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RESEARCH ARTICLE

Evaluation of genetic damage in pesticides applicators from the province of Córdoba, Argentina

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Abstract

The impact evaluation of pesticide exposure is conducted using combined data from biomonitoring and environmental monitoring. Damage to the human genome is, probably, the leading cause of chronic-degenerative disorders, reproductive toxicology, and developmental problems. Although the general population is exposed to pesticides, workers in the agrochemical industry and farmers represent a high-risk group due to the occupational and environmental exposure. The aim of this study is to determine whether occupational exposure to agrochemicals in Córdoba (Argentina) constitute a factor of genotoxic damage. The study was conducted in 30 pesticide applicators from the province of Córdoba. Chromosomal aberrations (CAs), micronuclei (MN), and comet assays (CO) were performed. The current study shows that occupational exposure to pesticides increases values of CAs, MN, and DNA fragmentation biomarkers, all indicators of damage to the genetic material. Evidence suggests that chronic exposure to pesticides is a potential risk to workers health.

Keywords Occupational exposure · Pesticides · Genotoxicity · Biomonitoring · Chromosomal aberrations · Micronuclei · Comet assays

Introduction

The effects on health of pesticide applicators have been reported in the literature for several years and they include acute and persistent injury to the nervous system, lung damage, and injury to the reproductive organs, dysfunction of the immune and endocrine systems, birth defects, and cancer (Mansour 2004).

The impact evaluation of pesticide exposure is conducted using combined data from biomonitoring and environmental monitoring. Biomonitoring is defined as the repeated, controlled measurement of chemical or biological markers (biomarkers) in fluids, tissues, or other accessible samples from subjects exposed to chemical substances, or biological, chemical, or physical risk factors in the workplace and in the environment in general (Silins and Högberg 2011).

The important development that agriculture has had in the last decades in Argentina, mainly in relation to the cultivation of genetically modified soya, has led to the expansion of the agricultural frontier, from 40,000 ha planted in the 1970s to more than 39 million in the currently, between oilseeds, cereals, and other crops (Ministerio de Agroindustria de la Nación, Dirección de Estimaciones Agrícolas y delegaciones, 2018).

The Córdoba province's with an area of 165,321 km2 does not escape this reality mainly because it is located in an area of the country characterized by having the most favorable soils for agriculture. In the last campaigns, almost eight million hectares of the six main crops produced in the province were planted: soy, corn, wheat, sorghum, peanuts, and sunflower. Therefore, exposure to pesticides is increasing both in rural workers and in the population in general.

Although general population is exposed to pesticides, workers in the agrochemical industry and farmers represent

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a high-risk group due to the occupational and environmental exposure. Occupational exposure to pesticides in agricultural work place occurs during the preparation (mixing and loading) and application (spraying) of pesticides (Khan et al. 2013). Moreover, it is difficult to identify the individual effects of pesticides since complex mixtures are often used, either in the application of different chemical agents simultaneously or by the presence of additives in commercial forms. The effects of long-term occupational exposure at low pesticide doses are hard to diagnose as they include temporary and non-specific health outcomes (García-García et al. 2016). Chronic exposure to pesticides could be associated with some alterations, such as immunological disorders, impaired reproduction and development, carcinogenic effects, and neurotoxicity (Simoniello et al. 2010; Coalova et al. 2013).

Weichenthal, Moase, and Chan (Weichenthal et al. 2012) and Mostafalou and Abdollahi (2013) have compiled many publications that linked several types of neoplasias (leukemia, myeloma, melanomas, lung cancer, colon, rectum, pancreas, and bladder) with different groups of pesticides, although some of these studies have differed in their conclusions. Occupational exposure to pesticides have also been associated with risks of developing cancer in the skin, lips, brain, prostate, stomach, in the lymphohematopoietic system, ovaries, and breast (Alavanja et al. 2005; Partanen et al. 2009; Santamaría 2009; Koutros et al. 2010).

Other effects on human health caused by exposure to pesticides have been associated with certain events such as damage to the genetic material (Porcel de Peralta et al. 2011). The importance of the determination of the effects of the substances used refers mainly to the toxicological classification. The glyphosate in the Argentine toxicological classification is category IV, the lowest and therefore allows its spraying at less than 500 m from homes and for applicators allows its handling with few measures of prevention for your health.

Genotoxic potential is a primary risk factor for long-term effects, such as carcinogenic and reproductive toxicology and degenerative diseases (Bolognesi et al. 2011).

Bibliographic reviews show that the most commonly used biomarkers to evaluate the genotoxic effect in human populations occupationally exposed to pesticides are chromosome aberrations (CAs), micronuclei (MN), sister chromatid exchanges (SCEs), and DNA fragmentation. This is based on studies conducted by researchers from many countries around the world, providing scientific evidence that suggest positive correlations between exposure time, doses, and high frequencies of CAs, MN, SCEs, and DNA fragmentation (Martinez-Valenzuela and Gómez-Arroyo 2007; Aiassa et al. 2012).

The changes in the genetic material induced by these chemical substances can be identified through genotoxicity studies, even in the early stages, before cancer occurs or damage could be transmitted to the offspring if germinal cells are affected.

The study of populations occupationally exposed to genotoxic agents is a powerful tool to obtain information that allows promoting and guiding health policies, to prevent and reduce genetic damage.

In view of the foregoing, the aim of this study is to determine whether occupational exposure to agrochemicals in Córdoba (Argentina) constitute a factor of genotoxic damage.

Materials and methods

A descriptive-correlational study was carried out. The rural workers whose blood samples were studied are terrestrial applicators of pesticides in extensive crops (they regularly perform loading, mixing, or spraying tasks), residents of the province of Córdoba. The sample size was established based on (Preston and Hoffmann 2008) who suggest that "study groups with 20 or more individuals may be a reasonable surrogate for exact agreement given that confounding factors will have a lesser impact on chromosome aberration or mutation."

According to the Ethics Committee of Biomedical Research of the IMBICE and the Research Ethics Committee of the UNRC (Exp 03/2013), it is contemplated that the working protocol includes written information for the participants and informed consent form for adults.

The population sample consists in exposed individuals selected considering labor activity (applicators of pesticides in agriculture), number of applications sprayed in the area per year (≥ 3), age (18–65 years), and exposure time ≥ 3 years.

The reference group was formed considering: place of residence away from areas sprayed with agrochemicals ($\geq$ 1000 m), without any contact with pesticides used in agriculture, age (18–65 years), and a lifestyle habit similar to that of the exposed people. There is no demonstrable distance in the literature to ensure that there is no exposure by inhalation; therefore, a distance is chosen as far as possible from the source of contamination (fields where it is sprayed) which for this case is > 1000 m and work activity in rural areas.

Each participant filled in a questionnaire (environmental-clinic history) that included general information such as age, gender, diet, medical conditions, and pathological history including: recent illnesses, radiation exposure, radiation therapy and chemotherapy; exposure to environmental toxics, signs and symptoms of acute poisoning, occupational backgrounds and type of trades, exposure time at work, use of personal protection equipment, hygiene measures and industrial safety, extra-occupational exposures, and toxicological background like smoking habit and alcohol consumption.

This information is essential to discard any factor that may cause confusion regarding the obtained results. Both populations were considered exposed to pesticide degradation residues through food. Although the group referring not to be in direct contact with chemical substances do not exhibit the

same exposure through dermal and inhalatory routes (main entry routes) than the exposed group.

Spraying season begins in late June and runs through March, with a duration of 2 to 8 months per year. In April and May, the activity decreases. The amount of applications goes from 4 to 30 days per month. The analysis of damage to the genetic material was carried out sampling 5 ml of heparinized peripheral blood, collected between March and April.

Biomarkers of exposure were analyzed chromosomal aberrations, micronuclei, and DNA fragmentation through the comet assay and as a biomarker of effect the plasma cholinesterase enzyme.

Chromosome aberrations test (Moorhead et al. 1960; OECD 1997; Møller 2006)

Whole blood is grown were cultured for 72 h at 37 °C according to conventional methods (Moorhead et al. 1960); after this time, smears were prepared and stained with 10% Giemsa solution. Afterwards, smears were examined under the optical microscope and all the chromosome aberrations were rescreened by a second observer. Mitotic index (MI) was calculated for each treatment and 100 metaphases were analyzed per participant and classified according to the ISCN (2013).

Micronucleus test (OECD 2004)

Whole blood is grown were conducted as described for CAs assay. After 44 h of incubation, 4 μg/ml of cytochalasin B was added to the culture and they were analyzed after 72 h. A total of 1000 binucleated cells were scored for each individual to determine the micronuclei frequency, following the criteria established for selecting binucleated cells and micronuclei in human cell cultures.

Binucleated cells

The cytoplasm must be clearly distinguished. The cytoplasmic and nuclear membrane must be intact. The nuclei must have a similar degree of chromatin condensation. They must be of equal size, shape (oval), and coloring pattern. They can be linked by nucleoplasmic bridges. They can touch but not overlap. None of the nuclei should be in the stage of apoptosis.

Micronuclei

The diameter should be between 1/16 and 1/3 of the average diameter of the main core. They should not be refractories. The intensity of staining should be similar to that of the main nuclei. The shape should be similar to the nuclei of the binucleated cell. They cannot be connected to any of the nuclei of the cell. They can touch the nuclei of the binucleate cell but not overlap.

Comet assay (Singh et al. 1988; Møller 2006)

The assay was performed using low melting point agarose and normal melting point agarose, cold lysis solution, and electrophoresis with alkaline solution. Then, slides were submerged in neutralization buffer (Tris pH 7.5). Slides were stained with 50 μl ethidium bromide (20 μg/ml) and immediately examined under the fluorescent microscope, in a dark room. At least 100 cells were photographed per individual and the images were analyzed employing the Comet Score 1.5 software. The parameter used to quantify the damage was TailMoment, the most widely used according to the available literature.

Plasma butyrylcholinesterase

Plasma cholinesterase levels were measured in U/l according to Ellman's spectrometric method (Ellman et al. 1961). The activity of the enzyme, considering the reference range of normality 3200–9000 U/l.

Statistical analysis

The Kolmogorov–Smirnov test was performed to verify whether the results follow a normal distribution and the differences between exposed and control group were analyzed applying Student t test, the level of significance was set at $p < 0.05$.

Age, diet, and exposure, using indicators of work seniority, the use of protective equipment, the habit of smoking and the consumption of alcohol, were considered independent variables. The dependent variables were the effects on health from the manifested symptomatology, the frequency of CA, the frequency of MN, DNA fragmentation, and plasma cholinesterase levels.

Results

Fifty-two individuals participated in the study, of which 30 were pesticide applicators males and 22 were referents males, the mean ages were 38.0 $\pm$ 2.2 years for the first group and 34.1 ± 2.3 years for the second one. All the participants were involved voluntarily, after having been informed about the objectives of the study and their consent was required to participate and to use the information obtained.

No significant differences were found between the two groups regarding the lifestyle habits in general, and particularly the diet, even though the consumption of red meat was higher in the exposed group. Therefore, the diet of the studied population was considered homogenous, eliminating confounding variables and allowing to determine the risk associated with occupational exposure. 60% of the pesticides

applicators had been preparing and spraying the chemical substances for 3–10 years, 3% for 11–20 years, and the rest had more than 20 years of exposure.

The active ingredients of the most used pesticides are glyphosate, cypermethrin, and chlorpyrifos (this information was not known a priori and arises from the survey implemented in the applicators). Ground spraying machines are used for the applications. Thirty-seven percent of pesticide applicators wore at least two personal protection elements (gloves and mask) while preparing the mixture and during spraying; 23% only wore gloves, 17% wore three elements (gloves, mask, and glasses), and 23% did not wear any protective element.

Thirty-seven percent of the exposed people expressed that they suffered headaches and eye irritation or watering at the time of spraying the pesticide and after.

Twenty-seven percent indicated that they had respiratory allergies and/or skin reactions, 10% suffered digestive problems (stomach pain, gastritis), persistent symptoms with no apparent cause.

Thirteen percent manifested that they had acute intoxication at least once after work.

Individuals that ingested alcoholic drinks in both groups did it sporadically and in moderate amounts, thus, this was not considered a confounding factor.

The exposed group included six smokers: 3 of them smoked 10 cigarettes per day, one of them smoked 15 cigarettes per day, and 2 individuals smoked 20 cigarettes daily. Control group was non-smoker.

People of both groups stated that they had not ingested any medication or psychotropic drug and they were not exposed to x-rays recently (within the last 6 months).

The genotoxicity tests performed in the pesticide applicators showed a significant increase in the mean of CAs, MN, and DNA fragmentation ($p < 0.05$) relative to the reference group (Table 1).

Chromosome aberrations observed in both groups were chromatids and chromosome gaps, acentric fragments, chromosome and chromatids breaks, and endoreduplications, of which the last two showed statistically significant difference between the groups. The mean values for fragmentation of the DNA in the group of reference group were 269, while for the group of applicators, they were approximately 3206 units, that is, more than 10 times higher.

Table 2 presents the frequency of different chromosome and chromatids aberrations types in applicators and referents.

The results of the Pearson correlation where it was found a statistically significant association ($p < 0.005$) between the parameters: CAs with spaces ($r^2 = 0.99$), CAs without spaces ($r^2 = 0.81$), and MN ($r^2 = 0.44$), and the exposure time are shown in Figs. 1a, b and 2. This association was not found in DNA fragmentation or in the other variables studied.

Regarding the biomarkers of effect, the activity of butyrylcholinesterase showed values within the reference range of normality (3200–9000 U/l) for both groups of subjects, with average values (DE) and (minimum-maximum) equal to 5454.84 (1065.02) (3349.58–8886.56) and 4875.09 (865.17) (3292.10–7289.48) U/l in exposed and not exposed, respectively ($p = 0.11$).

Discussion

Regarding the occupational exposure, the pesticides applicators from Córdoba (Argentina) are exposed to agrochemicals from 2 to 8 months per year, such substances contain the main ingredients: glyphosate, cypermethrin, and chlorpyrifos rated as toxicity category III (slightly hazardous), II (moderately hazardous) and Ib (highly hazardous), respectively. According to Baldi et al. (1998), while the available literature emphasizes the long-term effects of most commonly used pesticides, they cannot be considered harmless to human health.

There is no information regarding the effects of interaction between different types of pesticides and human health; however, it is known that interaction among substances within the same chemical group enhances the damage (Sanborn et al. 2002).

The frequency of acute intoxication in the study population agrees with the available literature. The high incidence of acute poisoning due to pesticides in Argentina resembles that in other developing countries. The protection elements used by the pesticides applicators that participated in the current study are insufficient; they do not wear impermeable coveralls

Table 1 Chromosome aberrations, micronuclei, and comets values in peripheral blood of pesticides applicators

Parameter	Applicators ($n = 30$)	Minimum value	Maximum value	Referents ($n = 22$)	Minimum value	Maximum value
CAs with gaps/100 cél(*)	7.27 ± 0.65	1	14	3.73 ± 0.49	0	7
CAs without gaps/100 cél (*)	4.97 ± 0.62	0	11	2.18 ± 0.36	0	5
MN/1000 cél (*)	12.40 ± 0.98	4	25	6.91 ± 0.36	5	12
Comet-tail moment (*)	3206 ± 785.4	199.4	22800	269.7 ± 67.91	117.45	1520.9

*$p < 0.05$ statistically significant difference (applicators vs referents)

Table 2 Frequency of chromosomal aberrations

Group	Ctg	Csg	*Ctb (*)*	Csb	Ace	*Endo (*)*	*Aberrant cells with gaps (*)*	*Aberrant cells without gaps (*)*
Applicators $N=30$	1.43 ± 0.23	1.17 ± 0.24	*1.93 ± 0.36*	0.93 ± 0.24	0.66 ± 0.15	*1.23 ± 0.31*	*7.27 ± 0.65*	*4.97 ± 0.62*
Referents $N=22$	0.95 ± 0.20	0.59 ± 0.16	*0.86 ± 0.20*	0.45 ± 0.13	0.41 ± 0.11	*0.45 ± 0.13*	*3.73 ± 0.49*	*2.18 ± 0.36*

*$p < 0.05$ statistically significant difference (applicators vs referents)

Ctg, chromatid gap; *Csb*, chromosome break; *Ctb*, chromatid break; *Csg*, chromosome gap; *Ace*, acentric fragment; *Endo*, endoreduplications

or chemical resistant gloves for work. The minimum personal protection equipment includes impermeable coveralls, gas mask, and chemical resistant gloves (Lanteri et al. 2009).

None of the participants wore impermeable clothes, which is one of the major measures to prevent contact with the substances due to the low penetration through them. In the workplace, the first entrance path for substances is the skin followed by the liver. The main routes of exposure to the contaminants are dermal or cutaneous mucosa and by inhalation (Turnbull et al. 1985; Al-Saleh 1994), being the first, the most important one considering the amount of substance absorbed (Vitali et al. 2009).

These flaws in the correct use of protective measures may be due to different factors, including: little or no knowledge of current legislation; lack of law enforcement; limited perception of risk (even inability to understand the warning labels) due to a poor education; and the use of products that are prohibited in industrialized countries, as Lanteri et al. (Lantieri et al. 2009) and Gentile et al. (2015) have previously stated.

For all the above, it is possible to indicate that this sample of applicators analyzed is exposed directly by dermal and inhalation routes to mixtures of chemical substances used in food production, whose main components are the active ingredients of glyphosate (herbicide) and cypermethrin and chlorpyrifos (insecticides).

The symptoms manifested during the manipulation of the substance and/or the ones that occurs with no apparent cause are consistent with those associated with long-term exposure to pesticides in the working population (Vallebouona 2007; Butinof et al. 2015).

The results of the genotoxicological monitoring in pesticide applicators of the Province of Córdoba allow two main aspects to be discussed: the methodology used and the quality

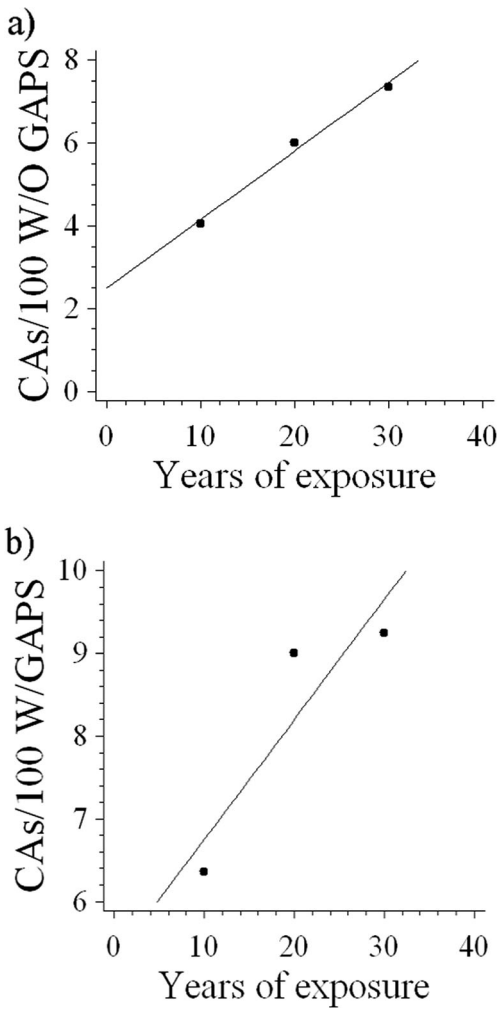

Fig. 1 Correlation between **a** chromosome aberrations without gaps and exposure time and **b** chromosome aberrations with gaps and exposure time

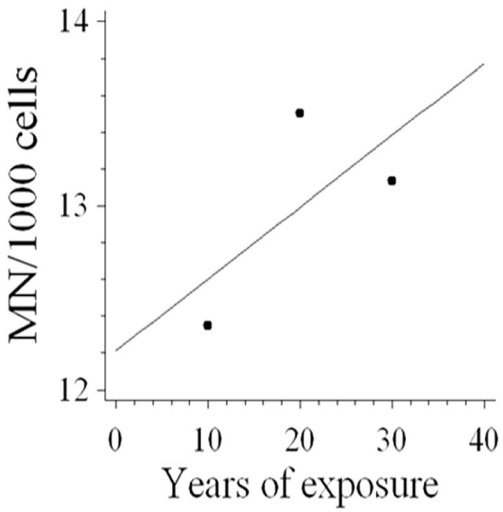

Fig. 2 Correlation between MN and exposure time

of life of the applicators of pesticides related to working conditions.

The studies of genotoxicological monitoring in human populations occupationally exposed to various agents are, nowadays, the main topic of several research (Gil and Hernandez 2015; Singaravelu and Sellappa 2015).

Methodologically, the study was conducted in clinically healthy people and with lifestyles that did not include the most relevant confounding factors since the available bibliographic evidence shows that the existing pathologies and some lifestyle habits are the most significant factors affecting the results, whether in isolation or combined (Fenech and Bonassi 2011).

Results obtained in the CA and MN tests have shown that the blood culture time during 72 h (prolonged time) was not affected. Some reviews exclude studies with 72 h of culture since at this time, many cells could pass through three cellular divisions after sampling, and therefore, CA cells could be lost and the effectiveness of DNA repair mechanisms could influence the results, which would lead to false negative data (Bull et al. 2006).

The current study shows that occupational exposure to pesticides increases values of CAs, MN, and DNA fragmentation biomarkers, all indicators of damage to the genetic material. Evidence suggests that chronic exposure to pesticides is a potential risk to workers health.

Although genotoxocity studies performed before registering the product are designed to identify potential in-vivo genotoxins, there is a concern that exposure to agrochemicals may cause long-term adverse effects. Several papers reveal a correlation between the increase of damage in the genetic material of rural workers and chronic exposure to pesticides (Martinez-Valenzuela and Gómez-Arroyo 2007; Aiassa et al. 2012).

The detection of groups exposed to diverse agents that provoke chromosomal abnormalities is extremely important, since these have been related with the occurrence of neoplasia in a variable period of time after exposure (Bonsái et al. 1995; Bonassi et al. 2000).

Both chromosome aberrations with gaps and without gaps exhibit a statistically significant increase when compared between exposed and control groups; however, chromosome and chromatid gaps are not considered to be representative of a cytogenetic effect (Scott et al. 1990). The presence of chromosome aberrations is associated with a carcinogenic effect.

The study of Mañas et al. (2009) analyzed CAs in a small sample of rural workers from the province of Córdoba, and the values reported are not significantly different from those obtained in the current work.

The high frequency of Ctb (chromatide breaks) founded in the population of pesticides applicators may be due to the action of residues of genotoxic substances accumulated inside the cells. Such damaging effect is manifested as CA, originated during S-phase of cell cycle (Albertini et al. 2000).

The number of micronuclei observed was lower than those reported by Gentile et al. (2012), whose work analyzed the MN parameter in pesticides applicators from Córdoba. Such difference is possible due to the sample sizes and/or the type of substance manipulated.

A long-term follow-up study (or a retrospective study of exposed people) would determine whether occupational exposure is related with the occurrence of neoplasia or not, and which tissues are susceptible of developing the disease.

Regarding confounding factors, the smoking habit may affect the frequency of genetic damage due to the mutagenic and carcinogenic risk of many cigarette components. The comparison between exposed people and referents indicates that smoking habit did not significantly increase the frequency of biomarkers. The fact that no adverse effect of smoking was detected can be attributed to the small proportion of smokers in relation to the total sample and to the consumption of tobacco (with a mean of 14.17 ± 2.01 cigarettes a day per person). According to the criteria establish by Calderón-Ezquerro et al. (2007), people that consume $< 19 \pm 3.88$ cigarettes per day are considered light smokers; participants are included in this category because the number of cigarettes consumed does not cause an adverse effect to lymphocytes. Moreover, Ergene et al. (2007) and Fenech and Bonassi (2011) report no damaging effects of smoking habit in workers exposed to pesticides and with a daily consumption of 22–30 cigarettes per day.

In this study, it was not possible to identify the damage caused by each pesticide in contact with the workers, mainly because they are exposed to complex mixtures of agrochemicals. However, the genotoxic effect of the following substance is well known: glyphosate (Mañas et al. 2007; Barbosa et al. 2017), cypermethrin (Kocaman and Topaktaş 2009), and chlorpyrifos (Vindas et al. 2004). It is also known that toxic agents may differ in the type and number of induced DNA lesions, and in their biological effects.

Most genotoxic and carcinogenic substances are highly electrophilic and can form covalent bonds with biological molecules such as DNA and RNA. In organophosphorus compounds, like chlorpyrifos, phosphoryl group is a potential electrophilic site able to react with DNA and the alkyl group can interact with nucleophilic centers of DNA, such as guanine-N7 (Vindas et al. 2004). Thus, chlorpyrifos might function as an alkylating agent (AA). When alkylating agents interact with DNA, they can react with highly nucleophilic sites (nitrogen atoms) or poorly nucleophilic sites (oxygen atoms). Oxygen alkylation is associated with mutagenic and oncogenic effects, while nitrogen alkylation causes cytotoxicity (Qiao et al. 2003).

However, it is not unusual to find discrepancies between the results of different studies possibly due to variations in people ages, use of pesticide mixtures, genetic polymorphism, application methods, genotoxicity category of compounds, place of spraying (enclosed area or open field), or interaction among these properties.

Despite an abundance of genotoxicological studies performed in rural workers (Aiassa et al. 2012; Arroyo et al. 2013), most of them did not examine the effects in human health, becoming impossible to compare and perform meta-analysis among similar studies. This situation is particularly critic in Latin America where a large number of published studies makes clear the immediate need to draw global conclusions.

Research in the area of genetic toxicology applied to public health has a major social significance as they allow the early recognition of carcinogenic, mutagenic, and teratogenic effects in individuals occupationally exposed to genotoxic agents.

Although the reported results serve to describe risk contexts and health impacts in the occupational exposure to pesticides, there are certain limitations to be able to determine the contribution of other variables not contemplated in this work (the type of food, genetic susceptibility, and other eating habits of everyday life). This analysis could offer greater robustness to the evidence provided and is indicative of the direction in which future research should be oriented.

In this regard, the results obtained emphasize the need to consider genotoxicologic monitoring as an integral part of a good medical surveillance in people exposed to pesticides and, ultimately, implement measures for the early identification of genetic risks (Martinez-Valenzuela and Gómez-Arroyo 2007).

In addition, this work shows that there is a genetic risk associated with exposure due to an intensive use of pesticides and it reinforces the need to develop educational programs for applicators, intended to minimize the use of chemical products in agriculture and, in the meanwhile, implement strict security measures.

Compliance with ethical standards

According to the Ethics Committee of Biomedical Research of the IMBICE and the Research Ethics Committee of the UNRC (Exp 03/2013), it is contemplated that the working protocol includes written information for the participants and informed consent form for adults.

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