Mutation Research 752 (2013) 28–33

Contents lists available at SciVerse ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Genetic damage in soybean workers exposed to pesticides: Evaluation with the comet and buccal micronucleus cytome assays

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ARTICLE INFO

Article history:
Received 26 October 2012
Received in revised form
28 December 2012
Accepted 3 January 2013
Available online 22 January 2013

Keywords:
Soybean farm workers
Pesticides
Human monitoring
Comet assay
Buccal micronucleus cytome assay

ABSTRACT

Soybean cultivation is widespread in the State of Rio Grande do Sul (RS, Brazil), especially in the city of Espumoso. Soybean workers in this region are increasingly exposed to a wide combination of chemical agents present in formulations of fungicides, herbicides, and insecticides. In the present study, the comet assay in peripheral leukocytes and the buccal micronucleus (MN) cytome assay (BMCyt) in exfoliated buccal cells were used to assess the effects of exposures to pesticides in soybean farm workers from Espumoso. A total of 127 individuals, 81 exposed and 46 non-exposed controls, were evaluated. Comet assay and BMCyt (micronuclei and nuclear buds) data revealed DNA damage in soybean workers. Cell death was also observed (condensed chromatin, karyorhectic, and karyolitic cells). Inhibition of non-specific choline esterase (BchE) was not observed in the workers. The trace element contents of buccal samples were analyzed by Particle-Induced X-ray Emission (PIXE). Higher concentrations of Mg, Al, Si, P, S, and Cl were observed in cells from workers. No associations with use of personal protective equipment, gender, or mode of application of pesticides were observed. Our findings indicate the advisability of monitoring genetic toxicity in soybean farm workers exposed to pesticides.

1. Introduction

Across the world, pesticides have been widely used since the 1940's [1]. In Brazil, the use of chemical substances in agriculture has increased significantly, and the country is considered to be one of the largest consumers worldwide, with sales increasing by 160% between 1991 and 1998. According to the Brazilian Agricultural and Livestock Confederation, in 2003, sales of pesticides amounted to 375,000 tons of commercial product, equivalent to 182,400 tons of active ingredients [2].

Brazil is the world's second largest producer and exporter of soybean. The area dedicated to soybean culture has increased from 11.5 million ha in 1990 to 21.7 million ha in 2009. Five states are responsible for 80% of Brazil's soybean production; one of these is the state of Rio Grande do Sul, in the south region [3]. The significant increase in soybean production entails the use of several pesticides for crop protection and pest control. Farm workers are exposed simultaneously to a complex mixture of insecticides, such as organophosphates, pyrethroids, and organochlorines [4], as well as fungicides and herbicides employed in the preparation and application of these chemicals [2].

The risks to human health that may be associated with chronic exposure to pesticides should be addressed in more detail. The effects of long-term exposure to low doses of pesticides are often difficult to assess, since associated signs and symptoms may not manifest clinically [5]. Pesticides and fertilizers are extensively used in agriculture; formulations, combinations, and interactions between chemical compounds and multiple exposures are a rule, not an exception, in agricultural practice. Different formulations are often used simultaneously in complex mixtures, including a significant number of genotoxic compounds [6]. Thus, information about toxicity of pesticides may not be sufficient to evaluate risk of adverse health effects. Some of these compounds are considered possible initiators of cancer, and can lead to a higher incidence of chronic diseases, degenerative diseases, and congenital malformations, as a consequence of their genotoxic effects [6–11]. These toxic effects vary considerably, depending on the degree of poisoning, absorption pathway, specific characteristics of pesticides or cultivation practices, and individual factors, such as age, gender, nutritional status, and general health [4,12–14].

In the present study, we have used the comet assay in peripheral leukocytes and the human buccal micronucleus (MN) cytome

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http://dx.doi.org/10.1016/j.mrgentox.2013.01.001

assay (BMCyt) in exfoliated buccal cells to assess whether prolonged exposure to complex mixtures of pesticides could lead to an increase in cytogenetic damage in soybean farm workers.

2. Materials and methods

2.1. Study population and sample collection

This study was approved by the Brazilian National Committee on Research Ethics – Comissão Nacional de Ética em Pesquisa – CONEP and written informed consent was obtained from each individual before the research began.

Subjects from Espumoso were sampled from January to February 2008 and from January to February 2009, periods with intensive use of pesticides. In total, 127 individuals (46 non-exposed controls and 81 occupationally exposed to pesticides) took part in this study. For an appropriate assessment of the exposed group, individuals were screened by the Institute of Technical Assistance and Rural Extension of Rio Grande do Sul (EMATER), which listed the farming communities where pesticides are intensely used. Two different groups of exposed individuals were formed, according to the mode of spraying pesticides: (a) those that made use of tanks installed in tractors and (b) those that made use of tanks installed in tractors associated with use of hand pumps. Hands pumps are considered as useful tools in successive applications of pesticides in small areas and where tractor access is difficult. Apart from spraying plantations, soybean farm workers also prepare the pesticide mixtures and refill the tanks.

The control individuals were office employees living in the same region as the exposed individuals. None of the control individuals was recently exposed to agrochemicals or any other suspected genotoxic agents, and they had no previous occupational exposure to genotoxins.

All individuals in the study were asked to answer a Portuguese version of a questionnaire from the International Commission for Protection against Environmental Mutagens and Carcinogens [15] and to participate in a face-to-face interview, which included standard demographic data (age, gender, etc.), as well as questions concerning medical issues (exposure to X-rays, vaccinations, medication, etc.), lifestyle (smoking, coffee and alcohol consumption, diet, etc.) and occupation (number of working hours per day, personal protective equipment – PPE). All individuals in this study were intentionally selected to be non-smokers, so as to eliminate confounding factors.

Blood samples were collected by venipuncture using vacutainers with heparine and EDTA and processed as quickly as possible. Buccal samples were obtained by rubbing the inside of the cheeks with a cytobrush for analysis of BMCyt and PIXE elemental analysis. Blood and buccal samples were transported to the laboratories at or below 8 °C and processed within 20 h of collection. The samples were stored at 4 °C.

2.2. Comet assay

The alkaline comet assay was performed as described by Singh [16] with the modifications suggested by Tice et al. [17]. Blood samples (5 μL) were embedded in 0.75% low melting point agarose, in 95 μL, and after the agarose solidified, slides were placed in lysis buffer (2.5 M NaCl, 100 mM EDTA and 10 mM Tris; pH 10.0–10.5) containing freshly added 1% (v/v) Triton X-100 and 10% (v/v) dimethyl sulphoxide for a minimum of 1 h and a maximum of one week. After treatment with lysis buffer, the slides were incubated in freshly prepared alkaline buffer solution (300 mM NaOH and 1 mM EDTA; pH > 13) for 20 min, and the DNA electrophoresed for 20 min at 25 V (0.90 V/cm) and 300 mA. The buffer solution was subsequently neutralized with 0.4 M Tris (pH 7.5), and the DNA was stained with silver nitrate. The electrophoresis procedure and the efficiency of each electrophoresis run were assessed using negative and positive internal controls consisting of whole human blood collected in the laboratory, with the negative control being unmodified blood and the positive control 50 μL blood mixed with 13 μL (8×10^{-5} M) methyl methane sulphonate solution (CAS 66-27-3; Sigma, St Louis, MO, USA) and incubated for 2 h at 37 °C. Each electrophoresis run was considered valid only if the negative and positive controls yielded the expected results. Slides were randomized and coded to blind the scorer. Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analyzed for each individual using bright-field optical microscopy at a magnification of 200–1000. Two parameters were evaluated: (i) the damage index (DI), in which each cell was assigned to one of five classes (from no damage = 0 to maximum damage = 4) according to tail size and shape such that the values obtained for each individual could range from 0 (0×100) to 400 (4×100) and (ii) the damage frequency (DF in %), it was calculated for each sample based on the number of cells with tail versus those without. International guidelines and recommendations for the comet assay consider the visual scoring of comets to be a well-validated evaluation method [17,18].

2.3. Buccal micronucleus cytome assay (BMCyt assay)

Buccal cell samples were obtained by gently rubbing the inside of the cheeks (right and left side) with a cytobrush, which was immersed in 5 mL cold saline (0.9% (w/v) aqueous NaCl) in a conical tube and transported under refrigeration to the laboratory, where the saline was centrifuged at 1500 rpm for 8 min and the sedimented buccal cells were washed twice more with saline and once more with Carnoy's fixative (methanol and glacial acetic acid 3:1) under the same centrifugation conditions. The cell suspension was dropped onto a slide and allowed to air at room temperature. The slides were stained with 2% Giemsa solution for 10 min, rinsed in distilled water, and air dried. For each individual, the frequency of the various cell types in the assay is represented as the number of cells in 2000, as suggested by Thomas et al. [19]. The BMCyt assay has been used to measure biomarkers of DNA damage (micronuclei and/or elimination of nuclear material by budding – buds), cytokinetic defects (binucleated cells) and proliferative potential (basal cell frequency) and/or cell death (condensed chromatin, karyorrhectic, pyknotic and karyolytic cells). For each volunteer, 2000 buccal cells (1000 from each of the duplicate slides) were scored using bright-field optical microscopy at a magnification of 1000.Non-specific choline esterase activity (BChE)

BChE activity determination was performed using the kit from Wiener™ Laboratory, according recommendations of Ellman et al. [20]. The final quantities (per liter) of reagents in the test were: 7.0 mmol butyrylthiocholine iodide as the substrate, 50 mmol phosphate buffer pH 7.7, and 0.25 mmol 5.5-dithio-bis-(2)-nitrobenzoic acid. The procedure included the addition of plasma sample (10 μL) to 1.5 mL reaction solution containing the above reagents. The absorbance of the reaction was measured at 405 nm using a Cobas Mira spectrophometer, according to a kinetic method, where the results were compared to reference values established by the test methodology. This is the most widely used test to determine acute intoxication by organophosphates and carbamates.

2.5. Chemical analysis

The inorganic elements content of the cytome buccal samples was analyzed through the Particle-Induced X-ray Emission (PIXE) technique [21]. 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). Briefly, buccal samples were diluted in 2 mL distilled water, and were filtered under pressure across 30-mm diameter filters, pore size 0.22 μm. These filters were subsequently placed in the target holder inside the PIXE reaction chamber. A 3 MV Tandetron accelerator provide a 2.0 MeV proton beam with an average current of 5 nA at the target. The X-rays produced were detected by a Si(Li) detector [22,23] and the spectra were fitted to obtain the elemental concentrations using the GUPIXWIN software package [24]. The results are expressed in parts per million (mg/kg).

2.6. Statistical analysis

The normality of variables was evaluated using the Kolmogorov–Smirnov test. Student's *t* test was used to test the characteristics of the population. The statistical differences between the damage observed in the comet assay and micronucleus test, and the differences between exposed and non-exposed individuals concerning different characteristics, as well as PIXE analyze were carried out using the non-parametric Mann–Whitney (for independent samples). Correlations between different variables were determined by Spearman rank correlation test, when applicable. The critical level for rejection of the null hypothesis was considered to be a *P* value of 5%, two-tailed. Analysis values were calculated using the software Graphpad Prism (Graphpad Inc., San Diego, CA).

3. Results

The non-exposed group consisted of 19 males and 27 women, between 22 and 67 years of age (mean age: 49.5 ± 11.6), with no known exposure to genotoxic agents. The group of exposed workers included 65 males and 16 women, between 23 and 73 years of age (mean age: 48.0 ± 10.5). These individuals were farm workers directly involved in the preparation and application of pesticides in soybean fields exposed simultaneously to a complex mixture of pesticides since childhood. In total, 80% of agricultural workers in this study did not use any kind of protection during pesticide preparation and application (gloves, breathing masks, protective goggles, impermeable boots, etc.) and presented some symptoms related with pesticides exposure such as headaches, abdominal pain, nausea, and vomiting.

Table 1 shows the main pesticides used. The agricultural workers included in this study were exposed to complex mixtures of pesticides, such as herbicides (18%), fungicides (16%), and mainly insecticides (66%), most of which were organophosphorous (17%), carbamates (16%), pyrethroids (17%) and organochlorines (16%) compounds.

The result of analysis of DI and DF of the comet assay in exposed and non-exposed individuals is shown in Table 2. Significant

Table 1
List of pesticides used by exposed group and their hazard classification.

Pesticides	Compounds	Chemical class	ANVISA[a]
Herbicides	Alachlor	Chloroacetanilide	III
	2,4 Dichlorophenoxy-acetic acid (2,4 D)	Phenoxy-carboxylic-acid	I
	Flumioxazin	N-phenylphthalimide	IV
	Glyphosate	Glycine derivative	IV
	Lactofen	Diphenyl ether	III
	Paraquat	Bipiridilio	I
Insecticides	Alpha cypermethrin	Pyrethroid	II
	Beta cypermethrin	Pyrethroid	III
	Cypermethrin	Pyrethroid	II
	Chlorpyrifos	Organophosphorus	II
	Deltamethrin	Pyrethroid	III
	Parathion	Organophosphorus	Uncategorized
	Methomyl	Carbamate	I
	Monocrotophos	Organophosphorus	Uncategorized
	Methamidophos	Organophosphorus	Uncategorized
	Malation	Organophosphorus	III
	Permethrin	Pyrethroid	III
	Carbosulfan	Carbamate	I
	Fipronil	Pyrazole	II
	Endosulfan	Organochlorine	I
Fungicides	Carbendazim	Benzimidazol	III
	Captan	Dicarboximide	III
	Maneb	Dithiocarbamates	Uncategorized
	Tebuconazole	Triazole	IV
	Thiram	Dithiocarbamates	II

[a] Hazard classification from National Agency of Sanitary Surveillance (ANVISA: Agência Nacional de Vigilância Sanitária). I = extremely hazardous, II = highly hazardous, III = moderately hazardous, IV = slightly hazardous.

increase in these two parameters was observed in both male and female exposed individuals, compared to non-exposed controls. Fig. 1 illustrates the distribution of damage classes for each group. Non-exposed individuals presented higher frequencies of class 0 cells ($P < 0.001$), while exposed groups had higher numbers of cells classes 1, 2, 3 and 4 ($P < 0.001$).

The results of the BMCyt assay are shown in Table 3. Evaluation of epithelial cells revealed a higher frequency of micronucleus in differentiated cells ($P < 0.001$), as well as nuclear buds and binucleated cells ($P < 0.01$), compared to the non-exposed group. As a whole, exposed groups showed a significantly higher frequency of condensed chromatin ($P < 0.05$), karyorhectic ($P < 0.01$) and karyolitic ($P < 0.05$) cells, when compared to the non-exposed group.

No correlation was found for age and exposure time in terms of the different parameters of comet assay and BMCyt assay (data not shown).

The mean level of BChE activity did not differ significantly across exposed workers ($8231 \pm 1368\,U\,L^{-1}$) and non-exposed controls

Table 2
Mean values (±standard deviation) of DNA damage in non-exposed and exposed.

Groups (*n*)	Comet assay (100 leukocytes/subject)	
	Damage index (0–400)	Damage frequency (%)
Non-exposed (46)	19.6 ± 10.3	13.3 ± 6.4
Male (19)	17.7 ± 10.2	12.2 ± 6.1
Female (27)	20.9 ± 10.3	14.1 ± 6.5
Exposed (81)	38.5 ± 19.9***	23.1 ± 9.4***
Male (65)	38.8 ± 19.2***	23.5 ± 9.4***
Female (16)	37.4 ± 23.0*	21.4 ± 9.4*

n = number subjects.
* Significant in relation to non-exposed controls at $P < 0.01$.
*** $P < 0.001$ (Mann–Whitney test).

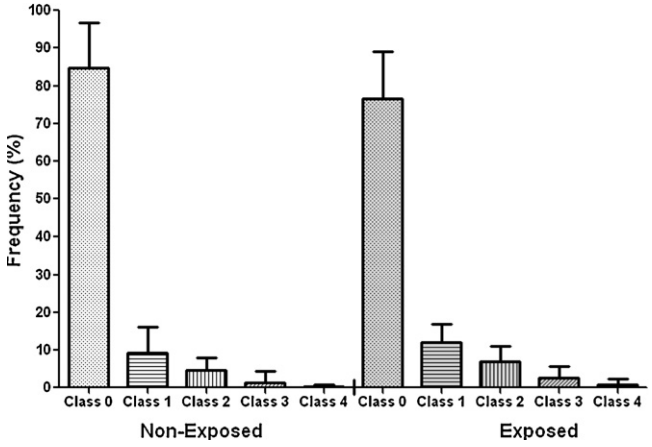

Fig. 1. Frequency of DNA damage observed for non-exposed and exposed groups.

($8068 \pm 920\,U\,L^{-1}$). Moreover, no significant association was found between gender and comet assay (Table 2), BMCyt assay (Table 3), and cholinesterase activity (exposed: male = $8249 \pm 1420\,U\,L^{-1}$ and female = $8133 \pm 1044\,U\,L^{-1}$; non-exposed: male = $8051 \pm 890\,U\,L^{-1}$ and female = $8081 \pm 943\,U\,L^{-1}$).

Inorganic elements content in buccal samples of non-exposed and exposed groups to pesticides was analyzed by PIXE. Fig. 2 show content of trace elements (ppm; mean ± standard error) in buccal samples of the non-exposed and exposed groups. Among these elements, higher concentrations of Mg, Al, Si, P, S, and Cl were observed in the cells from workers than in those from control individuals. Despite this, no significant difference was observed between exposed and non-exposed groups for all inorganic elements.

Two modes of spraying pesticides were observed: the use of tanks installed in tractors, and the use of tanks installed in tractors associated with hand pumps. DI and DF results obtained in the comet assay, BMCyt assay and BChE activity were not significantly higher in workers using tractors associated with hand pumps, compared to those that apply pesticides using tractors alone (Table 4).

Mean DI and DF obtained by the comet assay showed no significant differences in terms of the use of PPE, similarly to the analysis of BMCyt and serum cholinesterase activity.

4. Discussion

Large volumes of pesticides are used in Brazilian agriculture, and the expansion of soybean plantations accounts for approximately 45% of all pesticides sales in the country. The increased use of pesticides, many of which are toxic for humans as well as the

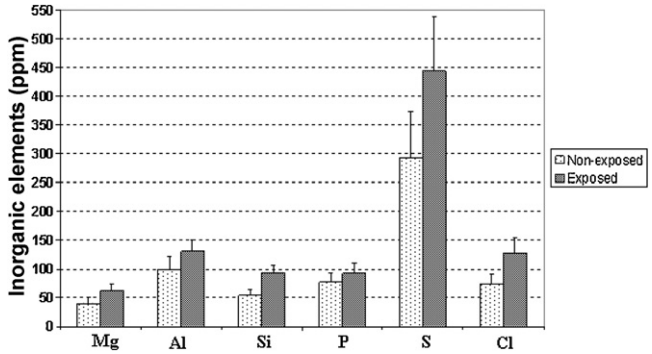

Fig. 2. Content of trace elements (ppm; mean ± standard error) in buccal samples of the non-exposed and exposed groups.

Table 3
The human buccal micronucleus cytome assay (BMCyt) from non-exposed and exposed groups (mean ± standard deviation). For each volunteer 2000 buccal cells (1000 from each of the duplicate slides) were scored.

Parameters	Groups (n = number subjects)					
	Non-exposed			Exposed		
	Males (19)	Females (27)	Group (n = 46)	Males (65)	Females (16)	Group (n = 81)
Basal cell layer						
Basal	17.4 ± 10.7	12.0 ± 9.8	14.2 ± 10.4	12.2 ± 6.4	9.9 ± 6.1	11.8 ± 6.3
Differentiated cell layer						
Micronuclei	1.1 ± 1.1	1.8 ± 2.0	1.5 ± 1.7	3.3 ± 2.4[c]	4.0 ± 3.4[a]	3.4 ± 2.5[f]
Nuclear buds	2.3 ± 2.7	4.5 ± 4.4	3.6 ± 3.9	5.9 ± 5.1[b]	7.9 ± 6.0	6.2 ± 5.3[e]
Binucleated	6.1 ± 6.5	7.6 ± 7.2	7.0 ± 6.9	8.4 ± 6.1[a]	8.4 ± 6.8	8.4 ± 6.2[e]
Condensed chromatin	7.1 ± 5.8	5.1 ± 4.2	5.9 ± 4.9	9.2 ± 8.1	11.2 ± 9.9[a]	9.5 ± 8.4[d]
Karyorrhectic	6.6 ± 7.0	6.5 ± 5.1	6.6 ± 5.9	10.4 ± 6.6[a]	10.1 ± 5.3[a]	10.4 ± 8.0[e]
Pyknotic	1.9 ± 2.6	4.4 ± 4.8	3.4 ± 4.2	4.3 ± 4.0[a]	4.9 ± 4.8	4.4 ± 4.1
Karyolitic	9.7 ± 4.6	6.9 ± 6.4	8.0 ± 5.9	11.5 ± 8.1	9.4 ± 5.5	11.2 ± 7.7[d]

[a] Significant in relation to non-exposed control group, same gender, at $P < 0.05$ (Mann–Whitney test).
[b] Significant in relation to non-exposed control group, same gender, at $P < 0.01$ (Mann–Whitney test).
[c] Significant in relation to non-exposed control group, same gender at $P < 0.001$ (Mann–Whitney test).
[d] Significant in relation to non-exposed control group, data from group, at $P < 0.05$ (Mann–Whitney test).
[e] Significant in relation to non-exposed control group, data from group at $P < 0.01$ (Mann–Whitney test).
[f] Significant in relation to non-exposed control group, data from group at $P < 0.001$ (Mann–Whitney test).

environment, has been the main object of attention of the Brazilian Health Surveillance Agency (ANVISA) and, in 2008, ANVISA planned a review of 14 active ingredients used in agriculture [3].

Here, the comet assay in peripheral blood shows that exposure to pesticides induces DNA damage, observed as the increase in DI and DF. The same was observed using the buccal micronucleus (MN) cytome assay (BMCyt), which detected increased occurrence of cells with micronuclei, nuclear buds and binucleated cells, as well as cell death (increased condensed chromatin, karyorrhectic and karyolitic cells).

Similarly to our comet assay data, Maroni et al. [13] and Vrhovac and Zeljezic [25] reported the occurrence of DNA damage associated with the exposure to herbicides and insecticides such as parathion, malathion, 2,4 D, and atrazine. Also, Bolognesi [6] recognizes methamidophos, monocrotophos, glyphosate, and endosulfan as pesticides that induce DNA damage. Carbosulfan (carbamate) and organophosphate and pyrethoid pestices have been shown to pose a significant risk of adverse DNA effects [26,27]. Moreover, organophosphates, pyrethroids, organochlorines, and carbamates have been reported to be genotoxic, generating free radicals that react with cell membranes and initiate the process of lipid peroxidation. The accumulation of these radicals can cause oxidative stress, depending on the antioxidant capacity of individuals exposed to these pesticides [5,28]. The metal ions present in some pesticides and fertilizers [29] may interfere with DNA repair and produce reactive oxygen species (ROS), leading to oxidative damage [6]. Among the inorganic elements detected by PIXE in this study, higher concentrations of Mg, Al, Si, P, S, and Cl were observed in the cells from workers than in those from control individuals. The inorganic elements found in buccal samples in general are present in chemical composition of different pesticides and fertilizers that workers are exposed. The toxicity of inorganic elements depend of capacity in degraded by living organisms and also may accumulate up to harmful levels. In general, metal genotoxicity is caused by indirect mechanisms through the physicochemical properties. Recently, Beyersmann and Hartwing [30] described three predominant mechanisms of metal genotoxicity: interference with cellular redox regulation and induction of oxidative stress, which may cause oxidative DNA damage or trigger signaling cascades that lead to the stimulation of cell growth; inhibition of DNA repair systems that results in genomic instability and the accumulation of critical mutations; and deregulation of cell proliferation by the induction of signaling pathways or the inactivation of growth controls, e.g., tumor suppressor genes.

The comet assay evaluates recent exposure in general; in this sense, we found lesions in DNA probably associated to oxidative damage, which may be leading to mutagenic processes, as observed with the formation of MN and nuclear buds, indicating persistence

Table 4
Comet assay, human buccal micronucleus cytome assay (BMCyt) and BChE (cholinesterase activity) parameters from exposed group (mean ± standard deviation).

Parameters	Form of application of pesticides[a]		Personal protective equipment (PPE)	
	Tractor (n = 34)	Tractor + hand pump (n = 47)	Without (n = 65)	With (n = 16)
Comet assay				
Damage index (0–400)	33.9 ± 18.1	41.2 ± 20.9	36.9 ± 18.0	40.0 ± 20.6
Damage frequency (%)	20.8 ± 8.7	24.6 ± 9.6	22.4 ± 9.1	24.1 ± 8.9
BMCyt				
Basal cell	12.6 ± 6.7	11.2 ± 6	11.7 ± 6.4	12.3 ± 6.1
Binucleated	9.7 ± 6.5	7.2 ± 5.6	8.4 ± 6.4	8.2 ± 5.4
Micronuclei	3.2 ± 2.4	3.6 ± 2.7	3.4 ± 2.6	3.2 ± 2.5
Nuclear buds	7.0 ± 5.3	5.5 ± 5.2	6.5 ± 5.4	5.1 ± 4.7
Condensed chromatin	7.4 ± 7.6	11.2 ± 8.7	9.3 ± 8.7	10.2 ± 6.9
Karyorrhectic	8.6 ± 6.2	12.0 ± 9.1	10.0 ± 7.3	11.8 ± 10.7
Pyknotic	5.2 ± 4.5	3.7 ± 3.6	4.4 ± 4.3	4.2 ± 3.5
Karyolitic	10.9 ± 7.4	11.4 ± 8.2	10.8 ± 7.4)	12.6 ± 9.2
BChE	8.259 ± 1.292	8.195 ± 1.500 (n = 31[b])	8.157 ± 1.473 (n = 55[b])	8.502 ± 944.6 (n = 15[b])

[a] Tractor = use of tanks installed in tractors, and tractor + hand pump = use of tanks installed in tractors associated with hand pumps.
[b] Levels of cholinesterase activity for some individuals were not possible verify; n = number subjects.

of lesions or occurrence of incorrect repairs [31,32]. Continuous exposure and persistence of unrepaired genotoxic damage induced by pesticides and the formation of free radicals can lead to a higher level of cytogenetic alterations [6,9].

Here, the results of the BMCyt assay revealed DNA damage in soybean farm workers manifested as increased micronuclei, nuclear buds and binucleated cells in individuals exposed to pesticides, compared to non-exposed controls. Cell death was also observed (condensed chromatin, karyorhectic, and karyolitic cells). Farm workers that spray pesticides using tanks installed in tractors and that also use hand pumps did not demonstrate significant increase in BMCyt parameters, compared to those that use only tractors to spray pesticides. Although those who use tanks installed in tractors and who also use hand pumps can be more intensely exposed to pesticides than those who use only tractors, both are exposed to high quantity of agrochemicals; this factor may have resulted in no significant difference between both groups of exposure. The use of dithiocarbamates, atrazine, alachlor, cyanazine, 2,4-D, and malathion for extended periods of time can lead to chromosomal breaks, acentric fragments, dicentrics, sister chromatid exchange and micronucleus frequency [33]. The mechanism triggering nuclear bud formation is unknown, but may be related to chromosomal instability and gene amplification [34–36]. Binucleated cells were also found to be more frequent in the exposed group to pesticides. The precise significance of these cells is not known, but they may be indicative of the failure of cytokinesis due to aneuploidy [36]. Furthermore, the morphological analysis of cells allows us to evaluate the oral mucosa, chromosomal instability, cell death and regeneration potential of the epithelium [19,23,35,36]. Condensed chromatin, karyorrhexis, pyknotic and karyolytic cells are cell death biomarkers [19,36–38]. These anomalies are intrinsic to the squamous epithelium, in particular because of the chronic effect of the masticatory process on the oral mucosa, and the constant action of mutagenic agents increases the rate of cell deaths, as indicated by any significant rise in the frequency of this anomaly [37]. Karyolytic cells are associated with cytotoxicity, which is also evident in necrotic cells and, karyorrhetic cells accompanies apoptosis [37] and is thought to be a late stage of apoptosis [36], a process under genetic control. Our results may suggest that the genetic damage caused by exposure to pesticides could lead to an increase in the induction of cell death, as mechanisms of elimination of genetically damaged cells. In a previous study, Kehdy et al. [38] observed an increase in MN and cells related to cell death in individuals exposed to pesticides. The HUMNXL Project evaluated a database of 5424 subjects with buccal micronucleus values obtained from 30 laboratories worldwide, which compiled and analyzed the influence of several conditions affecting micronuclei frequency [36]. Variables affecting BMCyt biomarkers included pesticides exposure, and demonstrate that significant increases were seen in MN, nuclear bud, binucleated cells and karyorrhetic cells.

Furthermore, the exposure to multiple pesticides may cause various cells injuries and DNA damage, which depends on the application form and use of PPE [5–7,13,14,39]. In a review, Bull et al. [8] discussed genotoxicity in workers who apply pesticides and highlighted the importance of personal protective equipment. In our study, most workers (80%) do not adopt all protective measures. Nevertheless, we noticed an increase in DNA damage and cell death among workers, with no difference between those who do not wear any protective equipment. Similar to this study, other authors have not demonstrated association between DNA damage and appropriate use of PPE [14,40], maybe due to accuracy in workers answers or because workers do not always renew or clean their PPE. If the equipment is rarely changed or cleaned, the protection they afford falls short of what is expected.

Although occupational exposure generally characterizes the combined use of several classes of pesticides in soy production, in our study, the interviews revealed an increased use of carbamates and organophosphates, both of which are inhibitors of BChE. Since these compounds act through the inhibition of acetylcholinesterase they may act directly on this enzyme, without biotransformation, while others act indirectly by inhibiting processes and require metabolic transformation for subsequent absorption. However, this biomarker presents limited significance in long-term exposure, and can produce conflicting results [5]. No significant difference in the BChE activity between non-exposed and worker subjects was found, remaining within the normal range in the groups. Similarly to our results, Shadnia et al. [41] did not identify association between chronic exposure to organophosphates and cholinesterase inhibition. Additionally, it should be noted that, after soybean planting and harvesting, the farm workers that participated in the present study also take part in the management of other cultures during the year, such as corn, wheat and oats, increasing these individuals' exposure to some type of pesticide throughout this period and characterizing chronic exposure.

Our study demonstrated that soybean farm workers are exposed to a mixture of substances with cytotoxic, genotoxic and mutagenic potential, which were demonstrated by the comet assay and the BMCyt assay. The DNA damage observed in the soybean workers may be a consequence of oxidative damage resulting from their exposure to complex mixtures, including inorganic elements. Genotoxic evaluation using these tests is useful and necessary to ensure good occupational conditions and the health of workers. By assessing genotoxic modifications in individuals, those who are at risk to develop diseases such as cancer may be identified and greater care may be recommended.

Conflict of interest statement

None declared.

Acknowledgements

We are grateful to Hospital São Sebastião from Espumoso, EMATER (Instituto de Assistência Técnica e Extensão Rural do Rio Grande do Sul). This investigation was supported by CNPq, FAPERGS, and Universidade Luterana do Brasil (ULBRA).

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