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Environmental/Occupational Exposure to Pesticides of Pregnant Women Living in a Countryside Municipality

Exposição Ambiental/Ocupacional aos Agrotóxicos em Gestantes Residentes em um Município Rural

Exposición Ambiental / Ocupacional a los Agrotóxicos en Gestantes Residentes en un Municipio Rural

*Maria Isabel Gonçalves da Silva[1]; Anna Maria Siebel [2]; Maria Assunta Busato[3]; Clodoaldo Antônio De Sá[4]; Vanessa da Silva Corralo[5]**

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ABSTRACT

Objective: The study's goal has been to analyze if environmental or occupational exposure to pesticides can produce changes in pregnant women living in a countryside municipality. **Methods:** The participants of this study were twenty-three pregnant women, who both answered a questionnaire and donated biological material in order to perform Micronucleus (MN) Tests in lymphocytes, oral epithelial cells, and also for measuring the enzyme activity of erythrocyte acetylcholinesterase. **Results:** Considering the total analyzed samples, the following was found: an average of 8 ± 2.92 MN/1000 oral epithelial cells from urban participants; an average of 6.82 ± 3.43 MN/1000 oral epithelial cells from rural participants; and 100% of the microscope slides contained cells with two MN, which shows high intensity lesions to the DNA. There was found a high frequency of spontaneous abortions (34.8%), greater than in Brazil. **Conclusion:** The exposure of pregnant women living in a countryside municipality to pesticides may increase the rate of spontaneous abortions, as well as the chances of mutagenic effects.

Descriptors: Abortion, Acetylcholinesterase, Agrochemicals, Occupational Exposure, Mutagenicity Tests.

[1] Pharmacy Graduate by the *Universidade Federal de Santa Catarina (UFSC)*, Specialist's Degree in Applied Toxicology by the *UNOCHAPECÓ*, MSc in Health Sciences by the *UNOCHAPECÓ,* PhD student in Health Sciences by the *UNOCHAPECÓ.Universidade Comunitária da Região de Chapecó (UNOCHAPECÓ)*, Brazil.

[2] Biological Sciences Graduate by the *Universidade Feevale*, MSc and PhD in Molecular and Cellular Biology by the *Pontifícia Universidade Católica do Rio Grande do Sul (PUC-RS)*, Coordinator and Professor of the Environmental Sciences Postgraduate Program (Stricto Sensu) at the *UNOCHAPECÓ Universidade Comunitária da Região de Chapecó (UNOCHAPECÓ)*, Brazil.

[3] Biology Graduate by the *Universidade de Passo Fundo (UPF)*, Specialist's Degree in General Biology by the *Fundação Educacional Severino Sombra (FESS)*, MSc in Tropical Diseases by the Universitat de València, PhD in Biosciences by the Universitat de Barcelona, Professor of the Health Sciences Postgraduate Program (Stricto Sensu) at the *UNOCHAPECÓ. Universidade Comunitária da Região de Chapecó (UNOCHAPECÓ)*, Brazil.

[4] Physical Education Graduate by the *Universidade Federal de Santa Maria (UFSM)*, MSc and PhD in Science of Human Movement by the *UFSM*, Coordinator and Professor of the Health Sciences Postgraduate Program (Stricto Sensu) at the *UNOCHAPECÓ. Universidade Comunitária da Região de Chapecó (UNOCHAPECÓ)*, Brazil.

[5] Pharmacy Graduate by the *Universidade de Cruz Alta (UNICRUZ),* MSc and PhD in Toxicological Biochemistry by the *UFSM,* Professor of the Health Sciences Postgraduate Program (Stricto Sensu) at the *UNOCHAPECÓ. Universidade Comunitária da Região de Chapecó (UNOCHAPECÓ)*, Brazil.

RESUMO

Objetivo: Analisar se a exposição ambiental ou ocupacional aos agrotóxicos causa alterações em gestantes residentes em um município rural. **Métodos:** Compuseram a amostra 23 gestantes, que responderam a um questionário e doaram amostras biológicas para a realização dos testes de micronúcleos (MN) em linfócitos, em células do epitélio oral, e para a dosagem da atividade da enzima acetilcolinesterase eritrocitária. **Resultados:** Obteve-se uma média de 8 ± 2,92 MN/1000 células do epitélio oral analisadas em amostras de participantes da zona urbana, 6,82 ± 3,43 MN/1000 de participantes da zona rural, e 100% das lâminas continham células com dois MN, o que demonstra lesões ao DNA de maior intensidade. Encontrou-se uma frequência elevada de casos de abortos espontâneos (34,8%), superior à encontrada no Brasil. **Conclusão:** A exposição de gestantes residentes em um município rural aos agrotóxicos eleva a taxa de abortos espontâneos, bem como as chances de ocorrência de efeitos mutagênicos.

Descritores: Aborto, Acetilcolinesterase, Agroquímicos, Exposição Ocupacional, Testes De Mutagenicidade.

RESUMEN

Objetivo: Analizar si la exposición ambiental o ocupacional a los agrotóxicos causa cambios en gestantes residentes en un municipio rural. **Métodos:** Compusieron la muestra 23 gestantes, que respondieron a un cuestionario y donaron muestras biológicas para la realización de las pruebas de micronúcleos (MN) en linfocitos, en células del epitelio oral, y para la dosificación de la actividad de la enzima acetilcolinesterasa eritrocitaria. **Resultados:** Se obtuvieron una media de 8 ± 2,92 MN / 1000 células del epitelio oral analizadas en muestras de participantes de la zona urbana, 6,82 ± 3,43 MN / 1000 de participantes de la zona rural, y el 100% de las láminas contenían células con dos MN, lo que demuestra lesiones al ADN de mayor intensidad. Se encontró una frecuencia elevada de casos de abortos espontáneos (34,8%), superior a la encontrada en Brasil. **Conclusión:** La exposición de gestantes residentes en un municipio rural a los agrotóxicos eleva la tasa de abortos espontáneos, así como las posibilidades de ocurrencia de efectos mutagênicos.

Descriptores: Aborto, Acetilcolinesterasa, Agroquímicos, Exposición Ocupacional, Pruebas De Mutagenicidad.

INTRODUCTION

Exposure by environmental contaminants such as pesticides promotes a series of biochemical changes in the human organism, such as variations in hormonal activities, oxidative parameters, inhibition or enzymatic activation, and cause DNA damage.[1] In this sense, biomonitoring studies, made from the analysis of biological parameters that allow the detection of biochemical alterations, play a fundamental role in the evaluation of the health of populations exposed to environmental contaminants.

In view of the importance of agricultural activity for the Brazilian economy and the growing demand for food production around the world, consumption of agrochemicals has also increased, especially in the country.[2] In addition to the harmful effects on the environment, pesticides pose a risk to exposed humans as they are composed of biologically active molecules capable of causing genotoxic and mutagenic damage. They affect, in particular, agricultural workers and rural residents, as well as the general population consuming food products contaminated by residues of these substances.[3]

Pregnant women and neonates are among the most vulnerable populations, since pesticides, considered as endocrine disruptors, may affect the development of tissues and organs during the gestational period as they modulate hormonal action.[4] Exposure to these compounds can begin early in the maternal womb when the pesticides cross the placenta or in the perinatal phase, a fact that increases the risk of disease development due to the immaturity of the physiological systems.[1]

Biomarkers of effect can be used to verify the occurrence of pathological changes to health, due to exposure to chemical compounds. Among the techniques used for this purpose is the Micronucleus Test, an assay belonging to the genetic toxicology field, which allows identifying the increase in the frequency of cellular mutations, then expressing chromosomal damage.[5]

Micronuclei appear as secondary nuclei in the cytoplasm and consist of small amounts of DNA from the fragmentation of chromosomes that were not included in the daughter cell's main cellular nucleus at the time of cell division.[5] Another widely used biomonitoring technique is the enzymatic activity analysis of the erythrocyte acetylcholinesterase, an important indication of exposure to pesticides from the class of carbamates and organophosphates.[6]

Considering that in the South of Brazil the annual commercialization of agrochemicals reaches the order of 285 million kilos,[2] and no work assessing biomarkers of effect in pregnant women possibly exposed to these products was found, this study aimed to analyze if environmental and/or occupational exposure to agrochemicals can produce changes in pregnant women living in a countryside municipality.

METHODS

This research is characterized as an analytical cross-sectional study with a quantitative approach. Pregnant women living in the rural and urban areas of a countryside municipality were evaluated. It should be noted that, according to the Instituto Brasileiro de Geografia e Estatística (IBGE) [Brazilian Institute of Geography and Statistics], municipalities can be classified as predominantly rural when they have a population corresponding to 3,000 and 10,000 citizens in an occupation area, with an urbanization index of less than 75%.[7]

The pregnant women underwent prenatal follow-up at the city's Basic Health Unit over the period from January to April 2017. Twenty-three pregnant women within the age

group from 18 to 35 years old, who agreed to participate, were included in the sample. The average age was 25.50 ± 4.06 years old among rural women, and 25.64 ± 7.99 years old among urban women, while the average weight (kg) was 60.55 ± 7.76 and 69.83 ± 9.96, respectively.

While waiting for their prenatal consultations, invited pregnant women who agreed to participate in the study were individually directed to an adequate space in the Basic Health Unit, which had separate rooms for both the collection of biological samples and interviews. Initially, the biological samples were collected for the tests proposed here. Then, the pregnant women interviewed answered a questionnaire, containing sociodemographic information, referring to lifestyle and contact with pesticides.

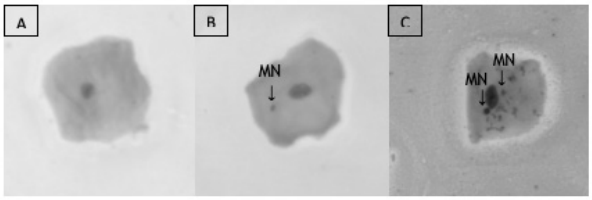
In this study three tests were carried out involving analysis of biomarkers of effect: Micronucleus (MN) Test in peripheral blood lymphocytes, in cells of the oral epithelium, and also the measurement of erythrocyte acetylcholinesterase (AChE) enzyme activity.

In order to perform the Micronucleus Test in lymphocytes, 4 mL of peripheral venous blood was collected from the venous puncture in an antecubital fossa, then put in a tube with heparin. The smear technique was then performed with 5 µL of blood in duplicate, leaving the histological slides to dry protected from light, and then stained by the method established by Romanowski using the LB® Quick Panotic Kit.[8] After drying, the slides were taken to the Olympus® optical microscope for analysis and counting of MN in the lymphocytes. The samples were analyzed under a microscope with 1000 x magnification. For each slide containing the sample, 100 cells were counted and classified according to the amount of MN present.

MNs can be recognized according to some criteria: they do not present refractions, they have round or oval shapes, a diameter of 1/16 to 1/3 if compared to the diameter of the main nuclei, they do not bind to or overlap the main nuclei, but have the same color intensity of the nucleus.[9]

In order to perform the Micronucleus Test in oral epithelial cells, the sample was collected by means of a previous oral rinse with water and with the aid of a cytobrush, immersed in a test tube containing 5 mL of 0.9% physiological solution for the dispersion of cellular content in the liquid medium. The samples were processed according to the protocols described by Sarto et al. (1987),[10] as well as fixed according to Stich and Rosin (1983).[11] The samples were centrifuged at 1500 rpm for 10 minutes, and the supernatants discarded thereafter. Then, the fixation process was performed by adding to each tube, 4 mL Carnoy's solution and performing another centrifugation. The fixation procedure was repeated, and the supernatant removed. The material was resuspended in 2 mL Carnoy's solution and for each sample, in duplicate, either 2 or 3 drops of the material were dripped onto a microscope slide, which was allowed to dry at room temperature. The slides were stained by the May Grünwald Giemsa method (MGG), allowed to dry, and then counted and graded according to the microscope, following the same parameters used for the micronucleus test on lymphocytes, as aforementioned. For each slide containing the sample, 1000 cells were counted (**Figure 1**).

Figure 1 - (A) A epithelial cell from the oral mucosa of the sample studied without the presence of micronucleus. (B) A epithelial cell from the oral mucosa with the presence of a micronucleus (MN). (C) A epithelial cell from the oral mucosa with the presence of two micronuclei.



With regards to the analysis of the AChE activity, 4 mL of peripheral vein blood was collected from each pregnant woman, packed in a tube containing EDTA, sent to a third-party laboratory, which used the potentiometric method to analyze it. The reference values considered normal are in the range of 0.56 to 0.94 ΔpH/hour for women.[12]

Concerning the ethical aspects, it should be noted that all the women were informed about the objectives and procedures of the study, furthermore, they all signed the Informed Consent Form (ICF) authorizing their participation in this research. One of the routes was evaluated and the other with the researcher. The data is on guard for the responsible researchers. This study was approved by the Research Ethics Committee involving Human Beings, under Legal Opinion No. 1 877 262.

For data analysis, descriptive statistics, average and standard deviation, and frequency distribution were used. Student's t-test for independent samples was used for comparisons of AChE concentrations between rural and urban environments. Pearson's Chi-square test or Fisher's exact test was used for the association between the variables, depending on the frequency observed in the categories analyzed. The Statistical Package for Social Sciences (SPSS®), version 24.0 was used for all analyzes, and the significance level adopted was 5% ($p<0.05$).

RESULTS AND DISCUSSION

The participants of this study were twenty-three pregnant women living in rural and urban areas, whose sociodemographic characteristics are presented in **Table 1**. Among the evaluated ones, 52.2% lived in the urban area, 60.9% self-referred to the skin color as brown, 91.3% were married, 52.2% completed high school and 13% were female.

Table 1 – The sociodemographic characteristics of pregnant women residing in a countryside municipality according to the living area, taking into consideration the period from January to April 2017.

Parameter	Rural Area n (%)	Urban Area n (%)
Skin color		
White	6 (26.1)	3 (13.0)
Brown	6 (26.1)	8 (34.8)
Marital status		
Married	11 (47.8)	10 (43.5)
Single	0 (0.0)	2 (8.7)
Education		
Pre-elementary school	0 (0.0)	1 (4.3)
Complete elementary school	3 (13.0)	2 (8.7)
High school	6 (26.1)	6 (26.1)
College	2 (8.7)	3 (13.0)
Occupation		
Housewife	6 (26.1)	7 (30.4)
Farmer	3 (13.0)	0 (0.0)
Other (exposed to chemical compounds)	1 (4.4)	3 (13.4)
Other (not exposed to chemical compounds)	1 (4.4)	2 (8.7)

Regarding the nutritional habits, all pregnant women reported daily fruits and vegetables included in the diet, and 91.3% did not consume alcoholic beverages. The smoking habit was cited by only one pregnant woman (4.4%), while 26.1% were passive smokers. Pregnant women used drugs such as multivitamins, folic acid, and ferrous sulfate, and 13% were taking other drugs such as antidepressants, antibiotics, and analgesics.

Although 47.8% of the participants in this study were rural residents at the time of the survey, it was found that 78.3% lived in the rural area previously, and 39.1% worked in the field. It was observed that 39.4% of the pregnant women had farmers' spouses, and they were responsible for the hygiene of the clothes used by them during farming work. Moreover, 30.4% of the interviewees stated that they work near crops, such as corn, soybeans, beans, tobacco and eucalyptus plantations.

About the data on possible exposures to pesticides still, it was verified that 21.7% of the interviewees confirmed that pesticides were applied in the gardens or orchards of their residences. They also reported that annual disinfection was carried out at their homes (52.2%). Only three pregnant women (13%) were aware of pesticides used in crops or even those used at home. The insecticide thiamethoxan (neonicotinoid, toxicological class III), glyphosate herbicide (substituted glycine, toxicological class IV) and chlorpyrifos insecticide (organophosphate, toxicological class II) were mentioned.

When assessing the data related to neonatal outcomes, a case of congenital malformation (anencephaly) and one case of prematurity were detected. The number of previous pregnancies and abortions occurred were also accounted for, as shown in **Table 2**.

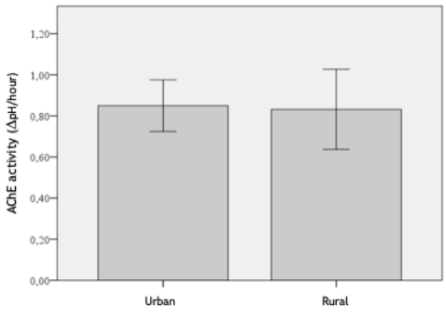
Table 2 - The number of previous pregnancies and abortions occurred in pregnant women residing in a countryside municipality according to the living area, taking into consideration the period from January to April 2017.

Parameter	Rural Area n (%)	Urban Area n (%)
Number of previous pregnancies		
None	7 (30.4)	5 (21.7)
1	2 (8.7)	2 (8.7)
2	3 (13.0)	3 (13.0)
3	0 (0.0)	1 (4.4)
Number of abortions		
None	8 (34.8)	10 (43.5)
1	1 (4.4)	2 (8.7)
2	1 (4.4)	0 (0.0)
3	1 (4.4)	0 (0.0)

The pregnant women were also questioned about the existence of a family history of congenital defect or hereditary disease. Among those interviewed, 34.8% reported having relatives with congenital diseases such as Down's Syndrome, Turner's Syndrome, deafness and malformations in the upper limb, such as syndactyly (21.7% were in the rural area and 13.0% in the urban area).

Considering the analysis of the erythrocyte acetylcholinesterase enzyme activity, the results indicated that there was no change in enzyme activity, as shown in **Figure 2.**

Figure 2 – Measurement of the erythrocyte acetylcholinesterase enzyme activity in pregnant women residing in a countryside municipality according to the living area, taking into consideration the period from January to April 2017.



*Results shown as averages ± SD.

In relation to the micronucleus test in peripheral blood or exfoliative cells of the oral epithelium, no statistically significant differences were found between pregnant women living in the rural and urban areas (p>0.05) (Table 3). All samples of oral epithelial cells presented micronuclei and 78.4% of the pregnant women produced samples of epithelial cells with two MN each.

Table 3 - Comparisons of the micronuclei averages in lymphocytes and oral epithelial cells of pregnant women living in a countryside municipality, taking into consideration the period from January to April 2017.

Parameter	Urban Area			Rural Area			
	n	Average	SD	N	Average	SD	p-value
Lymphocytes[a]	11	0.05	0.15	10	0.10	0.21	0.500
Oral epithelial cells[b]	12	8.00	2.92	11	6.82	3.43	0.387

aAverage of MN/100 lymphocytes. bAverage of MN/1000 oral epithelial cells.

An important finding evidenced in this study was the high number of abortion cases in the sample, which represented 34.8%. This percentage is higher than that found for Brazil (25%) and among the factors associated with the etiology of abortions is exposure to xenobiotics, such as pesticides.[13] Of the eight cases of abortion detected, six were found to occur between pregnant women living in the rural area and two in the urban area.

The pesticides may have an influence on the reproductive systems due to their performance as endocrine disruptors. Chronic exposure is related to reproductive dysfunctions such as decreased fertility in both sexes, as well as increasing the risk of abortion up to three times.[14]

Data analysis showed that among pregnant women living in rural areas, only 13% reported working with agricultural activities, and of these, none were under conditions of direct occupational exposure to pesticides during the gestational period, because they were away from work and did not handle such products.

Although no cases of acute poisoning have been reported, chronic contamination cannot be ruled out, as there are other sources of contact with these substances, even to a lesser extent, over a long period of time. It was observed that 39% of the interviewees had previously worked as agricultural workers, and approximately 30% were currently working near crops, which could expose them to pesticides dispersed in the environment through inhalation. Furthermore, 39.4% of pregnant women reported washing the clothes their spouses used in agricultural activities, thus allowing dermal exposure.

All women confirmed daily fruit and vegetable intake during gestation, which could lead to exposures by absorbing residues of these substances, in addition to which part of the pregnant women consumed what they grew in orchards and vegetable gardens of their residences, where pesticides were used (21.7%).

It is considered that this practice is a possibility of contamination, and pesticides also pose risks to the health of the population, through the absorption of residues of these compounds present in foods.15 Another factor to be considered is the exposure of pregnant women to contaminated water with pesticide residues, since the pollution of water from surface water sources, shallow and deep wells, by agrochemical residues in the country is high.[16]

It is also noted that 34.8% of pregnant women reported having relatives with congenital diseases. Although the cause of these diseases is unknown, environmental factors such as maternal exposure to teratogens, such as pesticides, cannot be neglected, especially when contact occurs in the first months of gestation. They are important risk factors for the appearance of congenital anomalies because they live near crops where pesticides are applied, have housing up to 1 km away from crops and store the pesticides at home.[17]

In regards to the assessment of the AChE activity, the values found were within normal limits and were similar among pregnant women in rural and urban areas. It is known that the AChE undergoes changes in its activity through acute exposure to agrochemicals of the class of carbamates and organophosphates. Due to the ability of these compounds to inhibit it, they prevent the hydrolysis of acetylcholine (ACh), causing an accumulation of this substance in the synaptic cleft and, consequently, signs of cholinergic hyperstimulation.[6]

Therefore, the level of the cholinesterase enzyme in the blood becomes an important biomarker for the prevention or diagnosis of pesticide poisonings. The lower the value of enzyme activity, the greater the degree of intoxication.[18]

It should be noted that if pregnant women were exposed to compounds of different classes, such as glyphosate, one of the most used herbicides in the region, there would be no interference in the results. It is considered that the care of pregnant women in not exposing themselves to pesticides can explain the results found. Moreover, the decrease in AChE activity may remain for up to 120 days after the last contact with the pesticides,[19] and it should be considered that the sample consisted of pregnant women of different gestational ages, including more than three months gestation. In this case, by not exposing themselves to pesticides from the beginning of the gestational period, there would be no detectable reductions in the activity of the enzyme.

In addition to the effects on human reproduction and gestation, pesticides stand out as potentially genotoxic and mutagenic agents. Some studies correlate the use of pesticides with this type of damage, measured by techniques such as the micronucleus test and the comet assay.[20-1] It is important to know the action of these agents and to verify possible alterations to the genetic material, both in the evaluation of environmental impacts, as well as on the influence of the population's lifestyle on genomic stability.[5]

Herein, the micronucleus test in lymphocytes and in the cells of the oral epithelium of pregnant women was used as mutagenicity parameters. No statistically significant differences were found by associating the residence areas with the frequency of micronuclei in the two tests.

As a less invasive alternative to the micronucleus test

in lymphocytes, the test performed with oral epithelial cells also stands out as an analysis of damage to the genetic material, being able to detect chromosomal mutagenesis, constituting an early biomarker of diseases with genetic bases.[22] The maintenance of healthy growth, development and aging of cells depends on the genomic stability as well as the gene expression profile of basal stem cells and is fundamental in the processes of regeneration of tissues and organs of the body.[23]

When a genotoxic substance reaches the basal layer of the oral mucosa, which has cells in mitotic activity, cytogenetic damage can occur that originate the micronuclei during the process of cell division. When these lesioned cells advance to the surface of the epithelium, they can be collected and observed from appropriate trials.[24]

Another pertinent result found in this study was the frequency of micronucleated cells of the oral epithelium, with an average of 8 ± 2.92 MN/1000 cells analyzed in samples of participants from the urban area, and 6.82 ± 3.43 MN/1000 in samples from rural participants. It should be noted that all slides contained cells with two MN, which demonstrates the possibility of lesions to DNA of higher intensity in the sample studied. Similar frequencies of micronucleated cells were found in research conducted with populations exposed to pesticides in the country.[25-6]

It is observed that the frequency of micronuclei detected from the test performed with oral epithelial cells was higher than that found when counting lymphocytes. This situation can be explained by the fact that the oral mucosa, as well as the skin in general, is one of the first contact barriers of the genotoxic agent with the organism.[27]

Some variables may influence MN averages found in research, such as age, population genetic profile and intensity of exposure to chemical agents, and there is no baseline measure in the literature that establishes a reference standard or normality.[28]

Changes in the number of micronuclei may be related to the effects of DNA repair mechanisms, at a high oxidative stress level, and as a consequence of higher genotoxicity caused by exposure to xenobiotics. Hence, the MN test is widely used in biomonitoring of mutagenic effects caused by environmental pollutants.[29]

Given this framework, differences between the results obtained from biomonitoring techniques in populations exposed to pesticides may reflect different exposure conditions, and consideration should be given to the intensity of the exposure, the genotoxic potential of the compound, as well as the potential for its absorption, endogenous factors, and environmental factors.[25]

Considering the place of study, it can be observed that the differences between residing in the rural and urban areas of rural municipalities are tenuous. As the territorial extension is small and the distance is short, many people move to work in the rural area, but they live in the urban area, and vice versa, making it difficult to divide the population between the areas of residence aiming to know in which environment the pregnant women would be most vulnerable to exposure by agrochemicals. In small rural municipalities, it can be seen from the sample profile that there were no relevant distinctions regarding the intensity of exposure to pesticides, and all pregnant women were exposed to these compounds in a chronic manner.

It is considered that there is a close relationship between urban and rural environment for municipalities with a population of less than 25,000 people, therefore, the results found here can be extrapolated to the rest of the country, since 75% of the Brazilian municipalities fall into this category.[30]

CONCLUSIONS

A exposição ambiental e/ou ocupacional aos agrotóxicos de gestantes residentes nas zonas rural e urbana indica uma elevada taxa de abortos espontâneos, assim como uma média considerável de micronúcleos obtida a partir do teste realizado com células do epitélio oral.

Estes resultados demonstram a necessidade de efetuar novas avaliações, que objetivem investigar os possíveis fatores etiológicos para estes desfechos adversos, de maneira mais aprofundada, com o intuito de minimizar os riscos a esta população.

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***Corresponding Author:**
Vanessa da Silva Corralo
Servidão Anjo da Guarda, 295-D
Efapi, Santa Catarina, Brasil
E-mail address: vcorralo@unochapeco.edu.br
Telephone number: +55 49 3321-8215
Zip Code: 89809-900

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