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Markers of genotoxicity and oxidative stress in farmers exposed to pesticides

Carolina Hilgert Jacobsen-Pereira[a,b,*], Claudia Regina dos Santos[c,e], Flora Troina Maraslis[a,b], Luisi Pimentel[d], Ana Júlia Lobo Feijó[d], Clarice Iomara Silva[e], Guilherme da Silva de Medeiros[a,b], Rodrigo Costa Zeferino[f,g], Rozangela Curi Pedrosa[f], Sharbel Weidner Maluf[b]

[a] Postgraduate Program in Pharmacy, Federal University of Santa Catarina, Florianópolis, SC, Brazil
[b] Laboratory of Genetics, University Hospital, Federal University of Santa Catarina, Florianópolis, SC, Brazil
[c] Department of Pathology, Health Science Center, Federal University of Santa Catarina, Florianopolis, SC, Brazil
[d] Faculty of Pharmacy, Federal University of Santa Catarina, Florianópolis, SC, Brazil
[e] Laboratory of Toxicology, University Hospital, Federal University of Santa Catarina, Florianópolis, SC, Brazil
[f] Laboratory of Experimental Biochemistry, Department of Biochemistry, Federal University of Santa Catarina, Florianópolis, SC, Brazil
[g] Postgraduate Program in Biochemistry, Federal University of Santa Catarina, Florianópolis, SC, Brazil

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ABSTRACT

The effects of chronic exposure to pesticides can lead to the development of several diseases, including different types of cancer, since the genotoxic and mutagenic capacity of these substances can be observed. The objective of this study is to investigate the relation between the occupational exposure to various pesticides and the presence of DNA damage and oxidative stress. Blood samples from 50 rural workers (41 men and 9 women) exposed to pesticides, 46 controls (20 men and 26 women) from the same city (Antônio Carlos, Santa Catarina state, Brazil) and 29 controls (15 men and 14 women) from another city (Florianópolis, Santa Catarina state, Brazil), were evaluated using the comet assay and the cytokinesis-block micronucleus (CBMN) technique for genetic damage, and the test of thiobarbituric acid reactive substances (TBARS) and catalase (CAT) activity for the oxidative stress. Cholinesterase activities were also determined, but there was no statistical difference among exposed workers and controls. Significant differences were found in DNA damage among groups. The comet assay performed on peripheral blood lymphocytes of these individuals had a significantly higher DNA damage index in the exposed group comparing to controls ($p < 0.0001$). MNi ($p < 0.001$), NBUDs ($p < 0.005$) and NPBs ($p < 0.0001$) were also found to be significantly higher in the exposed group. The TBARS values were significantly higher comparing to the Florianopolis control group ($p < 0.0001$). Even though CAT values were higher than controls, there was no statistical difference. Thus, it is concluded that the exposed individuals, participants of this study, are more subject to suffer genetic damage and, consequently, more susceptible to diseases resulting from such damages.

1. Introduction

Many in vitro and in vivo studies, as well as epidemiological approaches, have demonstrated the ability of certain pesticides to produce genomic toxicity. This genotoxicity is considered a primary risk factor that will trigger effects over the years, such as carcinogenic, neurological and reproductive processes, due to frequent exposures. Genetic alterations may occur due to mutagenic and non-mutagenic processes, caused by the use of pesticides. Some studies have shown a strong relationship between occupational exposure and some proto-oncogenes in the exposed populations, due to the cytogenetic effects of pesticides (Bolognesi et al., 2011; George and Shukla, 2011). It is attributed a higher frequency of cancer risk involving the brain, skin, esophagus, lung, kidney, bladder, prostate, testis, thyroid, cervix, rectum and soft tissues, as well as leukemia and non-Hodgkin's lymphoma (Blair and Freeman, 2009). Due to the evidence of carcinogenic effects caused by pesticides and the frequency of increased risk in the development of malignancies in occupationally exposed populations, there is a growing need for studies of these populations (Singh et al., 2011).

Cytogenetic methods, including the quantification of micronucleus, have been widely used for the biological monitoring of populations exposed to mutagenic and carcinogenic agents (da Silva, 2016). Micronuclei (MNi) are acentric fragments or complete chromosomes that do not bind to the mitotic spindle during cytokinesis and are excluded from the nuclei. Different mechanisms may be involved in the formation of micronucleus (Heddle et al., 1983; Tucker and Preston, 1996),

* Corresponding author at: Laboratory of Genetics, University Hospital, Federal University of Santa Catarina, Florianópolis, SC, Brazil.
E-mail address: carolina.hil@hotmail.com (C. Hilgert Jacobsen-Pereira).

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including chromosome rupture (clastogenesis) and spindle rupture (aneugenesis).

The comet assay provides a rapid method for quantitatively assessing DNA damage in isolated cells. This method is based on the electrophoresis of incorporated cells and lysed on agarose on a microscope slide. The underlying mechanism is based on the organization of DNA in large coiled structures that can be relaxed by electrophoresis by breaks of ribbons, forming a comet tail like effect. Since the comet method is a relatively simple, inexpensive and rapid technique that can be performed using samples obtained from in vitro or in vivo studies, the assay is suitable for use in biomonitoring of farmers exposed to genotoxic hazards (Kaur et al., 2011). Under alkaline conditions, the comet assay detects cleavages of DNA strands and labile alkaline sites (Singh et al., 1988).

Oxidative stress has been proposed as a mechanism linking exposure to pesticides to increased risk for the development of diseases such as cancer and neurodegenerative diseases. In addition to increasing the production of free radicals, exposure to pesticides can also affect antioxidant capacity and defense mechanisms, as well as increase lipid peroxidation (Abdollahi and Karami-Mohajeri, 2012; Astiz et al., 2011).

Chronic and acute exposures to pesticides are assessed by the levels of their biomarkers, which are cholinesterase enzymes. Reports of current studies demonstrate that cholinesterase activity in rural workers is decreased relative to control subjects (Singh et al., 2011). Plasma cholinesterase (BChE) is reduced more rapidly and intensely than erythrocyte cholinesterase (AChE), reflecting acute exposure to toxic agents. AChE is, in fact, a more accurate biomarker of chronic and low-intensity exposures (ATSDR, 2017).

This study aimed to investigate the relationship between occupational exposure to pesticides and the presence of DNA damage and oxidative stress. Farmers exposed to mixtures of pesticides for at least 15 years were evaluated. In order to evaluate genetic damage, the comet assay and the cytokinesis-block micronucleus (CBMN) technique were performed. Oxidative stress was evaluated by the dosage of thiobarbituric acid reactive substances (TBARS) and catalase activity (CAT). In addition to these tests, the activities of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were also evaluated.

2. Materials and methods

2.1. Study populations

A retrospective cohort study was carried out in which 50 individuals (41 men and 9 women) from the rural population of the city of Antônio Carlos, Santa Catarina state, Brazil, were exposed to a mixture of pesticides for at least 15 years. Farmers were selected from the records of the health center of the city. Their working places were visited and there were held the invitation to participate in the study. The application of a questionnaire was conducted to assess the exposure conditions, use of personal protective equipment, age and smoking (Table 1), in addition to the most commonly used pesticides (Supplementary materials). Peripheral blood samples were collected in Becton Dickinson (Franklin Lakes, Nova Jersey, EUA) tubes and stored in thermal insulation conditions for a maximum of 6 h until analysis. 46 controls (20 men and 26 women) with no history of occupational exposure to agrochemicals, which were matched in terms of age, smoking status and place of residence were evaluated. To consider the environmental exposure, we evaluated 29 controls (15 men and 14 women) with no history of occupational exposure to agrochemicals living in the city of Florianópolis, Santa Catarina state, Brazil, was also evaluated for purposes of comparison between the two locations.

This study was approved by the research ethics committee of the Federal University of Santa Catarina, and all participants gave their informed consent to participate in the study.

2.2. Micronucleus assay

For the micronucleus assay, an aliquot of blood in heparin (0.3 mL) was added to 5.0 mL of RPMI 1640 medium containing 20% fetal bovine serum and phytohemagglutinin (PHA, 0.2%). All reagents are from GIBCO, Grand Island, NY, EUA. The vials were cultured at 37 °C. At 44 h after the start of lymphocyte culture, Cytochalasin B (Sigma-Aldrich, São Paulo, SP, Brazil) was added at a concentration of 4.5 μg mL $^{-1}$, according to the method of Fenech and Morley (1985). The cell suspension was fixed in methanol 3:1 acetic acid (both from Merck KGaA, Rio de Janeiro, RJ, Brazil) under hypotonic treatment and dropped onto clean slides. The slides were then stained in Giemsa (Merck KGaA, Rio de Janeiro, RJ, Brazil). One thousand binucleated cells per individual were evaluated for the presence MNi, nuclear buds (NBUDs) and nucleoplasmic bridges (NPBs) according to Fenech (2007).

2.3. Comet assay

The protocol used was based on the technique proposed by Singh et al. (1988) and adapted by Tice et al. (2000). The slides were prepared by mixing 5.0 μL of whole blood with 90.0 μL of low melting point agarose (0.5%), which was added to a slide completely covered by normal melting point agarose (Sigma-Aldrich, São Paulo, SP, Brazil). A cover slip was immediately placed on the blood and the slide was placed in a refrigerator for 5 min (until the agarose layer hardened). The coverslip was carefully removed and the slide was placed in a freshly prepared cold lysis solution: 2.5 M NaCl, 100 mM EDTA (Química Moderna, Barueri, SP, Brazil), 10 mM Tris (Sigma-Aldrich, São Paulo, SP, Brazil), 1% Na Sarcosinate to which Triton X-100 at 1% and 10% DMSO (Neon Comercial, Suzano, SP, Brazil). After at least 1 h at 4 °C, the slides were removed from the lysis solution and excess liquid was removed by absorbent paper. The slides were placed in the ice-wrapped electrophoresis vessel and filled with fresh electrophoresis buffer: 300 mM NaOH (Neon Comercial, Suzano, SP, Brazil), 1 mM EDTA, pH 13.0, until the liquid completely covered the slides, which were then left for 30 min before power has been turned on. Electrophoresis was performed for 20 min at 25 V and 300 mA. All steps above were performed in the absence of white light to prevent induction of DNA damage. After electrophoresis, the slides were carefully removed from the vessel. Neutralizing buffer: 0.4 M Tris, pH 7.5 (Sigma-Aldrich, São Paulo, SP, Brazil) was added dropwise to the slides 3 times at 5 min intervals. The slides were dried at room temperature for 2 h. To fix the material, it was immersed for 10 min in the fixative solution: 15% trichloroacetic acid, 5% zinc sulfate heptahydrate and 5% glycerol (Neon Comercial, Suzano, SP, Brazil), washed 3 times with distilled water and set to dry for at least 2 h at 37 °C. Prior to staining, the slides were rehydrated for 5 min in distilled water. Coloring solutions followed by the method described by Nadin et al. (2001). Coloring solution A: sodium carbonate 5% (Química Moderna, Barueri, SP, Brazil) and B: ammonium nitrate 0.02% (Química Moderna, Barueri, SP, Brazil), silver nitrate 0.02% (Cennabras, Guarulhos, SP, Brazil), tungstosilicic acid 0.1% (Sigma-Aldrich, São Paulo, SP, Brazil), 0.05% formaldehyde (Neon Comercial, Suzano, SP, Brazil) were mixed immediately prior to use and the slides were placed in cuvettes containing this working solution for approximately 20 min. After the slides had reached a grayish coloration, they were washed with distilled water and placed in the stop solution: 1% acetic acid (Merck KGaA, Rio de Janeiro, RJ, Brazil) for 5 min. After washing again with distilled water, the slides were dried at room temperature. The analysis then proceeded.

For evaluation of DNA damage, 100 cells per subject were analyzed at a magnification of 200x under an optical microscope. The cells were visually evaluated and received scores of 0 (undamaged) to 4 (maximally damaged) according to tail intensity (size and shape). Thus, the total score for 100 cells ranged from 0 (all undamaged) to 400 (all with maximum damage) (Bagatini et al., 2008).

Table 1
General characteristics of the study population.

	Exposed group (N = 50)	Control AC (N = 46)	Control Fp (N = 29)
Gender	82% male 18% female	43% male 57% female	51% male 49% female
Age (mean ± SD*)	51.47 ± 14.23	48.83 ± 14.23	27.46 ± 10.43
Smoking	14% smokers 86% non-smokers	10% smokers 90% non-smokers	2% smokers 98% non-smokers
Years of exposure (mean ± SD*)	34.64 ± 16.09	____	____
Use of PPE**	72% yes 28% no	____	____
Last exposure (days)	up to 2: 29.10% 2 to 5: 16.67% 6 to 10: 22.92% 11 to 15: 16.67% more than 15: 14.58%	____	____

AC: Antônio Carlos city; Fp: Florianópolis city.
* SD: standard deviation.
** PPE: personal protective equipment.

2.4. Determination of catalase

Catalase activity was determined according to the method described by Aebi (1984). The method determines the rate of decomposition of hydrogen peroxide for 20 s by the enzyme catalase present in the sample. On the day of the analysis, a working solution composed of 10 nM hydrogen peroxide in 50 nM phosphate buffer pH 7.0 was prepared and titrated. In a microplate, 2.0 µL of peripheral blood was added in EDTA diluted 1:20. 200 µL of the working solution was added, with the help of multichannel micropipette, immediately before reading at 240 nm, in the Spectramax Paradigm Multileague. The values were expressed in mmol min^{-1} mL^{-1}.

2.5. Evaluation of lipid peroxidation (TBARS levels)

The assay is based on the detection of substances resulting from lipid peroxidation which react with thiobarbituric acid according to the method of Bird and Draper (1984). 1.0 mL of 12% trichloroacetic acid was added in test tubes. This was followed by the addition of 100 µL of plasma and then the tube was homogenized in vortex. 0.9 mL of 60 nM Tris-HCl buffer, pH 7.4 (0.1 mM DTPA) and 1.0 mL of 0.73% thiobarbituric acid were added. After further homogenization, the tube was incubated at 100 °C in a water bath for 1 h. At the end, the tube with the material was cooled for 30 min at 4 °C and then centrifuged at 5 min (1500 × g). With micropipettor, 200 µL of the supernatant was transferred to a microplate and followed with reading at 535 nm in the Infinite M200 TECAN Multileveler. The analyses were performed in triplicate. The result was expressed as nmol mL^{-1}.

2.6. Cholinesterases

The principle of the method consists in the hydrolysis of an acetylcholine substrate by AChE, in the first stage of the reaction, forming acetate and thiocoline. In the second step, the thiocholine is reacted with dithionitrobenzoic acid (DTNB) yielding the yellow anion 5-thio-2-nitrobenzoic acid (TNB) (Worek et al., 1999). For evaluation of AChE, 100 µL of EDTA peripheral blood sample (Vacuette®Tube K3EDTA, Greiner Bio-One, Americana, SP, Brazil) were diluted in 10 mL of hemolyzing Buffer composed by (150 µL of Triton X-100 in 500 mL of 0.1 M phosphate buffer pH 7.4 (both from Sigma-Aldrich, São Paulo, SP, Brazil)). The method, validated by the Laboratory of Toxicology of the University Hospital of the Federal University of Santa Catarina, allowed eliminating interferences of hemoglobin present in the sample. First, a reading by UV-1800 Spectrophotometer (Shimadzu, Barueri, SP, Brazil) at 546 nm was performed to determine hemoglobin concentration using 1.5 mL of hemolysate and 1.5 mL of transformation solution composed by 0.2 g of potassium ferrocyanide (Dinâmica, São Paulo, SP, Brazil), 0.05 g of potassium cyanide, 1.0 g sodium bicarbonate (Both from Vetec, Rio de Janeiro, RJ, Brazil) and 150 µL of Triton X-100 in 1000 mL of ultra-pure water (Medica DV 25, ELGA LabWater, United Kingdom). The AChE activity was then determined and all reagents are from Sigma-Aldrich, São Paulo, SP, Brazil. To 1.0 mL of hemolysate, 10.0 µL of 6 mM ethopropazine, (to inhibit BChE), 2.0 mL of 0.1 M phosphate buffer pH 7.4 and 100 µL of 10 mM DTNB solution were added. The tube was then maintained in a water bath at 37 °C for at least 10 min. Subsequently, the 28.3 mM acetylthiocholine substrate was added. The kinetic reading was performed by UV-1800 Spectrophotometer at 436 nm. All analyzes were performed in triplicate. In the evaluation of BChE, hydroxylation of butyrylthiocholine by BChE, with formation of butyrate and thiocoline, reduces the color reagent 2,6-dichlorophenolindofenol (DCFI), originally blue, rendering it colorless. The variation in absorbance at 600 nm is directly proportional to the BChE activity present in the sample and is measured by a bichromatic rate technique (600 and 700 nm). For this determination was used Reagent Dimension®Flex® Quantitative Determination Pseudocholinesterase (Siemens, Pirituba, SP, Brazil) in Dimension® RxL Max® equipment (Siemens, Pirituba, SP, Brazil).

2.7. Statistical analysis

The data from this study were analyzed by the IBM program SPSS 20.0. The Mann-Whitney tests, Kruskal-Wallis and one-way ANOVA were chosen for comparisons among groups. Spearman Correlations were performed. Values of $p < 0.05$ were considered statistically significant.

3. Results and discussion

The results obtained in this study indicate the presence of genotoxic and mutagenic effects in the exposed group compared to AC and Fp control groups. The effect of continuous and low dose exposure to complex mixtures of pesticides is associated with single and double strand breaks of DNA, oxidative stress and crosslinks. DNA damage, when not repaired or repaired incorrectly, can persist and accumulate, triggering mutagenic processes and causing important cytogenetic changes (Benedetti et al., 2013).

Fig. 1A shows the significant increase in MN frequency in the exposed group comparing to control groups. The three groups were statistically different in relation to the MN frequency. Previous studies carried out by our research group have shown that in cities with strong agricultural activity, the inhabitants of urban areas are also exposed to pesticides. In addition to the vicinity of crops, the use of agrochemicals

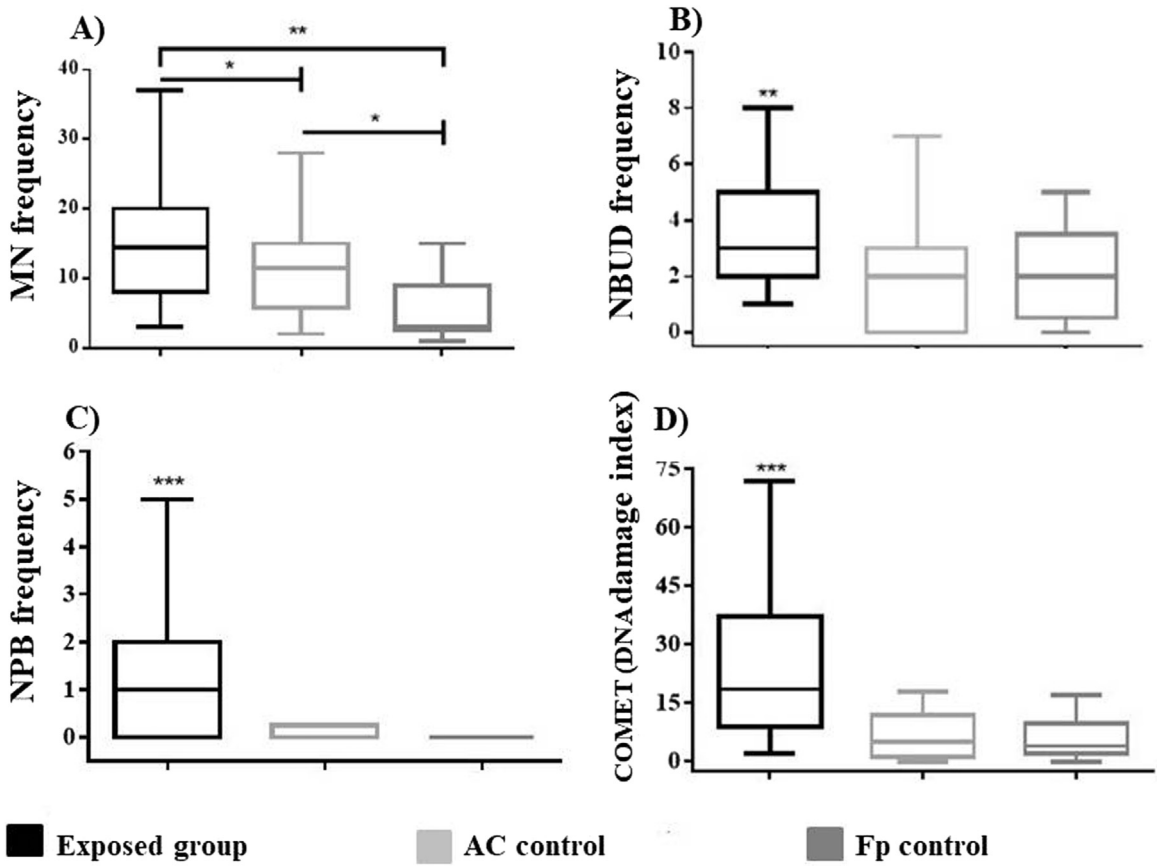

Fig. 1. Frequency of MN, NBUD, NPB and DNA damage index in each of the groups evaluated. The internal horizontal lines in each group represent the medians; boxes and whiskers represent, respectively, the interquartiles and lowest/highest ranges. Exposed Group (N = 50); AC control group (N = 46); Fp control group (N = 29). (*) Represents the statistical difference with $p < 0.001$ (Kruskal-Wallis); (**) Represents the statistical difference with $p < 0.005$ in comparison with control groups (Kruskal-Wallis); (***) Represents the statistical difference with $p < 0.0001$ in comparison with the control groups AC: Antônio Carlos city; Fp: Florianópolis city.

in urban areas, such as squares, parks and in the private courtyards of the houses is observed, without the proper control of the authorities. Therefore, we chose to carry out a second control group of an urban area of a city that does not have a significant role in agricultural production.

Statistical difference was found in the higher frequency of MN, NBUD (Fig. 1B) and NPB (Fig. 1C) in cells submitted to the CBMN technique. A statistically significant increase in DNA damage index was also observed in cells submitted to the comet assay (Fig. 1D). In a review article on MN and exposure to pesticides, Bolognesi et al. (2011) recognized several pesticides such as metamidofos, monocrotophos, glyphosate and endosulfan as agents capable of causing DNA damage. Exposure to herbicides and insecticides such as paration, malathion, 2,4 D and atrazine was also associated with the increase in damage index assessed by the comet test by Maroni et al. (2000), in addition to Garaj-Vrhovac and Zeljezic (2000). Another article that corroborates with the results of this work is the one of Benedetti et al. (2013) that evaluated farmers exposed to the mixture of pesticides and demonstrated to have genotoxic and mutagenic effects linked to the manipulation of these products. The pesticides most commonly used by the subjects of this study can be observed in Supplementary materials section.

The techniques used in this study to detect levels of DNA damage are complementary. While the comet assay detects primarily single strand breaks or structural DNA changes that are induced to evolve into breaks, which are DNA lesions that can be effectively repaired by cells, the CBMN assay detects the result of lesions such as MNi, NPBs or NBUDs, after cell division and DNA repair.

A number of studies have associated occupational exposure to complex mixtures of pesticides with DNA damage using the comet assay (Benedetti et al., 2013; Bhalli et al., 2009; Costa et al., 2014; Da Silva et al., 2014; Khayat et al., 2013; Simoniello et al., 2008; Undeger and Basaran, 2002) and CBMN (Ali et al., 2008; Coskun et al., 2011; Gentile et al., 2012; Marquez et al., 2005; Tope et al., 2006). However, there are studies that have not found such associations with the comet test (Piperakis et al., 2006, 2003) or with the CBMN technique (Joksic et al., 1997; Lucero et al., 2000; Pastor et al., 2003, 2002; Venegas et al., 1998).

As for the other factors that may influence the levels of DNA damage, there was a significant increase in the frequency of MN ($p < 0.03$; Mann Whitney Test) for females compared to males. It can be explained by the tendency to loss of the X chromosome in women. The lost X chromosome would form micronucleus (Fenech and Bonassi, 2011). The other markers of genomic instability were also higher for women, but the differences were not significant. There was a positive correlation between all markers of genetic damage: comet, MN, NBUDs, NPBs (Table 4). The age of the individuals demonstrated to have influence in the increase of MN, being verified a positive correlation between them. This result is also corroborated by other studies (Ali et al., 2008; Fenech et al., 2011; Marquez et al., 2005; Pastor et al., 2003). The time of exposure to pesticides (in years), the time elapsed since the last exposure (in hours or days), the use of personal protection equipment and smoking was evaluated, but in this study none of these factors were shown to have influence in markers of genetic damage (Table 4). Although Fenech et al. (2011) questioned gender, advanced age, malnutrition and other individual conditions lead to an increase in the amount of DNA damage, a large part of the studies with rural workers do not observe such correlations (Joksic et al., 1997; Lucero et al., 2000; Pastor et al., 2002; Tope et al., 2006; Venegas et al., 1998).

Table 2
TBARS and catalase activity in each of the groups evaluated.

	Exposed group (N = 50)	AC control (N = 46)	Fp control (N = 29)
TBARS (nmol mL^{-1})	6.77 ± 2.93***	5.95 ± 2.98***	1.93 ± 1.02
CAT (mmol min^{-1} mL^{-1})	14.22 ± 14.55	8.33 ± 6.83	6.54 ± 5.64

Data are represented as Mean ± Standard Deviation. (***) represents the statistical difference with $p < 0.0001$ in comparison with the Fp control (one-way ANOVA) TBARS: thiobarbituric acid reactive substances; CAT: catalase; AC: Antônio Carlos; Fp: Florianópolis.

Table 3
Cholinesterase enzymes in each of the groups evaluated.

	Exposed group (N = 50)	Control group (N = 46)	Reference value
BChE (U/L)	13908.00 ± 2528.52*	15204.35 ± 2858.82	7000 a 19000
AChE (U/mmol Hb)	500.58 ± 63.86	496.66 ± 58.11	352 a 779

Data are represented as mean ± standard deviation. (*) Represents the statistical difference with $p < 0.02$ in comparison with the control group. BChE: butyrylcholinesterase; AChE: acetylcholinesterase.

The farmers evaluated in this study had higher TBARS values than the AC and Fp control groups, but there was statistical difference only in relation to the Fp Control (Table 2). In addition, age also showed a positive correlation with TBARS, making interpretation of the result uncertain. However, other studies have demonstrated increased values of TBARS in workers exposed to pesticide mixtures (Arnal et al., 2011; Simoniello et al., 2010a; Surajudeen et al., 2014). Lukaszewicz-Hussain (2010), in a review published in 2010, demonstrated that oxidative stress expressed as changes in antioxidant parameters (antioxidant enzyme activity) is the main mechanism of acute and subacute intoxication from organophosphate pesticides.

Pyrethroid insecticides have already been evaluated as products that affect the activity of antioxidant enzymes and increase lipid peroxidation in rats (Raina et al., 2009). Exposure to neonicotinoid imidacloprid may increase levels of lipid peroxidation and is associated with high enzyme activities, including superoxide dismutase, catalase, glutathione peroxidase and glutathione s transferase (El-Gendy et al., 2010). The activity of catalase, oxidative stress marker antioxidant enzyme, was higher in the exposed group compared to controls in this study. However, the difference was not statistically significant (Table 2). Studies evaluating catalase activity in farmers exposed to pesticides present controversial results. There are studies that show a significant inhibition of CAT activity (Hernandez et al., 2013; Murussi et al., 2014; Simoniello et al., 2010a). Oxidation of biomolecules containing thiol groups (sulfhydryl – SH) may be of great importance in pesticide induced cytotoxicity, since these groups can be depleted by free radicals in the presence of oxidative stress. Consequently, enzymes containing this group, such as CAT, are critical targets of the process. Other studies show an increased activity of this enzyme, such as that of Ogut et al. (2011).

In addition, Hernandez et al. (2013) suggested that there is an adaptive response of CAT in certain genotypes of the enzyme paroxonase, which acts on the hydrolysis of certain pesticides, which would explain the differences between studies in the determination of oxidative stress enzymes. The researchers demonstrated a decrease in BChE associated with an increase in CAT levels in certain genotypes, a result also obtained in this research. Studies have indicated genetic polymorphisms as determinants of individual susceptibility to xenobiotics, these include pesticides. Genetic alterations in enzymes that metabolize pesticides may help to clarify the greater or lesser toxicity suffered by individuals. In addition, there is evidence that inhibition of AChE correlates with symptoms induced by pesticide toxicity (Simoniello et al., 2010b). Reports of current studies demonstrate that cholinesterase activity in rural workers is decreased relative to control subjects (Singh et al., 2011). Therefore these enzymes should be monitored in individuals with occupational exposure, and whenever possible, the baseline values for each individual should be determined too, since the individual variability of these parameters is very high. In addition, inhibition of approximately 25% of cholinesterase may occur without significant physiological consequences for the individual (ATSDR, 2014). In our study, both the AChE and BChE activity values of the exposed group were below the values of the Control AC group (Table 3).

However, only in the evaluation of BChE this difference was statistically significant. The results of BChE are in agreement with other studies (Ali et al., 2008; Astiz et al., 2011; Hernandez et al., 2005; Remor et al., 2009). BChE (plasma cholinesterase) is reduced more rapidly and intensely than AChE (erythrocyte cholinesterase), reflecting acute exposure to toxic agents. The half-life of BChE is 8 days and its recovery starts within 72 h after exposure, so it has little value in the identification of chronic intoxications (Maroni et al., 2000). A fact illustrating this characteristic is the negative correlation found in this study between BChE and the injury indexes by the comet assay, another marker of recent exposure (Table 4). Thus, it is suggested that farmers have made use of pesticides in the days prior to sample collection. AChE levels, on the other hand, can last for up to 100–120 days, which is the estimated life expectancy of red blood cells. Among the results of this research is the correlation between AChE and the hours of monthly application of agrochemicals by farmers. AChE is, in fact, a more accurate biomarker of chronic and low-intensity exposures (ATSDR, 2014). Such correlations were not found in similar studies. Although there were differences in the values of AChE and BChE activities among rural workers and controls, all values were within the reference range.

Pesticides have been reported as genotoxic, free radical generators that react with cell membranes and initiate the process of lipid peroxidation. The accumulation of these radicals can cause oxidative stress, depending on the individual antioxidant capacity. There are several

Table 4
Correlations between the variables.

	MNi	NBUDs	NPBs	Comet	TBARS	BChE	Age	DLE	MHU
MNi	ρ 1.000	ρ 0.568**	ρ 0.326**	ρ 0.438**	ρ 0.468**	ρ −0.190	ρ 0.212*	ρ 0.245	ρ 0.304
NBUDs		ρ 1.000	ρ 0.436	ρ 0.299**	ρ 0.204*	ρ −0.092	ρ−0.065	ρ −0.193*	ρ 0.184
NPBs			ρ 1.000	ρ 0.309**	ρ 0.319**	ρ −0.103	ρ−0.013	ρ −0.316*	ρ 0.103
Comet				ρ 1.000	ρ 0.349**	ρ −0.274**	ρ 0.108	ρ −0.326*	ρ 0.047
TBARS					ρ 1.000	ρ −0.223**	ρ 0.338**	ρ −0.218	ρ 0.144
BChE						ρ 1.000	ρ−0.012	ρ 0.208	ρ−0.317
Age							ρ 1.000	ρ 0.375**	ρ 0.217
DLE								ρ 1.000	ρ−0.284**
MHU									ρ 1.000

MNi: micronuclei; NBUDs: nuclear buds; NPBs: nucleoplasmic bridges; BChE: butyrylcholinesterase; DLE: days of last exhibition; MHU: monthly hours of use. ρ: Spearman correlation coefficient; (**) the correlation is significant at the 0.01 level; (*) the correlation is significant at the 0.05 level.

studies with lipoperoxidation products and antioxidant enzymes in animals exposed to more than one pesticide, but few studies with humans bring detailed reports (Astiz et al., 2011). In this study, a positive correlation was found between the TBARS values and the frequency of MNi, NBUDs and NPBs (Table 4), suggesting that oxidative stress may be one of the factors responsible for the DNA damage.

4. Conclusion

Farmers exposed to pesticides showed a significant increase in the frequency of MNi, NBUDs and NPBs. The damage index assessed by the comet assay was also significantly higher in these individuals. Therefore, it can be stated that the exposed group is suffering damage from recent exposure that can be repaired, however, they suffer mutations as well. These mutations may be caused by the direct exposure of the DNA to pesticides or by the oxidative stress generated from the exposure, since the markers of oxidative stress are also altered. Thus, it is concluded that individuals exposed to pesticides, participants in this study, are more subject to genetic damage and, consequently, more susceptible to diseases resulting from these damages. The authors emphasize the importance of carry out a second control group of an urban area of a city that does not have a significant role in agricultural production, since in cities with strong agricultural activity, the inhabitants of urban areas are also exposed to pesticides.

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Conflict of interest

None of the authors have any potential financial conflict of interest related to this manuscript.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2017.10.004.

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