Assessment of the level of damage to the genetic material of children exposed to pesticides in the province of Córdoba

Natalí Bernardi, B.S.,[a] Natalia Gentile, B.S.,[a] Fernando Mañas, M.D.,[a] Álvaro Méndez, M.D.,[a] Nora Gorla, M.D.,[a] and Delia Aiassa, M.D.[a]

ABSTRACT

Introduction. In the past decades, several authors have investigated the genotoxicity caused by exposure to chemicals, but there are no reports on studies analyzing such effects on children in Argentina. The objective of this study was to establish the micronucleus frequency in exfoliated buccal mucosa cells in children from urban areas with environmental exposure (through inhalation) and to compare it with the micronucleus frequency in children from urban regions far from areas subjected to spraying.

Population and Methods. Fifty children living in the town of Marcos Juárez (Córdoba) at different distances from pesticide spraying areas and twenty-five children from the city of Río Cuarto (Córdoba), who are considered not exposed to pesticides, were studied; the micronucleus assay in buccal mucosa cells was used.

Results. A significant difference was observed between exposed children living less than 500 m from areas subjected to spraying and those who were not exposed. Forty percent of exposed children suffer some type of persistent condition, which may be associated with chronic exposure to pesticides.

Conclusions. Results indicate that genotoxicity is present in a group of children compared to the other one, and highlight the importance of the micronucleus assay in buccal mucosa cells for genetic biomonitoring and public health surveillance. This assay is capable of detecting a level of damage that can be reversible.

Key words: *micronuclei, genotoxic induction, buccal mucosa, pesticide exposure, monitoring.*

http://dx.doi.org/10.5546/aap.2015.eng.126

a. Genetics and Environmental Mutagenesis (GeMA) Research Group. Department of Natural Sciences. Universidad Nacional de Río Cuarto.

E-mail Address:
Delia Aiassa, M.D.: daiassa@exa.unrc.edu.ar; delia.aiassa@gmail.com

Funding:
This study was financed with a grant from the Department of Science and Technology of Universidad Nacional de Río Cuarto. Resolution Rec. 852/11. Project: "Assessment of the level of damage to the genetic material of humans exposed to agrochemicals and its implications on education and legislation."

Conflict of Interest:
None.

Received: 05-19-2014
Accepted: 11-10-2014

INTRODUCTION

Genotoxicity monitoring in humans is a useful tool to estimate the genetic risk of exposure to a compound or complex chemical mixtures[1] and is an early warning system for genetic conditions and/or cancer.[2] It identifies risk factors so that control measures can be implemented.[3]

Scientific research on human genotoxicity monitoring was first published around 1985, and has undergone an exponential increase to date. Between 1980 and 2000, monitoring assays on human populations exposed to chemicals focused mainly on cytogenetic testing, such as chromosome aberration (CA), micronuclei (MN) and sister-chromatid exchange (SCE) assays; all these tests are done on blood.[4]

In this regard, micronucleus frequency in exfoliated buccal mucosa cells is a minimally invasive and very useful method to monitor genetic damage in humans.[5] The Human Micronucleus Project has launched an international validation process for the micronucleus assay in buccal mucosa cells, similar to the process used previously with human lymphocyte tests.[6]

Between 2000 and 2008, the micronucleus assay in buccal mucosa cells was first used in populations exposed to pesticides in Brazil, Poland, Mexico, Spain, Hungary, Costa Rica and other European populations,[7,8] mobile phone users,[9] smoker students in Bolivia,[10] and individuals exposed to different mutagenic agents.[11]

This assay was also used by Peñaloza and Jaraba[12] in children with dietary deficiencies, and by Benítez-Leite, et al.,[13] Unal, et al.,[14] Minicucci, et al.,[15] Holland, et al.[6] and Gómez-Arroyo, et al.[16] in children exposed to pesticide mixtures.

In Argentina, results on genotoxicity monitoring in adults exposed to pesticides through inhalation and/or dermal absorption have been reported by Larripa, et al.[17] and Dulout, et al.,[18] in the province of Buenos Aires, and by Simoniello, et al.,[19] in the province of Santa Fe. Among others, Aiassa, et al.,[8,23] Mañas,

et al.,[20-22], Peralta et al.,[24] and Gentile, et al.[25] have reported results obtained on exposed individuals. These studies used the CA, MN and comet assays in peripheral blood.

The proximity between houses and pesticide-treated agricultural fields has been suggested as a factor that is closely related to pesticide environmental exposure.[26] There are no reports on MN frequency in the buccal mucosa of children environmentally exposed to pesticides (through inhalation).

Therefore, the micronucleus frequency in exfoliated buccal mucosa cells in children from urban areas with environmental exposure (through inhalation) was studied and compared with the micronucleus frequency in children from urban regions far from areas subjected to spraying, i.e., without environmental exposure to pesticides (through inhalation).

POPULATION AND METHODS

Design: observational, cross-sectional and analytical.

Population

Group 1: healthy children aged 4 to 14 years old who have lived in Marcos Juárez for 4 or more years. In turn, this group was divided based on the limits established by Article 59 of Law 9164 of the province of Córdoba: individuals living less than 500 meters from areas where pesticides are sprayed and individuals living more than 500 meters and up to the geographic town borders.

Marcos Juárez is located in the East of the province of Córdoba. It has 27 004 inhabitants (INDEC, 2010). The town is surrounded by crops.

Group 2: healthy children aged 4 to 14 years old who have lived in Río Cuarto for 4 or more years, far from areas where pesticides are sprayed (≥3000 m) and whose lifestyles were similar to those in group 1.

Río Cuarto is located in the South of the province of Córdoba. It has 155 911 inhabitants (INDEC, 2010).

The sample size was established based on Preston and Hoffmann,[27] 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."

Samples were obtained at two different time points: one in March/April 2012, the end of the continuous spraying season (which lasts between 4 and 6 months), and the other in August/September 2012, previous to the continuous spraying season (no spraying for at least 5 months).

The assay was repeated in 19 children living in Marcos Juárez who were randomly selected from the first sample.

The study protocol was approved by the Health Research Ethics Committees (*Comités de Ética de Investigación en Salud*, CIEIS) of Universidad Nacional de Río Cuarto and the Ministry of Health of the Province of Córdoba. It includes basic information on the study, informed consent and assent forms.

The study was disseminated by the research group through briefings open to the community. Only children and parents who agreed to voluntarily participate in the study were included.

Children whose parents or relatives were smokers, consumed coffee or tea in excess, used chronic medications, have been exposed to X-rays in the past 6 months, have been exposed to contaminants in drinking water or to any other environmental pollutants close to their place of residence[28] were excluded because all these factors are considered potentially confounding for results.

Both populations were considered exposed to pesticide degradation residues through food.

Before collecting buccal mucosa cells, an environmental-medical record was taken, which included demographic data, type of pesticide exposure, persistent conditions and symptoms, and lifestyle.

The micronucleus assay was performed on buccal mucosa cells collected using sterile swabs scraped inside the cheeks for 30 seconds, upon rinsing the mouth with drinking water in order to eliminate any food residue. Smears were performed as per the modified method described by Tolbert, et al.[29]

Observations corresponded to 1000 cells per individual. Inclusion criteria for micronucleus consideration were those suggested by Budak, Diler and Ergene.[11]

Statistical analysis: data in the medical-environmental record were analyzed using descriptive statistics (frequency and percentages).

The Kolmogorov-Smirnov test was used to establish the normal distribution of micronucleus data, followed by Student's t test ($p < 0.05$), using the GraphPad Prism software, version 5.02.

RESULTS

The mean age of participating children was 9.06 ± 0.39 in group 1, and 9.92 ± 0.54 in group 2.

The total sample size was 75 children (n= 75), 31 boys and 44 girls. Group 1 consisted of 27 children who lived less than 500 meters from areas where pesticides were sprayed (166.7 ± 11.62 m) and 23 children who lived more than 500 meters from such areas (1095 ± 146.4 m); group 2 included 25 children who lived more than 1500 meters from these areas (3320 ± 192.7 m). None of the children refused to participate.

The following data was obtained based on medical-environmental records:

- The most commonly used pesticides in the region are liquid or granular glyphosate and liquid formulations of cypermethrin and chlorpyrifos.
- Of all exposed children, 20 (40%) had varying persistent symptoms: 9 had respiratory symptoms (repetitive sneezing, respiratory distress, cough and/or bronchospasm); 9 had respiratory symptoms associated with skin itching or stains, and nose itching or bleeding; and 2 had respiratory symptoms associated with lacrimation, and eye and ear burning or itching. None of the participants who had not been exposed described any persistent symptoms.
- The duration of such symptoms ranges from six months to the entire year.
- The most recent three reports on drinking water microbiological, physical and chemical analysis, requested by the municipality to the official agency (18 months prior to sampling), indicate that bacterial count and tests for ammonia, arsenic, chloride, calcium carbonate, fluoride, nitrate, nitrite, sulfate and bicarbonate comply with the specifications of the Argentine Food Code and Resolution 608/93 issued by the Provincial Department of Water and Sanitation.

Table 1 shows mean micronucleus values and standard error in the studied population. *Figure 1* shows buccal mucosa cells with micronuclei.

The micronucleus frequency in children with symptoms of chronic pesticide exposure is 5.35 ± 0.97 (n= 20), while it is 5.13 ± 0.73 (n= 30) in those without symptoms; therefore, there are no statistically significant differences between both groups.

The mean micronucleus frequency per 1000 buccal mucosa cells was higher in children from Marcos Juárez than in those from Río Cuarto. Mean micronuclei found per 1000 cells were 5.20 ± 0.58 in the Marcos Juárez sample, and 3.36 ± 0.63 in the Río Cuarto sample.

The analysis comparing mean micronucleus frequency between participants from the first and the second samples (March/April 2012 and August/September 2012, respectively) indicated a mean micronucleus frequency of 5.78 ± 1.27 micronuclei per 1000 cells in children living less than 500 meters from areas where spraying was used and sampled in the first period and of 16.67 ± 2.37 micronuclei per 1000 cells in children included in the second sample. This is a highly significant difference (p= 0.0009), with a greater frequency observed in the second sample when compared to the first one.

A comparison of exposed children who live more than 500 m from areas where pesticides are sprayed between the first and the second sample also shows highly significant differences (p= 0.0006), with greater frequencies in the second period. The mean value for children in the first sample was 3.60 ± 0.99 micronuclei per 1000 cells, while in the second sample it was 19.70 ± 3.75 micronuclei per 1000 cells.

DISCUSSION

Symptoms observed in exposed children are mainly respiratory (repetitive sneezing, respiratory distress, cough and/or bronchospasm), skin itching or stains, nose itching or bleeding, lacrimation, and eye and ear burning or itching. Forty percent of children exposed have some sort of persistent condition

TABLE 1. *Mean micronucleus frequency per 1000 buccal mucosa cells in studied groups*

Group and place of residence	Distance from areas where spraying was used	n	Distance from place of residence (mean ± SD)	MN/1000 cells
Group 1 Marcos Juárez	Up to 500 m	27	166.7 ± 11.62	5.59 ± 0.75*
	More than 500 m	23	1095 ± 146.4	4.74 ± 0.91
Group 2 Río Cuarto	More than 3000 m	25	3320 ± 192.7	3.36 ± 0.63*

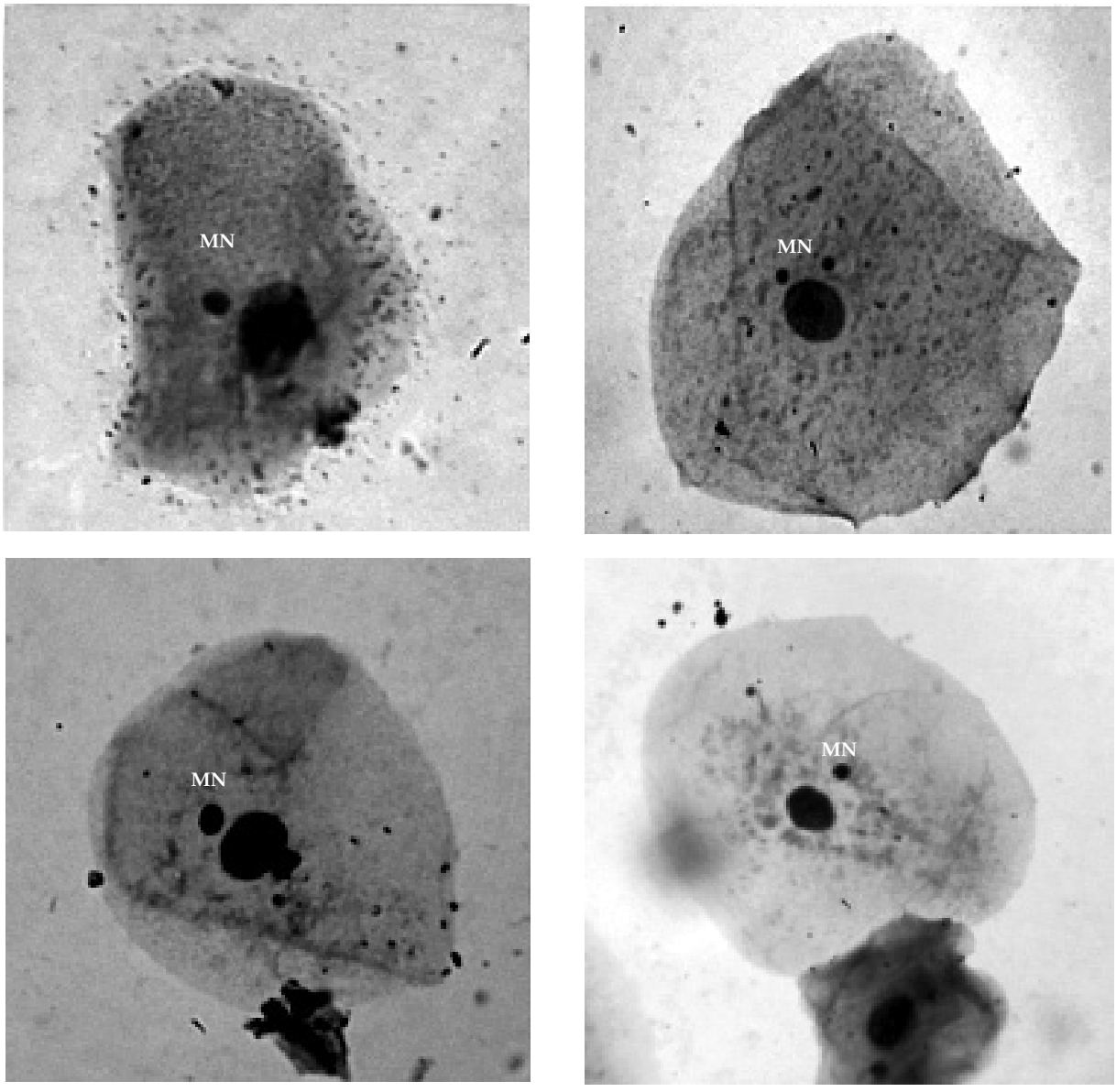
* Statistically significant compared to group 2.
MN: micronuclei.

which may be associated with chronic pesticide exposure.[30-33] In 1993, the International Labour Organization (ILO) proposed that symptoms associated with pesticide exposure may take the form of malaise, weakness, skin irritation, lacrimation, eye burning or itching, nausea, vomiting, abdominal pain, allergy, diarrhea, headache, and mood alterations, among others.

Jointly, the micronucleus frequency observed in Marcos Juárez (group 1), in relation to the distance of houses from areas where spraying was used (less than 500 m and 500-1500 m), showed no significant differences between both sub-groups. Since this is a relatively small town, such result evidences that sprayed pesticides may reach the entire town through the air and that the vulnerable children population is subjected to an extremely high and continuous pesticide exposure because they live in an area surrounded by crops.

Considering that there are no differences between children in the study groups regarding the distance from spraying areas up to 1095 m, this information should be considered when

FIGURE 1. *Micronuclei in buccal mucosa cells*



MN: micronuclei.

establishing environmental protection measures in any town surrounded by crops where pesticides are sprayed.

Micronucleus frequency in the group of exposed children (less than 500 m from spraying areas) is significantly higher ($p < 0.05$) than the one observed in the group of children who live more than 1500 m from such areas. These data are consistent with the available literature.

As in this study, Gómez-Arroyo, et al. [16] made the same association when studying genetic damage in children from El Porvenir, Ahome, Sinaloa. Their results suggest that exposure to pesticide mixtures is possibly the cause of significant differences observed in terms of micronucleus frequency in this population. In addition, Benítez-Leite, et al.[13] analyzed two populations from Paraguay (San Lorenzo and Ñemby) and found a significantly higher micronucleus frequency in the group of potentially exposed children versus non-exposed children. Similar studies were conducted by Unal, et al.[14] Minicucci, et al.[15] and Holland, et al.[6] which showed increased genetic damage in children exposed to pesticide mixtures.

When comparing values observed for the group who lives 500-1500 m from areas where pesticides are sprayed and the group who lives more than 1500 m from such areas, no statistically significant differences were observed; however, there was a remarkable increase in the micronucleus frequency of exposed children (4.74 in Marcos Juárez versus 3.36 in Río Cuarto), which may indicate a greater genetic damage in children from Marcos Juárez: 44% higher than in children from Río Cuarto. Likewise, the comparison of mean micronucleus frequency between children from Marcos Juárez and from Río Cuarto indicates that it is 58% higher (5.2 for Marcos Juárez versus 3.36 for Río Cuarto), suggesting that the genetic damage in children from Marcos Juárez is greater.

Few studies have assessed the association between agricultural pesticides used near population houses and pediatric diseases. Reynods, et al.[34-36] and Rull, et al.[37] demonstrated a relationship between childhood leukemia and the use of pesticides near living areas.

The analysis of results obtained for the first and the second samples (March/April 2012 versus August/September 2012) showed a highly significant difference ($p = 0.0009$ and $p = 0.0006$) for both the group who lives less than 500 m and the one who lives 500-1500 m from spraying areas, respectively, with a higher frequency in the second sample when compared to the first one. Spraying ends in March/April; therefore, the second sample (August/September) was collected following a period of approximately 5 months of low exposure, i.e. of less contact with pesticides, which should translate into a decreased micronucleus frequency. However, results show an increased micronucleus frequency for the second sample. Such increase might be explained by the burning of waste dumps during April, June and July of 2012, which affected all of Marcos Juárez. Such burnings were more intense on July 10th, 19th, 21st and 29th. In addition, it rained following the burnings, on August 6th (personal communication from Doctor Méndez, Environmental Primary Care Facility, Marcos Juárez). As a result, all town inhabitants were exposed to several pollutant gases, such as dioxin, carbon dioxide, carbon monoxide, sulfur dioxide, among others, not less than 4-5 days before samples were collected. Given that buccal mucosa cells are regenerated every 7-21 days, it is theoretically possible that genotoxic effects of acute exposure are observed approximately 7-21 days later, which may account for the elevated increase in mean micronucleus frequency observed in the second sample. Such findings evidence the importance of micronucleus assay in buccal mucosa cells for genetic biomonitoring and public health surveillance.

These results indicate that one of the groups of children is actually exposed to genotoxic agents when compared to the other. The level of damage detected by this marker is one that can be reversible. Therefore, effect markers should be used for follow-up to establish whether biological markers of cell damage continue to be present.

The importance of detecting an early genetic damage through the micronucleus assay lays in that it allows to take the necessary measures to reduce or eliminate exposure to damaging agents when still reversible, thus preventing and reducing the risk of malignancies and other pathological alterations.

The health status of a society can be judged based on its children's health. This implies an early identification of preventable risks and the immediate translation of such knowledge into effective interventions and protection policies. ■

REFERENCES

1. Bolognesi C. Genotoxicity of pesticides: a review of human biomonitoring studies. *Mutat Res* 2003;543(3):251-72.
2. Sailaja N, Chandrasekhar M, Rekhadevi PV, Mahboob M, et al. Genotoxic evaluation of workers employed in

pesticide production. *Mutat Res* 2006;609(1):74-80.
3. Kassie F, Parzefall W, Knasmüller S. Single cell gel electrophoresis assay: a new technique for human biomonitoring studies. *Mutat Res* 2000;463(1):13-31.
4. Albertini RJ, Anderson D, Douglas GR, Hagmar L, et al. IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. International Programme on Chemical Safety. *Mutat Res* 2000;463(2):111-72.
5. Torres-Bugarín O, Ramos-Ibarra ML. Utilidad de la prueba de micronúcleos y anormalidades nucleares en células exfoliadas de mucosa oral en la evaluación de daño genotóxico y citotóxico. *Int J Morphol* 2013;31(2):650-7.
6. Holland N, Bolognesi C, Kirsch-Volders M, Bonassi S, et al. The micronucleus assay in human buccal cells as a tool for biomonitoring DNA damage: The HUMN project perspective on current status and knowledge gaps. *Mutat Res* 2008;659(1-2):93-108.
7. Ceppi M, Biasotti B, Fenech M, Bonassi S. Human population studies with the exfoliated buccal micronucleus assay: statistical and epidemiological issues. *Mutat Res* 2010;705(1):11-9.
8. Aiassa D, Mañas F, Bosch B, Gentile N, et al. Biomarcadores de daño genético en poblaciones humanas expuestas a plaguicidas. *Acta Biol Colomb* 2012;17(3):485-510.
9. Hintzsche H, Stopper H. Micronucleus frequency in buccal mucosa cells of mobile phone users. *Toxicol Lett* 2010;193(1):124-30.
10. Ayarde Romero B, Cuti M, Ascarrunz González ME, Tirado Bustillos N. Efecto genotóxico del consumo de tabaco en estudiantes de la Facultad de Medicina de la UMSA que habitan en la altura. *Biofarbo* 2008;16:67-71.
11. Diler SB, Ergene S. Nuclear anomalies in the buccal cells of calcite factory workers. *Genet Mol Biol* 2010;33(2):374-8.
12. Peñaloza M, Jaraba V. Determinación del daño genético por desnutrición en niños de los centros educativos de la periferia de Pamplona, usando el ensayo de micronúcleos en células epiteliales de la mucosa bucal. *Iatreia* 2011;23(4-S):S36.
13. Benítez-Leite S, Macchi ML, Fernández V, Franco D, et al. Daño celular en una población infantil potencialmente expuesta a pesticidas. *Pediatr (Asunción)* 2010;37(2):97-106.
14. Unal M, Celik A, Ates NA, Micozkadioglu D, et al. Cytogenetic biomonitoring in children with chronictonsillitis: micronucleus frequency in exfoliated buccal epithelium cells. *Int J Pediatr Otorhinolaryngol* 2005;69(11):1483-8.
15. Minicucci EM, Ribeiro DA, de Camargo B, Costa MC, et al. DNA damage in lymphocytes and buccal mucosa cells of children with malignant tumours undergoing chemotherapy. *Clin Exp Med* 2008;8(2):79-85.
16. Gómez-Arroyo S, Martínez-Valenzuela C, Calvo-González S, Villalobos-Pietrini R, et al. Assessing the genotoxic risk for mexican children who are in residential proximity to agricultural areas with intense aerial pesticide applications. *Rev Int Contam Ambient* 2013;29(3):217-25.
17. Larripa I, Matos E, Vinuesa ML, Salum SB. Sister chromatid exchanges in a human population accidentally exposed to an organophosphorus pesticide. *Rev Bras Genet* 1983;6(4):719-27.
18. Dulout FN, Grillo CA, Seoane AI, Maderna CR, et al. Chromosomal aberrations in peripheral blood lymphocytes from Andean women and children from northwestern Argentina exposed to arsenic in drinking water. *Mutat Res* 1996;370(3-4):151-8.
19. Simoniello MF, Kleinsorge EC, Carballo MA. Evaluación bioquímica de trabajadores rurales expuestos a pesticidas. *Medicina (B Aires)* 2010;70(6):489-98.
20. Mañas F, Peralta L, Gorla N, Bosch B, et al. Aberraciones cromosómicas en trabajadores rurales de la Provincia de Córdoba expuestos a plaguicidas. *BAG J Basic Appl Genet* 2009;20(1):9-13.
21. Mañas F, Peralta L, Raviolo J, García Ovando H, et al. Genotoxicity and oxidative stress of glyphosate: in vivo and in vitro testing. *Environ Toxicol Pharmacol* 2009;28:37-41.
22. Mañas F, Peralta L, Raviolo J, García Ovando H, et al. Genotoxicity of AMPA, the environmental metabolite of glyphosate, assessed by the Comet assay and cytogenetic tests. *Ecotoxicol Environ Saf* 2009;72(3):834-7.
23. Aiassa D, Mañas F, Bosch B, Peralta L, et al. Los plaguicidas. Su relación con la salud humana y ambiental en la Provincia de Córdoba. *Exp Med* 2010;28(1):39-44.
24. Peralta L, Mañas F, Gentile N, Bosch B, et al. Evaluación del daño genético en pobladores de Marcos Juárez expuestos a plaguicidas: estudio de un caso en Córdoba, Argentina. *DiáLogos* 2011;2(1):7-26.
25. Gentile N, Mañas F, Bosch B, Peralta L, et al. Micronucleus assay as a biomarker of genotoxicity in the occupational exposure to agrochemicals in rural workers. *Bull Environ Contam Toxicol* 2012;88(6):816-22.
26. Fenske RA, Kissel JC, Lu C, Kalman DA, et al. Biologically based pesticide dose estimates for children in an agricultural community. *Environ Health Perspect* 2000;108(6):515-20.
27. Preston RJ, Hoffmann GR. Genetic toxicology. En Klaassen CD, ed. *Cassaret & Doull´s Toxicology: The Basic Science of Poisons*. 7th ed. Nueva York: Mc Graw Hill; 2008. Págs. 381-413.
28. Holland N, Fucic A, Merlo DF, Sram R, et al. Micronuclei in neonates and children: effects of environmental, genetic, demographic and disease variables. *Mutagenesis* 2011;26(1):51-6.
29. Tolbert PE, Shy CM, Allen JW. Micronuclei and other nuclear anomalies in buccal smears: methods development. *Mutat Res* 1992;271(1):69-77.
30. Solomon G, Ogunseitan OA, Kirsch J. Pesticides and human health: a resource for health care professionals. Santa Mónica CA: Physicians for Social Responsibility; 2000. Available at: http://www.psr-la.org/files/pesticides_and_human_health.pdf. [Accessed on: November 10, 2014].
31. Miller RL, Chew GL, Bell CA, Biedermann SA, et al. Prenatal exposure, maternal sensitization, and sensitization in utero to indoor allergens in an inner-city cohort. *Am J Respir Crit Care Med* 2001;164(6):995-1001.
32. Salameh PR, Baldi I, Brochard P, Raherison C, et al. Respiratory symptoms in children and exposure to pesticides. *Eur Respir J* 2003;22(3):507-12.
33. Salam MT, Li YF, Langholz B, Gilliland FD. Early-life environmental risk factors for asthma: findings from the Children´s Health Study. *Environ Health Perspect* 2004;112(6):760-5.
34. Reynolds P, Von Behren J, Gunier RB, Goldberg DE, et al. Childhood cancer and agricultural pesticide use: an ecologic study in California. *Environ Health Perspect* 2002;110(3):319-24.
35. Reynolds P, Von Behren J, Gunier R, Goldberg DE, et al. Agricultural pesticides and lymphoproliferative childhood cancer in California. *Scand J Work Environ Health* 2005;31 Suppl 1:46-54.
36. Reynolds P, Von Behren J, Gunier RB, Goldberg DE, et al. Agricultural pesticide use and childhood cancer in California. *Epidemiology* 2005;16(1):93-100.
37. Rull RP, Gunier R, Von Behren J, Hertz A, et al. Residential proximity to agricultural pesticide applications and childhood acute lymphoblastic leukemia. *Environ Res* 2009;109(7):891-9.