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Abstract This paper aims to evaluate the genotoxic effect of agrochemicals in rural workers occupationally exposed by the micronucleus assay in peripheral blood lymphocytes and to promote the development of health and environmental preventive and protective practices. A total of 30 blood samples from 20 individuals occupationally exposed to different agrochemicals and 10 unexposed persons, who formed the reference group, were analyzed. We found statistically significant differences ($p < 0.0005$, Student's *t* Test) in the frequency of micronuclei between the two groups (7.20 ± 1.55 and 15.15 ± 5.10 CBMN for reference and exposed groups respectively). The analysis of age showed a positive correlation (Pearson Correlation Test) with the frequency of micronuclei in exposed population ($p < 0.05$; $r^2 = 0.47$), in contrast with smoking habits and years of exposure. Micronucleus assay allows an early detection of populations at higher risk of having genetic damage, allowing us to implement strategies of intervention for the purpose of contributing to reduce that risk.

Keywords Biomarkers · Genotoxicity · Micronuclei · Pesticides · Rural workers

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The Food and Agriculture Organization of the United Nations (FAO 2003) defines a pesticide as any substance or mixture of substances intended for preventing, destroying or controlling any plague, including vectors of human or animal diseases, unwanted species of plants and animals that cause harm or interfere in any other way in the production, processing, storage, transportation or commercialization (marketing) of food, agricultural products, wood and its derivates (Martínez Valenzuela y Goméz Arrollo 2007).

The benefits obtained by the use of pesticides are certainly numerous, but the dissemination of large amounts of these compounds to the environment, has led to a series of problems affecting both the environment and human health (Bhalli et al. 2006). Particularly, in agricultural activities, agrochemicals are widely used products, and its use without the necessary protection can lead to genetic alterations and the possible development of some types of neoplasia (Alavanja et al. 1997; Kohen et al. 2002).

The use of pesticides has also been associated to the induction of congenital malformations and reduced fertility in women (Bolognesi 2003), increases in spontaneous abortions (Pastor et al. 2001b), and other reproductive effects in men like sperm toxicity (Santivañez et al. 2002). In recent years, the use of pesticides has been associated with neurodegenerative diseases like Parkinson's and Alzheimer's (Bhally et al. 2006).

The International Agency for Research on Cancer (IARC), has classified a great number of insecticides, fungicides, herbicides and other similar compounds as carcinogenics in laboratory animals (Bolognesi 2003; Costa et al. 2006). Several human studies, have reported a provable role of the exposure to pesticide in the development of non-Hodgkin lymphoma (McDuffie et al. 2001), lung, pancreatic, bladder, stomach and liver cancer, multiple myeloma, leukemia, prostate and brain cancer (Lucero et al. 2000; Pastor et al. 2001a, b; Sailaja et al. 2006).

For these reasons, genetic human biomonitoring is a useful tool to estimate the genetic risk of an integrated exposure to a compound or complex mixtures of chemicals (Bolognesi 2003) and is an early warning system for genetic diseases or cancer (Sailaja et al. 2006). It also allows the identification of risk factors, while control measures can still be put into practice (Kassie et al. 2000).

In particular, the cytokinesis-block micronucleus assay has gained increased attention in recent years, and a large number of studies have been published applying this method. It is widely used for environmental monitoring studies on plants and animals susceptible to environmental contamination, and is currently applied in mutagenesis research lines, to learn about the in vitro genotoxic effect of new chemical agents, as well in the environmental area with the use of new pesticides, as in the health area with the use of new cytostatic drugs in antitumor treatments (Zalacain et al. 2005). It has been proved that repeated exposure to cytostatic agents can induce genetic damage and alter cell division mechanisms, therefore it can significantly increase the number of micronuclei (MN) (Hongping et al. 2006). In our country, biomonitoring of genotoxicity in people occupationally exposed to chemicals is limited to studies carried out in the Province of Buenos Aires (Larripa et al. 1983; Dulout et al. 1985, 1987), Santiago del Estero (Gorla et al. 1988, 1989); and in Córdoba the first reports are done by Mañas et al. (2009).

Within a regional context and considering the current problem on the potential prejudicial effects of pesticides on human health, our aims were to identify the level of knowledge on the type and toxicity of pesticides; habits and risk behavior in handling them and to evaluate the genotoxic effect on rural workers occupationally exposed to agrochemicals, in order to promote the development of preventive, permanent and protective practices for the environment and health.

agrochemicals, whose age and gender were analogous to those of rural workers. The study excluded people with health problems and/or exposed to other known contamination agents.

Before the field work started, the research team met with health professionals and workers who were part of the sample. The objectives of this meeting were to inform about the study and to select workers who met the inclusion criteria. Workers, who agreed to participate voluntarily and signed a written consent, were surveyed.

Open and closed questions were combined in the survey since we considered necessary that surveyed people answer with absolute freedom, while in other questions we sought to assess their response on the basis of series of predetermined criteria and according to characteristics listed by Badia and Carne (1988). The survey data included characteristics of their work and lifestyle. Then, we proceeded to take biological samples of blood.

A cell culture of human lymphocyte was carried out for 72 h at 37°C according to conventional methods (Moorhead et al. 1960). After 44 h of culture, 0.15 ml of Citocalasina B was added to each tube at a concentration of 5 µg to block cytokinesis (Fenech y Morley, 1985). After (at) 72 h of incubation, it was added a hypotonic solution of ClK 0.075 M and it was fixed with a solution of Methanol: Acetic Acid (3:1). Finally, smears were colored with a 10 % Giemsa solution for 10 min.

We determined the frequency of binucleated cells with micronuclei (CBMN) and total number of micronuclei (MNL) in lymphocytes, analyzing 1000 binucleated cells per donor (OECD 2004). The count was performed by a single person and all preparations were previously coded. There were also counted 500 lymphocytes to evaluate percentage of cells with 1, 2, 3 and 4 nuclei, and it was calculated the cell proliferation rate with cytokinesis block (CBPI) to estimate cytotoxic effects. In order to calculate CBPI the following formula was applied:

$$\mathrm{CBPI} = \frac{\mathrm{noofmononuclearcells} + 2 \times \mathrm{noofbinucleatedcells} + 3 \times \mathrm{noofmultinucleatedcell}}{\mathrm{Total\ number\ of\ cells}}$$

Materials and Methods

A case–control study design was used, with a random population sample, pondered by age and length of occupational exposure to pesticides, consisting of 20 male rural workers whose work activity was mainly crop spraying in the Rio Cuarto Department, Province of Cordoba; and a reference group of 10 individuals not exposed to

The statistical analysis was performed using the software GraphPad Prism version 5.02. To determine the normal distribution of data, the Kolmogorov–Smirnov test was performed.

The Student's *t* Test was used to compare the results obtained for the exposed and the reference group.

The Pearson Correlation Test was carried out to determine possible relationships between MN values and possible mistaken factors such as age, smoke habit, etc. In all cases, the level of significance selected was less than 5 % ($p < 0.05$).

Results and Discussion

The characteristics of individuals regarding age, years of exposure and smoking habits are summarized in Table 1. The mean age of the individuals was similar in both groups under study; there were not statistically significant differences between them (t test $p > 0.05$). In the reference group, the observed range of age was from 18 to 62 (mean 37.00 ± 12.86), in the exposed group age was from 21 to 62 (mean 36.25 ± 12.25). Rural workers exposed to pesticides had an exposure period of 1–45 years (mean 9.93 ± 11.64). Concerning the smoking habit, only in the exposed group, smokers were observed; they represented 41.66 % of the individuals and smoked from 10 to 20 cigarettes per day (mean 13.00 ± 4.47).

From the analysis of the surveys, it comes out that rural workers were exposed to different types and mixtures of pesticides, mainly insecticides and herbicides. Table 2 summarizes the agrochemicals mostly used in the localities under study, the chemical group they belong, their frequency of use (%) and toxicological classification (WHO 2004).

The percentage of the rural workers who applied pesticides in spring-summer season (September to March) was 66.66 %. Due to weather conditions, 16.66 % applied them from June-July to January-March, and the remaining workers usually crop sprayed all year long.

Regarding protective measures, 75 % of the surveyed rural workers reported to use some of the personal protective equipments (25.00 % used masks, gloves and safety goggles; 25.00 % gloves and masks and 25.00 % gloves only), the remaining workers used no protection when handling the agrochemicals.

Concerning the disposal of the equipment (containers, cans, buckets, clothing, etc.), the most frequent risk behaviors

Table 1 Characteristics of the groups under study

Characteristics	Reference group (n: 10)		Exposed group (n: 20)	
	N° (%)	Mean ± SD (range)	N° (%)	Mean ± SD (range)
Age		37.00 ± 12.86 (18–62)		36.25 ± 12.25 (21–62)
Years of exposure				9.93 ± 11.64 (1–45)
Smoking habit				
Smokers			5 (25.00)	
Non smokers	10 (100.00)		15 (75.00)	
Cigarettes per day				13.00 ± 4.47 (10–20)

Table 2 Main pesticides used by rural workers in the localities under study

Type	Product	Group	Use %	Toxicological Classification (OMS)
Insecticides	Cypermethrine	Pyrethroid	83.30	Class II
	Chlorpyrifos	Organophosphate	58.30	Class II
	Endosulfan	Organochlorine	33.30	Class II
	Dimethoate	Organophosphate	16.60	Class II
	Fipronil	Phenyl pyrazole	25.00	Class II
	Lambda-cyhalothrin	Pyrethroid	16.60	Class II
Herbicides	Glyphosate	Phosphonate	75.00	Class III
	Atrazine	Triazine	58.30	Class III
	2, 4D	Chlorophenol	41.60	Class II
	Haloxyfop	Haloxifop-R-Methyl_Ester	16.60	Class II

WHO World Health Organization (2005) classification: Class I = Extremely hazardous; Class II = Moderately hazardous; Class III = Slightly hazardous; Class IV = Usually is not hazardous of exposed workers while applying pesticides are the following: A percentage of 8.33 of the surveyed people leave the equipment at home, 16.66 % isolate it by wrapping, 8.33 % place it in a deposit near their home, another 8.33 % sell it, 25.00 % do not pay much attention and 33.33 % of the individuals do not know.

As an indication of the risk of exposure to pesticides, 58.33 % of the exposed individuals reported poisoning at some point of their working life. The most common symptoms were headache, in 12.96 % of the cases, stomachache in 12.96 %, burning and itching on the skin in 25.92 % and eye tearing in 6.48 %. The percentage of surveyed rural workers who showed and still show diseases such as allergies, asthma and gastritis related to the use of these chemicals, was 33.33 %.

Table 3 summarizes the cytogenetic variables, CBMN and MNL, analyzed in both the group of individuals exposed to pesticides and in the reference group, including the CBPI. In all the cases, results are expressed as Mean ± standard deviation (SD). We want to emphasize that even though we make reference to CBMN and MNL, CBMN cells is a more appropriate parameter than MNL, to interpret the results, since it takes into account cells that have suffered damage. However, the obtained results show no obvious differences between the two variables. The statistical analysis was performed both for CBMN and MNL, obtaining similar results at the end.

The analysis performed reveals a statistically significant increase of CBMN (Fig. 1a) and MNL (Fig. 1b), in the group of rural workers exposed to pesticides, compared with the reference group ($p < 0.05$). Regarding the CBPI, no significant differences were observed between the two groups ($p > 0.05$).

Concerning the dependent variables CBMN, MNL and CBPI, no statistically significant differences were observed between smokers and non smokers from the exposed group.

The results of Pearson's Correlation show an association between the age of exposed people and the variables CBMN, $r = 0.69$ ($p < 0.05$; $r^2 = 0.47$) (Fig. 2a) and MNL, $r = 0.65$ ($p < 0.05$; $r^2 = 0.42$) (Fig. 2b), indicating that with increasing age of individuals exposed, both the frequency of binucleated cells with micronuclei (CBMN) and the total amount of micronuclei increase. This association was not demonstrated between ages and CBPI, $r = 0.25$ ($p > 0.05$; $r^2 = 0.06$), ruling out cytotoxicity as responsible for this increase.

We also compared these variables with age in the reference group, and no significant association was observed between them: CBMN, $r = 0.40$ ($p > 0.05$, $r^2 = 0.16$), MNL, $r = 0.37$ ($p > 0.05$, $r^2 = 0.14$); CBPI, $r = 0.004$ ($p > 0.05$, $r^2 = 0.00002$).

Pesticides comprise a diverse group of chemical products that individuals use to protect themselves and protect animals and plants from negative effects of other living beings that can turn into a plague due to their action and proliferation. However, there is no doubt that the overuse and misuse of them causes environmental and health

Table 3 Dependent variables analyzed (Mean ± standard deviation)

Dependent variables	Reference group	Exposed group	Smokers	Non smokers
CBMN	7.20 ± 1.55	15.15 ± 5.10***	13.20 ± 1.83	12.43 ± 1.60
MNL	7.40 ± 1.35	16.60 ± 5.66***	15.80 ± 2.27	13.00 ± 1.62
CBPI	1.24 ± 0.03	1.26 ± 0.07	1.26 ± 0.02	1.25 ± 0.01

Frequency of binucleated cells with micronuclei (CBMN), total number of micronuclei in binucleated lymphocytes (MNL) and cell proliferation rate with cytokinesis block (CBPI)

*** P < 0.0005

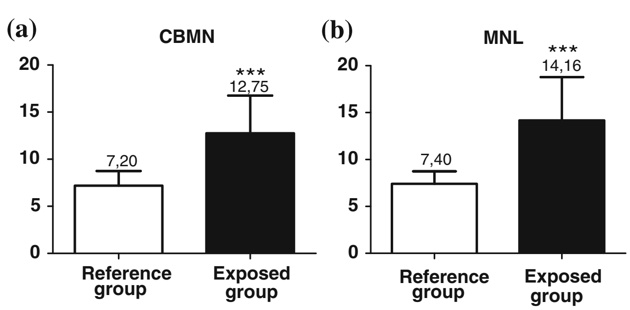

Fig. 1 Comparison of Mean values of (**a**) CBMN and (**b**) MNL between the reference and the group exposed to pesticides

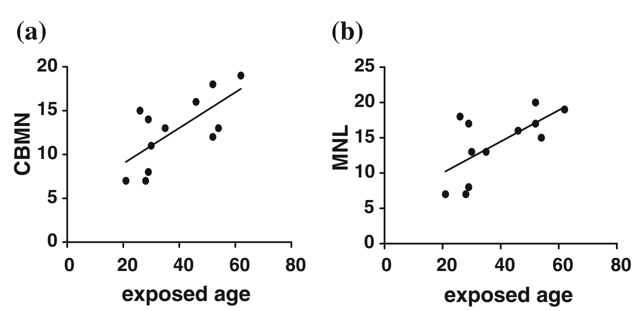

Fig. 2 Correlation between (**a**) CBMN and age of the exposed group, (**b**) MNL and age of the exposed group

problems for diverse exposed populations (Castro et al. 2004). As a consequence, biomonitoring of human populations occupationally exposed to different agents, including pesticides, is currently the main objective of numerous studies (Ergene et al. 2007; Remor et al. 2009).

The average length of employment for the exposed group was 9.93 ± 11.64 years, which reveals a chronic exposure to different chemicals that can cause long term health effects.

Cypermethrine, belonging to the pyrethroids family, is the most used insecticide among the farmers under study, followed by glyphosate, the most commercialized herbicide in Argentina Cypermethrine has been classified by the EPA (United States Environmental Protection Agency) and the IARC (International Agency for Research on Cancer) as possible or probable carcinogenic and/or mutagenic agent (Pastor et al. 2001b).

It is important to note that several studies report that mixtures are more toxic for humans than exposure to a single pesticide (Martínez Valenzuela y Gómez Arrollo, 2007). In this study it was observed that all surveyed rural workers used different types and mixtures of agrochemicals, most of them classified by the WHO as moderately hazardous (class II). In fact, according to surveys the most widely used pesticide is cypermethrine, a pyrethroid insecticide considered by the EPA and the IARC as a possible or probable carcinogenic and/or mutagenic agent.

Seventy five percent of the exposed people report wearing some type of protection while applying pesticides, being gloves the most frequently worn garment, leaving evidence about the unawareness on the routes of entry of pesticides. Therefore, it could indicate that there is a lack of knowledge in the group of workers under study, about the protective measures required when these products are handled, which make obvious the need to intensify efforts in continuous training and updating of day laborers and farmers, as well as strengthen preventive and educational actions towards the community. There were no statistically significant differences in the frequency of MN between the rural workers who used some type of protection and those who did not use any protective measures ($p > 0.05$).

Similarly, there were flaws in the final disposal of the containers, since most of the rural workers throw and/or accumulate them in inappropriate places, without realizing that this can lead to contamination of the environment and the members of the family group.

In this study we found that the frequency of binucleated cells with micronuclei (CBMN) and the total number of micronuclei in binucleated lymphocytes (MNL) in the exposed population are significantly higher than in the reference population. This could indicate a clastogenic and/or an aneugenic effect related to pesticide exposure. A similar increase was observed in investigations conducted in persons who crop sprayed and employees who work in pesticide production (Ascarrunz et al. 2005; Bhalli et al. 2006; Costa et al. 2006) although other studies did not report significant differences between the exposed group and the reference group (Lucero et al. 2000; Pastor et al. 2001a, b).

The basal frequency of micronuclei in human lymphocytes obtained in this study is consistent with frequency of micronuclei achieved by other populations reported by Surrallés and Natarajan (1997).

The fact that there is evidence of increased genotoxic damage, reflected in the increased frequency of micronuclei in rural workers, who used some protective measures may be because the measures have been insufficient to reduce the degree of exposure, taking into account that half of the individuals only wore gloves and in some cases masks. The limited use or misuse of protective measures is also reflected in the amount of individuals who have suffered poisoning, being mostly through skin contact or inhalation, and in the occurrence of diseases related to the use of pesticides, like asthma and allergies.

On the other hand, CBPI is considered as an indirect measure of cytotoxicity, making possible to estimate the existence of mitotic delays. However, the analysis of CBPI values between both groups, exposed and reference does not reveal statistically significant differences. Consequently, exposure to pesticides does not seem to influence the kinetics of cell proliferation. This agrees with the results obtained by other biomonitoring studies in human populations (Lucero et al. 2000; Pastor et al. 2001b).

Confounding factors that can affect the frequency of micronuclei in a population are very varied. Those considered in this study were age, smoking habit and years of exposure.

Age is one of the confounding factors more widely studied. Several studies relate the increase of micronucleus frequency with the increase of age (Fenech y Morley, 1985; Bolognesi 2003; Norppa y Falk, 2003; Zalacain et al. 2005). This increase, in spontaneous chromosomal instability with age, is associated with an accumulation of DNA damage due to a progressive deterioration of the repair capacity and the increase of highly reactive free radicals in the cells (Costa et al. 2006). In this study we observed a direct relationship between age and frequency of CBMN and MNL, both increases with the age of exposed individuals. Regarding CBPI, we did not observe a significant association between this variable and the increase of age, but it was evident a little tendency to diminution.

As a confounding factor, cigarette smoking is closely related to the increase in the frequency of various cancers, mainly in lungs; and also with respiratory and cardiovascular diseases. Genotoxicity studies on tobacco effects indicate that it can induce a higher frequency of micronuclei in peripheral blood lymphocytes (Di Giorgio et al. 1995, Zalacain et al. 2005. However, the analysis of our data has not been able to confirm this type of effect. Besides, it should also be noted that this factor was analyzed only in the group exposed, due to the absence of smokers in the reference group. Therefore, it could be possible to consider that the increase in the frequency of micronuclei in rural workers could be affected by cigarette smoking. However, no statistically significant differences are evident between smokers and nonsmokers in the exposed group, once again placing pesticides as the main possible cause of the increase of genotoxic damage in this population. Costa et al. (2006) points out in his work that the effective concentration of chemical substances in cigarette smoke is lower in blood compared with other organs, therefore, it is insufficient to detect any genetic damage through MN assay in peripheral blood lymphocytes. The results of this study also reveal no differences in the CBPI when comparing smokers and nonsmokers. This is consistent with other studies (Lucero et al. 2000, Pastor, et al. 2001b), where the consumption of cigarettes doesn't seem to reveal a statistically significant cytotoxic activity.

It has also been established a significant positive correlation in the occurrence of MN, with respect to years of exposure to pesticides (Bolognesi 2003, Martinez and Gomez Valenzuela Arroyo, 2007). Studies performed in Pakistan indicated that MN frequency in individuals with exposure time from 3 to 18 years, increased in relation to years of exposure (Bhalli et al. 2006). The results presented here do not reveal a statistically significant association with CBMN, MNL and CBPI, but in all cases it is evident a little tendency to the increase of CBMN and MNL, and a diminution of CBPI, with increasing years of exposure. This absence of association has not been observed in other studies (Lucero et al. 2000, Pastor, et al. 2001b). It should be noticed that, in this work, age does not show positive correlation with years of exposure.

For these reasons, cytogenetic monitoring should be considered as an integral part of a good medical surveillance on people in contact with pesticides, since it allows assessing the potential risk of occupational exposure, which, in turn, would allow taking the necessary measures for early identification of genetic risk.

In conclusion, the exposure to pesticides in the group of rural workers under study could induce levels of genetic damage detectable by the micronucleus assay in peripheral blood lymphocytes. Age is a factor that increases the frequency of binucleated cells with micronuclei and the total number of micronuclei. Others, like the years of exposure, do not affect these variables. However, even with the above, all the factors must be taken into account when performing a cytogenetic evaluation.

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