Investigation of potential biomarkers for the early diagnosis of cellular stability after the exposure of agricultural workers to pesticides

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ABSTRACT

Agricultural workers involved in the harvest of tobacco crops are regularly exposed to large quantities of pesticides. In order to determine how this exposure to pesticides induces genetic alterations in these workers, blood samples were obtained from 77 exposed individuals, as well as from 60 unexposed subjects. DNA damage was analyzed by the Comet assay and by the micronucleus (MN) test. The antioxidant profile was evaluated by activity of superoxide dismutase (SOD), and the polymorphism of gene *PON1* was used as a susceptibility biomarker. The content of inorganic elements in the blood samples was determined by PIXE analysis. Our results demonstrated that the damage frequency, damage index, the MN frequency, and the SOD activity were significantly elevated in the exposed relative to the unexposed group. A modulation of the MN results for the *PON1* gene was observed in the exposed group. The concentrations of inorganic elements in the exposed group were higher compared to those of the unexposed group. In this study, we observed that genetic damage, and change in oxidative balance were induced by the exposure of workers to complex mixtures of pesticides in the presence of inorganic compounds, whereby an influence of the genotype was evident.

Key words: pesticides, Comet assay, micronucleus test, superoxide dismutase activity, *PON1* gene.

INTRODUCTION

Globally, the use of pesticides has increased in recent years. Among the groups involved in the preparation and the final distribution of pesticide mixtures, farmers and agricultural workers are

commonly the most exposed individuals (Bolognesi 2003). Many of these pesticides are classified as "carcinogenic" by the International Agency for Research on Cancer (IARC). Various pesticides interacting with the DNA have been identified to induce miscarriages, degenerative diseases, immune disorders, and cancer (Bolognesi 2003,

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Au et al. 1999). Although it is difficult to establish a correlation between the exposure to pesticides and the prevalence of cancer, especially due to the large number of compounds involved, some studies suggested a higher prevalence of certain types of cancer in population groups exposed to pesticides. According to Buckley et al. (2000) and Meinert et al. (2000), increased incidences of leukemia and non-Hodgkin lymphomas can be observed for individuals exposed to pesticides. A meta-analysis showed that farmers were at risk of specific tumors including leukemia and multiple myeloma (Bolognesi 2003, Bolognesi et al. 2011). Bull et al. (2006) and Bolognesi et al. (2011) argued that the assessment of cytogenetic effects in exposed subjects might act as an early indicator for an increased risk to develop cancer, although the evidence presented was somewhat contradictory.

Numerous reports on chromosomal aberrations (CA; Au et al. 1999, Zeljesic and Garaj-Vrhovac 2001), sister chromatid exchange (SCE; Shaham et al. 2001, Zeljesic and Garaj-Vrhovac 2002), micronucleous (MN; Falck et al. 1999, Da Silva et al. 2008) and Comet assay (Zeljesic and Garaj-Vrhovac 2002, Da Silva et al. 2012a, Da Silva et al. 2008, Grover et al. 2003) observed significant increases in these biomarkers posterior to exposure to pesticides, providing suggestive evidence for the genotoxic effects induced by these chemicals. However, the conclusions drawn with respect to the genotoxic damage attributed to these pesticides remain conflicting. Some investigations indicated a significant increase in MN, SCE, and CA frequencies, while others did not observe any significant differences (Gauthier et al. 2001, Bolognesi 2003, Bull et al. 2006, Da Silva et al. 2012a, b). The responses reported in these studies were found to depend predominantly upon the type of pesticide, the exposure period, and the use of personal protective equipment.

Genotypes responsible for interindividual differences in the ability to activate or detoxify genotoxic substances are recognized as biomarkers

of susceptibility towards mutations, cancer, and other diseases (Zeljesic and Garaj-Vrhovac 2001, Bolognesi 2003, Bolognesi et al. 2011). Paraoxonases (PON) are responsible for the metabolism of organophosphate-based insecticides (Bolognesi 2003), and many enzymatic isoforms, such as e.g. *PON1* were suggested to contribute to the individual cancer susceptibility, as they act as genetic risk modifiers in the development of cancer after the exposure to pesticides (Bolognesi 2003).

In order to evaluate potential biomarkers of exposure to pesticides for the early diagnosis of cellular stability, agricultural workers exposed to pesticides were investigated by the MN frequency test in exfoliated buccal cells, and the Comet assay in peripheral leukocytes. In order to evaluate, if genetically determined individual variations in xenobiotic metabolizing capacity might modify the individual susceptibility to the potential genotoxic effects induced by pesticides, subjects were genotyped for *PON1*. Moreover, SOD activity, hematological parameters, and content levels of inorganic compounds were examined in the blood samples of these workers.

MATERIALS AND METHODS

STUDY POPULATION AND SAMPLE COLLECTION

With an annual production in excess of 687,180 tons, cultivation of tobacco (*Nicotiana tabacum*) generates employment for over 223,000 farmers in Brazil (AFUBRA 2011). The municipality of Santa Cruz do Sul (Rio Grande do Sul, Brazil) is an important production area for tobacco leaves. In order to improve crop characteristics and yield, tobacco farmers usually distribute several pesticides (insecticides, herbicides, fungicides, and plant growth regulators) over tobacco plants by hand pumps.

Between 2007 and 2008 blood and buccal samples were collected from 77 agricultural workers on a tobacco plantation (collected during the peak periods of pesticide application), and

60 unexposed individuals to pesticides from the same region (control group). Individuals who had smoked more than five cigarettes/day for at least one year were considered smokers.

Selected population groups were contacted by community health agents, in order to set an appointment with a general practitioner, where a medical interview was conducted and the individual occupational history was recorded. Clinical data were collected and evaluated by medical personnel. Workers applying pesticides also completed an occupational questionnaire, which included questions related to work activity, duration of the pesticide application, types of pesticides applied, and potentially implemented protective measures. All individuals gave informed consent in writing, and the study was approved by the *Brazilian National Committee on Research Ethics* (*Comissão Nacional de Ética em Pesquisa – CONEP*).

The individuals examined in this study were asked to fill out a Portuguese version of a questionnaire of the *International Commission for Protection against Environmental Mutagens and Carcinogens* (Carrano and Natarajan 1988), before being asked to participate in a face-to-face interview, which included questions related to age, gender, medical issues (they should not have diabetes or cancer or receiving medication treatment), life style (e.g. smoking), and occupation (e.g. working hours per day, exposure times to organic solvents, use of personal protective equipment).

Blood samples were obtained by venopuncture using vacutainers and were processed as quickly as possible, in order to prevent storage-related damage. Blood-cell samples were transported to the laboratory facilities at or below 8 ºC, and processed within 8 h of collection.

EVALUATION OF GENOTOXICITY

Micronucleus Test (MN) in exfoliated buccal cells

Buccal cell samples were obtained by gently rubbing the inside of the subjects' cheeks (right and left side) with a cytobrush, which was subsequently immersed in cold, aqueous saline solution (5 mL; 0.9% NaCl w/v) in a conical tube, before being transported under refrigeration to the laboratory. The suspension was centrifuged (1,500 rpm; 8 min), the supernatant decanted, and the deposited sediment of buccal cells was washed twice with saline (5 mL), and once with Carnoy's fixative (methanol and glacial acetic acid; 3:1 v/v) using the same centrifugation conditions. The cell suspension was dropped onto a slide and allowed to dry in air. The slides were stained with 2% Giemsa solution for 10 min, rinsed with distilled water and dried in air. Thus, 2,000 cells were scored per subject and used to determine the MN frequency. The criteria used for MN analysis were those defined by Tolbert et al. (1992) and Titenko-Holland et al. (1998).

For each subject, 2,000 buccal cells (1,000 on each of the duplicate slides) were analyzed by two different scorers using identical microscopes (Axiophot, Carl Zeiss, Oberkochem, Germany), whereby no significant variability was observed between scorers. All slides were coded for blind analysis, and exposed/unexposed groups were analyzed simultaneously.

Comet assay

The alkaline Comet assay was carried out as described by Singh et al. (1988), whereby the modifications suggested by Tice et al. (2000), Da Silva et al. (2000) and Collins et al. (2008) were implemented. For that purpose, blood cells (5 µL) were embedded in 95 µL of 0.75% low-melting point agarose. After solidification of the agarose, slides were placed for a minimum of 1 h and a maximum of 2 weeks in lysis buffer (2.5 M NaCl, 10 mM EDTA, 10 mM Tris; pH 10.0-10.5), containing 1% (v/v) of freshly added Triton X-100 and 10% (v/v) dimethyl sulfoxide (DMSO). After treatment with lysis buffer, slides were incubated for 20 min in a freshly prepared alkaline buffer

(300 mM NaOH, 1 mM EDTA; pH >13), before DNA was submitted to electrophoresis (20 min; 25 V; 0.90 V/cm; 300 mA). Following electrophoresis, slides were neutralized (400 mM Tris; pH 7.5), fixed (15% w/v trichloroacetic acid, 5% w/v zinc sulfate, 5% glycerol), washed in distilled water, and dried overnight at room temperature. Gels were rehydrated for 5 min in distilled water, and subsequently stained (15 min; 37 ºC) with a solution containing 34 mL of solution B (0.2% w/v NH_4NO_3, 0.2% w/v $AgNO_3$, 0.5% w/v tungstosilicic acid, 0.15% v/v formaldehyde, 5% w/v Na_2CO_3) and 66 mL of solution A (5% Na_2CO_3). The staining was stopped with 1% acetic acid and the gels were dried in air (Nadin et al. 2001).

Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analyzed for each test subject using a fluorescence microscope equipped with a 12 nm BP546 excitation filter and a 590 nm barrier filter. Two parameters were evaluated: (1) damage index (DI), in which each cell was categorized according to tail size and shape (Heuser et al. 2007) into one of five classes 0-4 (no damage = 0; maximum damage = 4). DI values thus obtained for each individual ranged from 0 (0×200) to 400 (4×100); and (2) damage frequency (DF), calculated as the percentage of damaged cells. All slides were coded for blind analysis, whereby exposed and unexposed groups were analyzed simultaneously by two different scorers.

ENZYMATIC ACTIVITY OF SUPEROXIDE DISMUTASE (SOD)

The activity of superoxide dismutase was determined spectrophotometrically in serum samples by measuring the inhibition of the autocatalytic formation rate of adrenochrome at 480 nm. The reaction medium of these samples was adjusted to a pH value of 10.0 and contained a final concentration of 1 mM/L adrenaline (pH 2.0) and 50 mM/L glycine (pH 10.2). Both reagents were purchased from E. Merck. The reaction was allowed to proceed for 3 min at a constant temperature of 30 ºC. The enzymatic activity is expressed in SOD units per g of protein. One unit is defined as the amount of enzyme that inhibits the rate of adrenochrome formation by 50%. Total protein levels were analyzed spectrophotometrically at 545 nm according to the Biureto method (Total Protein Kit-labtest Diagnostica S.A., Brazil).

HEMATOLOGICAL EVALUATIONS

The hematological evaluation was carried out in a commercial laboratory (ENZILAB Laboratory, Santa Cruz do Sul-RS, Brazil) according to standard methods.

DNA EXTRACTION AND GENOTYPING

Genomic DNA was isolated from whole blood samples (collected using vacutainers with EDTA) by the salting out method (Lahiri and Nurnberger 1991). The genotyping of five polymorphic markers was accomplished by using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.

The polymorphism of *PON1* in amino acid 192 (*Gln192Arg*) was examined by PCR/RFLP according to Humbert et al. (1993). For that purpose, an aliquot of the PCR product was digested with *Alw I*, and the genotypes were resolved in agarose gel. The *Arg* allele (G or B type) is present in the high activity allozyme, and the *Gln* allele (A type) is present in the low activity allozyme at position 192 of the protein (*Gln* = 99 bp fragment; *Arg* = 69 bp and 30 bp fragments).

CHEMICAL ANALYSIS

The content of inorganic elements in the blood samples was analyzed by particle-induced X-ray emission (PIXE) (Johansson et al. 1995). Blood samples were briefly dried (t = 6 h; T = 60 °C), ground into a fine powder, homogenized, and pressed into approximately 3 mm thick pellets, before being placed in the target holder inside the reaction chamber. During the experiments, the

pressure inside the reaction chamber was about 10^{-5} mbar. The experiments were carried out at the Ion Implantation Laboratory of the Physics Institute of the Federal University of Rio Grande do Sul (IF-UFRGS). A 3 MV Tandetron accelerator provided a 2.0 MeV proton beam with an average current of 5 nA at the target. The X-rays produced in the samples were detected by a Si(Li) detector with an energy resolution of ca. 160 eV at 5.9 keV. The spectra were analyzed with the GUPIX software package and the data are expressed in parts per million (ppm) (Campbell et al. 2000).

STATISTICAL ANALYSIS

The normality of the variables was evaluated using the Kolmogorov-Smirnov test. To compare the parameters of the study population, c^2 Student t-, and Mann–Whitney U non-parametric tests were used. The critical level for rejection of the null hypothesis was considered to be at $P < 0.05$.

RESULTS

The subjects were classified by gender and age, and divided into smokers and non-smokers. The unexposed group consisted of 60 subjects (38 males and 22 females; mean age: 45.34 ± 13.19 years), whereby 15 subjects were smokers; the exposed group consisted of 77 subjects (49 males and 28 females; mean age: 44.27 ± 12.44 years), whereby 20 subjects were smokers. The mean age did not differ significantly between groups, and about 30% of the male agricultural workers in each group were smokers (no female smokers).

In the exposed group, all agricultural workers were regularly exposed (ca. 2-3 times per week) to complex mixtures of pesticides, the composition of which depends on weather conditions (~600 h/year). The list of pesticides used is shown in Table I. Agricultural activities were conducted by the subjects mainly in open fields, and the main crop was tobacco. As a rule, pesticides were applied onto the tobacco plants at a level above the workers' heads. All pesticide-exposed workers (100%) confirmed that they had not used any kind of personal protection equipment (e.g. gloves, breathing masks, protective goggles, impermeable boots) during the preparation or application of the pesticides. During the exposure period, all individuals presented symptoms related to pesticide poisoning and green tobacco sickness (GTS), such as headaches, abdominal pain, nausea, and vomiting.

The results of the cytogenetic parameter analysis are presented in Table II. No significant

TABLE I
List of pesticides used by exposed group in tobacco cultivation.

Pesticides	Compounds	Chemical Class	Hazard Classification[a]
Fungicides	Mancozeb	Dithiocarbamate	Moderately hazardous
	Propinebe	Carbamate	Moderately hazardous
	Iprodione	Dicarboximide	Slightly hazardous
Insecticides	*O,S*- Dimethyl acethylphosphoramidothiate	Organophosphate	Moderately hazardous
	Chlorpyrifos	Organophosphate	Highly hazardous
	Imidachlopride	Neonicotinoid	Moderately hazardous
	Carbofuran	Carbamate	Extremely hazardous
	Magnesium aluminum phosphide	Inorganic	Highly hazardous
	Deltamethrin	Pyrethoid	Moderately hazardous
Herbicides	Glyphosate	Organophosphate	Slightly hazardous
	Sethoxydim	Organophosphate	Moderately hazardous
	Clomazone	Isoxazolidinone	Moderately hazardous
	Pendimethalin	Dinitroaniline	Moderately hazardous

[a]Hazard classification from National Agency of Sanitary Surveillance (ANVISA: Agência Nacional de Vigilância Sanitária).

differences were found between males and females in the unexposed or exposed groups, both in the Comet assay [unexposed: DI = 9.72 ± 7.50 (males: 9.34 ± 7.79; females: 10.36 ± 7.08); exposed: DI = 28.01 ± 21.43 (males: 30.37 ± 21.83; females: 23.74 ± 20.39)] and MN test [unexposed: MN = 1.33 ± 1.86 (males: 1.42 ± 1.72; females: 1.17 ± 2.09); exposed: MN = 7.14 ± 6.49 (males: 6.3 ± 5.4; females: 8.6 ± 7.9)]. Therefore, samples were combined and divided into exposed and unexposed workers. The analysis of the Comet assay exhibited a significant increase (ca. threefold) in DI and DF in the exposed group compared to the unexposed group (Table II). The exposed group also presented a ca. fivefold increased number of MN in exfoliated

buccal cells relative to the control group. No significant difference was observed between smokers and non-smokers.

Figure 1 shows the effect of the *PON1* (*Gln/Gln*, or *Gln/Arg* or *Arg/Arg*) genotype on the level of different biomarkers evaluated in the exposed individuals. No deviations from Hardy–Weinberg expectations were detected, either relative to the polymorphism or the sample group. Similar genotype and allele distributions (*PON1 192Arg/-* = 36.6%) were observed in the control and the exposed group. A significant increase in MN frequencies was observed for *PON1 Gln/Gln* individuals in the exposed group compared to *PON1 Arg/-* individuals (P < 0.01; Student *t*-test).

TABLE II
Mean values (mean ± standard deviation) obtained in the cytogenetic analysis in unexposed and exposed groups.

Group (n)	Comet assay (100 leukocytes/subject)		MN in Exfoliated buccal cells (2000 cell/subject)
	Damage Index	**Damage Frequency**	
Unexposed (60)	9.72 ± 7.50	6.75 ± 4.73	1.33 ± 1.86
Smokers (15)	11.45 ± 10.01	6.82 ± 5.40	1.31 ± 2.50
Non-smokers (45)	9.33 ± 6.88	6.73 ± 4.63	1.32 ± 1.65
Exposed (77)	28.01 ± 21.43[*]	19.54 ± 13.03[*]	7.14 ± 6.49[*]
Smokers (20)	32.24 ± 21.24[*]	22.76 ± 13.05[*]	5.22 ± 5.64[*]
Non-smokers (57)	24.36 ± 20.12[*]	17.29 ± 12.47[*]	7.73 ± 6.66[*]

[*]Significant difference as compared to the respective control group (unexposed) at P < 0.001 (Student *t*-test).
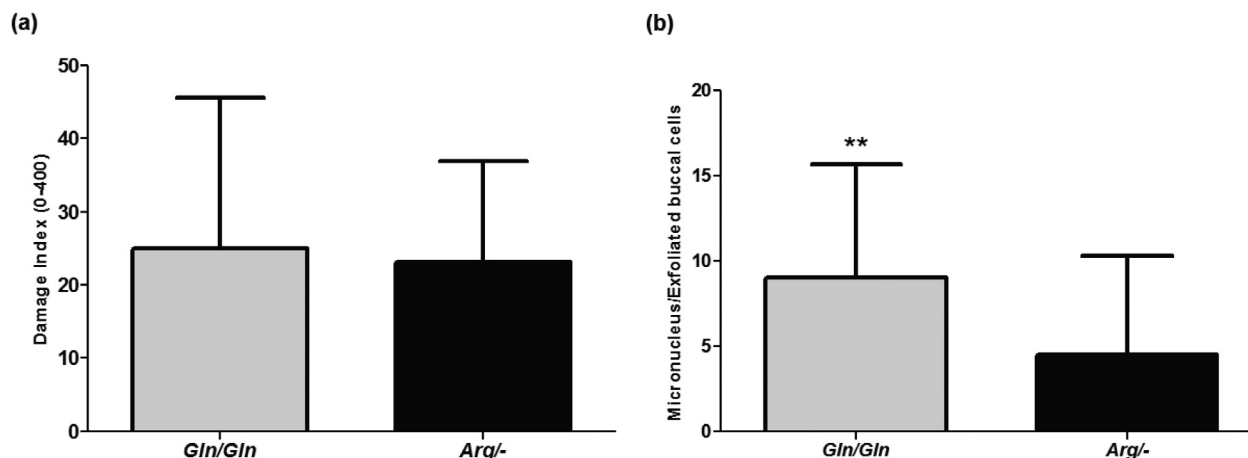

Figure 1 - Effect of individual genotype (*PON1*) on the level of different biomarkers evaluated in exposed group (mean ± S.D.): **(a)** Comet assay and **(b)** Micronucleus test. **Significant at P<0.01 in relation to *PON1 Arg/-* genotype; *t*-Student Test.

The SOD activity is shown in Figure 2. After exposure to pesticides, a drastic increase in SOD activity was observed for the exposed group relative to the unexposed control group (P < 0.001; Student *t*-test).
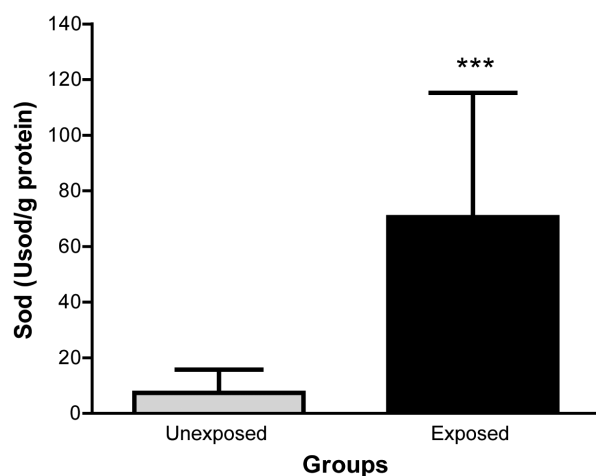

Figure 2 - Superoxide dismutase activity in the unexposed and exposed groups. ***Significant at P<0.001 in relation to unexposed group; Student *t*-Test.

Mean values for the hematological parameters of unexposed and exposed groups, and reference values are presented in Table III. In this analysis, the values obtained were within the normal limits.

In the first sample (unexposed group), the number of eosinophils of two females and one male was higher than the reference values. With the exception of one subject, all volunteers presented normal eosinophil counts in the second sample (exposed group). Nevertheless, significant differences were observed with respect to band neutrophils and monocyte counts between the exposed and the unexposed group (P < 0.05; Student *t*-test).

The content of inorganic elements in blood samples of unexposed and exposed groups was analyzed by PIXE, and the presence of eight elements was detected (Table IV). The inorganic elements that appear in significantly increased concentrations in the blood samples of exposed subjects are: zinc (Zn), magnesium (Mg), and aluminum (Al). Moreover, higher levels of chlorine (Cl) were detected relative to the unexposed group.

DISCUSSION

Pesticides are a heterogeneous category of chemicals, especially designed to control pests, weeds, and plant diseases. The abuse or misuse of pesticides can lead to significant levels of exposure, particularly among those occupationally exposed. The potential cytogenetic damage caused by these

TABLE III
Mean values of hematological parameters (± standard deviation) analyzes of unexposed and exposed groups to pesticides.

Parameters	Unexposed group	Exposed group	Reference values
Hematocrit	44.17 ± 3.50	42.71 ± 3.95	39.00-53.00 g/dl
Hemoglobin	14.22 ± 1.18	14.51 ± 1.43	12.80-17.80 %
White blood cells	6872.72 ± 1794.74	6621.05 ± 1780.94	3.600-11.500/mm^3
Band neutrophils	2.68 ± 1.08	1.78 ± 1.31*	0-5%
Segmented neutrophils	62.7 ± 6.57	60.57 ± 7.52	45-70%
Eosinophils	5.40 ± 11.40	2.94 ± 3.36	1-7%
Basophils	0.00 ± 0.00	0.05 ± 0.22	0-3%
Lymphocytes	30.22 ± 6.61	29.84 ± 6.90	20-50%
Monocytes	1.31 ± 0.64	4.78 ± 1.35*	2-10%
Red blood cells	4.87 ± 0.31	4.74 ± 0.32	4.50-6.10 million/mm^3
Mean Corpuscular Volume	90.51 ± 3.22	89.81 ± 3.15	80.00-98.00m^3
Red cell Distribution Width	12.65 ± 0.52	12.70 ± 0.51	Max. 15.00%
Platelets	206,545.50 ± 47,336.41	233,894.70 ± 50,094.24	150,000-450,000/mm^3

*Significant difference as compared to the control group (unexposed) at P < 0.05 (Student *t*-test).

TABLE IV
Inorganic element detection by PIXE analyses (ppm; mean values± standard deviation)
in the blood samples of unexposed and exposed groups to pesticides.

Inorganic element	Unexposed	Exposed
Zinc	99 ± 28	207 ± 67[*]
Iron	2077 ± 165	2033 ± 189
Magnesium	562 ± 134	1019 ± 271[*]
Aluminum	76 ± 4	648 ± 83[*]
Phosphorus	1599 ± 341	1510 ± 221
Sulfur	3705 ± 155	3664 ± 161
Chlorine	9081 ± 2192	9670 ± 1242
Bromide	12 ± 4	13 ± 8

[*]$P<0.05$ in relation to unexposed group. Mann-Whitney *U*-test.

compounds has been investigated by researchers in several countries, and literature reviews on this subject (Bolognesi 2003) report that most studies found an increase in genotoxicity-biomonitoring end points in subjects using or applying pesticides.

Working environment, personal protective equipment, exposure times and conditions are described in the literature as factors that may affect cytogenetic damage levels (Bolognesi 2003). The agricultural workers in our study did not use any protective measures. In the present study, Comet assay and MN test results showed significantly higher values for the exposed group compared to the unexposed group. Accordingly, this study demonstrates a significant genotoxic and mutagenic effect of exposure to pesticides in tobacco farmers, which is consistent with previous reports. The most frequently exposed agricultural workers are those distributing pesticides by spraying. For these subjects, positive findings were obtained in 18 of 27 studies, representing a 1.12-7.67 times higher exposure rate compared to other workers (Bolognesi 2003). The agricultural workers in this study were exposed to a large number of pesticides, some of which are classified as "hazardous" by the World Health Organization (WHO), e.g. glyphosate, sethoxydim, and chlorpyrifos (Table I). In another study from our group involving tobacco workers from same region in Rio Grande do Sul (Brazil), an

increase in DNA damage was observed by various early biological biomarkers of exposure (Da Silva et al. 2012a, b). It is noteworthy that no difference between males and females was observed by Comet assay or MN test in the unexposed and exposed groups. This result is supported by a recent study of Costa et al. (2006), where no significant differences between males and females regarding chromosome aberrations and SCE was observed.

The Comet assay detects recent repairable lesions, such as breaks and alkali-labile sites, while the MN test detects non-repairable damage, such as clastogenic and aneugenic lesions (Buckley et al. 2000, Collins 2004, Iarmarcovai et al. 2008). In this study, we observed predominantly damage classes related to single strand breaks (classes 1, 2, and 3; data not shown), i.e. some reminiscent lesions or incorrect rejoining of the DNA molecules, which could potentially lead to MN formation (Videla et al. 2003, Iarmarcovai et al. 2008). Some evidence has been presented, suggesting that metal ions contained in some pesticides may interfere with distinct steps of various DNA repair systems and produce reactive oxygen species (ROS), leading to oxidative DNA damage (Videla et al. 2003). It is also noteworthy that in our study, the MN frequency was observed to be increased fivefold between exposed and unexposed individuals. In a review about biomonitoring DNA damage by

MN in human buccal cells, studies about pesticide exposure have shown a ca. onefold increase in MN frequency (Holland et al. 2008).

In addition, many pesticides used extensively on tobacco leaves have metal-based formulations, some of which are associated with DNA damage. Pesticides classified as "moderately hazardous", such as Mancozeb and Propinebe contain zinc (Zn) and manganese (Mn). Furthermore, the insecticide magnesium aluminum phosphide that is classified as "highly hazardous" (Table I) contains magnesium (Mg) and aluminum (Al). In the whole blood samples of tobacco farmers analyzed in this study, significantly increased levels of Zn, Mg, and Al, and chlorine (Cl) were detected by PIXE in the exposed group (Table IV). However, this only confirms exposure to these chemicals. In general, the genotoxicity of metals proceeds via indirect mechanisms. Recently, Beyersmann and Hartwig (2008) described three predominant mechanisms for the genotoxicity of metals: (1) interference with cellular redox regulation and induction of oxidative stress, which may cause oxidative DNA damage or trigger signaling cascades leading to stimulation of cell growth; (2) inhibition of major DNA repair systems, resulting in genomic instability and accumulation of critical mutations; (3) deregulation of cell proliferation by induction of signaling pathways or inactivation of growth controls such as tumor suppressor genes. In addition, several other studies in the literature relate exposure of individuals to inorganic elements with cancer, including those observed in this study (Beyersmann and Hartwig 2008, Khlifi and Hamza-Chaffai 2010, Tokar et al. 2011, Zhao et al. 2014). A previous study from our group investigating tobacco farmers was able to associate DNA damage with increased blood levels of Mg, Al, Zn, and Cl (Da Silva et al. 2012a).

We also noticed a significantly increased SOD activity (Figure 2) in the agricultural workers exposed to pesticides. SOD is an important antioxidant enzyme that metabolizes the superoxide anion into peroxide. The increased activity of this enzyme suggests an increased generation of the superoxide anion by exposure to pesticides. Shadnia et al. (2005) previously demonstrated that chronic exposure to pesticides is associated with increased SOD activity. Other studies have reported that exposure to different categories of pesticides, e.g. organophosphates, carbamates, or pyrethroids leads to oxidative stress in individuals spraying pesticides (Prakasam et al. 2001, Da Silva et al. 2012a).

A significant increase in MN frequency was observed for the *PON1 Gln/Gln* genotype (Figure 1). Enzymes from the *PON1* genes have been reported to be responsible for the metabolism of pesticides, suggesting that the 'unfavorable' alleles could have increased the body load of reactive genotoxic agents in the exposed subjects (Bolognesi 2003, Da Silva et al. 2008). The hydrolysis of paraoxon is catalyzed by serum paraoxonase (*PON1*) / arylesterase, an enzyme associated with the lipoprotein fraction of the serum. Our study revealed that the MN frequency increased in *PON1 Gln/Gln* individuals compared to *PON1 Arg/-* individuals. It has been suggested that individuals with low enzyme levels may be more susceptible to the toxic effects of organophosphates (Bolognesi 2003). This was also observed in our study, where the exposed *Gln/Gln* homozygote individuals presented higher levels of genotoxicity, i.e. higher MN frequencies as a result of exposure to these pesticides. Da Silva et al. (2008) showed that the mean MN frequency in a group of farmers from Caxias do Sul (Rio Grande do Sul, Brazil) exposed to organophosphates was significantly higher for *Gln/Gln* homozygote individuals relative to other genotypes. No increase in DI or DF (repairable damage) was observed for *PON1 Gln/Gln*, which allows the conclusion that the micronuclei observed may be due to a deficiency in the DNA repair mechanisms.

In conclusion, our study demonstrates the presence of genotoxic effects in blood cells and

exfoliated buccal cells of agricultural workers exposed to pesticides. DNA damage may be a consequence of oxidative damage, resulting from exposure to complex pesticide mixtures containing metals. These genotoxic effects were observed during the high-exposure period, when pesticides are applied on a daily basis. However, it is important to emphasize that protective measures are essential to avoid potential health risks. In addition, some effects of genetic polymorphism in the *PON1* gene were observed in the modulation of MN results in the exposed group. Application of new techniques to the occupational exposure to pesticides may facilitate a better understanding of the mechanisms involved in toxicity and carcinogenesis of inorganic elements. Such studies may eventually result in the development of molecular markers for pesticide exposure, toxicity, and carcinogenesis. However, a risk assessment of pesticide users is crucial to prevent long-term health hazards leading to the development of cancer and other degenerative diseases.

RESUMO

Os agricultores envolvidos na colheita do tabaco estão regularmente expostos a grande quantidade de agroquímicos. Para determinar como esta exposição a pesticidas induz alterações genéticas nestes agricultores, amostras de sangue foram obtidas de 77 indivíduos expostos e de 60 indivíduos não expostos. O dano ao DNA foi analisado pelo Ensaio Cometa e pelo Teste de Micronúcleos (MN). A capacidade antioxidante foi avaliada pela atividade da enzima superóxido dismutase (SOD), e a influência do polimorfismo do gene *PON1* foi usada como um biomarcador de susceptibilidade. O teor de elementos inorgânicos foi determinado nas amostras de sangue pela análise PIXE. Nossos resultados mostraram que a freqüência de danos, índice de danos, freqüência de MN e a atividade da SOD foram significativamente mais elevados no grupo exposto em relação ao não exposto. O gene *PON1* demonstrou influenciar a frequência de MN observada no grupo exposto. As concentrações de elementos inorgânicos foram maiores no grupo exposto em relação ao não exposto. Neste estudo observamos que danos genéticos e alterações no balanço oxidativo foram induzidos pela exposição dos agricultores a misturas complexas de pesticidas na presença de componentes inorgânicos, e pelas quais a influência do genótipo foi evidente.

Palavras-chave: pesticidas, ensaio Cometa, teste de micronúcleos, atividade da superóxido dismutase, gene *PON1*.

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