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Biomarkers of cellular reaction to pesticide exposure in a rural population

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Abstract

In the present study we report data obtained from the evaluation of subjects occupationally exposed to pesticide mixtures from Santa Fe province, Argentina, using biomarkers for butyrylcholinesterase (BChE) and acetylcholinesterase (AChE) activities, catalase (CAT), lipid peroxidation (by TBARS assay) and the Damage Index Comet Assay (DICA). Our results showed an AChE inhibition (25% and 15% in directly and indirectly groups, respectively) in relation to controls with no significant modifications in BChE. TBARS levels were higher (51%) in pesticide sprayers while CAT activity was reduced in both, applicators (61%) and non-applicators (43%). DICA was significantly increased in direct (83%) and indirect (98%) exposed groups, compared with controls. These results showed modifications in lipid peroxidation, antioxidant defence system, and DNA damage in lymphocytes of exposed workers. Further investigations are suggested in order to link our findings with adverse health effects observed in chronic pesticide toxicity, where oxidative damage plays a pathophysiological role.

Key words: *Oxidative stress; genotoxicity; comet assay; pesticides; human biomonitoring*

Introduction

Pesticides are widely used in intensive agriculture to improve production, protect stored crops and control disease vectors. Although pesticide use has benefits, health risks have been suggested in humans who are occupationally and environmentally exposed to these agrochemicals. Occupational exposure occurs during the preparation of the mixtures, loading and/or washing equipment and spraying on crops (Prakasam et al. 2001, Abdollahi et al. 2004). Chronic exposure to pesticides is associated with damage to health including carcinogenesis, neurotoxicity, reproductive and development alterations and immunological effects (McCauley et al. 2006). In these cases, individuals are often exposed to different pesticides or pesticide mixtures, either simultaneously or in series, making it difficult to identify the effects of each one separately. Thus, exposure and effect biomarkers can be used to detect changes induced by pesticides in human tissues, occurring before the identification of adverse health effects.

The existence of two types of cholinesterases has been proved: one is acetylcholinesterase (AChE) or 'true cholinesterase', which is found in erythrocytes and in cholinergic nerve terminals; the other is butyrylcholinesterase (BChE) or pseudocholinesterase, found in plasma, liver, smooth muscle and fat cells. It is well known that AChE can be an effect biomarker of organophosphorous (OP) and methyl-carbamic (MC) compounds. Also, there is evidence that AChE inhibition correlates with OP-induced symptoms of toxicity (Ranjbar et al. 2002).

Recently, oxygen free radicals (OFRs) have also been involved in pesticide toxicity. Measurement of

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lipid peroxidation products, such as malondialdehyde (MDA), as well as modifications in endogenous OFR scavengers including superoxide dismutase (SOD) and catalase (CAT), are used as effective biomarkers to study OFR effects (Kamel & Hoppin 2004).

In the last decade several studies have shown that pesticides can modify oxidative system balance by inducing reactive oxygen species (ROS) and oxidative damage to tissues (Banerjee et al. 1999). However, there are few reports available that evaluate enzymatic and non-enzymatic antioxidants in human populations considering the use of different pesticides simultaneously (Prakasam et al. 2001, Shadnia et al. 2005, Hernandez et al. 2006).

The aim of this work was to find a possible correlation between DNA damage (evaluated through the comet assay), modifications in oxidative balance (CAT activity and lipid peroxidation) and exposure biomarkers (cholinesterase enzymes) in groups of individuals occupationally exposed to agrochemicals mixtures.

Materials and methods

Chemicals

Acetylthiocholine iodide, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), hydrogen peroxide (H_2O_2), trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), potassium dihydrogen phosphate (KH_2PO_4), potassium hydrogen diphosphate (K_2HPO_4), sodium chloride (NaCl), 1,1,3,3'-tetraethoxypropane, ethylenediaminetetraacetic acid (EDTA), trypan blue, RPMI-1640, agarose, low melting agarose, Tris–HCl, sodium hydroxide (NaOH), acridine orange and ethidium bromide were from Sigma Chemical Co. (St Louis, MO, USA) Plasmatic cholinesterase was processed with Wiener Lab® kit (Rosario, Argentina).

Study subjects

The José M. Cullen Provincial Hospital Ethical Committee established the regulations for the development of the study and informed consent was given by each individual prior to the beginning of the study. A face-to-face questionnaire was completed to obtain information on: (1) standard demographic datum (age, gender, etc.); (2) individual lifestyles (diet, medicine consumption, smoking and alcohol habit); (3) occupational questions (working hours/days, years of exposure, use of protective measures, etc.); and (4) pesticides used in case of sprayers.

The study involved 124 subjects divided into three groups. The first group, directly exposed, consisted of 18 pesticide sprayer workers, the second group, indirectly exposed, included 23 non-applicator agricultural workers and farmers, and the control group consisted of 82 individuals (two unexposed subjects were sought for each exposed one) from the same area, without current or previous exposure to pesticide in their workplace. In this retrospective cohort study, all subjects exposed to pesticide mixtures, in the age range of 18–65 years, worked with horticultural crops. They had been occupationally exposed to a pesticides mixture directly and indirectly in the last month, with a minimum work history of 1 year and a maximum of 25 years.

Sample collection

A blood sample (10 ml) from each subject was obtained from the brachial vein with a heparinized syringe and divided into two vials. The samples were transported on ice to the laboratory and processed within 2 h. The content of one vial was centrifuged at 1000*g* for 10 min for erythrocyte separation. The buffy coat was removed and the remaining erythrocytes were drawn from the bottom and washed three times in cold saline solution (9.0 g l^{-1} NaCl). Aliquots of erythrocytes were kept at–70°C, except those for BChE and AChE assays which were used within 8 h from separation time. The second vial of whole blood was used for the study of DNA damage by means of the comet assay.

Acetylcholinesterase activity in erythrocytes (EC 3.1.1.7)

An aliquot of washed erythrocytes were haemolysed by adding demineralized water at a 1:10 dilution. The hydrolysis rate of acetylthiocholine iodide (substrate) in the erythrocyte dilution was measured at 405 nm with a spectrophotometer, by the reaction with DTNB at pH 7.6, 25°C, to give the yellow 5-thio-2-nitrobenzoate anion. Enzyme activity was expressed as U l^{-1} of red blood cells (RBC) (Ellman 1961).

Plasmatic cholinesterase (EC 3.1.1.8)

Plasmatic cholinesterase was processed using the Wiener Lab® kit. The hydrolysis rate of butyrylthiocholine (substrate) in plasma was measured at 405 nm with a spectrophotometer by the reaction of thiocholine iodide with DTNB at 25°C, to give the yellow 5-thio-2-nitrobenzoate anion. Enzyme activity was expressed as U l^{-1} (Ellman 1961).

Haemoglobin

The conversion of haemoglobin to cyanomethaemoglobin by Drabkin reagent was measured against a

standard curve. The results are expressed as g 100 ml^{-1} haemoglobin.

Catalase activity in erythrocytes (EC 1.11.1.6)

Erythrocytes were haemolysed by adding ice-cold demineralized ultrapure water (Milli Q plus reagent grade) at a 1:100 dilution. CAT activity in haemolysate erythrocytes was measured spectrophotometrically by monitoring the decrease in H_2O_2 concentration over time (Aebi 1984). Sample aliquots (10 µl) were added to 3 ml 50 mM phosphate buffer, pH 7.0, in a quartz cuvette. H_2O_2 was added to a final concentration of 54 mM and absorbance was measured at 240 nm, 25°C during 60 s in the spectrophotometer. The specific activity of each sample was calculated on the basis that one unit of enzyme activity is defined as the activity required to degrade 1 mole of hydrogen peroxide during 60 s g^{-1} Hb.

Lipid peroxidation in erythrocytes

MDA as a marker for lipid peroxidation in RBC was determined by measuring the production of the colour generated during the reaction of TBA with MDA (TBARS assay), according to a modification of the method of Beuge and Aust (1978). Erythrocytes were lysed (dilution 1:4) with ice-cold demineralized ultrapure water (Milli Q plus reagent grade) and mixed thoroughly with four volumes of a solution (15% w/v TCA, 0.375% w/v TBA, 0.25 mol l^{-1} HCl acid) and 4% BHT to inhibit peroxidation stimulated by Fe^{3+}, without affecting the formation of the MDA-TBA chromogen. The mixture was heated in a glycerine bath at 95°C for 45 min. After cooling, the flocculent precipitate was removed by centrifugation at 12 000*g* for 10 min at 4°C. Sample absorbance was determined at 535 nm and TBARS concentration was calculated using the extinction coefficient 1.56×10^5 M^{-1} cm^{-1}. To avoid interferences, iron-free tubes and deionized water were used for the assay. MDA concentration in erythrocytes was expressed as nmol g^{-1} Hb. To obtain precision parameters, 1,1,3,3'-tetraethoxy propane was used.

Alkaline comet assay

The standard procedure originally described by Singh et al. (1988) with modifications was used and two slides were processed for each sample. Negative and positive (H_2O_2 50 µM) controls were included in each electrophoresis run, in order to minimize intralaboratory variation and to allow the comparison of results with those of other population studies. A freshly prepared suspension of 50 µl blood in 950 µl of RPMI 1640 at 37°C was centrifuged at 1000*g* for 4 min. Cell pellets were mixed with 200 µl of a 1% low-melting point agarose solution at 43°C and were spread onto two frosted slides precoated with 1% NMP agarose. To lyse cellular and nuclear membranes of the embedded cells, the key-coded slides were immersed in ice-cold, freshly prepared, pH 10 lysis solution (2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM trizma base, 1% Triton X-100 and DMSO 10%) and left at 4°C overnight. The slides were then placed in an electrophoresis alkaline solution (300 mM NaOH and 1 mM Na_2EDTA, pH >13) for 20 min to allow DNA unwinding. Electrophoresis was performed in the same alkaline solution at 0.75 V cm^{-1} (25 V, 300 mA) for 20 min at 4°C (the temperature of running buffer did not exceed 12°C). After electrophoresis, the slides were neutralized with 0.4 M Tris, pH 7.5, stained with 50 µl of ethidium bromide (2 µg ml^{-1}) and covered with cover slips. All steps were conducted in darkness to prevent additional DNA damage.

One hundred randomly selected 'nucleoids' (50 from each of two duplicated gels) were analysed visually on a scale of 0–4 (zero category is considered without damage and not included in the final score; 1–4 categories, which depend on DNA damage level, are included). The overall score, between 100 and 400 arbitrary units, is related to DNA break frequency and a comet-like image indicates the presence of DNA breaks (Rodriguez Ferreiro et al. 2002). Damage Index Comet Assay (DICA) was calculated for each sample.

Cell viability using fluorescent dyes

The same cell suspension used in the comet assay was mixed with fluorescent DNA-binding dyes and examined by fluorescent microscopy to visualize and count cells with aberrant chromatin organization. A dye-mix working solution of 100 µg ml^{-1} acridine orange and 100 µg ml^{-1} ethidium bromide was prepared in Ca^{2+} and Mg^{2+} free PBS. A volume of 4 µl of this mixture was added to 100 µl of cell suspension. This mixture was examined with a 40x objective using a fluorescent microscope, Olympus CX-40, equipped with an excitation filter (Olympus U-RFLT 50). A minimum of 200 total cells was counted, recording the number of viable cells (V) and nonviable cells (NV) (Mercille & Massie 1994). The percentages of each of these cellular states in relation to the total cells were obtained.

Statistical analysis

The samples were coded at the time of preparation and scoring. They were decoded before statistical analysis for comparison. All experiments were carried out in duplicate. The average values were used for determination of mean ± SD. The distribution of variables was evaluated by the Kolmogorov–Smirnov test. Differences between control and pesticide worker endpoints means were

analysed using the Mann–Whitney non-parametric test and was used to compare the demographic characteristics of studied populations. Correlations between different variables were determined by the Spearman rank correlation test when applicable. A significant level was considered at a *p*-value of 5%, two tailed. All analyses were performed with the SPSS/PC statistical software package (SPSS 11.5 for Windows).

Results

Demographic features of both exposed groups and controls are shown in Table 1. Groups were similar regarding age and smoking habits.

The levels (mean ± SD) of the comet assay, BChE and AChE assay, CAT activity and TBARS assay in control and exposed workers are shown in detail in Table 2.

Statistical evaluations of the two exposed groups were compared in all cases with the population control. AChE showed a significant decrease (p <0.01) with an inhibition of 25 and 15% in the directly and indirectly exposed groups, respectively. BChE showed an inhibition of 4 and 10% in the directly and indirectly exposed groups, respectively, but the decrease was not significant (p >0.05).

A significant increase (51%) in the levels of TBARS was found in pesticide sprayers (p <0.001), but no difference was observed in indirectly exposed group (p >0.05).

CAT activity decreased in the whole pesticide-exposed population (applicators and non-applicators). CAT reduction was 61% in the directly (p <0.0001) and 43% in the indirectly exposed group (p <0.05). Analysis of the comet assay values (mean ± SD) indicated a significant increase in DICA in both the directly and indirectly exposed groups (p <0.001), in relation to controls

Table 1. Demographic characteristics of controls and exposed workers.

Parameter	Controls (n = 82)	Pesticide applicator workers (n = 18)	Non-pesticide applicator workers (n = 23)
Age (years), mean ± SD	37.70 ± 14.07	40.66 ± 11.44	33. 78 ± 11.26
Sex, *n* (%)			
Female	37 (45)	6 (33)	13 (56)
Male	45 (55)	12 (67)	10 (44)
Smoking, *n* (%)			
Yes	20 (25)	3 (17)	3 (13)
No	62 (75)	15 (83)	20 (87)
Alcohol, *n* (%)			
Yes	41 (50)	13 (72)	13 (56)
No	41 (50)	5 (28)	10 (44)

Table 2. Comet assay (DICA), butyrylcholinesterase (BChE) and acetylcholinesterase (AChE) assay, catalase (CAT) activity and assay of thiobarbituric acid reactive substances (TBARS), in control and exposed workers. Values are presented as mean ± SD.

Parameter	Controls (n = 82)	Pesticide applicator workers (n = 18)	Non-pesticide applicator workers (n = 23)
Comet assay	113.56 ± 16.01	212.94 ± 14.79[a]	224.73 ± 20.56[a]
BChE assay (U l^{-1})	6993.31 ± 1131.92	6777.77 ± 1281.84	6313.86 ± 1268.26
AChE assay (U l^{-1} erythrocytes)	9045.54 ± 2191.56	6740.33 ± 1454.48[a]	7651.52 ± 2062.07[a]
CAT activity (kU g^{-1} Hb)	187.12 ± 23.71	72.60 ± 30.48[a]	106.12 ± 37.15[a]
TBARS assay (nmol g Hb)	151.14 ± 30.26	192.74 ± 42.13[a]	138.90 ± 31.89

[a]p <0.05 (Mann–Whitney test). DICA, Damage Index Comet Assay.

Table 3. Overall Spearman correlation among the erythrocyte enzymes studied and DNA damage in direct and indirect pesticide workers.

	Pesticide applicator workers					Non-pesticide applicator workers				
Parameters	DICA	BChE	AChE	CAT	TBARS	DICA	BChE	AChE	CAT	TBARS
DICA	-					-				
ChE	0.457	-				-0.279	-			
AChE	0.001	-0.194	-			0.587**	0.131	-		
CAT	-0.181	0.127	0.002	-		0.110	-0.128	-0.255	-	
TBARS	0.261	0.344	-0.454**	-0.285	-	0.645**	0.208	-0.375*	-0.396	-

DICA, Damage Index Comet Assay, BChE, butyryl cholinesterase; AChE, acetylcholinesterase; CAT, catalase; TBARS, thiobarbituric acid reactive substances.

*p <0.05; **p <0.01.

(87% and 98%, respectively). Cell viability was evaluated and expressed as a proportion of living cells, being >85% in all samples.

The Spearman correlation analysis showed a significant inverse correlation between erythrocyte TBARS and AChE in both exposed groups. On the other hand, the comet assay showed a positive correlation with TBARS in the indirectly exposed group (Table 3). For those variables that showed a significant Spearman correlation (TBARS-AChE) we also carried out a linear regression. Figure 1 shows significant linear regression between AChE and TBARS in pesticide applicators (r = -0.33, p <0.05)

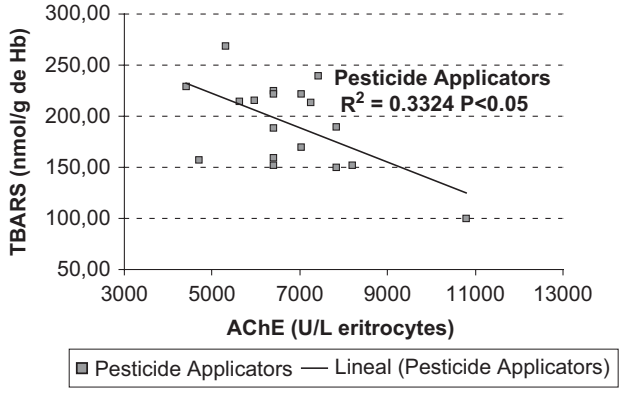

Figure 1. Correlation of biochemical parameters in blood samples. AChE, acetylcholinesterase; TBARS, thiobarbituric acid reactive substances.

Table 4 summarizes the mean data of variables studied stratified by confounding factors in applicators. No significant difference was obtained for any confounding factors.

Personal protective equipment (PPE) was analysed, considering the use of chemical-resistant gloves, breathing masks, glasses, impermeable boots and protective clothing. A total of 93% of the pesticide-exposed workers reported using only one kind of protection during the preparation and application of pesticides.

A significant correlation was found between age and CAT in agricultural and farmer workers (indirectly exposed), but no significant difference was obtained for other confounding factors (Table 5).

Table 6 summarizes the pesticides most commonly used (questionnaire answers) by the exposed subjects, CAS number, International Agency for Research on Cancer (IARC) classification (IARC 2008), Environmental Protection Agency (EPA) classification (EPA 2006) and World Health Organization (WHO) hazard classification (WHO 2004).

Discussion

Different studies have recognized the invaluable role of AChE monitoring in rural workers at high risk for exposure to OPs and MC pesticides (McCauley et al. 2006). However, it is well recognized that this is a relatively

Table 4. Comet assay, butyryl cholinesterase (BChE), acetylcholinesterase (AChE) assay, catalse (CAT) activity and thiobarbituric acid reactive substances (TBARS) assay, in applicator workers in relation to confounding factors.

Parameters	AChE (U l^{-1} erythrocytes) (mean ± SD)	BChE (U l^{-1}) (mean ± SD)	DICA (mean ± SD)	CAT (kU g^{-1} Hb) (mean ± SD)	TBARS (nmol g^{-1} Hb) (mean ± SD)
Age(years)					
<35	6907.00 ± 2227.99	6907.00 ± 2227.99	208.40 ± 17.01	88.76 ± 27.18	179.21 ± 34.53
≥35	6676.23 ± 1151.66	6676.23 ± 1151.66	214.69 ± 14.21	66.38 ± 20.01	197.94 ± 31.94
Sex					
Female	6910.00 ± 2220.99	6999.00 ± 2128.99	223.62 ± 10.56	68.57 ± 33.75	199.08 ± 34.04
Male	6760.94 ± 1496.54	6668.82 ± 1232.38	211.53 ± 13.94	60.91 ± 31.65	193.00 ± 43.40
Smoking					
Yes	6633.33 ± 875.23	5828.33 ± 1166.53	198.00 ± 13.11	65.75 ± 37.65	199.40 ± 25.38
No	6761.73 ± 1567.32	6967.66 ± 1252.60	215.93 ± 13.56	73.97 ± 36.26	191.40 ± 45.29
Alcohol consumption					
Yes	6778.64 ± 1648.68	6589.28 ± 1350.08	212.57 ± 14.56	56.35 ± 25.91*	187.15 ± 45.87
No	6606.25 ± 422.51	7437.50 ± 816.32	214.25 ± 17.85	129.44 ± 35.23*	212.28 ± 16.74
Time					
≤7 days	6640.60 ± 1015.86	6335.40 ± 339.46	208.40 ± 14.35	71.50 ± 34.13	197.50 ± 33.10
>7 days	6865.00 ± 1943.94	7330.75 ± 1371.05	218.62 ± 14.16	73.97 ± 42.30	186.78 ± 43.18
PPE					
No	6476.42 ± 990.59	6423.00 ± 1021.96	216.09 ± 12.42	73.97 ± 35.32	184.49 ± 34.64
Yes	6908.27 ± 1711.10	7003.54 ± 1422.14	208.00 ± 15.85	71.72 ± 30.99	197.98 ± 47.10

DICA, Damage Index Comet Assay, BChE, butyryl cholinesterase; AChE, acetylcholinesterase; CAT, catalase; TBARS, thiobarbituric acid reactive substances; PPE, personal protective equipment.
*p <0.05 (Mann–Whitney test).

Table 5. Comet assay, butyryl cholinesterase (BChE), acetylcholinesterase (AChE) assay, catalse (CAT) activity and thiobarbituric acid reactive substances (TBARS) assay in non-applicator workers in relation to confounding factors.

Parameters	AChE (U l^{-1} erythrocytes) (mean ± SD)	BChE (U l^{-1}) (mean ± SD)	DICA (mean ± SD)	CAT (kU g^{-1} Hb) (mean ± SD)	TBARS (nmol g^{-1} Hb) (mean ± SD)
Age (years)					
<35	7362.28 ± 2268.43	6011.71 ± 1228.99	220.00 ± 20.83	130.32 ± 36.18*	139.78 ± 34.08
≥35	8101.44 ± 1719.71	6783.88 ± 1249.23	232.11 ± 18.90	68.49 ± 38.72*	137.54 ± 30.11
Gender					
Female	7356.84 ± 526.33	6064.00 ± 1187.08	216.84 ± 16.88	98.62 ± 39.89	150.97 ± 33.17
Male	8034.60 ± 2303.02	6638.70 ± 1358.50	35.00 ± 21.14	115.88 ± 39.34	123.21 ± 23.16
Smoking					
Yes	8370.00 ± 1980.63	6645.66 ± 1035.74	239.66 ± 32.92	115.06 ± 39.64	136.44 ± 38.59
No	7543.75 ± 2101.64	6264.10 ± 1315.12	222.50 ± 18.30	104.78 ± 37.94	139.27 ± 31.94
Alcohol consumption					
Yes	7598.35 ± 2285.69	6055.35 ± 1078.97	225.78 ± 21.44	96.83 ± 36.74	144.55 ± 33.78
No	7734.22 ± 1786.39	6716.00 ± 1494.21	223.11 ± 20.28	120.54 ± 40.96	130.11 ± 28.28

DICA, Damage Index Comet Assay, BChE, butyryl cholinesterase; AChE, acetylcholinesterase; CAT, catalase; TBARS, thiobarbituric acid reactive substances; PPE, personal protective equipment.
**p* <0.05 (Mann–Whitney test).

Table 6. List of pesticides used in the study area.

Pesticides	Compound	CAS number	Chemical class	IARC	US EPA	WHO
Fungicide	Captan	133-06-2	Thiophthalimide	3	NL	U
	Copper	7440-50-8	Inorganic-copper	NL	D	NL
	Mancozeb	8018 01 7	Dithiocarbamate-inorganic zinc	NL	B2	U
Insecticide-nematicide	Chlorpyrifos	2921-88-2	Organophosphorus	NL	E	II
	Carbofuran	1563-66-2	Carbamate	NL	NL	Ib
Insecticide	Cypermethrin	67375-30-8	Pyrethroid	NL	NL	II
	Dimethoate	60-51-5	Organophosphorus	NL	C	II
	Endosulfan	115-29-7	Organochlorine	NL	NL	II
	Imidacloprid	105827-78-9	Chloronicotinyl	NL	NL	II
	Malathion	121-75-5	Organophosphorus	3	Suggestive	III
	Methamidophos	10265-92-6	Organophosphorus	NL	E	Ib
	Parathion	56-38-2	Organophosphorus	3	C	Ia
	Permethrin	54774-45-751877-74-8	Pyrethroid	3	Suggestive	II
Herbicide	Glyphosate	1071-83-6	Phosphonoglycine	NL	NL	U

IARC classification: 3, not classifiable as to carcinogenicity to humans; NL, not listed. US EPA classification: group B, probable human carcinogen; B2, sufficient evidence of carcinogenicity from animal studies; group C, possible human carcinogen; group D, not classifiable as to human carcinogenicity; group E, evidence of non-carcinogenicity to humans. WHO hazard classification: Ia, extremely hazardous; Ib, highly hazardous; II, moderately hazardous; III, slightly hazardous; U, unlikely to pose an acute hazard in normal use.

insensitive indicator of an absorbed dose of OP. Blood cholinesterase activity needs at least a 15% decrease from an individual's normal level of plasma or erythrocyte enzyme activity to be considered indicative of pesticide overexposure. In our research AChE levels showed a significant decrease in workers directly and indirectly exposed to pesticides, in agreement with different previous reports (Ranjbar et al. 2002, Singh et al. 2007). Measuring BChE activities is a frequent marker of exposure in pesticide sprayers, is easier to assay and more widely available than AChE. In our study BChE activity was not significantly inhibited in either exposed groups compared with the control. These results may be related to the differential profiles of cholinesterase inhibition that can be observed depending on the particular OP compound, e.g. chlorpyrifos and malathion are preferential inhibitors of BChE while dimethoate preferentially inhibits AChE. Moreover, each kind of cholinesterase has a different mechanism of response. On one side, plasma cholinesterase is generally more rapidly inactivated by exposure to organophosphates and it can be regenerated relatively quickly, being produced in the liver. After exposure, BChE could result in high levels because of the rebound phenomenon. On the other hand, AChE responds later that BchE and is *de novo* synthesized only 20–30 days after efficiently

destroying the Ach in excess. It is also necessary to define fully the interaction profile of a group of pesticides in relation to the composition of the mixture, as the sequence and timing of exposure can have a marked influence on the toxicity profile. We considered that these findings were a consequence of differences in the pesticide mixtures to which workers had been exposed and also because of interindividual variation. Oxidative damage is thought to be an important effect mechanism of several pesticides (Banerjee et al. 1999, Prakasam et al. 2001). In blood, normal erythrocyte function depends on an intact cell membrane, which is the target for many toxic materials, including pesticides. The results of the present study indicate that the CAT activity decrease was significant in both the pesticide applicators and non-applicator groups (p <0.001). The available data on experimental animals (Seth et al. 2001), *in vitro* studies in human cells (Gultekin et al. 2000, Prasanthi et al. 2005) and *in vivo* studies in exposed humans (Ranjbar et al. 2002, Lopez et al. 2007) indicate that the enzymes associated with the antioxidant defence mechanisms change under the influence of pesticides. These enzymes efficiently scavenge toxic free radicals and are partly responsible for protection against lipid peroxidation due to pesticide exposure (Banerjee et al. 1999). Hence, the elevated level of TBARS observed in this research could be due to decreased antioxidant activity caused by exposure to pesticide mixtures, which results in an increased peroxidation of membranes. Different OPs such as phosalone, chlorpyrifos ethyl and diazinon, have been reported to induce oxidative stress as shown by enhancement of MDA production (Gultekin et al. 2000, Prakasam et al. 2001, Altuntas et al. 2003, Catalgol et al. 2007). Carbamate pesticides may induce oxidative stress leading to the generation of free radicals and variation in antioxidant enzymes or OFR scavenging enzymes (Seth et al. 2001, Dettbarn et al. 2006). Some pyrethroids affect the flow of the erythrocyte membrane due to increased lipid peroxidation (Kale et al. 1999, Gabbianelli et al. 2002; Nasuti et al, 2003). It is likely that the production of O_2^- or direct action of pyrethroid on the generation of glutation peroxidase is the cause of oxidative damage (Prasanthi et al. 2005, El Demerdash 2007). The correlation between TBARS and AChE activity found in the present study (Table 3, Figure 1) is in agreement with the results obtained by other authors (Ranjbar et al. 2002, Akhgari et al. 2003, Singh et al. 2007).

Different pesticides used in the region of our study (Table 6) match up with those mentioned by Rodriguez and Lenardon (2007), who evaluated the same area in relation to the incidence of agrochemicals in health and environment of applicators. Measurement of urinary metabolites is not invasive. Although there are few guidance values for urinary metabolites, it requires knowledge of the metabolism and toxic kinetics of the material and the obtaining of specific timed samples of blood and urine for analysis and accurate calculation of absorbed doses of the parent material. Arburckle and Ritter (2005) measured urinary concentrations of different herbicides in 125 women living on farms where herbicides had recently been used for the first time in that growing season. Approximately 80% of the women had no detectable level of either herbicide in their urine. In other study, Acquavella et al. (2006) showed that correlations were lower when algorithm scores were based on participants' self-reports. These results demonstrate the importance of collecting the type of pesticide formulation and suggest that a generic exposure assessment is likely to result in appreciable exposure misclassification for many pesticides.

Several different pathways have been proposed for the occurrence of oxidative DNA damage, including chemical modification of nucleotides (Cicchetti & Argentin 2003), direct action of ROS on DNA or indirect lipid peroxidation degradation products (Collins 1999). Previous studies have used the comet assay to determine the extent of DNA damage in leukocytes from rural workers occupationally exposed to pesticide mixtures. They have reported a significant increase of damage (Garaj-Vrhovac & Zeljezic 2000, Shadnia et al. 2005, Remor et al. 2009, Simoniello et al. 2008).

Although several studies report that pesticide sprayers (applicators) represent the most exposed group of agricultural workers, in our study non-applicators were included in the exposed group because they were present during all working activities, including pesticide applications. This can be due to the misconception that non-applicators are not as exposed as applicators. Our study revealed similar frequencies in the comet assay when considering applicators and non-applicators, in agreement with previous literature research (Costa et al. 2006). Therefore, occupational exposure to pesticides can fluctuate in time and the skin constitutes a significant exposure route of absorption mainly in agriculture. On the other hand, some reports consider para-occupational or take-home exposure as agricultural chemicals move from the work place to residential environments through the activities of farm workers (Curl et al. 2002).

Our results show (Table 2) that pesticide-spraying workers and farmers presented a significant increase of DICA when compared with control subjects (p <0.0001 in both cases). The spraying group exhibited a marginally significant difference ($p = 0.05$) in DICA when we use the PPE worn by individuals as comparison factor. This may be due to the fact that less than 7% of the applicators had used complete equipment and therefore the number of individuals in this population

is very small. Interestingly, studies that evaluated damage where the majority of workers had used protective measures (>60%) reported negative results (Bolognesi et al. 2003, Piperakis et al. 2006), suggesting the importance of the PPE for preventing exposure. Moreover, applicator workers may be affected by the lack of available work-site laundering facilities, prolonging their exposure to pesticides and other farm chemicals.

Tobacco consumption did not affect the results for DNA damage in this study (Tables 4 and 5). In agreement with other studies, the effect of smoking on the comet assay is controversial (Moller et al. 2000).

A significant correlation was found between age and CAT in indirectly exposed workers. This finding could be explained through random deleterious effects of free radicals produced during aerobic metabolism that cause damage to DNA, lipids and protein and accumulate over time (Valko et al. 2007). However, this difference was not observed in pesticide applicators, where CAT values obtained from young applicators are as low as those obtained in the older groups of both applicators and non-applicators. It is essential in this regard to extend sample size in future studies.

We consider that this research contributes to the subclinical evaluation in relation to the potential damage generated by exposure to agrochemicals in our country. Our study shows that subjects directly and indirectly exposed to pesticides have enzymatic changes, modifications in oxidative balance and genotoxic damage when compared with controls, under the conditions of this work. It is essential to enlarge the size of the sampling and perform systematic follow-ups of the populations exposed to pesticide mixtures in our country, using effect and exposure biomarkers.

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