

## ASSESSING THE GENOTOXIC RISK FOR MEXICAN CHILDREN WHO ARE IN RESIDENTIAL PROXIMITY TO AGRICULTURAL AREAS WITH INTENSE AERIAL PESTICIDE APPLICATIONS

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Key words: micronuclei, exfoliated cells, child risk, pesticides, cytogenetic biomarkers

### ABSTRACT

Pesticide ambient exposure is a potential risk when children live in or near a field aerial sprayed with such chemicals as is the case in Sinaloa state in the northwest of Mexico. In this study the possible genotoxic risk assessment was evaluated in two groups: 125 children (52 females and 73 males) living in residential proximity to areas of intensive agriculture and exposed to agricultural activity of pesticide mixtures, in addition to 125 control youngsters (57 females and 68 males) living in the city of Los Mochis, Sinaloa. The risk assessment was done through the use of micronuclei (MN) in exfoliated buccal cells as biomarkers. The age range in both groups was 1 to 13 years. Microscopic analysis was performed in 3000 buccal epithelial cells for each sample. Significant increment of MN frequencies was observed when the odds ratios (OR) values were calculated (3.11 and 95 % CI 2.70 and 3.50), indicating high health risk to the exposed children. Other nuclear abnormalities associated to cytotoxicity or genotoxicity as binucleate cells, nuclear buds, karyorrhexis and karyolysis were detected; in all cases the differences were significant in relation with the control group. The MN assay in exfoliated cells was useful, and a minimally invasive method was followed for monitoring cytogenetic damage in the children who live in residential proximity to areas of intensive agriculture treated with large amounts of pesticide mixtures.

Palabras clave: micronúcleos, células exfoliadas, riesgo infantil, plaguicidas, biomarcadores citogenéticos

### RESUMEN

La exposición ambiental a plaguicidas constituye un riesgo potencial para los niños que viven en o cerca de campos agrícolas asperjados de forma aérea con estas sustancias, como es el caso en el estado de Sinaloa en el noroeste de México. En este estudio el posible riesgo genotóxico fue evaluado en dos grupos: 125 niños (52 mujeres y 73

hombres) cuyas casas están cercanas a zonas de intensa actividad agrícola que son asperjadas con mezclas de plaguicidas y en 125 niños testigos (57 mujeres y 68 hombres) que viven en la ciudad de Los Mochis, Sinaloa, utilizando como biomarcador los micronúcleos (MN) en células exfoliadas de mucosa bucal. El rango de edad en ambos grupos fue de 1 a 13 años. El análisis microscópico fue realizado en 3000 células del epitelio bucal para cada muestra. Se observó incremento significativo en la frecuencia de MN. También fue calculada la razón de momios (RM) (3.11 y 95 % CI 2.70 y 3.50) indicando los valores alto riesgo a la salud de los niños expuestos. Otras anormalidades nucleares asociadas a citotoxicidad o genotoxicidad como células binucleadas, yemas nucleares, cariorresis y cariólisis fueron detectadas; en todos los casos las diferencias fueron significativas con relación al grupo testigo. El ensayo de MN en células exfoliadas resultó un método útil y poco invasor para el monitoreo del daño citogenético en los niños que viven en o cerca de las áreas agrícolas que son intensamente tratadas con grandes cantidades de mezclas de plaguicidas.

## INTRODUCTION

Pesticides are among the chemical products most extensively used to control agricultural pests. The development of agrochemical industries in the 20<sup>th</sup> century originated a great number of highly aggressive and toxic compounds against humans, and that altered the equilibrium in ecosystems. To a high or low degree, human populations are unavoidably exposed to environmental pollutants in physical, chemical or biological forms through products presented or degraded in air, water, soil or food. Environmental exposure to pesticides causes a potential risk to humans; when children are exposed, the risk is heightened. Children are more sensitive to toxic effects because of immature metabolism and liver functions (National Research Council 1993). There are many associated factors that affect children's health. Children interact with the environment in a way unlike adults, they are more sensitive to physical and biological factors, and present a different behavior (Faustman *et al.* 2000, Garry 2004, Neri *et al.* 2006). Children's health is at a higher risk than that of adults of being affected by exposure to pesticides, considering primarily that the immunological system and the metabolic capacity in children are not mature (National Research Council 1993). Thus, a fundamental principle of pediatric medicine to bear in mind is that "children are not little adults", a fact which is particularly important when the discussion is centered on children and their exposure to pesticides. Children are at risk through diverse sources and are vulnerable to a higher degree than adults under similar conditions of exposure (Neri *et al.* 2006). Furthermore, the respiratory rate, heart rate, and metabolism of children are significantly different from those of

adults (Bearer 1995). Wassels *et al.* (2003) provide evidence that factors as body surface/body weight ratio, circulatory flow rates, as well as intake of water, milk and fruits are greater in children than in adults. Additionally, consumption of large quantities of fresh food (possibly contaminated) and the high frequency of hand-to-mouth habits also increase the susceptibility of children to pesticide exposures (Rössner *et al.* 2002).

In the northwest of the Mexican Republic, in Sinaloa state, agriculture is a predominant activity. The climatic conditions have favored the development of pests and plant diseases, which has given rise to a preventive practice related with the use of pesticides. Likewise, children living in or near treated crop lands can be exposed in the course of agricultural application (Carozza *et al.* 2008), as in the north of Sinaloa state where large amounts of pesticides are aerial sprayed in the fields.

Epidemiological studies have described associations between childhood cancer and either parental or child exposure to pesticides (Daniels *et al.* 1997, Zahm and Ward 1998, Thompson *et al.* 2003, Nasterlack 2006). Pesticide spraying by parents has been associated with acute lymphocytic leukemia, tumors in the central nervous system and non-Hodgkin's lymphoma (Daniels *et al.* 1997, Zahm and Ward 1998, Flower *et al.* 2004).

In Mexico, the mortality caused by leukemia in individuals younger than 20 years of age represents a serious health problem, and therefore an early diagnosis is important for health prevention (Rizo-Ríos *et al.* 2005). For this purpose the use of biomarkers such as micronucleus (MN) is appreciated in showing signs of chromosomal damage and affording a marker of an early-stage of chronic diseases as cancer; such markers also reveal an increase in micronuclei fre-

quency predicting cancer risk in humans (Bonassi *et al.* 2005, 2007).

Micronuclei are formed by chromosomal damage in the basal cells of the epithelium; when these cells divide, chromosomal fragments or entire chromosomes that lack an attachment to the spindle apparatus are excluded from the main nuclei in the daughter cells and they appear as Feulgen-specific bodies in the cytoplasm called micronuclei. Later, these cells mature and exfoliate (Rosin 1992).

Nevertheless, cytogenetic biomarkers are known to be useful as endpoints in the study of subjects occupationally exposed to genotoxic agents. The micronucleus (MN) assay in human exfoliated cells has been widely used to detect the genotoxic effects of environmental mutagens (Titenko-Holland *et al.* 1994, Bolognesi *et al.* 2011). The MN test in epithelial cells has several advantages as has been suggested by Bonassi *et al.* (2009): it is a non-invasive method (particularly important in studies which involve children), and the exfoliated epithelial cells from the mouth can be collected easily and rapidly to make the cytogenetic analysis, among other benefits. MN assays in buccal epithelial cells have been used in the biomonitoring of agricultural workers exposed to pesticides, and the data reported indicate both positive (Gómez-Arroyo *et al.* 2000, Ergene *et al.* 2007, Bortoli *et al.* 2009, Martínez-Valenzuela *et al.* 2009, Remor *et al.* 2009) and negative correlations (Lucero *et al.* 2000, Pastor *et al.* 2001a, b, 2002).

The aim of the present study was to assess the genotoxic risk in children who live in residential proximity to areas of intensive agriculture treated with pesticide mixtures in the north of Sinaloa; this was done through the use of micronuclei (MN) in exfoliated buccal cells as biomarkers.

## MATERIALS AND METHODS

### Study population

Sinaloa state is situated in the northwest of the Mexican Republic. At the beginning of the study, all of the children's parents were informed of the purpose of the work; the parents consented and signed a document in which they agreed to participate in the study and to complete a standardized questionnaire that contained personal data with a history of recent illness, medical treatment and food habits, as well as to inform about the pesticides used in these agricultural areas (**Table I**). The parents interviewed said that the area is continually aerial sprayed during at

least two annual periods. This study was performed in accordance with the principles of the Declaration of Helsinki. The study protocol and sample collection procedure were reviewed and approved by the local Pediatric Committee. The MN test was carried out on the buccal epithelial cells. The samples were obtained from March to November 2008 of 125 children (52 females and 73 males) whose parents were agricultural workers living and working in the north of Sinaloa, in addition to 125 control youngsters (57 females and 68 males) who lived in the city of Los Mochis, Sinaloa. The age range in both groups was 1 to 13 years. At the moment of the sampling none of the children were under medical treatment or exposed to radiation, none had infectious diseases, and none had had recent vaccines.

The houses of the children involved in the research were in all cases located near the agricultural fields where mango, corn, sorghum, beans, among other products, are cultivated almost all year long; thus, when the pesticides were aerial sprayed on the fields, the houses were also sprayed with the applied pesticides and the substances dripped on the sheet of the metal roofs. The children that participated in this study as well as the control group did not live in the vicinity of busy roads, waste sites, nor of industrial emissions, and in most of the cases the children's parents mentioned that they did not smoke inside their houses, which suggested that they are not passive smokers. All of them corresponded to the same socioeconomic status. Two important factors involve the pesticide behavior: first, the area studied is a valley without barriers that prevent the movement of volatilized pesticides whose molecules can go several kilometers; secondly, the climate exhibits mainly high temperatures during the summer up to 48 °C and relative humidity of 85% (INEGI 2005). For this reason children wear minimal clothing, and often walk or play outside the house, thereby increasing the pesticide contact and dermal absorption; also, given the fact that these communities lack recreational activities, the children spend many hours playing in the open space behind their house.

With respect to the general dietary habits, all of the children were found to be omnivores. They consumed meat, fish and fruit at least once a week and they ate vegetables daily, including the corn "tortilla" which is the basis of the Mexican diet.

### Micronuclei procedure in buccal exfoliated cells

After the children had rinsed their mouth with water, sample cells were obtained from the buccal

**TABLE I.** PESTICIDES COMMONLY USED IN THE NORTH OF SINALOA STATE

Organochlorines	Organophosphorus	Carbamates	Others
<b>Insecticides</b>			
Endosulfan (II, UM)	Parathion methyl (3, Ia, UM)	Aldicarb (Ia)	Fenvalerato (II)
Chlordane (III, 2B)	Azinphos methyl (Ib)	Carbofuran (Ib)	Cypermethrin (III)
Aldrin (3, AIOP)	Gusathion (Ib)	Oxamyl (Ib)	
	Monocrotophos (Ib)	Lannate (Ib)	
	Diazinon (II, UM)	Vydate (Ib)	
	Chlorpyrifos (II)	Propoxur (II)	
	Malathion (3, III)	Carbaryl (III)	
		Carbosulfan (III)	
<b>Herbicides</b>			
		Molinate (II)	Paraquat (II)
		Butylate (U)	2,4,D (III)
		Aminocarb (AIOP)	Glyphosate (U)
			Atrazine (U, UE)
			Dinoseb (AIOP)
<b>Fungicides</b>			
Pentachlorophenol (Ib)		Benomyl (U)	Captan (U)
		Mancozeb (U)	Cupravit (U)
		Maneb (3, U, UE)	

IARC (2012) 2B= possible carcinogenic to humans, 3= not classify as to its carcinogenicity to humans. WHO (2004) hazard classification: Ia= extremely hazardous, Ib= highly hazardous, II= moderately hazardous, III= slightly hazardous, U= unlikely to present acute hazard in normal use, AIOP= active ingredients believed to be obsolete or discontinued for use as pesticides.

CICOPLAFEST (2012) UE = possible endocrine effects, UM = probably teratogen effects

mucosa with a wooden spatula. The sample was then applied to a clean microscope slide. Smears were air dried and fixed in methanol-acetic acid (3:1). The cell smears were stained using the Feulgen technique described by Stich and Rosin (1984) and modified as follows: the cell smears were pretreated with 1 N HCl for 10 min at room temperature, placed in 1 N HCl for 10 min at 60 °C, rinsed in distilled water, put in Schiff's reagent for 90 min and washed with running tap water. The frequency of micronucleated cells was estimated according to the criteria of Stich and Rosin (1984), and Livingston *et al.* (1990). For each child 3000 epithelial cells were scored to determine the MN frequency and other nuclear anomalies as nuclear buds also known as "broken eggs" (nuclei appear cinched with a Feulgen-negative band), karyolysis (nuclear dissolution, in which a Feulgen-negative, ghost-like image of the nucleus remains), karyorrhexis (disintegrated nuclei) and binucleate cells (presence of two nuclei within a cell), which were classified according to Tolbert *et al.* (1992) and Holland *et al.* (2008). All the slides were coded before scoring to avoid bias.

#### Statistical analysis

The U of Mann-Whitney test was applied to the results of MN and the other nuclear anomalies. The

correlation between different parameters was established by means of the Spearman correlation test.

For evaluating and determining the risk factors for children exposed to environmental conditions, probability distribution of two binary random variables using logistic regression was applied and the odds ratios (OR) values were calculated (Wassertheil-Smoller 2003). The odds higher than 1.5 and the confidence interval (CI) higher than 1.5 indicated risk for the studied children.

## RESULTS

**Table II** shows the mean of MN per 1000 cells in the children living in the areas of high agricultural activity and the mean of the control group; in this table we observe that the mean of the cells in the former group is six times higher than in the control group. The range of micronuclei observed is of 0 to 10 % in the former and 0 to 0.4 % in the control. The difference was significant for the U of Mann-Whitney test  $p < 0.0001$ . The calculated OR to evaluate risk for health of exposed children shows 3.11 with 95 % CI 2.70 and 3.50, which explains the significant risk for children living in contaminated agricultural areas.



**TABLE II.** FREQUENCIES OF MICRONUCLEI AND OTHER NUCLEAR ANOMALIES IN BUCCAL EXFOLIATED CELLS OF CHILDREN THAT LIVING ON OR NEAR OF FIELD SPRAYED WITH PESTICIDES<sup>1</sup> AND CONTROL<sup>2</sup>

	A	B	OR	95 %	CI
Micronuclei	3.6 ± 0.79*	0.6 ± 0.15	3.11*	2.70,	3.50
Binucleate	1.3 ± 0.56*	0.2 ± 0.32	4.30*	3.90,	4.60
Karyolysis	2.8 ± 0.25*	1.2 ± 0.30	2.63*	2.57,	2.69
Karyorrhexis	1.8 ± 0.28*	0.2 ± 0.19	17.80*	14.80,	20.80
Nuclear buds	1.1 ± 0.52*	0.6 ± 0.47	1.53*	1.45,	1.80

<sup>1,2</sup>In 125 children

n= 3000 cells in each individual

A Exposed mean + SD per 1000 cells

B Controls means + SD per 1000 cells

\*Significance p < 0.001

When the MN frequencies were compared between female and male as well as with age, in children living in the fields sprayed with pesticides, no significant differences were found. No correlation was observed between MN frequency and low or no consumption of meat, fish or fruit.

The analysis of exfoliated cells of the buccal mucosa also provided evidence of other nuclear anomalies such as binucleate cells, nuclear buds, karyorrhexis and karyolysis; in all the cases the difference was significant in relation with the control group for the U of Mann-Whitney test  $p < 0.0001$  (Table II).

Table II presents the OR values for other nuclear anomalies such as binucleate cell (OR = 4.30, 95 % CI 3.90, 4.60), karyolysis (OR= 2.63, 95 % CI 2.57, 2.69), nuclear buds (OR= 1.53, 95 % CI 1.45, 1.80) and the highest karyorrhexis (OR= 17.80 95 % CI 14.80, 20.80).

## DISCUSSION

When MN frequency was compared between the samples obtained from male and female children whose residence involved contact with pesticides, no significant differences were observed. These results agreed with Ganguly (1993), Barale *et al.* (1998) and Shi *et al.* (2000) who found that MN frequencies in male and female children were not significantly different. However, Bolognesi *et al.* (1993) did find higher MN frequency in women than in men; in both cases, individuals had been exposed to pesticides.

No effect of age was observed in the children who lived in the north of Sinaloa and consequently were under pesticide exposure. These results agreed with Holland *et al.* (2001) who did not observe an age-dependant increase in MN in exfoliated buccal cells

of children aged 5-12. However, other studies have shown an MN increase in lymphocytes (Ganguly 1993) and in urothelial cells (Holland *et al.* 2001).

The MN test in epithelial cells of the buccal mucosa allowed the conclusion that the exposure to pesticides significantly increased MN frequencies. This implied that the tissue was damaged at the chromosomal level, and that it had undergone chromosome breakage and/or mitotic spindle alterations, along with other nuclear abnormalities such as pycnosis, karyolysis, karyorrhexis and nuclear buds. According to Tolbert *et al.* (1992), pycnosis and karyorrhexis are associated with both cytotoxicity (necrosis and keratinization) and genotoxicity, while karyolysis is only related with cytotoxicity. In contrast, the nuclear bud is perhaps related to anaphase bridges, which occur as a result of chromosome aberrations; however, the origin of the nuclear bud and its significance are even now unknown (Titenko-Holland *et al.* 1994, Holland *et al.* 2008). In our research, results for the children with residential proximity to agricultural areas with intense aerial pesticide applications showed that all the frequencies of nuclear anomalies were significantly different from those of the control group, a finding that agrees with other studies in which pesticides caused this kind of abnormalities in adults (Gómez-Arroyo *et al.* 2000, Ergene *et al.* 2007, Martínez-Valenzuela *et al.* 2009).

The comparison of exposed and control children through OR calculations is a basic epidemiological measure of risk assessment, and a powerful statistical tool to determine if an exposure is associated with a prospective condition following two groups, one exposed and one unexposed. The magnitude of the difference in the MN frequency between the two groups is a consequence of the pesticide contact, and the MN OR value evidences a possible health risk for exposed children. In all the other nuclear anomalies analyzed, the OR values were higher than 1.5; that means that the pesticide exposition probably caused cytotoxic and genotoxic effects.

The analysis in epithelial cells is relevant because approximately 92 % of cancer cases are of epithelial origin (Rosin 1992). The heightened risk of childhood cancer is associated with residence in agriculturally intense areas in the United States of America. Children living in areas of great agricultural activity may be exposed to higher levels of pesticides than others (Carozza *et al.* 2008). Thus, through this simple and sensitive analysis, the damage in target organs can be evaluated.

The study of Carozza *et al.* (2008) indicates an increased risk of different types of childhood cancers as leukemia and central nervous system tumors related

with pesticide exposure, generally related to the residence of children in places with greater agricultural pesticide use. In Sinaloa state, parents working in the fields are exposed to pesticides as well as children living in places where the crops are aerial sprayed with pesticides; because of a shorter life period, the youngsters did not present signs of cancer. The positive MN test is a warning because other children not included in the study did show leukemia and because family members of the children included in the study had other types of cancer; in fact, several young women had breast cancer. The questionnaire indicated that about 40 % of the families of the children living in a field sprayed with pesticides had at least one family member with cancer, while in the control group about 18 % of the families had a member with cancer. The MN show signs of chromosomal damage and afford a marker of an early-stage of chronic diseases as cancer (Bonassi *et al.* 2005).

In the present study, parents of the children exposed to pesticide mixtures said that in their area the most frequently used compounds contained different active ingredients, mainly organophosphorus and carbamates. Some of the mixtures included two compounds which, according to the WHO (2004) classification, are “extremely hazardous” (parathion methyl and aldicarb) and eight which are “highly hazardous” (azinphos methyl, monocrotophos, gusathion, oxamyl, carbofuran, lannate, vydate and the fungicide pentachlorophenol). According to the IARC (2012), chlordane and pentachlorophenol are considered as possibly being carcinogenic to humans; as indicated by CICOPAFEST (2012), endosulfan, parathion methyl, diazinon, carbofuran, propoxur, carbaryl, benomyl, 2,4,D, atrazine and captan are classified as possibly having teratogen effects, and maneb and atrazine as possibly having endocrine effects.

The possible genotoxic damage caused by each pesticide in the children is associated with residence in agriculturally intense areas that are treated with these compounds in the north of Sinaloa, but this cannot be determined mainly because the substances are applied as complex mixtures. Moreover, the genotoxicity of some of these pesticides is unknown. The significant increase in the frequency of MN could be due to the accumulative effects of pesticides with clastogenic activity as is the case of endosulfan, paraquat, malathion, parathion methyl and 2,4, D (Titenko-Holland 1997, Zeljezic and Garaj-Vrhovac 2001). Nevertheless, the activity of other pesticides which have an aneugenic effect, as those associated to the use of glyphosate and carbofuran (Mladinic *et al.* 2009), can probably be shown.

The pesticide amounts are difficult to evaluate due to the fact that the children live in the proximity of the fields that are aerial sprayed almost all year around. In this way, it is important to mention that during the 2007 agriculture cycle in the Ahome place in which children were living, 65 171.29 ha of corn, 15 979 ha of sugar cane, 15 258 ha of sorghum, 7850.39 ha of bean, 2938 ha of mango, and 2694.56 ha of alfalfa, among others, were sown (INEGI 2008). A personal communication of the Technical Coordinator of the Agricultural Association of Río Fuerte Sur, Sinaloa, indicates that only for the mango culture during the 2007-2008 period, the following were applied: chlorpyrifos (II) 3 L/ha, chlordane (III, 2B) 3 L/ha, lannate (Ib) ½ kg/ ha, endosulfan (II, UM) 1.5 L/ha, vydate (Ib) 3 L/ha (which is a liquid that in contact with water forms a gas), atrazine (U, UE) 2 L/ha, 2,4 D (III) 2 L/ha (this herbicide has been forbidden but it is still applied, despite its high toxicity), molinate (II) 3 L/ha, benomyl (U) 3 kg/ha, captan (U) 1.5 kg/ha, mancozeb (U) 1.5 kg/ha, and paraquat (II) 1.5 kg/ha. As an example, in 5000 ha of cultures about of 6500 ton of captan were applied.

Considering this data and the information supplied by the children parents about the time the youngsters remain outdoors, we can establish that the exposure should be considered as constant and that it is highly probably the cause of the observed cytogenetic effects.

It is well known that pesticide toxicity may be caused not only by the active ingredients and the associated impurities, but also by solvents, carriers, emulsifiers, and other constituents of the formulated product (Al-Saleh 1994). This is so, due to the presence of solvents and surfactants which penetrate the lipid skin easily as a result of the alterations in cellular permeability; likewise, the pesticides may contain impurities such as dioxins, or solvents as naphthalene and benzene all of which are also carcinogenic.

Another important factor considered in our study was the dietary intake, given that in child population vulnerability to the toxic effect of pesticides increases due to malnutrition (Faustman *et al.* 2000, Lu *et al.* 2000, Suk *et al.* 2003, Garry 2004, Neri *et al.* 2006). However, in the children of Sinaloa with residence in agriculturally intense areas, malnutrition may not be a confounding factor influencing the susceptibility to pesticides; this statement is based on the fact that no correlation was found between the low intake or lack of consumption of meat, fish or fruit and the MN frequency.

The results obtained in the present study confirm the importance of designing strategies to protect

mainly children. The reason is that they are the most vulnerable sector of the population; children in the north of Sinaloa state are at risk of harm to their health when they are living exposed to pesticides associated with intensive agriculture activities.

It is for this reason that we make the following recommendations for reducing child exposure:

- The possibility of sending the planned schedule to spray pesticides to all of the inhabitants in the vicinity of the agricultural areas (it is important to circulate the information at least 24 hours before fumigation) must be considered.

- The person responsible in charge of the fumigation should be prepared to afford specific information and to avoid the pesticide application in the presence of pregnant women and of children particularly during class hours. Fumigations on Friday nights or during the end of the week are advisable.

- It is important to restrict the passing of children and women into the fumigated areas until the pesticides dry as well as to follow the instructions for users.

- It is appropriate to inform all individuals involved, as parents, children, day laborers, and farmers, about the risk of exposure to this type of compounds and how to be better protected, or if possible advise them to move away.

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