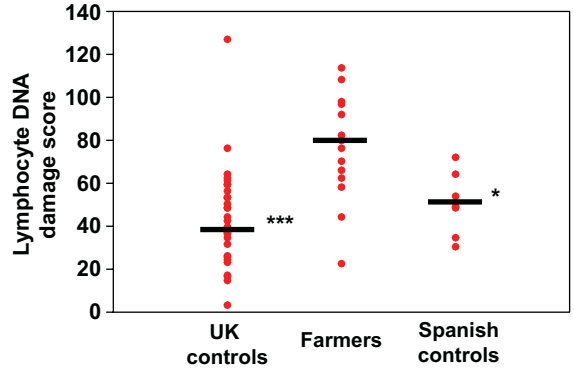

Figure 2. DNA damage scores for lymphocytes obtained from UK controls (n=32), farmers (n = 14) and Spanish controls. Individual values are the mean of 50 cells evaluated for DNA damage by the Comet method. The farmers had significantly higher DNA damage than both the UK controls ($p < 0.001$) and Spanish controls ($p < 0.05$) (Mann–Whitney U test).

Exposure to OPs: relationship with DNA damage

The pesticide logbook information is given in Table 4 and shows that in the weeks of the study, the farmers

Table 4. Pesticides used by the horticultural farmers in Spain between 22nd May and 10th November, 2006.

Pesticide Type	Common Name	IARC	EPA	WHO
Fungicides	Azoxystrobin	NL	E	U
	Carbendazim	NL	C	U
	Pyrimethanil	NL	E	U
	Procymidone	NL	B2	U
Insecticides	Imidacloprid	NL	E	II
	Malathion	3	C	III
	Pirimiphos-methyl	NL	UC	III
	Chlorpyrifos	NL	E	II
	Buprofectin	NL	NL	NL
	Pyridaben	NL	E	III
	Pyriproxifen	NL	NL	NL

IARC (2003) classification of carcinogenicity to humans: 3 = not classifiable; NL = not listed.
EPA classification of carcinogenicity to humans: C = possible; B2 = probable; UC = unclassifiable; E = unlikely; NL = not listed.
WHO classification of acute hazard to humans: II = moderately; III = slightly; U = unlikely; NL = not listed.

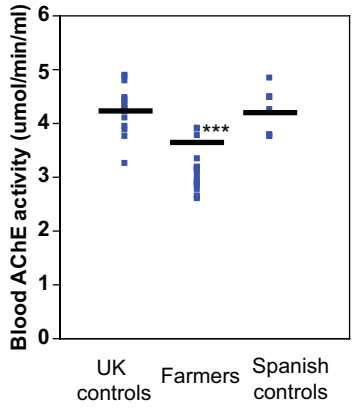

Figure 3. Acetylcholinesterase (AChE) activity (μmol min^{-1} ml^{-1}) for blood samples obtained from UK controls ($n = 32$), farmers ($n = 20$) and Spanish controls ($n = 7$). Taken as a group, the farmers' AChE activity was about 30% lower than both control groups, indicating moderate OP exposure. AChE activity was significantly ($p < 0.0001$) lower for the farmers compared to both the Spanish and UK controls (Mann–Whitney *U* test).

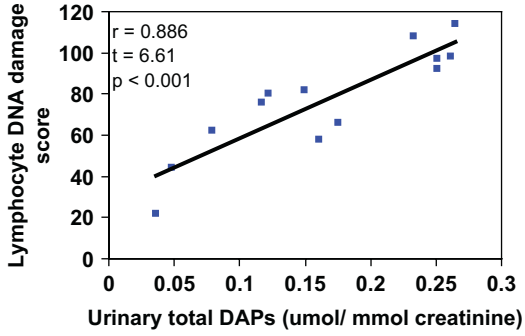

Figure 4. The correlation between lymphocyte DNA damage score and urinary total dialkylphosphate (DAP) metabolite levels (μmol $mmol^{-1}$ creatinine) for 14 farmers. Individual values for DNA damage are the mean of 50 cells evaluated for DNA damage by the Comet method. Total dialkylphosphate metabolites is the sum of dimethyl-phosphate (DMP), dimethyl-thiophosphate (DMTP), diethyl-phosphate (DEP) and diethyl-thiophosphate (DETP). There was a significant ($p < 0.001$) positive relationship between DAP levels and DNA damage scores with >95% power.

were exposed to three OPs (malathion, pirimiphos-methyl and chlorpyrifos) as well as other pesticide classes. The logbook showed that the farmers had been using pirimiphos-methyl (and imidacloprid) for about a week before and at the time of the July sampling, while in November they had been using pirimiphos-methyl, malathion and chlorpyrifos (and imidacloprid, pyridaben and carbendazim) for about a week before and at the time of sampling. Although the pesticide logbook provided information about the pesticides used at the sampling periods, the farmers did not know which pesticides they were using and we could not obtain any information about which pesticides were used by which farmer and when.

The AChE activities of the farmers ($n = 17$), Spanish controls ($n = 7$) and UK controls ($n = 32$) (mean ± SD) were 3.11 ± 0.39, 4.20 ± 0.43 and 4.26 ± 0.44 μmol ml^{-1} min^{-1}, respectively. AChE activity was significantly ($p < 0.0001$) lower for the farmers compared with both the Spanish and UK controls (Figure 3). As a group, the farmers' AChE activity was about 30% lower than both control groups, indicating moderate OP exposure. As a group, plasma BuChE activity was similar for the farmers and the Spanish controls (Table 3).

Exposure to OPs was also measured by determining urinary DAP metabolite levels, which is a specific marker of these pesticides. The sum of the dimethyl-containing (DMP and DMTP) and diethyl-containing (DEP and DETP) urinary DAPs for the farmers as a group were 0.054 ± 0.01 and 0.150 ± 0.035 μmol $mmol^{-1}$ creatinine (mean ± SEM), respectively. The Spanish controls had levels at the analytical limit of detection of the method (0.6–1.1 ng ml^{-1}). The diethyl-DAPs were present at higher levels ($p < 0.01$) than the dimethyl-containing compounds. 'Total' urinary DAP levels (DMP + DMTP + DEP + DETP) was 0.204 ± 0.02 μmol $mmol^{-1}$ creatinine (mean ± SEM) for the farmers as a group and there was a significant ($p < 0.001$) positive relationship between individual 'total' DAP levels and DNA damage score (Figure 4) and power analysis of this relationship showed it to be greater than 95%.

Comparison of the results across the two sampling periods showed that in July the farmers had moderate DMP + DMTP levels (0.06 ± 0.03, mean ± SD), presumably from pirimiphos-methyl exposure, but they also had high DEP + DETP levels (0.232 ± 0.19). In November, when the farmers had been using pirimiphos-methyl, malathion and chlorpyrifos (and imidacloprid, pyridaben and carbendazim), DMP + DMTP (0.05 ± 0.03) remained at moderate levels and DEP and DETP

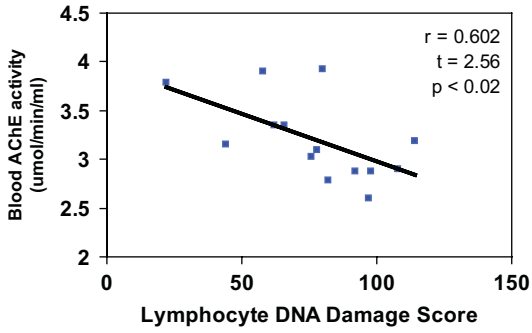

Figure 5. The correlation between lymphocyte DNA damage score and blood acetylcholinesterase (AChE) activity (μmol min^{-1} ml^{-1}) for 14 farmers. Individual values for DNA damage are the mean of 50 cells evaluated for DNA damage by the Comet method. There was a significant (p <0.02) negative relationship between DNA damage score and blood AChE activity.

excretion (0.07 ± 0.05) had greatly decreased compared with July. In July, when DEP + DETP levels were high, the median (range) DNA damage score for the farmers was 97.5 (92–108, n = 4) compared with 68.0 (22–114, n = 10) in November when the metabolite levels were much lower. This was significant at p <0.05.

The relationship between OP exposure and DNA damage was substantiated as there was a significant negative relationship between blood AChE activity and DNA damage score (Figure 5, p <0.02) for the farmers as a group. Moreover, AChE activity was significantly (p <0.01) lower for the farmers in July (2.86 ± 0.20 μmol ml^{-1} min^{-1}, mean ± SD) compared with November (3.35 ± 0.39 μmol ml^{-1} min^{-1}). Although, as a group, plasma BuChE activity was similar for the farmers and both control groups, it was significantly lower (p <0.05) when comparing activity in July (2.53 ± 0.20 μmol ml^{-1} min^{-1}, mean ± SD) with November (3.11 ± 0.56 μmol ml^{-1} min^{-1}). The two biomarkers of OP exposure, blood AChE activity and urinary DAP concentration, had a significant negative relationship (Figure 6, p <0.05). These associations make it likely that OP exposure strongly influenced the level of DNA damage.

Exposure to OPs: relationship with oxidative stress

Urinary levels of 8OHdG were determined as a biomarker of oxidative DNA damage and oxidative stress. There was a significant (p <0.02) relationship between OP exposure (total urinary DAP levels) and urinary 8OHdG levels for the farmers as a group (Figure 7). Urinary 8OHdG levels were higher (p <0.01) for the farmers (1.17 ± 0.697 pmol $mmol^{-1}$ creatinine, mean ± SD) than the Spanish controls (0.515 ± 0.314 pmol $mmol^{-1}$ creatinine). In July, when DEP + DETP levels were high, the median (range) 8OHdG level was 1.28 pmol $mmol^{-1}$ creatinine (0.49–3.03, n = 10) compared with 0.59 pmol $mmol^{-1}$ creatinine (0.45–1.50, n = 6) in November (p = 0.06) when the metabolite levels were much lower.

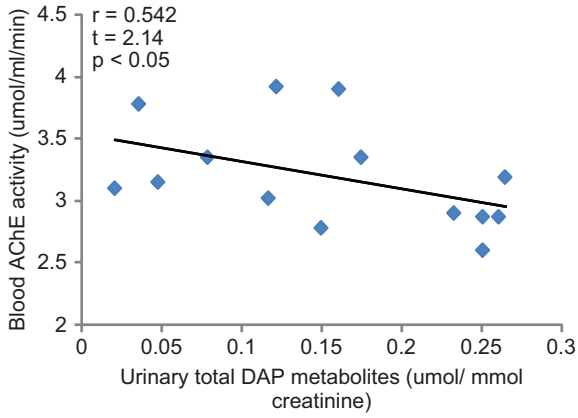

Figure 6. The correlation between urinary total dialkylphosphate (DAP) metabolite levels (μmol $mmol^{-1}$ creatinine) and blood acetylcholinesterase (AChE) activity (μmol min^{-1} ml^{-1}) for 20 farmers. Total dialkylphosphate (DAP) metabolites is the sum of dimethyl-phosphate (DMP), dimethyl-thiophosphate (DMTP), diethyl-phosphate (DEP) and diethyl-thiophosphate (DETP). There was a significant (p <0.05) negative relationship between DAP levels and blood AChE activity.

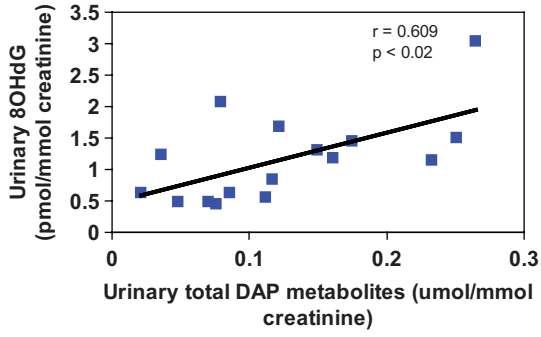

Figure 7. The correlation between urinary total dialkylphosphate (DAP) metabolite levels (μmol $mmol^{-1}$ creatinine) and urinary 8-hydroxy-2′-deoxyguanosine (8OHdG) levels (pmol $mmol^{-1}$ creatinine) for 16 farmers. Total dialkylphosphate (DAP) metabolites is the sum of dimethyl-phosphate (DMP), dimethyl-thiophosphate (DMTP), diethyl-phosphate (DEP) and diethyl-thiophosphate (DETP). There was a significant (p <0.02) positive relationship between urinary DAP metabolite levels and urinary 8OHdG levels.

Discussion

Several studies have suggested that chronic, subacute exposure to OPs can cause DNA damage and adverse long-term health effects, although literature reports often conflict (Bolognesi 2003). The inconsistency in the literature may reflect the type of worker investigated (farmers, pesticide applicators, floriculturists, etc.), the working practices of the workers (use of protective clothing, handling of pesticide concentrate),

the pesticide mixtures used, the individuals' genetic make-up (metabolising and DNA repair enzymes, etc.) and/or the cytogenetic measurements utilised (including the robustness of the methodology), among others. The present study is important, even though the cohorts were small, because it is the first to show significant relationships between DNA damage and two measures of OP exposure (blood AChE activity and urinary DAP metabolite levels) in an occupational group.

We report that, as a group, the farmers had increased DNA damage and elevated oxidative stress at a time when blood AChE activity was lower than control by about 30% in parallel with raised urinary DAP levels. In support that OPs may cause DNA damage, AChE activity was lower in July, when diethyl metabolite levels were high, compared with November when they were much lower. Moreover, although blood BuChE activity was not lower than the controls when looking at the farmers as a group, levels were lower in July compared with November. BuChE is a less specific marker of OP exposure and expression varies widely within the healthy population due to genetic and environmental influences. Conversely, AChE is not influenced by genetics or diet making measurement of inhibition of this enzyme more useful for monitoring purposes (Mutch et al. 1992). The two enzymes have different capacities to bind to individual OPs, in particular chlorpyrifos is known to preferentially inhibit BuChE. Although only speculative at this stage, and contrary to the logbook information, it is likely that the farmers were exposed to relatively high levels of a diethyl-containing OP in July which influenced the DNA damage seen.

It is possible that factors other than OP exposure influenced the DNA damage and elevated oxidative stress seen for the farmers. Smoking has often, but not always, been identified as a confounder of DNA damage and oxidative stress (Zhu et al. 1999, Grover et al. 2003, Muniz et al. 2007) and this study indicated that, for similar OP exposure levels, the farmers that smoked tended to have higher oxidative stress and DNA damage compared with the non-smokers. As expected, as none of the farmers was older than 47 years, age did not confound the DNA damage.

At the time of the study, the logbook showed that the farmers were exposed to the OPs malathion, pirimiphos-methyl and chlorpyrifos, as well as other pesticide classes. Of note, the farmers used carbendazim which has been has been shown to be an indirect genotoxin, but it does not cause DNA strand breaks that could be measured by the Comet assay (Vigreux et al. 1998). None of the pesticides used by the farmers, other than the OPs, had chemical structures enabling inhibition of AChE or production of DAP metabolites and therefore exposure to the non-OP pesticides would be unlikely to have influenced the relationships seen between OP exposure and DNA damage.

Many of the biomonitoring studies to date have been hampered by a lack of exposure information to the pesticides used. For example, although a study of workers manufacturing various OPs as well as other pesticides had higher DNA damage levels than controls using the Comet method, internal dose exposure was not determined (Grover et al. 2003). In another study of pesticide manufacturers (OPs and other pesticide classes), Comet DNA damage was increased in the workers after daily exposure to the compounds for 6 months and, although damage levels had decreased, they remained above control at 6 months after cessation of the work, but exposure was not determined (Garaj-Vrhovac & Zeljezic 2000).

In the present study, urinary 8OHdG was determined as an indicator of oxidative stress, reactive oxygen species (ROS) formation and oxidative injury to the body. All ROS have the potential to react with cellular components, including DNA bases and the deoxyribosyl backbone of DNA, to produce damaged bases and/or strand breaks, some of which are promutagenic (Lunec et al. 2002). Urinary 8OHdG levels were higher for the farmers than the Spanish controls and there was a positive association between urinary 8OHdG and DAP levels, suggesting that OP exposure increased the oxidative stress. In support that OPs may cause DNA damage by increasing oxidative stress, 8OHdG levels were higher in July than November, although this did not reach statistical significance. A previous study of 18 pesticide applicators that were mainly exposed to OPs also reported a positive relationship between urinary 8OHdG and DAP levels, although DNA damage was not measured (Lee et al. 2007). Another small study that did investigate DNA damage (Comet method) in parallel with DAP metabolites, found increased DNA damage for nine farmers and ten pesticide applicators who were believed to be primarily exposed to the OPs azinphos-methyl and chlorpyrifos compared with nine controls (Muniz et al. 2007). The methyl-containing DAPs were higher in the occupational groups than the controls but, in contrast with our study, Muniz et al. did not see a significant relationship between urinary DAP and 8OHdG levels.

It has been reported that the OP malathion inhibited mitochondrial respiratory chain activity in the hippocampus of rats exposed to low concentrations of this OP which led to increased oxidative stress (Delgado et al. 2006). The Delgado study is of importance to the present one because malathion was used by the farmers and their research appears to support that exposure to this OP can cause increased oxidative stress. Our research group has also shown that, at no more than 30% brain AChE inhibition, the OPs malathion, diazinon and parathion caused DNA damage to the hepatocytes (and

lymphocytes in some cases) of rats dosed intraperitoneally (Atherton et al. 2008). An alternative mechanism for the DNA damage seen in the rats and the farmers could therefore involve increased oxidative stress following hepatic metabolism of OPs by the CYP isoforms, as this is known to increase ROS formation (Imaoka et al. 2004).

In conclusion, this study of horticultural farmers occupationally exposed to pesticide mixtures has shown positive relationships between OP exposure and both increased DNA damage and oxidative stress. These data advance this important area of research; however, as the cohorts were small, the findings should be regarded as preliminary and warrant further investigation.

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