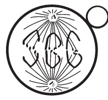

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Research Article

Occupational exposure of workers to pesticides: Toxicogenetics and susceptibility gene polymorphisms

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Abstract

Farm workers are often exposed to pesticides, which are products belonging to a specific chemical group that affects the health of agricultural workers and is mostly recognized as genotoxic and carcinogenic. The exposure of workers from Piauí, Brazil, to these hazardous chemicals was assessed and cytogenetic alterations were evaluated using the buccal micronucleus assay, hematological and lipid parameters, butyrylcholinesterase (BChE) activity and genetic polymorphisms of enzymes involved in the metabolism of pesticides, such as PON1, as well as of the DNA repair system (OGG1, XRCC1 and XRCC4). Two groups of farm workers exposed to different types of pesticides were evaluated and compared to matched non-exposed control groups. A significant increase was observed in the frequencies of micronuclei, kariorrhexis, karyolysis and binucleated cells in the exposed groups ($n = 100$) compared to controls ($n = 100$). No differences were detected regarding the hematological parameters, lipid profile and BChE activity. No significant difference was observed either regarding DNA damage or nuclear fragmentation when specific metabolizing and DNA repair genotypes were investigated in the exposed groups.

Keywords: human monitoring, buccal micronucleus test, pesticides, *PON 1*, DNA repair genes.

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Introduction

The extent and complexity of health hazards inherent to workers who live with injuries caused by some types of work activities have highlighted the need to develop a model study of the future of occupational health (Higashi, 2006). In this scenario, agriculture is one of the main activities deserving special attention with regard to workers' health, since most farmers handle or manipulate highly hazardous compounds such as pesticides.

Pesticides are chemicals used in agriculture to control weeds and pests and to fight plant diseases. Most pesticides are a mixture of several chemical components, characterizing them as a complex mixture (Bolognesi, 2003).

Pesticides are associated with the increase of various types of cancer at specific sites, such as lip, skin, prostate and brain tumors, non-Hodgkin lymphoma, Hodgkin's disease (Blair and Zahm, 1991; Georgellis *et al.*, 1999), leukemia (Purdue *et al.*, 2007), multiple myeloma (Khuder and Mutgi, 1997), cancers of the immune, nervous, reproductive and hematological systems (Beck, 1991; Mourad, 2005) and lipid discords (Remor *et al.*, 2009; Sharma *et al.*, 2010). Many studies have pointed to the action of these chemicals as inducers of chromosomal aberrations (CA) (Au *et al.*, 1999; Zeljezic and Garaj-Vrhovac, 2001), sister chromatid exchange (SCE) (Shaham *et al.*, 2001) and the formation of micronuclei (MN) (da Silva *et al.*, 2008).

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Among the techniques used in human biomonitoring toxicogenetics, the micronucleus assay is universally considered a validated methodology for assessing the genetic instability induced by genotoxic agents (Zalacain *et al.*, 2005). Micronuclei are corpuscles present in the cytoplasm that resemble the core in its structure and color. They are the result of loss of chromosomal fragments or whole chromosomes due to clastogenic and/or aneugenic events, respectively (Maluf and Erdtmann, 2000).

Individual susceptibility plays a critical role in the response to pesticide exposure, determining the onset or absence of clinical symptoms, as well as acute poisoning. This sensitivity is directly associated with polymorphisms of key metabolism enzymes such as paraoxonase 1 (PON 1) (Hernández-Jerez, 2006). Organophosphates are activated by cytochrome P450 and hydrolyzed by PON 1, present in the liver (Costa *et al.*, 2003). Two polymorphisms in *PON1* are common, affecting the efficiency of the protein and its catalytic function for different substrates. A consequence of these polymorphisms is the differential response regarding the incidence of DNA damage in individuals exposed to organophosphates (Morahan *et al.*, 2007). Additionally, polymorphisms that reduce the DNA repair capacity can be associated with enhanced mutagenic effects and should be investigated in order to understand the differences in susceptibility to pesticide exposure (Winkinsonz and Clapper, 1997).

The aim of this study was to assess the effects of human exposure to complex mixtures of pesticides. To evaluate the mutagenic effects of occupational exposure we used the buccal micronucleus assay in exfoliated cells of oral mucosa, besides investigating the polymorphisms of the *PON 1* and DNA repair *OGG1* (8-Oxoguanine glycosylase), *XRCC1* (X Ray Repair Cross-Complementing 1) and *XRCC4* (X Ray Repair Cross-Complementing 4) genes, biochemical parameters such as butyrylcholinesterase (BchE) enzyme, and hematological and lipid parameter activities.

Materials and Methods

Study population

This study was conducted in a setting of agricultural enterprises in the municipalities of Teresina, Nazária and José de Freitas, in the state of Piaui, Brazil. It was approved by the Research Ethics Committee of the Universidade Luterana do Brasil - ULBRA), and individual written informed consent was obtained from all participants. All subjects included completed a detailed questionnaire (a Portuguese version of the International Commission for Protection against Environment Mutagens and Carcinogens) (Carrano and Natarajan, 1988).

The sample used in this study comprised 100 male workers (out of a total of 150 registered workers found in the selected municipalities) exposed to pesticides, who were divided into two groups: group l: 80 individuals registered with the State Occupational Health Reference Center (Association), working with maize, bean and watermelon crops; group 2: 20 workers of a private company that grows lemons and mangoes. The control group was matched for age, sex, ethnic group, smoking and drinking habits. Individuals with a history of exposure to clastogens and aneugens were excluded, according to the protocol of Minozzo *et al.* (2004). A 10 mL blood sample was collected from each individual by venipuncture, using vacutainers with heparine and EDTA. Each blood sample was divided into two 5 mL aliquots, one to be used for DNA extraction and the other for analyzing biochemical, hematological and lipid parameter activities. The buccal samples for the micronucleus assay were obtained by rubbing the inside of the cheeks with a cytobrush. The samples were kept at 4 °C until processing.

Buccal micronucleus assay

An MN test in exfoliated epithelial cells of oral mucosa was performed according to the method described by Salaija *et al.* (2006). The buccal cells were collected from the inner cheeks of the subjects with a cytobrush, washed with 5 mL of cold 1% phosphate-buffered saline solution (PBS) (pH 7.4), centrifuged at 1,500 rpm for 8 min, and fixed with a 3:1 methanol-acetic acid solution. The cell suspension was dropped onto a slide and air-dried at room temperature. The slides were then stained with 2% Giemsa solution for 10 min, rinsed in distilled water, and air-dried. The nuclear abnormalities were evaluated as described by Holland *et al.* (2008). Biomarkers of DNA damage (micronuclei), cytokinetic defects (binucleated cells) and cell death (karyorrhectic and karyolytic cells) were scored in a total of 3,000 buccal cells of each subject. The analysis was performed by light microscopy with a magnification of 1000x.

DNA extraction and genotyping

Genomic DNA was isolated from whole blood according to the method described by Lahiri and Nurnberger Jr (1991), and stored at -20 °C. Four polymorphic markers were investigated by genotyping, using the PCR-RFLP (polymerase chain reaction - restriction fragment length polymorphism) method.

PON1Gln192Arg polymorphism

The *PON1Gln192Arg* polymorphism (location: 7q21.3) was genotyped by PCR/RFLP, as described by Humbert *et al.* (1993). An aliquot of the PCR product was digested with AlwI, and the genotypes were resolved on a 3% agarose gel stained with ethidium bromide.

OGG1Ser326Cys polymorphism

The *OGG Ser326Cys* polymorphism (location: 3p26.2) was genotyped using the primers and PCR condi-

tions described by De Ruyck *et al.* (2005). An aliquot of the PCR product was digested with Alw I, and the genotypes were resolved using on a 3% agarose gel stained with ethidium bromide.

XRCC1Arg194Trp polymorphism

The XRCC1Arg194Trp polymorphism (location: 19q13.2) was genotyped by PCR/RFLP according to Lunn *et al.* (1999). The *XRCC1194Arg* and *XRCC1 194Trp* alleles were detected after digestion with enzyme PvuII, and the genotypes were resolved on a 3% agarose gel stained with ethidium bromide.

XRCC4Ile 401Thr polymorphism

The *XRCC4*Ile 401Thr* polymorphism was genotyped using the primers and PCR conditions described by Relton *et al.* (2004). An aliquot of the PCR product was digested with BstNI, and the genotypes were resolved on a 3% agarose gel stained with ethidium bromide.

Determination of plasmatic BchE activity

The level of BchE activity was measured using the Dietz methodology with a Hitachi/Roche P800 automatic equipment to read absorbance in a spectrometer at 410 nm, in accordance with the protocol of Chaves (Chaves TVS, 2007, Master Thesis, Universidade Federal do Ceará. Master Thesis).

Hematological and lipid parameters

The hematological study was performed using an automatic analyzer (Sysmed KX21) to measure the following hematological parameters: leucocytes (granulocytes, lymphocytes and monocytes), erythrocytes, hematocrit, hemoglobin and platelets. The lipid parameters analyzed were LDL (low density lipoprotein) and HDL (high density lipoprotein) cholesterol, and triglycerides, using Labtest® methods.

Statistical analysis

The normality of the variables was evaluated by the Kolmogorov-Smirnov test, and Student's *t* test was used to compare the characteristics of the study population. Since the values of MN, karyorrhexis and karyolysis were not normally distributed, even after transformation, the non-parametric Kruskal-Wallis test was used to compare them. The gene frequencies were estimated by gene counting, and the Hardy-Weinberg equilibrium was evaluated using the chi-square test, adjusted for small samples. Differences between the genotypes in the exposed groups were tested by the non-parametric Mann-Whitney U-test. All analyses were made using the SPSS 17.0 / PC statistical software.

Results and Discussion

Previous studies have been conducted to investigate the genotoxicity of pesticides in human biomonitoring assessment (Bolognesi, 2003; Rohr *et al.*, 2011). However, there is little information in the scientific literature regarding the assessment of mutagenic occupational exposure using the buccal micronucleus assay associated with *PON 1* polymorphisms and DNA repair genes.

The characteristics of the study population of workers exposed to pesticides and the controls are summarized in Table 1. There were no statistically significant differences between groups 1 and 2 regarding age, smoking and drinking habits. However, the time of exposure was significantly longer in group 1 (Association), whereas group 2 (Private) showed significant differences only in the use of full personal protective equipment (PPE).

The two groups of workers included in this study were exposed to different types of pesticides. Those belonging to group 1 dealt basically with two organophosphates, considered extremely toxic, according to the World Health Organization (2005) (Table 2). The workers of group 2 were exposed to a greater number of pesticides, including two organophosphates, two triazines, a biological

Table 1 - Characteristics of the study population.

Characteristics	Control (n = 62)	Association (n = 80)	Private (n = 20)	p
Age (years)	38.68 ± 12.2	43.1 ± 14.9	35.6 ± 10.2	0.246
Exposure time (years)	-	17.9 ± 12.6	10.5 ± 5.0	0.021
Application				
Hand pump, n (%)	-	80 (100)	0	-
Tractor, n (%)	-	0	20 (100)	-
Full PPE, n (%)[a]	-	26 (32.5)	20 (100)	0.016
Smoking, n (%)[b]	13 (20.9)	20 (25)	4 (20)	0.363
Drinking, n (%)[c]	25 (40.3)	32 (40)	7 (35)	0.328

Data are presented as mean ± standard deviation. Mann-Whitney test.
[a]At least two kinds of PPE during pesticide application.
[b]Individuals who smoke more than 5 cigarettes per day.
[c]Individuals who drink more than two times/week.

Table 2 - List of pesticides used by the employees of the association (Group1) and of the private company (Group 2).

Group	Pesticides	Compound	Chemical Class
Association	Insecticides	Methyl parathion	Organophosphorus
		Dimethoate	Organophosphorus
Private	Fungicide	Copper oxychloride	Inorganic copper
	Insecticide	Abamectin	Biological insecticide
		Dimethoate	Organophosphate
	Herbicide	Glyphosate	Organophosphate
		Atrasine	Triazine
		Ametrine	Triazine

insecticide, and organic copper (Table 2). Of these compounds, only one - dimethoate - is classified as severely toxic (World Health Organization (WHO), 2009). Also concerning Table 2, only triazine is not listed as being carcinogenic in humans (International Agency for Research on Cancer (IARC), 2007).

Regarding the hematological markers (erythrocytes, leukocytes and platelets), both study groups presented normal values, according to Karazawa and Jamra (1989) and Remor *et al.* (2009), and no significant differences were observed between both exposed groups and the controls (Table 3).

Table 4 shows the lipid profiles of farm workers and controls. No significant difference was found between the two groups, and both presented normal lipid profiles, according to reference values of the Brazilian Consensus on Dyslipidemia. Additionally, no significant difference was found either in BChE activity values between non-exposed (9.530 ± 1.713 UL^{-1}) and exposed subjects (8.203 ± 2.865 UL^{-1}), which were within the normal range. Similar results were reported by Shadnia *et al.* (2005) and Benedetti *et al.* (2013), who did not find any association between chronic exposure to organophosphates and cholinesterase inhibition.

To assess the occupational risk of DNA damage, the buccal micronucleus test was performed in oral mucosa, using the following markers: MNi (chromosomal mutations), karyorrhexis (nuclear fragmentation), karyolysis (nuclear breakup) and presence of binucleated cells (defective cytokinesis). Group 1 (Association) and Group 2 (Private) were also compared with their respective negative controls (Figure 1 and 2), and the frequencies of MNi, karyorrhexis, karyolysis and binucleated cells were found to be significantly increased in groups 1 and 2. However, no significant differences were observed in the frequencies of cytogenetic

Table 3 - Hematological parameters in the study groups (mean ± SD).

	Control group (n = 100)	Farm workers (n = 100)
Leukocyte count (x10^3μL)	7.07 ± 1.66	6.86 ± 0.84
Granulocytes (%)	61.50 ± 8.01	52.62 ± 7.07
Lymphocytes (%)	32.55 ± 3.21	43.67 ± 6.65
Monocytes (%)	1.05 ± 0.83	1.27 ± 0.71
Erythrocyte count (x10^3/μL)	5.25 ± 1.02	4.80 ± 0.26
Hematocrit (%)	47.15 ± 8.18	44.18 ± 2.58
Hemoglobin (g/dL)	15.75 ± 2.73	13.99 ± 0.93
Platelets (x10^3/μL)	307.42 ± 76.13	285.80 ± 17.6

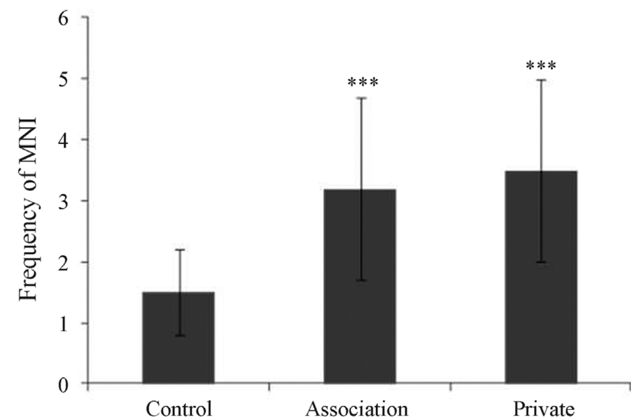

Figure 1 - Comparison of the MNi frequencies between groups of workers exposed to pesticides and the control group (in 1,000 cells). ***p < 0.001 (Kruskal-Wallis test).

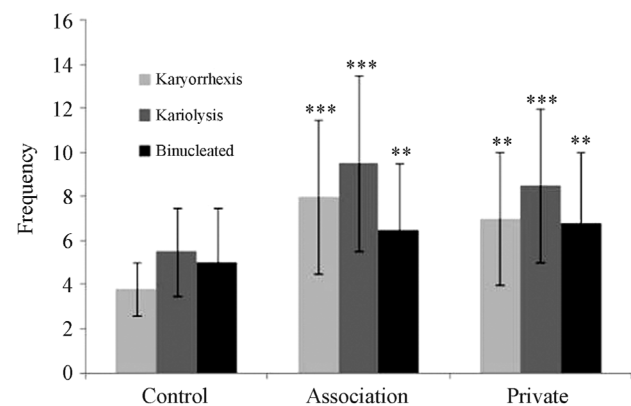

Figure 2 - Comparison between nuclear abnormality frequencies in groups of workers exposed to pesticides and the control group (in 1,000 cells). **p < 0.01; ***p < 0.001 (Kruskal-Wallis test).

Table 4 - Lipid profile of study groups (mean ± SD).

	Controls (n:100)	Farm workers (n:100)	Reference values*
Total cholesterol (mg/dL)			< 200 Desirable
	185.08 ± 27.92	193.18 ± 40.37	200-239 Borderline
			> 240 Increased
LDL (mg/dL)			< 130 Desirable
	116.75 ± 18.33	113.31 ± 25.41	130-159 Borderline
			> 160 Increased
HDL (mg/dL)	40.41 ± 4.05	49.43 ± 6.15	< 35 Desirable
Triglycerides (mg/dL)			< 200 Desirable
	145.51 ± 26.51	119.68 ± 23.65	
			> 200 Increased

*According to the Brazilian Consensus on Dyslipidemia.

parameters when group 1 was compared with group 2. In contrast to the frequencies of MNi and binucleated cells, there was an increase, although not significant, in the frequencies of karyorrhexis and karyolysis in group 1 compared to group 2 (Figure 2).

Some researchers have argued that workers exposed to pesticides can present genetic damage, which can be minimized if proper personal protective equipment is used (Bull *et al.*, 2006). Another factor that complicates comparisons between exposed groups is related to the different pesticides employed (da Silva *et al.*, 2008). In fact, the two groups of workers included in this study were exposed to different pesticides, and there were also significant differences in the use of PPE. Group 1 handled two organophosphates, which are applied from top to bottom, leading to a lower risk of spreading, but only 32.5% used PPE. The subjects of Group 2, exposed to two triazines and two organophosphates, used PPE (100%) and applied pesticides with the pump turned on. All these observations may explain the absence of significant differences in the induction of MN between the two groups.

Both groups 1 and 2 exhibited higher frequencies of cytogenetic alterations compared to their (non-exposed) controls. It is well documented in the scientific literature that organophosphates can induce oxidative damage as well as reduce the levels of cellular antioxidants and antioxidant enzyme activity (Norppa, 2004).Therefore, considering that subjects of groups 1 and 2 may carry an enhanced body burden of reactive genotoxic agents, such as that induced by organophosphates, increased frequencies of cytogenetic alterations should be expected. Even in group 2, in which 100% of workers used full PPE, an increased frequency of events related to DNA damage and cell death was observed. In fact, studies have demonstrated that the use of pesticides such as dithiocarbamates, atrazine and malathion for extended periods of time can cause chromosomal breaks, acentric fragments, dicentrics, sister chromatid exchange and micronucleus frequency (Battershill, 2005).

The presence of binucleated cells is indicative of events of aneuploidy and could lead to failure in cytokinesis after the last nuclear division (Bonassi *et al.*, 2011). Karyorrhexis and karyolysis in turn are related to cell death (Thomas *et al.*, 2008), the first being characterized by the appearance of chromatin aggregation (on the condensation of chromatin), due to fragmentation and nuclear disintegration. Yet, cells in karyolysis show only the shadow of the nucleus (the nucleus "ghost") and are at a late stage of the cell death process.

In order to investigate whether individual genetic variations in xenobiotic metabolizing and DNA damage repair could influence the individual susceptibility to different DNA damage effects of pesticides, the exposed subjects were genotyped for *PON1*, *OGG1*, *XRCC1* and *XRCC4* genes. PON1 is the serum enzyme responsible for the metabolism of organophosphates, and genotoxic metabolites could be increased due to unfavorable genotypes of the exposed individuals (Au *et al.*, 1999). While OGG1 and XRCC1 proteins act in the base excision repair (BER) pathway for the correction of oxidized bases and DNA adducts (Goode *et al.*, 2002; Marsin *et al.*, 2003), XRCC4 acts at the non-homologous end, joining (NHEJ) the repair pathway for the correction of DNA double strand breaks (DSBs).

Genotype distribution and variant allele frequencies in the subjects studied are presented in Table 5. No deviations from the Hardy-Weinberg equilibrium were observed. Table 6 shows the individual effect of genetic polymorphisms in xenobiotic metabolizing and DNA repair enzyme genes on different results of micronucleus, karyorrhexis and karyolysis findings in exfoliated epithelial cells of oral mucosa. No significant difference was observed for the parameters of DNA damage or nuclear fragmentation in relation to the genes investigated. Previous studies have provided evidence that significant DNA

Table 5 - Distribution of genotypes and variant allele frequencies in the exposed groups.

Gene	Variant alleles	N (%)	p
OGG1 Ser326Cys	*Ser/Ser*	77 (68.1)	
	Ser/Cys	36 (31.9)	0.370
	Cys/Cys	0 (0.0)	
XRCC1 Arg194Trp	*Arg/Arg*	97 (85.8)	
	Arg/Trp	16 (14.2)	0.367
	Trp/Trp	0 (0.0)	
XRCC4 Ile401Thr	*Ille/Ille*	90 (91.2)	
	Thr/Ille	10 (8.8)	0.369
	Thr/Thr	0 (0.0)	
PON1 Gln192Arg	*Gln/Gln*	37 (37)	
	GLn/Arg	46 (46)	0.156
	Arg/Arg	17 (17)	

p-value calculated using the Kruskal-Wallis test for comparing allelic frequencies.

Table 6 - Effects of individual genotypes for metabolism (*PON1 Gln192Arg*) and DNA repair genes (*OGG1 Ser326Cys* , *XRCC1 Arg194Trp* , *XRCC4 Ile 401Thr*) polymorphism on the frequencies of the biomarkers evaluated (micronuclei, karyorrhexis and karyolysis) in the exposed groups (mean ± S.D).

Biomarkers	Genotypes	Mean ± SD	p
Micronuclei	*PON1 Gln/Gln*	1.77 ± 1.72 (n = 36)	0.240
	PON1 Arg/-	2.89 ± 2.62 (n = 64)	
	OGG1 Ser/Ser	2.75 ± 2.76 (n = 58)	0.221
	OGG1 Cys/-	2.06 ± 1.68 (n = 29)	
	XRCC1 Arg/Arg	2.70 ± 2.61 (n = 74)	0.117
	XRCC1 Arg/Trp	1.60 ± 1.50 (n = 15)	
	XRCC4 Ille/Ille	2.60 ± 2.32 (n = 66)	0.186
	XRCC4 Thr/-	1.63 ± 1.50 (n = 11)	
Karyorrhexis	*PON1 Gln/Gln*	19.11 ± 11.52 (n = 36)	0.563
	PON1 Arg/-	21.09 ± 18.56 (n = 64)	
	OGG1 Ser/Ser	21.74 ± 17.83 (n = 58)	0.249
	OGG1 Cys/-	20.90 ± 14.60 (n = 29)	
	XRCC1 Arg/Arg	21.19 ± 17.31 (n = 74)	0.349
	XRCC1 Arg/-	16.73 ± 13.32 (n = 15)	
	XRCC4 Ille/Ille	19.14 ± 13.02 (n = 66)	0.400
	XRCC4 Thr/-	23.27 ± 24.25 (n = 11)	
Karyolysis	*PON1 Gln/Gln*	23.42 ± 12.35 (n = 36)	0.986
	PON1 Arg/-	23.47 ± 15.59 (n = 64)	
	OGG1 Ser/Ser	24.41 ± 16.17 (n = 58)	0.888
	OGG1 Cys/-	23.93 ± 12.60 (n = 29)	
	XRCC1 Arg/Arg	23.62 ± 14.91 (n = 74)	0.782
	XRCC1 Arg/Trp	22.47 ± 13.79 (n = 15)	
	XRCC4 Ille/Ille	22.68 ± 11.67 (n = 66)	0.489
	XRCC4 Thr/-	25.73 ± 21.73 (n = 11)	

p value calculated using the Mann-Whitney U-test in relation to a variant (heterozygous and/or homozygous) genotype from the same group.

damage induced by pesticide exposure was not associated with metabolizing or DNA repair enzyme genes (Sözmen *et al.*, 2007; Silva *et al.*, 2012). However, other studies have demonstrated that polymorphism in the *PON1* and BER pathways could modulate the susceptibility to DNA damage caused by pesticide exposure in the cytokinesis-blocked micronucleus (CBMN) assay in human lymphocytes (da Silva *et al.*, 2008, Rohr *et al.*, 2011). Further efforts should be made to characterize the combined effects of metabolizing and DNA repair variants on the response of individuals to pesticide exposure.

In this study, we determined the mutagenic and cell death potential of pesticides through the increased frequency of MNi and other nuclear abnormalities in exposed individuals. The individual metabolism and DNA repair genotypes of the exposed subjects did not show any effect on the investigated biomarkers.

These results also indicate that the buccal mucosa micronucleus test is useful for assessing genotoxic effects of pesticides. Hence, the association with genetic polymorphisms of enzymes involved in xenobiotic metabolism and DNA repair in populations exposed to genotoxic agents such as pesticides are part of the evaluation of individual gene modulation of the cytological response to chemical exposure. This integration is relevant to prevent the harmful effects of pesticides on workers.

Acknowledgments

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