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Markers of genotoxicity and oxidative stress in farmers exposed to pesticides

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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),

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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).