Cellular Changes in Buccal Mucosa from Farmers Exposed to Glyphosate

Alterações Celulares na Mucosa Bucal de Agricultores Expostos ao Glifosato

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ABSTRACT
This study evaluated the sociodemographic characteristics and behavior of the oral mucosa epithelium exposured to the herbicide glyphosate of family farmers in Cerro Largo, RS, Brazil. 120 individuals were selected for social data collection through interviews. According to the results, most of the interviewees uses glyphosate between 5-10 years, being exposed between 30 minutes to one hour each application and applying the herbicide 1-2 times a year. After the interview, we selected the subjects to the Micronucleus (MN) test. For this test, oral smears were performed in three distinct regions (cheek, mouth floor and tongue edges) of 10 test subjects (exposed to glyphosate, non-smoker and non-alcoholic) and 10 control subjects. Results showed that glyphosate exposure increased the frequency of MN in the test group ($p = 0.0002$), as well as the frequency of other cellular alterations, such as brokenegg ($p = 0.001$), binucleation ($p = 0.0001$) and karyolysis ($p = 0.0004$). Based on these findings, the extent use of glyphosate may be causing damage to the oral mucosa epithelium and this might respond adaptively through cellular modifications.

Keywords: Herbicide, **S**ociodemographical, Micronuclei, Mucosa.

RESUMO
Este estudo avaliou as características sociodemográficas e o comportamento do epitélio da mucosa oral exposta ao herbicida glifosato em agricultores familiares no município de Cerro Largo, RS, Brasil. 120 indivíduos foram selecionados para a coleta dos dados sociodemográficos através de entrevista. De acordo com o resultado, a maioria dos entrevistados usam o glifosato entre 5-10 anos, sendo exposto entre 30 minutos a 1 hora a cada aplicação e estes aplicam o herbicida 1-2 vezes ao ano. Após a entrevista, os indivíduos foram selecionados para o teste de Micronúcleo (MN). Para este teste, a coleta da mucosa bucal foi realizada em três regiões distintas (bochecha, assoalho da boca e bordas da língua) de 10 indivíduos (expostos ao glifosato, não fumantes e não alcoolicos) e 10 indivíduos controle. Os resultados mostraram que a exposição ao glifosato aumentou a frequência de MN no grupo teste (p=0,0002), bem como a frequência de alterações celulares, como *brokenegg* (p = 0.001), binucleação (p = 0.0001) e cariólise (p = 0.0004). Baseado nestes achados, o intenso uso do glifosato pode estar causando dano ao epitélio da mucosa oral e isto pode estar respondendo adaptativamente através de modificações celulares.

Palavras chaves: Herbicida, **S**ociodemográfico, Micronucleos, Mucosa.

1 INTRODUCTION

The use of pesticides has increased in the last decades mainly due to the incentive policies of their use, such as the "National Plan of Agricultural Development" that imposed on the farmer, when obtaining the "rural credit", the conditioning to the obligatory purchase of pesticide in each financing required. This policy induced the growth of the synthesis industry of these compounds in the country and the dependence of chemical compounds in agricultural production (Ferreira 1993). In addition, there was an increase in transgenic seeds, which lead to even greater use of pesticides, especially herbicides such as glyphosate (Zimmermann 2009).

Pesticides may have genotoxic or clastogenic characteristics when interacting with nuclear DNA promoting mutations or changes in chromosomes, which in the long term contribute to the establishment of tumor processes. Cancer, congenital anomalies and genetic diseases are among the most significant changes (Bertram 2001).

One of the agrochemicals whose mutagenic and / or carcinogenic potential is still quite controversial is the glyphosate herbicide, which belongs to the class of organophosphates (James 2011; Coutinho and Mazo 2005), being the active principle of some of the most widely used pesticides worldwide. It is a broad spectrum, non-selective post-emergence herbicide capable of controlling various weeds (Duke 2011).

The acute toxicity of this herbicide is considered low (class III) according to the World Health Organization. But there is controversy regarding acute toxicity and chronic

toxicity, so much so that this herbicide has recently been classified as a probable human carcinogen (Group 2A) by the International Agency for Research on Cancer (IARC) (Guyton et al. 2015). Studies have related glyphosate herbicide exposure to tumorigenesis and genetic alterations. The review work performed by Ghisi, Oliveira, and Prioli (2016) found, from a meta-analysis of 93 papers, that glyphosate exposure is related to the formation of Micronuclei (MN) in fish.

To determine genotoxic effects caused by agrochemicals several standardized tests are used by researchers. These tests detect chromosome damage and cell cycle changes in organisms exposed to them (Albertini et al. 2000; Dutra 2002). In humans this test can be used by analyzing oral mucosa cells, which are sensitive to substances that interact with DNA in the mitosis phase.

The MN test is the most well established and commonly used test to evaluate cytotoxicity and clastogenicity caused by a wide spectrum of substances. It shows great potential because it is easy to perform, inexpensive and a good indicator of chemical contamination in organisms. MN is classified as a small mass of chromatin found outside the nucleus originated from chromosome breakdown in the mitosis phase, rounded with the same appearance and refraction as nuclear material (Fenech 2007).

Although there is a baseline level of spontaneous MN formation in most species, exposure of organisms to clastogenic substances, such as some pesticides, has been shown to increase the frequency of MN in laboratory tests (Manas et al. 2009; Bombail et al, 2001; Grisolia 2005; Guilherme et al. 2010; Guise, Oliveira, and Prioli 2016; Stich, Rosin, and Vallejera 1984; Stich 1987).

Similarly, nuclear alterations (NA) such as sprouts (gene amplification), karyolysis (necrosis), cariorexis (final and early stages of apoptosis), condensed chromatin (apoptosis induction) and the presence of two nuclei (cytokinesis failure), can be considered biological markers of cell division kinetics (Holland et al. 2008).

The objective of this work was to evaluate whether the farmers exposion to glyphosate leads to elevated levels of chromosomal damage in cells of buccal mucosa through MN testing and NA analysis.

2 MATERIAL AND METHODS

Initially, with the objective of evaluating the relationship between glyphosate exposure and the MN index, the sociodemographic profile of family farmers living in the municipality of Cerro Largo, in the Missions region, northwest of Rio Grande do Sul

state, was determined. One hundred and twenty individuals were randomly selected and, after signing the free and informed consent form, were interviewed. This interview consisted of a questionnaire related to the sociodemographic characteristics of the interviewees, such as age and education and questions related to the use of glyphosate herbicide, exposure time, years of use and others. This project was approved by the UFFS Research Ethics Committee (CAAE: 65029417.0.0000.5564).

The study region is very agricultural, consumer of agrochemicals, producing intensively soy and other crops, especially wheat and corn, besides using a large amount of agrochemicals and having a high rate of cancer cases (Jobim et al. 2010).

After the interviews were completed (Table 1), for the MN test, 10 non-smoking and non-alcoholic individuals who were used and applied the glyphosate herbicide were selected for the test group (identified with the letter T) and 10 non-smokers, non-alcoholic and who have not used glyphosate in the last six months (identified with the letter C), between the ages of 25 and 50 years, of both sexes, were selected to the control group. The samples were collected between October and November 2017, which corresponds to the time of year where glyphosate herbicide application occurs.

Table 1 - Sociodemographic characterization of the farmers of Cerro Largo, RS.

Parameter (mean±SD)	Test (n=76)	Control (n=44)
Age (y)	51.84±12.56	53.25 ± 13.51
Education (y)	9.72±2.41	9.25±2.02
Number of smokers	9	8
Number of Alcohol drinker*	55	29
Glyphosate use (yes)	76	None
Glyphosate aplication (times a y)	2.22 ±1.35	None
Duration of exposure to glyphosate (years)	12.47±6.9	None
Duration of exposure to glyphosate (h /working day)	1.6±0.75	None

*At least one drink (0.5 L of beer or one glass (0.25 L) of wine) a week; y – year; n = number; h – hour

The buccal mucosa cells were collected with a wooden spatula by a single operator, who removed material from the internal region of each cheek, on the lips of the tongue and on the buccal floor of each individual. Three slides were made from each region of the mouth, totaling nine slides per individual. After each collection the slides were placed in slide holders with methanol at 0ºC.

After fixation in methanol the slides were treated with 1N hydrochloric acid for 12 minutes at 60ºC and then washed gently with distilled water for 10 minutes. Then, the slides were stained with Giemsa and 2000 cells were counted per slide with the use of 400x magnification Optical Microscope. The classification and counting of MN followed the protocol suggested by Tolbert, Shy and Allen (1992), in a blind test, where only cells

with intact and clearly visible nuclei, with smooth and distinct perimeter and well-defined cytoplasm were computed. For MN counting the criterion used was the presence of a homogeneous surrounding halo, which determines the nuclear membrane, less than 1/3 of the diameter of the central nucleus, same focal plane under microscopy and that had no connection with the nucleus.

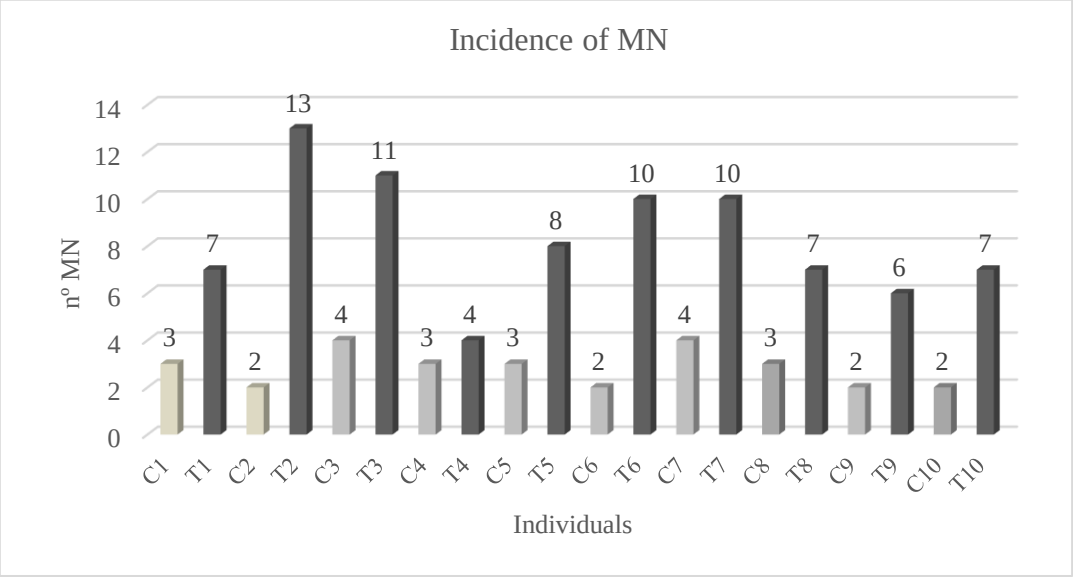
In addition to MN, AN were included, which also suggest damage to the processes of cell division and degeneration. The alterations analyzed were: brokenegg, binucleation, karyolysis and cariorhexis.

The results of the frequency of MN and AN were analyzed by the Kruskal-Wallis statistical method with a 5% significance level. The adjustments of the statistical test were performed in accordance with the collected data for better exposure of the results.

3 RESULTS

A total of 6000 cells were analyzed per individual. The MN frequency results obtained from this oral mucosa cell count of family farmers exposed to glyphosate (T) and control (C) are presented in Figure 1.

Figure 1: Number of MN in 6000 cells from the total of three buccal mucosa regions of farmers exposed to glyphosate herbicide (T) and control group (C) from Cerro Largo, RS.



Source: Prepared by the authors.

Note that in the test group there was a higher frequency of MN compared to the control group. In the test group, individual T2 had the highest number of NM (13), followed by individual T3 with 11 NM.

The result of applying the Kruskal-Wallis test to verify the frequency of NM in the test group and the control group is shown in Table 2.

Table 2: Comparison between the total of MN in the test group and in the control in 6000 analyzed cells submitted to the Kruskal Wallis statistical test with a confidence level of 0.05%.

Group	ẋ ± Dp	Md	Min	Max	p#
Test (n=10)	8.3±2,67	7,5	4	13	0.0002
Control (n=10)	2.8±0,79	3,0	2	4	

p for Kruskal Wallis test, n (individuals number); ẋ (average); Dp (standard deviation); Md (median); Min (lowest number found); Max (highest number found).

The Kruskal Wallis test result showed that the test group and the control group showed significant differences (P = 0.0002) regarding the frequency of NM.

Table 3 shows the cellular alterations binucleation, brokenegg, karyolysis and cariorhexis present in the oral mucosa of test group and control group farmers.

Table 3: Number of cellular alterations (Brokenegg, Binucleation, Karyolysis, Cariorhexis) in 6000 buccal mucosa cells of farmers exposed to glyphosate herbicide and control of the municipality of Cerro Largo, RS.

TEST GROUP										
	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
Brokenegg	0	4	4	1	2	3	5	4	3	3
Binucleation	13	23	19	12	15	18	21	13	17	16
Karyolysis	9	8	13	8	9	9	9	8	7	10
Cariorhexis	0	2	0	5	0	2	0	1	2	1
Total	22	37	36	26	26	32	35	26	29	30
CONTROL GROUP										
	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
Brokenegg	1	0	1	1	0	0	0	0	0	1
Binucleation	7	7	5	4	9	7	12	5	7	8
Karyolysis	7	5	6	7	6	5	6	4	8	7
Cariorhexis	0	0	1	0	0	0	0	0	0	0
Total	15	12	13	12	15	12	18	9	15	16

Based on the data in Table 3, it can be observed that the test group presented a higher frequency of cellular alterations in relation to the control; the individual T7 presented the largest amount of brokenegg (5) characterized by a micronucleus pre-event.

Regarding karyolysis in the test group, individual T3 presented the highest amount (13) and individual T9 the lowest (7). The cariorrex cellular alteration, which is characterized by apoptotic event, did not appear expressively in both the test and control groups and many individuals did not present such alteration.

Table 4 presents the Kruskal Wallis test data that establishes the cellular alterations found in individuals exposed to glyphosate herbicide in relation to the control group.

Table 4: Comparison between the total cell changes in the test group and the control group using the Kruskal Wallis statistical test in 6000 cells analyzed.

CELLULAR CHANGES							
TEST				CONTROL			
	Min	Max	ẋ	Min	Max	ẋ	P#
Brokenegg	0.0	5.0	7.2	0.0	1.0	0.4	0.001
Binucleation	12	23	16.7	4.0	12	7.1	0.0001
Karyolysis	7	13	9	4	8	5.9	0.0004
Cariorhexis	0.0	1.0	0.4	0.0	1.0	0.1	0.131

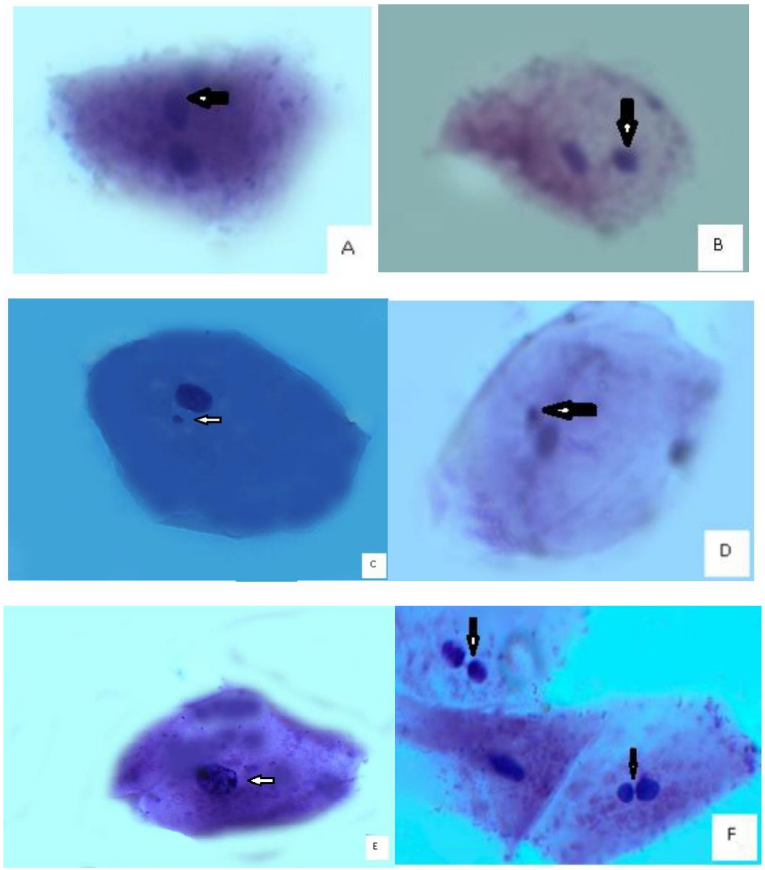
p for Kruskal Wallis test; ẋ (average); Min (lowest number found); Max (highest number found).

The mean number of brokenegg in the group of farmers exposed to glyphosate was higher (7.2) than in the control group (0.4), and this difference was statistically significant (p = 0.001).

The results of the Kruskal Wallis test showed that in relation to binucleation there was also a significant difference (p = 0.0001), as well as the average karyolysis was higher in the test group (9) than in the control group (5.9). significant difference (p = 0.0004).

The following photomicrographs (Figure 2) illustrate the types of changes found in exfoliated mucosal cells of the control and test subjects.

Figure 2: Exfoliated cells of the oral mucosa, stained by Giensa. Note: In A and B the arrow shows the presence of binucleation (1000x), in C and D the arrows indicates the presence of MN (1000x), in E karyolysis (1000x) and in F brokeneggs.



Source: Prepared by the authors

4 DISCUSSION

Considering the socio-demographic characterization, it is observed that the farmers living in the sampled communities are older, and most of the interviewed farmers are over 51 years old. It is important to note that several farmers are over 61 years old and handle pesticides. This practice is in breach of Brazilian Regulatory Standard for safety and health at work in agriculture, livestock forestry, forest exploitation and aquaculture, which prohibits the handling of pesticides for people over 60 years of age.

Ristow (2017) conducted a study with farmers in the municipality of Cerro Largo-RS and found that the predominant age group in the municipality is 51-60 years, corroborating the present study. The national average age range is 21-40 years (Araujo et al. 2007). It is in line with data from the Demographic Census, which shows that the age group of farmers in Cerro Largo municipality is 50 years or older, corresponding to 36%.

In this study it is possible to observe that the vast majority of farmers have low education. Corroborating these results, Mazzoleni and Nogueira (2006) state that in Brazil low education is predominant in the rural population, with few rural residents with secondary, technical or higher education. These results also corroborate Oliveira-Silva et al. (2001) who found the low education level of the interviewed farmers in the municipality of Magé, RJ.

Most respondents use glyphosate herbicide on their property mainly in soybean and corn crops (data not shown). Among those who use the herbicide, most use it for more than 05 years. Cabral (2012) points out in his study that most farmers (63.9%) have been using pesticides for over 10 years. In this study, only the use of glyphosate herbicide was explored, thus, the results showed that 26.66% have been using this herbicide for 5-10 years.

Regarding the time of exposure to the herbicide, it varies from 15 minutes to more than 2 hours and the frequency of application varies from one to more than 5 times a year, indicating the use of this product on a large scale in the studied region. Thus, the longer the preparation and application time, the longer the exposure time, consequently the greater the damage to health and the environment (Cerqueira et al. 2010).

Farmers are exposed to pesticides not only at the time of spray preparation or application, but since the purchase of the product. According to Abreu and Alonzo (2016) the work activities with potential risk of poisoning are: acquisition, transportation, storage, preparation and application, final destination of empty packaging and cleaning of contaminated clothing.

With these data collected it was possible to analyze the oral mucosa cellular quality of 10 individuals within this population that represents the individuals who met the terms of inclusion, ie, non-smokers and non-alcoholics and users of the herbicide.

Comparing the results obtained between the group exposed to the herbicide glyphosate and the control group, it is clear that the frequency of MN is statistically significant ($p = 0.0002$) compared to the control group. Given this, it is assumed that the glyphosate herbicide, or even the other components specified in the package insert as "inert", interact with the nucleus of oral mucosa cells in these individuals.

The number of MN found in the exfoliative mucosa of individual T2 is higher than other individuals in the same group, suggesting that it suffers greater exposure to glyphosate on collection days. The findings of Frison, Macedo, and Boeira (2005) shows that individuals with longer exposure time also had higher rates of MN.

These data are worrying since it is considered as normal event the emergence of one or at most two MN every 2000 cells (Ceppi et al. 2010) and in this case have values above these indices. We analyzed 6000 cells from the total of three regions of the mouth (edges of the tongue, buccal floor and cheek), thus, individuals who are suffering from xenobiotic interference in the mucosa present more than 6 MN in the analyzed cells. Thus, the data show that 90% of test subjects are subject to herbicide-induced genotoxic damage.

The material collected for this study purposely coincided with the time of herbicide use due to the beginning of soybean cultivation, so it is important to point out that the observed damages should be classified as acute. The period of cellular renewal of the oral epithelium is known to be approximately 25 days, so the formation of MN should be considered an acute and local cytogenetic damage (Suhas et al. 2004).

Fenech et al. (2011) reports that the evaluation of NM and other epithelial cell abnormalities has been used to identify genetic damage in humans who are in accidental or occupational exposure situations in order to evaluate early detection of diseases related to genetic disorders. Bloching et al. (2000) suggested that the presence of MN in the oral mucosa may be predictive of risk of upper airway digestive tract cancer, including premalignant phases such as oral leukoplakia.

It's important to point out that it is not yet clear in the scientific literature whether a high frequency of MN in the oral epithelium may be a predictor of increased risk of cancer in the oral cavity only limited to the upper digestive tract epithelium or may be projected for various cancers in other parts of the body (Holland et al. 2008). Given the

above assumptions, individuals in this research, exposed to the defensive glyphosate and with high MN count may be subject to develop tumor process over time.

López et al. (2012) report that glyphosate exposure can increase cancer incidence, affect embryonic and placental cells, induce endocrine disruption, produce mitochondrial damage, necrosis and programmed cell death, as well as damage to cell lines. Sanchez et al. (2017) analyzing different glyphosate formulations also found that glyphosate may cause acetylcholinesterase inhibition and loss of sperm quality in the fish species *Jenynsia multidentata*.

The study by Samsel and Seneff (2015) reported that rats undergoing glyphosate treatment over 26 months developed several tumors in the glands and organs. In addition, it has been shown that glyphosate exposure can increase the incidence of cancer in the general (Williams, Kroesb, and Munro 2000; Lopez et al. 2012), breast cancer (Thongprakaisang 2013; Mesnage et al. 2017) and Non-Hodgkin's Lymphoma (Chang and Delzell 2016; Hardell and Erikson 1999).

Our findings are in line with several other studies such as the results of Dutra (2002), who, when evaluating farmers from Ijuí and São Luiz Gonzaga, RS, found that the group exposed to pesticides, among them the herbicide glyphosate, had a frequent frequency. MN in oral mucosa epithelial cells than in the control group.

Similar data to the findings in this study, are verified in the work of Pacheco and Hackel (2002), who found a significant increase of cells with NM in the exposed group. This study aimed to determine the prevalence of MN in peripheral blood lymphocytes of agricultural workers exposed to pesticides, mainly herbicides and fungicides, in Passo Fundo, RS.

Gómez-Arroyo et al. (2000) analyzed oral mucosa cells and found increased frequency of MN in farmers exposed to various agrochemicals such as herbicides and fungicides. Similar results are described by Sailaja et al. (2006) who, when evaluating the frequency of MN in the oral mucosa of 54 individuals exposed to pesticides, among them the herbicide glyphosate, found a significant increase in the frequency of MN. In the study by Chaves (2011) the increase in the frequency of NM was significant ($p > 0.001$) in individuals exposed to pesticides, among them the herbicide glyphosate.

The nuclear alterations analyzed in this study aimed to observe if, besides the appearance of NM, the mucosal epithelium would also present lesions compatible with necrosis that suggest the presence of factors that lead to cell death. Cell necrosis can be induced by the production of free radicals, chemical agents and / or toxins that act directly

on enzymes or cell cycle, as well as direct aggression to the plasma membrane (Kumar, Abbas, and Aster 2018). Malignancy criteria for smear classification are known from the literature: nuclear contour irregularity, multinucleation, aberrant figures of mitosis, enlargement of nucleoli, presence of vacuoles in the cytoplasm, cellular pleomorphism, and increase in cell size (Carvalho 1995).

Comparing the glyphosate-exposed group with the control group, it is clear that the frequency of cellular alterations (brokenegg, binucleation, karyolysis, and cariorhexis) is higher in the exposed group than in the control group, and this difference is statistically significant for changes. brokenegg cells, binucleation and karyolysis ($p = 0.001$, $p = 0.0001$ and $p = 0.0004$, respectively) and not being significant for cariorrex cell alteration ($p = 0.13$).

Individual T2, as in the MN test, has a higher number of binucleated cells. This event is related to the final process of mitosis, specifically delayed telophase and cytokinesis. Alterations in mitosis can be induced by substances that directly interfere with the cell cycle as occurs with antineoplastic drugs (Kikushi and Pinto 2006) as well as in the events that regulate the various equivalence steps of genetic material.

The study by Ergene et al. (2007), is in line with the results presented in this paper, since, analyzing the frequency of chromosomal aberrations, MN, cariorrexe, karyolysis and binucleated cells, we observed an increase of such genetic alterations in the group of farmers exposed to pesticides in relation to the control group.

The results of Benedetti et al. (2013) also observed the occurrence of DNA damage in rural workers in the city of Espumoso, RS exposed to pesticides, which also showed an increase in the frequency of MN, binucleated cells and carriolysis.

The emergence of NM is an indicator of non-repairable clastogenicity and this is a significant fact when considering the establishment of error-bearing cell groups in a given tissue. Changes such as brokeneggs and binucleated cells are indicators of changes in mitotic processes induced by glyphosate exposure at that time. As these farmers are exposed, not only in short periods of life, but over several annual crops and over long years, it is clear that these errors recur in these individuals.

Biological markers may reflect exposure to carcinogens and their interaction with macromolecules such as DNA and how these interactions of chemicals with DNA are recognized as the first step in the initiation of tumorigenesis, greater emphasis should be given to methods that detect genotoxic activity in humans for risk assessment of these individuals as well as make use of them to establish monitoring strategies.

It will be important that further assessments of this risk group be made at pre-established periods in order to monitor the damage that may lead to the emergence of tumor lineages.

5 CONCLUSION

The results show that individuals exposed to glyphosate, during the period in which the samples were collected, presented higher cellular instability in the oral mucosa when compared to the control group. The observed events are indicative of changes and interferences in the cell cycle of these cells, suggesting that in this time frame, these cells show significant changes.

We cannot yet claim that glyphosate has potential for cancer formation, but we can consider that it actually has the potential to alter the efficiency of mitoses and induce errors in the anaphase process and DNA damage with potential for malignancy. Thus, we can use this cytogenetic test as a first tracker of cell damage in individuals exposed to glyphosate.

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CONFLICT OF INTEREST

There was no conflict of interest declared.

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