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Occupational exposure characterization in professional sprayers: Clinical utility of oxidative stress biomarkers

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ABSTRACT

The impact of involuntary exposure to pesticides was studied in a group of professional sprayers (S) (25 ± 5 years old) exposed to various agrochemicals for about 10 years. The results were compared with a group of non exposed people (C). S group showed hematological, renal, pancreatic and hepatic biomarkers within the reference values established for the general population, including cholinesterase activity. In spite of that, all the biochemical tests were statistically different compared to C. On the other hand, oxidative stress biomarkers (OSB) such as plasma tocopherol and the total reducing ability of plasma were significantly decreased, while protein carbonyls, thiobarbituric acid-reactive substances, total glutathione and the sum of nitrites and nitrates were increased in the exposed group. Results demonstrated that screening laboratory tests could not be fully sensitive in detecting sub-clinical exposure to pesticides, and also suggest that OSB could be validated and included in health surveillance protocols.

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1. Introduction

Pesticides include a wide variety of compounds such as insecticides, fungicides, herbicides and rodenticides among others. Occupational exposure to pesticides may occur in the process of manufacture (mixing, transport, loading and packaging), as well as in their storage, spraying and/or application. It is well known that the health hazards associated with pesticide handling are little understood by the sprayers themselves (Mekonnen and Agonafir, 2002). While acute toxic effects are easily recognized in these subjects, the effects resulting from long-term exposure to low doses are often difficult to be dis-

tinguished (Banerjee et al., 1999; He, 1999). In addition, farm workers from rural areas in most developing countries do not always pay attention to the use of protective equipment and correct manipulation of the pesticides (Pétille Remor et al., 2009).

For that reason we decided to investigate in more detail the risk associated to pesticide manipulation in a sample of asymptomatic professional workers involved in this agricultural activity. The sprayers studied in the present work were exposed to organophosphorus (OPs), dithiocarbamates, pyrethroids and copper-based fungicides frequently used worldwide to control plagues affecting citric orchards. The biological sample monitoring within this population is becoming

Abbreviations: ALP, alkaline phosphatase; ALT, alanine-aminotransferase; AST, aspartate-aminotransferase; BChE, butyrylcholinesterase; FRAP, ferric reducing ability of plasma; GGT, gamma-glutamyl transpeptidase; GSH, reduced glutathione; GSSG, oxidized glutathione; LDH, lactate dehydrogenase; LPO, lipid peroxidation; NOx, nitrate plus nitrite concentration; OPs, organophosphorus compounds; OS, oxidative stress; OSB, oxidative stress biomarkers; PrCs, protein carbonyls; ROS, reactive oxygenated species; RNS, reactive nitrogenated species; SD, standard deviation; TBARS, thiobarbituric acid-reactive substances.

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an increasingly important element to assess the risk of occupational exposure to pesticides (He, 1999) and to support those policies in favor of the elimination/reduction of exposure and proper information about primary protection on the application of pesticides. In Argentina, sprayer's health is evaluated by a number of routine analyses including hematological, biochemical, hepatic, pancreatic and renal function biomarkers among others. Also, plasma cholinesterase activity was included as the main indicator to monitor exposure to OPs and carbamates. Plasma cholinesterase (BChE) and red blood cell cholinesterase (AChE) reflect the inhibitory effect in the neuronal cholinesterase activity by the above mentioned pesticides. Thus, both isoforms could be used as exposure biomarkers (He, 1999; Singh et al., 2007; Pértile Remor et al., 2009). However, it is known that these biomarkers may not be enough in attempting to detect sub-clinic and sub-symptomatic effects of exposure to pesticides (López et al., 2007).

On the other hand, the production of reactive oxygenated and nitrogenated species (ROS/RNS) and the alteration in antioxidant enzymes have been involved in toxicity associated to pesticides (Prakasam et al., 2001; Muniz et al., 2008). Although studies on lipid peroxidation products and antioxidant status are available concerning experimental animals exposed to more than one pesticide (Astiz et al., 2009a,b), only few detailed reports provide data concerning human exposure to different categories of pesticides simultaneously (Prakasam et al., 2001; Singh et al., 2007). Typically, an oxidative stress biomarker (OSB) shows a change in a biological molecule that has arisen from ROS and/or RNS attack. This concept is applied equally to products derived from lipids, DNA, proteins and antioxidant consumption (Griffiths et al., 2002; Knudsen and Hansen, 2007). Most of these products have been proposed as risk evaluation biomarkers in agrochemical-exposed workers (De Zwart et al., 1999; Dalle-Donne et al., 2006).

Taking into account the previous comments, we aimed to investigate (i) OSB as non-traditional indicators of exposure in professional sprayers from a defined area of Entre Ríos province, and (ii) to evaluate their probable clinical utility in comparison with the usual biochemical analyses performed on this population for appropriate health surveillance.

2. Materials and methods

2.1. Chemicals

All chemicals used were of reagent grade and obtained from Sigma Chem. Co. (CA, or Buenos Aires, Argentina) or Merck Laboratories (Darmstadt, Germany). Organic solvents were from Carlo Erba (Milan, Italy).

2.2. Selection of subjects

This research was conducted over a large region of Entre Ríos Province (Argentina) which is typically involved in fruit plantation activities. The climate is characterized by alternating warm and cold seasons with a high relative humidity throughout the year which makes this area ideal for the production of many temperate crops, especially citrus. In coopera-

tion with local farmer owners we randomly enroll sprayers from 5 different zones (12,000 m² each on average each) with representative agricultural activities of the province. The initial selection criteria were based on a face-to-face questionnaire. From the started selected group, subsequent inclusion/exclusion criteria were applied (detailed below). Thirty-one pesticide applicators (19 males and 12 females) 26 ± 5 years old were recruited. They were specifically involved in controlling pests in citrus fruit plantations for the last ten years. They attended a mandatory health checkup in the middle of the outdoor spraying season (in the third month out of a total of six) and agreed to take part in this study after informed written consent. A control group consisted of 32 subjects (18 males and 14 females) 25 ± 5 years old. These subjects were randomly selected from the general population and hospital workers (nurses, physicians and administrative employees) that never performed spraying or any other activity with pesticides. They were recruited by means of advertising from the same geographical setting as that of applicators, and also they took part in the study after informed written consent. Experimental procedure was revised and approved by the local advisory Bioethical Committee of the School of Medicine, La Plata University (COBIMED; certify protocol #0800-002982/09-000) which follows the guidelines of Helsinki declaration, particularly in those aspects involved in non-invasive procedures for human studies.

Questionnaire data were collected for two staff members who were trained by the study investigation in participant recruitment, interview content and techniques, the safe handling of the biological samples, and ethical issues related to the study. Each interview was carried out on the day when the blood extractions were performed and required approx. 30 min and was administered (simultaneously on sprayers and control groups). The questionnaire includes personal and demographic information; specific agricultural activities, type of pesticide used and application methodology, socioeconomic variables and nutritional status (including questions related to the consumption of foods rich in antioxidants, following the recommendation of the Health Ministry of our country, <http://www.msal.gov.ar>). None of the subjects used antioxidants or any other type of nutritional supplements. The sprayers handled different categories of agrochemicals, mainly OPs (e.g. for the treatment of alphas and coccidia they use dimethoate 3% at doses of 20–30 kg/ha, acephate 75% at doses of 0.05–0.15%, ethion 47% at doses of 0.10–0.20% and chlorpyrifos 36% at doses of 0.15%), dithiocarbamates (e.g. for the treatment of *Alternaria alternata* pv *citri*, they use maneb 10% combined with copper oxychloride 30% and zineb 10%, at doses 0.30–0.50%), pyrethroids (cypermethrin 5%, and permethrin 25% are also used for the treatment of alphas at doses of 0.15% and 0.03% respectively) and copper-based pesticides (copper carbonate, sulphate and oxychloride), for the treatment of the whole tree they use malathion 50% at doses of 0.2% with an average of 5–7 L/tree. They were engaged in mixing the pesticides and loading them into the containers prior to application on the farm fields, as well as spraying according to routine procedures (8 h/day, 6 days/week). Pesticide spray schedules were essentially the same on the entire area subjected to study. The use of personal protective equipment (mainly breathing masks against inhalation, gloves, glasses,

boots and impermeable clothes for dermal exposure) was carefully checked during the period of exposure under study, and the results obtained were considered for statistical analysis.

Just before the extraction of the sample a complete medical interview was carried out in both selected populations. All the subjects included in this study were free from: neoplasias, osteoarticular degenerative diseases, any kind of autoimmune diseases, chronic infections of any etiology (viral, bacterial, fungal), allergy in any degree, nutritional disorders (such as dislipemias, malnutrition), neurodegenerative diseases (direct relatives of patients with diagnosed degenerative diseases were also excluded), heart diseases under pharmacological treatment, varicocele, or endocrine illnesses. Both groups were free of sportsmen/women, workers of paint, plastic, gasoline factories or occupationally exposed to these substances, target of any kind of transplant or in any post-surgical period, women with contraceptive treatment, patients consuming psychoactive drugs, dietary supplements (such as antioxidants supplements), vitamin formulations, anabolic steroids, alcohol and any other drug, people on extreme diets, and pregnant women (in any period and also 6 months after birth), patients recently vaccinated, convalescent of any physical trauma, patients with edema or fever. Excessive smokers (more than 10 cigarettes per day) were excluded; however, a person who smokes less than ten cigarettes per day was included but classified as “smoker”. Alcohol consumption was also registered and there was no significant difference between experimental groups. There was a different range of smokers between exposed and non-exposed groups. Thus, this confounder was considered during statistical treatment of data. None of the sprayers exhibited any clinical symptoms of pesticide exposure.

2.3. Sample collection

Morning fasting (8 a.m.) blood samples were collected in both groups on the same day using heparin as anticoagulant (10 UI/mL) in graduated ice-cold polypropylene test tubes. Plasmas were immediately separated by centrifugation (4000 × g, 10 min) and stored at –80 °C until analyzed. All samples were coded at the time of preparation.

2.4. Analytical methods

Albumin and total bilirubin in plasma as well as the activity of aspartate aminotransferase (AST), alanine aminotransferases (ALT), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), and lactate dehydrogenase (LDH) were determined to evaluate hepatic performance. Renal function was evaluated by plasma creatinine and urea concentrations, while pancreatic function by amilase activity, fructosamines and fasting glucose levels. The pesticide exposure biomarker butyryl-cholinesterase (BChE) was also measured. These determinations were performed using commercial kits from Wiener Lab. (Rosario, Argentina) following the instructions of the manufacturer. All of them, as well as the hematological profile, were the routine sprayer's health checkup in our country. Protein content was determined according to the method of Bradford (1976). Lipid peroxidation was

assayed as thiobarbituric acid-reactive substances (TBARS) with tetraethoxypropane as standard by the method of Yagi (1976) and was expressed as μmol malondialdehyde (MDA)/mg protein. The sum of nitrates and nitrites (NO_x) was measured as the main end-metabolite products of nitric oxide (NO) and peroxynitrite anion (ONOO^-) by the method of Miranda et al. (2001). Results were expressed as μmol of nitrites/mg protein. Protein carbonyls (PrCs) (expressed as nmol/mg of protein) were measured using the method of Reznick and Packer (1994) as a biomarker of oxidative damage to proteins. We also determined non-enzymatic biomarkers of the antioxidant defense system. The FRAP assay (Ferric Reducing Ability of Plasma), used to assess the whole antioxidant ability of samples, was determined by the method of Benzie and Strain (1996) and expressed as nmol/mg of protein of equivalent Trolox® or α -tocopheryl diacetate/L. The samples were previously treated with uricase dissolved in phosphate buffer 50 mM/EDTA 1 mM/glycerol 25 mM (pH 8.50) in a proportion of 1 IU enzyme/ μL plasma to avoid the contribution of uric acid to the total antioxidant ability of plasma. Vitamin E (α -tocopherol) was measured after a solvent extraction following the method of Buttriss and Diplock (1984) and using the HPLC technique of Bagnati et al. (1998). This method can detect and quantify α -tocopherol at μM concentration in plasma samples. Total glutathione (GSH plus GSSG) was measured by the enzymatic recycling method of Anderson and Meister (1984) and the results were expressed as mM concentration.

2.5. Statistical analysis

This was a transversal study designed to compare analytical data between two samples. Processing and scoring of the samples from exposed and control groups were immediately performed blind and concurrently. At the end of the study, the analytical data and the results obtained from the questionnaire were linked for statistical analyses. All data were expressed as the mean \pm standard deviation (SD). The Student *t*-test or the χ^2 test (depending on the type of variable tested) was used for analyzing the results. However, due to the fact that some biochemical parameters may not follow a normal distribution (as judged by Kolmogorov–Smirnov test) the non-parametric Wilcoxon–Mann–Whitney test was also employed (although with equivalent final conclusions). Correlation studies were performed on various parameters (Pearson's rank test and Spearman for normal and non-normal distributed data, as appropriate). The critical level for rejection of the null hypothesis was 5% ($p < 0.05$).

3. Results

Table 1 shows the main characteristics of the samples studied. No significant differences were observed between exposed and non-exposed people concerning age, male/female proportion, estimated diet parameters (including alcohol consumption and foods or supplements with especial antioxidant content) and ethnic constitution. However, the groups showed a different proportion of smokers. Due to the fact that tobacco smoke is a well-documented source of oxidative stress we

Table 1 – Characteristics of the study population.

	Groups	
	Control (C)	Exposed (S)
No. of subjects (n)	32	31
Males	18 (56%)	19 (61%)
Females	14 (44%)	12 (39%)
Age (mean ± SD)	25 ± 5	26 ± 5
Years of exposure (mean ± SD)	–	9.8 ± 3
Ethnia	Caucasian	Caucasian
Smoking status ^a		
Non-smokers	10 (31%)	16 (52%)
Smokers	22 (69%)	15 (48%)
Main dietary parameters ^b		
Type of diet	Balanced/omnivore	Balanced/omnivore
Cal/day	2310 ± 180	2088 ± 210
Percentage from proteins	16 ± 3	13 ± 5
Percentage from lipids	34 ± 6	30 ± 3
Use of antioxidants supplements	None	None
BMI (kg/m ²)	22.6 ± 3.5	20.1 ± 2.6
Personal protective equipment ^c		
Yes	–	23 (74%)
No	–	8 (26%)

^a Subjects who smoke less than 10 cigarettes per day.
^b Dietary parameters were estimated from the nutritional section of the evaluating questionnaire.
^c At least two or more kinds of protective equipment during mixing, loading and pesticide application.

analyzed all the biomarkers taking this question into account (data shown below).

In order to assess the exposure degree to organophosphorus compounds, the activity of butyrylcholinesterase (plasma cholinesterase, BChE) was measured as a typical marker. Table 2 shows the results of BChE activity in plasma from exposed and non-exposed groups considering the fact that professional sprayers may use or not the personal protective equipment (PPE) as stated in Table 1. The S group showed a significant decrease compared to C group indicating the exposure to OPs and carbamates. The sub-group of sprayers that do not use the PPE exhibited even lower levels of BChE activity compared to those who implemented at least two (or more) of the security implements during mixing, loading and spraying the pesticides. As indicated by the manufacturer of the kit, activities below 3200 UI/L were associated to a clinically relevant enzyme inhibition. However, none of the results were below this limit.

Results obtained from hepatic, renal, and pancreatic function biomarkers were listed in Table 3. Sprayer's values were compared with control ones and also with the reference range established by the kits manufacturer. The exposure to pesticides alter hepatic integrity and/or functionality because the levels of AST, ALT, GGT, ALP, LDH and total bilirubin were significantly increased ($p < 0.05$ or less) in the exposed group compared to controls. In addition, plasma level of albumin was diminished. The renal function was evaluated measuring the levels of urea and creatinine. Both parameters were significantly higher in the pesticide exposed group. Similarly, the levels of plasma fasting glucose, fructosamines and pancreatic amilase were incremented in S group compared with controls ($p < 0.01$). Thus, pesticide exposure at work seems to alter hepatic as well as renal and pancreatic functions; however, none of the data reached abnormal levels as compared with reference ranges considered for clinical purposes.

We also studied the hematologic profile in both groups. The hemoglobin content and the number of erythrocytes and lymphocytes were barely distinguishable in both groups (data not shown); however, the relative proportion of leukocytes was significantly modified in the exposed group showing increased segmented neutrophils (SN), basophils (B) and eosinophils (E) percentages with a concomitant decrease in lymphocytes (L) and monocytes (M) (Fig. 1).

The results for the determinations of oxidative stress biomarkers were shown in Table 4. NO_x measurement showed significantly higher values in the S group compared to

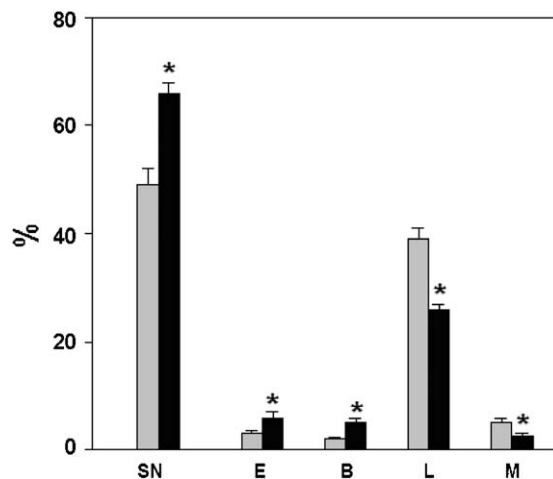


Fig. 1 – Relative proportion of peripheral leukocytes from controls (white bars) and exposed (gray bars) groups. Results were expressed as the mean ± SD. Values significantly different with respect to control group were indicated as * ($p < 0.01$). SN, segmented neutrophils; B, basophils; E, eosinophils; L, lymphocytes; M, monocytes.

Table 2 – Butyrylcholinesterase (plasma cholinesterase, BChE) activity in plasmas from control (C) and exposed (S) groups.

	Groups		
	C	S	
		+PPE	–PPE
BChE (U/L)	7231 ± 650	4910 ± 301 ^a	3574 ± 142 ^{a,b}
PC-C	–	–32.1 ± 2.8	–50.6 ± 3.0
PC-S + PPE	–	–	–27.2 ± 1.1

Data are expressed as the mean ± SD of the *n* indicated in Table 1. Analyses were performed using a commercial kit as detailed in Section 2.4. Mean value for the general population was established as 3200 U/L.

^a *p* < 0.05 respect to C.

^b *p* < 0.05 respect to S + PPE, PC-C, percent change respect to C; PC-S + PPE, percent change respect to S + PPE.

Table 3 – Routine laboratory determinations in control (C) and exposed (S) groups.

Biomarkers	C	S	RV
<i>Hepatic function</i>			
AST (UI/mL)	7.78 ± 1.6	13.06 ± 2.5*	<12
ALT (UI/mL)	9.13 ± 1.6	12.87 ± 3.8*	<12
GGT (U/L)	16.5 ± 6.3	32.0 ± 5.7*	5–38
ALP (UI/L)	112.1 ± 34.0	182.8 ± 46.6*	68–240
LDH (UI/L)	182.5 ± 36.0	267.5 ± 54.3*	160–320
Albumin (g/dL)	4.08 ± 0.3	3.78 ± 0.3*	3.5–4.8
Total bilirubin (mg/L)	4.38 ± 1.2	5.09 ± 1.8*	<10
<i>Renal function</i>			
Urea (g/L)	0.30 ± 0.06	0.33 ± 0.08*	0.20–0.45
Creatinine (mg/L)	11.2 ± 1.9	13.0 ± 2.1*	8–14
<i>Pancreatic function</i>			
Plasma glucose (g/L)	0.84 ± 0.08	0.94 ± 0.08*	0.7–1.10
Fructosamine (mmol DMF/L)	1.95 ± 0.42	2.93 ± 0.67*	1.9–2.9
Amlase (U/dL)	73.3 ± 23.9	88.3 ± 26.5*	<120

Data were expressed as the mean ± SD. Results were obtained using commercial kits as detailed in Section 2.4. Reference values (RV) are those established for this population for the manufacturer of the kits. Values significantly different compared to control group were indicated with * (*p* < 0.05 or less). AST, aspartate amino transferase; ALT, alanine amino transferase; GGT, gamma-glutamyl-transpeptidase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase. DMF, deoximorfolino-fructose used as standard.

controls, while α -tocopherol and the FRAP assay were both significantly decreased. We also measured GSH and its oxidized form (GSSG) as total glutathione (GSH + GSSG) using an enzymatic recycling method; however, no significant differences were observed between experimental groups. In addition, all the parameters that exhibited changes compared to controls also were different when comparing data within the exposed group that used or not the PPE (Table 4). These results were also

tested for the influence of tobacco consumption. We found that the smoking habit introduced a general elevation in the level of all biomarkers (8–14%, depending on the marker) that was insufficient to produce statistically significant differences between sub-groups (data not shown).

We also measured the levels of biomarkers of damage for lipids (TBARS) and proteins (PrCs) (Table 5). Lipid peroxidation end-products determined as malondialdehyde (MDA)

Table 4 – Oxidative stress biomarkers in plasma samples from control and exposed groups.

Oxidative stress biomarkers	Groups		
	C	S	
		+PPE	–PPE
NOx (μ mol/mg prot.)	0.31 ± 0.02	0.40 ± 0.03 ^a	0.46 ± 0.02 ^{a,b}
α -Tocopherol (μ M)	25.2 ± 1.6	18.1 ± 0.9 ^a	15.3 ± 0.5 ^{a,b}
Total glutathione (mM)	0.12 ± 0.02	0.11 ± 0.05	0.14 ± 0.02
FRAP (nmol/mg prot.)	9.85 ± 1.47	7.41 ± 0.9 ^a	6.60 ± 0.5 ^{a,b}

Data were expressed as the mean (of the *n* indicated in Table 1) ± SD. Analyses were performed according to the methods detailed in Section 2.4.

^a Significantly different (*p* < 0.01) compared to C.

^b *p* < 0.05 compared to S + PPE.

Table 5 – Oxidative damage to lipids (TBARS) and proteins (PrCs) in plasmas from control and sprayer groups.

Biomarkers	C	S	
		+PPE	–PPE
TBARS ($\mu\text{mol}/\text{mg prot.}$)	0.8 \pm 0.05	1.15 \pm 0.1 ^a	1.33 \pm 0.1 ^{a,b}
PrCs (nmol/mg prot.)	22.0 \pm 5.6	28.5 \pm 6.2 ^a	29.2 \pm 5.1 ^a

The results were obtained as described in Section 2.4 and expressed as mean (n indicated in Table 1) \pm SD.

^a Significantly different compared to C ($p < 0.01$).

^b Compared to S + PPE.

Table 6 – Correlation coefficients between various experimental variables.

	ALT	GGT	ALP	LDH	ALB	PFA	BChE	NOx	α -Toc	FRAP	TBARS	PrCs
AST	0.39	0.14	0.18	0.17	–0.05	0.08	0.36*	0.11	–0.06	–0.12	–0.15	–0.09
ALT		0.16	0.20	0.15	–0.07	–0.03	0.38*	0.05	–0.04	–0.09	–0.11	–0.12
GGT			0.15	0.37*	–0.40*	0.05	0.16	–0.15	–0.51*	–0.51*	–0.16	–0.11
ALP				0.14	–0.10	0.05	0.14	0.04	0.06	0.11	0.07	–0.04
LDH					–0.45*	0.10	–0.42*	0.36*	–0.36*	–0.44*	0.19	0.15
ALB						–0.12	–0.15	–0.08	–0.14	–0.08	–0.09	–0.04
PFA							–0.14	–0.10	–0.29*	–0.42*	0.14	0.10
BChE								–0.39*	–0.21	–0.15	0.15	0.15
NOx									–0.81*	–0.67*	0.73*	0.20
α -Toc										0.49*	–0.68*	0.18
FRAP											–0.51*	–0.17
TBARS												0.39*

Data were analyzed as described in Section 2.5. Significance was denoted by * ($p < 0.05$ or less). ALB, albumin; PFA, fructosamine. Other parameters exhibited not significant correlations (not shown).

concentration were higher in the exposed group than in controls, and even higher in the sub-group that did not use PPE. Also, PrCs were elevated within the sprayers group; however, they did not show any further elevation due to the absence of PPE (Table 5). Smoking habit did not introduce significant elevation in any of the markers studied (10 and 15% elevation with respect to C data). A clear negative correlation between the levels of α -tocopherol and MDA (in the S group but not within the C group) was also observed. Correlations between other experimental variables are summarized in Table 6.

4. Discussion

Results obtained from this work suggest a hepatic dysfunction (likely of the cholestatic type) probably induced by pesticide exposure. Previous works by other authors demonstrated that various pesticides (mainly OPs) affect mitochondrial membrane transport and disturb cytochrome P450 system in human liver (Singh et al., 2007). The levels of transaminases (ALT and AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and gamma-glutamyl transpeptidase (GGT) were increased indicating hepatic tissue damage. Despite the fact that values were within the interval assigned for normality, the results showed statistical differences compared with the non-exposed group ($p < 0.01$). GGT activity seemed to be the most sensitive biomarker with changes around 95% compared to control values. The other enzymes (AST, ALT, ALP) also increased by almost 50% with respect to control group. This finding was in agreement with previous reports where GGT was considered to be directly associated to oxidative stress conditions such as those induced by pesticide exposure

(Lee et al., 2004; Lim et al., 2004). GGT has a central role in glutathione homeostasis by initiating the breakdown of extracellular glutathione (GSH) during the hepatic re-synthesis process (Halliwell and Gutteridge, 1999). Lim et al. (2004) found an inverse association between the enzyme activity and the levels of many other antioxidants (α -carotene, β -carotene, β -cryptoxanthin, lutein/zeaxanthin and lycopene). In agreement with this, we found not only a direct correlation with LDH and indirect with albumin production, but also an inverse correlation between both GGT and α -tocopherol level and the general antioxidant status of the plasma estimated by FRAP assay. Other authors suggested that GGT activity could be used as an early biomarker of OS (Lim et al., 2004). Thus, this enzyme activity might have important clinical and epidemiological implications in monitoring human exposure to oxidative stress generators.

Renal function was also affected. The levels of urea and creatinine in sprayer's plasmas were significantly higher compared to controls. However, once again the levels of both parameters were within the reference intervals assigned for the population. This is another question of concern since the damage induced on renal function for pesticide exposure might be silent as judged by the values of the conventional markers.

Strong experimental evidence has demonstrated alterations in pancreatic and endocrine function ascribable to acute and chronic exposure to pesticides (Kamath and Rajini, 2007; Rahimi and Abdollahi, 2007). In fact, the hyperglycemia is one of the most common effects observed after acute OPs intoxication. The mechanisms involved in this alteration could be oxidative–nitrosative stress, or adrenal gland hyperstimulation among others (Gilon and Henquin, 2001; Abdollahi

et al., 2004; Kamath and Rajini, 2007; Rahimi and Abdollahi, 2007). Elevation of plasma glucose levels above normal (physiological) values could lead to non-enzymatic glycosylation of plasma proteins (fructosamine formation). We found that fructosamine levels were higher in sprayer's samples (almost 50% over control values) indicating that exposed subjects had asymptomatic hyperglycemia, probably during the post-prandial period due to the fact the fasting glucose levels were within the normal reference range of concentration. Fructosamine level is a strong biomarker because it reflects the average of glucose levels three weeks before sample collection (Misciagna et al., 2004), and the results were much more reliable than glycosylated albumin and/or hemoglobin (Lapolla et al., 2004). Protein glycosylation seemed to be directly associated to oxidative stress because there was a positive correlation between the decrease in plasma antioxidant status and the levels of oxidized LDL and glycosylated proteins (Willems et al., 1998). In agreement with these findings, amylase activity was also higher in sprayer's plasma showing pancreatic tissue damage. We also demonstrated that fructosamine levels inversely correlated with α -tocopherol and FRAP assay.

Interestingly, the exposed group showed some alterations in the relative proportion of leukocyte subtypes. The reason(s) for these differences are unknown; however, we could speculate that this could be derived from a pro-inflammatory condition that might result from the renal-, pancreatic- and/or hepatic-induced damages. However, this fact should be studied in detail and assessed by additional determinations such as acute phase reactants and other biomarkers (Wardle et al., 2008).

Plasma cholinesterase (BChE) has been widely used for monitoring exposure to OPs, and carbamates (He, 1999; Pértile Remor et al., 2009; Singh et al., 2007) because it is the main inhibitory target for all these chemicals (Banerjee et al., 1999; Hernández et al., 2005; López et al., 2007; Singh et al., 2007). In the exposed group BChE activity was significantly reduced compared to that in controls. This decrease was in agreement with that reported by other authors (Hernández et al., 2005; Pértile Remor et al., 2009); however, the result was above the lowest limit established for the reference interval of clinical significance. One important question that arises from this fact is whether the mandatory health checkup performed in the sprayers group is able to detect sub-clinical and/or sub-symptomatic effects of pesticide exposure. Another important conclusion is the supervision of the use of PPE due to the impact that it has on the exposure.

It has been widely recognized the direct association between pesticide exposure and oxidative stress (Prakasam et al., 2001; Di Monte, 2003; Paolini et al., 2004; Testa et al., 2005; Beard, 2006; Nielsen et al., 2006; López et al., 2007; Migliore and Coppèdè, 2008). Also, there is no doubt concerning the role of the xenobiotic-induced OS in the etiology of many diseases (Honig and Rosenberg, 2000; Zhou et al., 2004; Wang et al., 2006; Singh and Dikshit, 2007; Pope et al., 2008). For these reasons, OS biomarkers could be useful tools especially considering that various significant correlations were detected between OS and conventional markers that remain within the reference range for clinical significance. To validate these OS markers, some distracters should be evaluated, being tobacco

smoke one of them if not the most important to be considered. As mentioned before, the percentage of smokers was different between both groups. So far, tobacco smoke is recognized as an OS generator (Halliwell and Gutteridge, 1999). Thus, we analyzed each OS biomarker considering this difference. The results indicated that, smoking habit contributed only by 11% (on average) to the total difference observed between the groups, modifying almost all parameters related to OS in the same direction and with the same degree. Thus, from these results is possible to speculate that the differences observed in OSB could be due to pesticide exposure. In fact, even assuming an incidence of this condition on the results obtained, this incidence may reinforce the value of the conclusions because the possible effect (if any) of the tobacco smoking habit attempt against the differences found between groups.

A particular comment can be done in relation to the levels of NOx as the end-metabolites of nitric oxide and peroxynitrite production. It is well known that excessive RNS generation led to cellular macromolecule oxidative damage and also electron transport chain inhibition (Jackson et al., 2002; Moro et al., 2005; Navarro and Boveris, 2008). The method described by Miranda et al. (2001) is simple, fast and cheap, conditions that suggest it may be easily adapted as a routine laboratory determination (Tsikas, 2006). Interestingly, this biomarker has, respectively, a positive and negative correlation with LDH and BChE activities, which may reinforce the clinical significance of its determination in populations exposed to OS generators. A similar indicative value could be the FRAP assay that gives information regarding the total reducing ability of plasma in order to estimate the balance between pro- and antioxidants. In the exposed group the antioxidant ability was significantly lower than that in control group, probably due to a higher consumption of scavengers. This assay, performed in samples pre-treated with uricase, could also be a useful biomarker easily implemented in large populations. Probably, the main problem with this marker is its poor specificity; however, other authors have previously suggested the FRAP assay as a tool for clinical purposes (Ghiselli et al., 2000; Abuja and Albertini, 2001). By the way, low plasma levels of α -tocopherol (the main lipid soluble antioxidant obtained from the diet) are typically associated with increased fragility of red blood cell membranes, LDLs oxidation, and other deleterious effects related to oxidative alteration of membrane lipids (Halliwell and Gutteridge, 1999; Cadenas and Packer, 2002). In agreement with this fact, the exposed group showed increased lipid peroxidation and decreased α -tocopherol levels, evidencing a clear negative correlation. Also, as commented before, α -tocopherol correlates negatively with GGT and LDH activities and NOx levels, giving a context of interrelationships that reinforce the putative validation of these markers for clinical purpose.

Glutathione level was one of the OS parameters that produced no significant contribution as screening biomarker. This finding might suggest that the plasma concentration was maintained by means of a compensatory mechanism against the pro-oxidative condition induced by pesticide exposure. Liver produces and releases large amounts of reduced glutathione to maintain the physiological concentration in those situations of excessive antioxidant consumption. This

mechanism of endogenous (adaptative) response had previously been suggested by other authors (Cadenas and Packer, 2002).

Protein oxidative damage was also studied by measuring carbonyl formation in the aminoacid side chains (Dalle-Donne et al., 2003a). Previous studies from other laboratories have reported that PrCs are advantageous over lipid peroxidation products as biomarker of oxidative damage due to the fact that they are even more stable than TBARS (Dalle-Donne et al., 2003a,b). As in lipid peroxidation, the exposed group evidenced higher values of this parameter compared to those of controls. These two biomarkers (TBARS and PrCs) can be easily determined in clinical laboratories, have a direct correlation between them, and could be implemented as auxiliary biomarkers in situations of probable pesticide exposure. However, from our results; although TBARS are more unstable than PrCs they seem to be a more sensitive biomarker.

Oxidative stress biomarkers are typically associated with human pathologies such as several chronic diseases, including atherosclerosis, diabetes mellitus, rheumatoid arthritis, endocrine and neurodegenerative disorders (Kohen and Nyska, 2002; Elbaz and Tranchant, 2007; Clementi et al., 2008). So, the evaluation of oxidative stress generation due to pesticide exposure has become an essential question to be properly addressed for preventive purposes. The problem up to now is the lack of a clinical validation of OSB as laboratory techniques; these could be the first step to improve the screening power of the health checkup. Moreover, we suggest that the sampling scheme should be modified. Probably, serial determinations of the same parameter after and during the exposure period could be much more useful than a single measurement to analyze the health impact of pesticide exposure. As suggested by Roldán-Tapia et al. (2006), this conception may be reinforced examining the neurophysiological performance by tests assessing attention, memory, praxis and gnosis, motor coordination, naming and reasoning. Future research on this area may provide additional data concerning the real clinical utility of these determinations.

5. Conclusion

In summary, in agreement with other reports it seems that the occupational exposure to pesticides could alter the balance between plasma pro-oxidants and antioxidants in humans. These effects could have health consequences in the short- or long-term depending on the time, way, and intensity of the exposure, the kind of pesticides simultaneously used, and the attention paid to the use, renewal, and cleaning of the PPE among other factors. We suggest that the efforts may be canalized mainly to primary prevention by giving information to farm workers about possible hazards, improving work conditions, providing them with appropriate PPE and controlling the correct use of these implements. Also, a great effort should be directed to increase experimental works to clarify the complex relationship between chronic exposure and adverse health effects. This may allow us to prevent deleterious effects improving the assessment of preventive strategies such as ambient monitoring, biological monitoring of exposure, and early detection of effects. Taking into account that

routine laboratory tests may be not fully sensitive to assess the degree of damage, at least during the non-symptomatic (sub-clinical) exposure to pesticides, we suggest introducing another scheme of sampling and validation of OSB using them in conjunction with classical exams in order to increment the screening sensitivity of the routine health checkup.

Conflict of interest

The authors declare that there are no conflicts of interest.

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