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Cytogenetic, Hematological and Enzymes Levels Parameters in the Biomonitoring of Agricultural Workers Exposed To Pesticides in the State of Piauí, Brazil

Tatiana Vieira Souza Chaves[1], Fabrício Pires de Moura do Amaral[2*], Guilherme Castro Lima Silva do Amaral[2], Sarah Izabelly Alves Lemos[2], Aracelli de Sousa Leite[3], José Machado Moita Neto[4], Ana Amélia de Carvalho Melo Cavalcante[5],Samylla Miranda Monte[6], Manoel Odorico de Moraes[7], Maria Elisabete Amaral de Moraes[7]

[1]*Department of Health the State of Piauí – Board of Health Surveillance/Occupational Health Reference Center (DIVISA/CEREST), Teresina, Piauí, Brazil, and Federal University of Ceará (UFC), Fortaleza, Ceará, Brazil - Post Graduation Program in Pharmacology*
[2]*State University of Piauí (UESPI) - Laboratory of Molecular Biology and Biological Studies Slurs (LABMINBIO / UESPI), Teresina, Piauí, Brazil.*
[3]*Lutheran University of Brazil (ULBRA), Canoas, Rio Grande do Sul - Post Graduation Program in Genetics and Applied Toxicology*
[4]*Federal University of Piauí (UFPI) - Post Graduation Program in Chemistry;*
[5]*Federal University of Piauí (UFPI) - Program of Post Graduation Studies in Pharmaceutical Sciences;*
[6]*Center for Research in Biotechnology and Biodiversity (NPBio) of the State University of Piauí (UFPI) Teresina, PI, Brazil.*
[7]*Federal University of Ceará (UFC), Fortaleza, Ceará, Brazil - Post Graduation Program in Pharmacology*

ABSTRACT: *Studies have demonstrated genotoxic effects by the presence of micronucleus in exfoliated cells from the buccal mucosa of agricultural workers exposed to pesticides. This study has assessed the genotoxic effects of pesticides on 61 agricultural workers from the state of Piauí, Brazil. 31 individuals were exposed to pesticides and 30 are from the same area, but were not involved in pesticides application. Cytogenetic damage were evaluated through micronucleus test in cells from the buccal mucosa and some parameters such as hematological and levels of enzymes. Exposed individuals exhibited cytogenetic damage with increased number of micronuclei in cells from the buccal mucosa in comparison with subjects from the control group with significant statistical difference ($P < 0.01$). We perceive that there is a statistically no significant ($P > 0.05$) increase in levels of plasmatic and eritrocytaireacetylcholinesterase and no statistically significant increase of phosphatase alkaline were detected in exposed workers in relation to the control group. No association was found in relation to smoking habits, alcohol consumption, protection utensils and the biomarkers analyzed or the biochemical analysis. Analysis of variance revealed a correlation between occupational exposure to pesticides of workers in Piauí and the presence of micronuclei ($P < 0.05$).*

Keywords: *Micronuclei, Pesticides, Farmers.*

I. Introduction

The use of pesticides in agriculture represents a threat not only to the environment but also to the human populations exposed to them. Pesticides (fungicides, insecticides, and herbicides) form the largest group of poisonous compounds that are intentionally scattered through out the environment for combating agricultural damage. Pesticides are toxic substances, at both genetic and the metabolic levels. Many of these compounds are classified as carcinogenic by the International Agency for Research on Cancer (IARC) [1]. Some studies have demonstrated that pesticides can represent potential risks to human health, such as the presence of different symptoms, which includes neuritis, psychiatric manifestations, electroencephalograph changes [2], as well as neurological, immune, metabolic, and endocrine problems [3]. Occupational exposure to pesticides has been associated with genetic damage [4], lymphoma and leukemia [5], and cancer in the absence of reparation processes [6].

DNA damage and chromosomal aberrations are the most important critical events following the exposure to genotoxic and/or carcinogenic agents. Human biological monitoring is a tool of great interest in cancer risks assessment once it allows estimating genetic risks deriving from environmental exposure to pesticides (complex mixture). Biomarkers such as micronucleus (MN) test provide information about DNA damage. The increase in the frequency of MN as an intermediate endpoint of carcinogenesis has received much support in the specialized

studies, but stronger evidence is available concerning the association between rate of structural chromosome aberration and cancer risks [7, 8]. Monitoring studies using somatic cells have been extensively conducted to evaluate the possible genotoxic and occupational risks. MN test is a very promising biomarker to the predictability of cancer [9]. MN are defined as small, round, cytoplasmic bodies, containing DNA, formed during cell division by loss of both acentric chromatin fragments and whole chromosomes. This chromatin loss can be induced by different mutagens *in vivo* and *in vitro*. Thus, MN test can detect both structural and numerical chromosome aberrations [10].

The buccal epithelium is composed of four strata, including the basal cell layer, pickle cell layer, and intermediate and superficial layers, in which the cells reproduce them in a system of continuos cell renewal. In this system new cells produced by mitosis in the basal layer migrate to the surface to replace those that are dead. Thus exfoliated cells from the buccal mucosa are in constant contact with the environment, what suggests that oral epithelium is an important target site for inhaled toxicants. Assessment of cytogenetic damage in exfoliated cells from the buccal mucosa by MN test is a sensitive approach to monitor the toxic effects of pollutants in humans [9, 11], as well as a useful biomarker of genetic damage [12].

Agricultural workers to Piauí were exposed to great number of pesticides include the fungicides, insecticides and herbicides, some of them classified as being carcinogenic by US Environmental Protection Agency (US-EPA) (Available at:www.pan-uK.org/pestnews/pn51p18.htm. [13]and International Agency for Research on Cancer (IARC) [1].

The goal of this study was to analyze the effects of genotoxics (cytogenetic damage) caused by occupational exposure to pesticides in agricultural workers from Piauí, Brasil, by the measurement of MN in exfoliated cells from the buccal mucosa, as well as to measure the biochemical parameters and enzymes levels aiming at the monitoring of occupational risks due to the use of pesticides.

II. Material And Methods

2.1 Population studied and sample collection

The population studied consisted of 31 male workers exposed to pesticides in the agricultural explorations of Piauí flat lands and of a control group, comprised of 30 male workers selected from agricultural workers with no history of contact with pesticides or any particular environmental agent in their occupation. Each individual consented to take part in the research and filled in a detailed questionnaire, according to International Commission for Protection against Environmental Mutagens and Carcinogens (ICPEMC) [14]. The standard questionnaire covered general questions (ethnic group, age, smoking habits, alcohol consumption), occupational information (working hours/day, years of exposure, and protective measures), as well as medical information (exposure to X-rays, medication). The ethic committee of he Universidade Federal doCeará (UFC), Fortaleza, Ceará, Brazil approved the study. Informed consent was obtained from each individual prior to the beginning of the study.

2.2 Hematological study

Freshly collected blood samples were analyzed for hematological assay, using an automatic analyzer Sysmex-KX21, from Japan. The different tested hematological parameters were as follow: hemoglobin (Hb), measurement of the total Hb g/dl of blood, hematocrit (HCT), measurement of red blood cells percentage (RBCs) in whole blood and total RBC count, total number of RBC per ml of blood.

2.3 Enzyme analysis

Levels of acetylcholinesterase (AChE) plasmatic and AChEeritrocytaire and alkaline phosphatase (ALP) were measured in red blood cells, according to Maroni *et al*. [15]. To minimize the variability, the same researcher carried out all the microscopic analysis.

2.4 Protein contents

The protein concentration was determined by biuret assay [16]. The intensity of the purple color produced was measured at 520 nm, compared with a standard of bovine serum albumin, and submitted to electrophoresis. The intensity of the color is proportional to the total protein concentration in the sample determined from the calibration curve.

2.5 Micronucleus test (exfoliated cells from the buccal mucosa)

The micronucleus test was carried out on exfoliated cells from the buccal mucosa of 61 workers, according to Salaija*et al*. [17]. Buccal cell samples were obtained by rubbing the inside of the cheeks of each studied subjects with a toothbrush. The cells were collected in sample bottles containing 20 ml of buffer solution (0.1 M EDTA, 0.01 Tris–HCl, and 0.02 M NaCl, pH 7) and transported to our institute for processing. After three washes in the buffer solution, by centrifugation at 4000 rpm for 10 min, 50 ml of cell suspension was dropped onto preheated

(55ºC) slides and allowed to air dry for 15 min on a slide warmer. The slides were fixed at 80% cold methanol for 30 min, air-dried overnight at room temperature and stored at ~20ºC until use. The slides were stained by May-Grunwald-Giesma. A total of 3000 cells/subject (i.e. 1000 from each of the duplicate slides) were scored blind, on coded slides, by one observer using an optical microscopy at a magnification of 1000x. The total number of micronuclei (MN) in buccal cells was determined for each studied subject. MN scoring followed prior established criteria. MN were identified according to the following criteria: spherical cytoplasmic inclusions with a defined contour, diameter smaller than approximately 1/3 of the main nucleus, color and texture similar to the nucleus, and absence of contact with the nucleus. Cells with more than four MN were discarded to exclude apoptotic phenomena.

2.6 Statistical analysis

Data were analyzed using the SPSS package for Windows (Version 13). Values are expressed as mean± standard deviation (S.D.). The mean values of each group were compared through the use of One-way analysis of Variance (ANOVA), followed by Dunnett's Multiple Comparisons test. P-values of less than 0.05 were considered significant. Statistical differences between the control group and workers exposed to pesticides were considered significant at $P< 0.01$ or $P< 0.05$ by Student's T-test.

III. Results

The effects of occupational exposure to pesticides on the level of genetic damage of those workers who suffered the exposure to pesticides in Piauí and those individuals from the control group were assessed by micronucleus frequency in exfoliated cells from the buccal mucosa. In this biomonitoring study, we investigated whether an occupational exposure to a complex mixture of chemical pesticides produced a significant increase of micronuclei (MN) in cells from the buccal mucosa. Table 01 shows the main characteristics of the population studied.

Table 01: Characteristics of the studied population.

	Control group	**Exposed group**
Number of subjects	30	31
Age (years)[a]	38 ± 9.88	35 ± 12,9
Range (years)	22 - 55	20 - 61
Ethnic group Mixed race	 74.5%	 70 %
Smoking habits Smokers Non smokers	 37.5% 62.5%	 53.2% 46.8%
Alcohol drinkers	56.5%	35.3%
Protective measures	30 (100 %)	48,4%
Working hours/week	30 (100%)	29 (61.2 %)
[a]Mean ± S.D		

As shown in Table 01, the number of studied individuals in the exposed group (n = 31) and in the control group (n = 30).Agricultural workers included in this study were also exposed to a great number of different pesticides (Table 02) some of them classified as being carcinogenic by US Environmental Protection Agency (US-EPA) [13].

Results of this study indicated that occupational exposure to pesticides induced a significant increase in the level of genotoxicity on agricultural workers from Piauí. The workers exposed to pesticides revealed a significant ($P < 0.01$) induction of MN, when compared with those from the control group (0.4 versus 6.04, for those exposed for one year (Table 03), and 0.4 versus 7.04, for those exposed for ten years). Nevertheless, it is important to note that no significant ($P > 0.01$) increase is observed for MN frequency in relation to the exposed groups (for one or ten years).

Table 02: The most used pesticides by exposed group

Pesticides	**Compound**	**Chemicalclass**	**US EPA classification[a]**	**WHO clasification[b]**
Fungicides	Benomyl	BenzimidazoleThiophthalimide	Possible Human Carcinogen	Not available
	Captan	BenzimidazoleDithiocarbamate	Carcinogenicity in animal	C
	Carbendazim	Dithiocarbamate	Possible Human Carcinogen	C/M
	Mancozeb		Carcinogenicity in animal	M
	Mancozeb-metalaxyl M		Carcinogenicity in animal	M
Insecticides	Buprofezin	Triazine	-	C
	Butocarboxim	N-Methyl CarbamatePyretroid	-	-
	Deltamethrin	OrganophosphorusOrganochlori	Not likely	-
	Dimethoate	neOrganophosphorus	Possible Human Carcinogen	-
	Endosulfan	Chloro-nicotinyl	Not likely	M
	Fosmethilan		-	-

	Imidacloprid		Non-carcinogenicity to humans	M
Herbicides	Glyphosate Linuron	Phosphonoglycine Urea	Non-carcinogenicity to humans Possible Human Carcinogen	- C
[a]US Environmental Protection Agency (13). [b] World Health Organization. (29): Mutagenicity (M) and carcinogenitity (C) experimental data.				

Table 03: Effects of time of exposure on MN of buccal mucosa of agricultural workers from Piauí exposed to pesticides.

	N	**MN/3.000 cells**	**Micronucleus frequency (%)**
Control group	30	0.4 ± 0.8 (0-2)	0.0 ± 0.0
Exposed group	31	6.35 ± 3.7** (1-13)	0.21 ± 0.12
Exposed group for one year	21	6.04 ± 3.7** (1-10)	0.20 ± 0.12
Exposed group for ten years	10	7. 04 ± 4.3** (1-13)	0.23 ± 0.14
Means ± S.D. (*n* = number of individuals). Significantly different from control group (***P < 0.01*), according to Student's T-Test.			

No significant, statistical increase in number of MN was observed in those exposed to pesticides in relation to those from the control group, regarding the confounding factors: smoking, alcohol consumption, protective measures (data not shown). However, in relation to the time of exposure (one or ten years) a statistically significant (P < 0.05) increase in number of MN was observed in those who smoked and/or consumed alcohol, and were exposed for more than ten years (Table 04).

Table 04: Smokers, X-rays exposure, alcohol consumers and protective measures on MN in the population exposed to pesticides.

Parameters	Exposure 1 year			Exposure 10 years		
	n	MN/3.000 cells	Micronucleus frequency (%)	*n*	MN/3.000 cells	Micronucleus frequency (%)
Smoking	15	6.36 ± 4.07	0.20 ± 0.12	5	9.40 ± 4.00*	0.31 ± 0.13
No smoking	6	6.35 ± 3.57	0.20 ± 0.13	5	5.00 ± 3.80	0.16 ± 0.12
X-rays	12	6.33 ± 4.06	0.21 ± 0.13	2	5.00 ± 1.40	0.16 ± 0.04
No exposure to X-rays	9	5.86 ± 3.60	0.19 ± 0.12	8	7.75 ± 4.70	0.25 ± 0.15
Protective measures	10	6.36 ± 3.70	0.21 ± 0.12	5	7.60 ± 4.10	0.25 ± 0.13
No protective measures	11	6.18 ± 3.76	0.20 ± 0.12	5	6.80 ± 5.00	0.22 ± 0.16
Alcohol consumers	15	6.27 ± 3.37	0.20 ± 0.11	4	8.50 ± 4.79*	0.28 ± 0.15
Not alcohol consumers	6	5.63 ± 4.60	0.18 ± 0.15	6	6.33 ± 4.30	0.21 ± 0.14

Means ± S.D. (*n* = number of individuals). Significant differences were observed in workers exposed to ten years regarding drinking and smoking group* (*P < 0.05*), according to Student's T - Test.

Although not statistically significant, an increase of Biochemical study in exposed population in relation the date observed in negative control (Table 04). In this work, we found no significant (*P > 0.05*) increases in almost all biomarkers of haematological study in workers exposed (Table 05), enzyme analysis AChEs (Figure 1 and 2) and alkaline phosphatase (Figure 3) and total protein in comparisons the control group and maxim and minim normal reference, except by AChE plasmatic in 13, 22 and 23 individuals (Figure 1) and 7, 10, 12 e 13 individuals by AChEeritrocytaire (Figure 2) and 10 individual by alkaline phosphatase in comparison with minimal normal reference (*P < 0.05*). No association was found in relation to biochemical study, enzyme dosage, and total protein and frequency of micronuclei (P>0.05) in the groups exposed to pesticides.

Table 05: Biochemical study of the groups exposed to pesticides.

Biochemical Analysis	N[3]	Normal reference	Valor determined[4]	Men ± DV
Ureia[1]	28	15 a 40 mg/dl	19 a 46 mg/dl	34.40 ± 6.82
Ureia[2]	17	15 a 40 mg/dl	25 a 43 mg/dl	34.11 ± 4.96
Creatinin[1]	27	0.4 a 1.3 mg/gl	0.4 a 1.3 mg/gl	0.80 ± 0.27
Creatinin[2]	17	0.4 a 1.3 mg/gl	0.6 a 1.4 mg/gl	1.01 ± 0.23
TGO[1]	27	4.0 a 36 mg/dl	10 a 37 mg/dl	20.84 ± 6.45
TGO[2]	17	4.0 a 36 mg/dl	8 a 38 mg/dl	22.14 ± 7.92
TGP[1]	27	4.0 a 32 mg/dl	8 a 35. mg/dl	19.20 ± 6.57
TGP[2]	18	4.0 a 32 mg/dl	8 a 43mg/dl	20.11 ± 8.62
Gamaglobulin gt[1]	28	7 a 47 U/L	11.3 a 115 UL	28.34 ± 20.61
Gamaglobulin gt[2]	17	7 a 47 U/L	10.5 a 74 UL	27.87± 14.76
Total protein [1]	28	6 a 8 g/dl	6.4 a 9.1 g/dl	18.21 ± 16.59

Total protein [2]	17	6 a 8 g/dl	6.6 a 8.4 g/dl	7.59 ± 0.41
Albumin[1]	27	3.5 a 5.5 g/dl	2.8 a 4.9 g/dl	3.6593± 0.45
Albumin [2]	17	3.5 a 5.5 g/dl	3.6 a 4.8 g/dl	3.97±0.29
Leucocytos[1]	17	5000 K/UL	6700 - 9500 K/UL	8035.2941±844.79
Hemoglobin[1]	28	11.8 G/DL	8.61 - 6.40 G/DL	13.53±1.67
Hematocrit [1]	28	36%	23.90 – 52.40 %	44.31±5.45
Plaquetas[1]	28	150.000 K/L	223000 – 549000 K/L	363785.71±91803.87
Plaquetas[2]	15	150.000 K/L	230000 – 568000 KL	402400.0000±105237.69
[1]Data obtained in first collection; [2]Data obtained in first collection; [3]Number of individuals; [4]Mean of all individuals.				

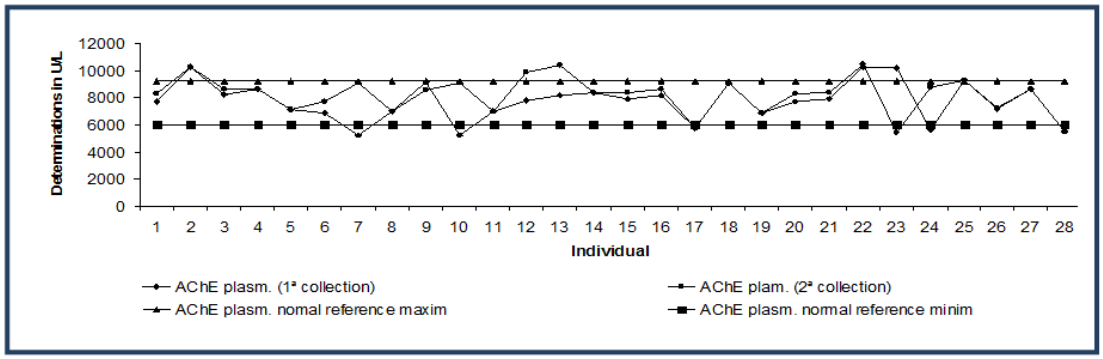

Fig. 1 Analysis of enzyme ACHE plasmatic in individuals from the exposed group.

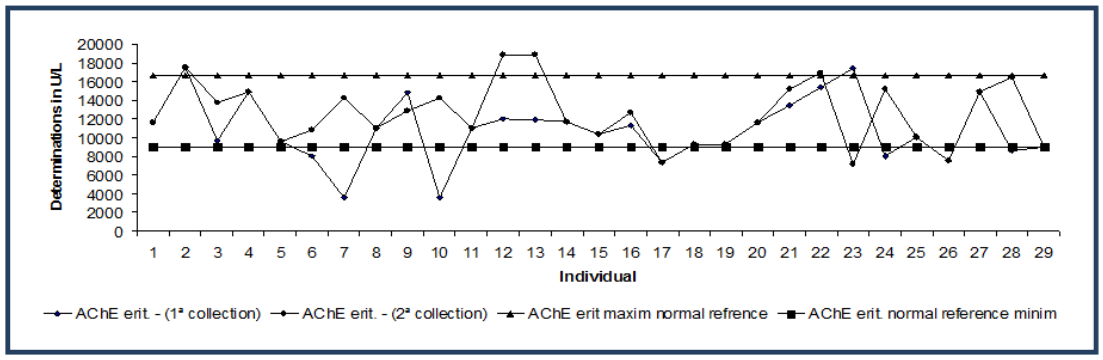

Fig. 2 Analysis of ACHE eritroplasmatic in individuals from exposed group.

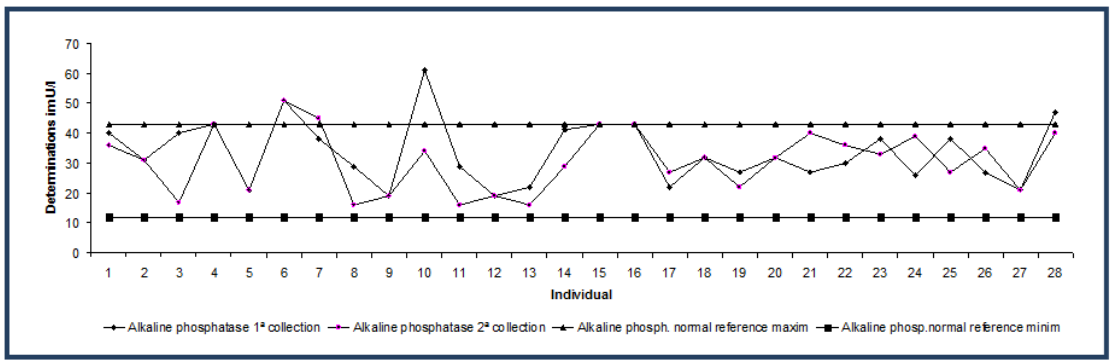

Fig. 3 Level of alkaline phosphatase in individuals from the exposed group.

IV. Discussion

In the present study, we evaluate whether or not occupational exposure to a complex mixture of pesticides results in a significant increase of micronuclei in buccal cells. Many of these compounds are capable of inducing mutationsin DNA, leading to several diseases including cancer. Individuals occupationally exposed to pesticides who are in direct contact with these chemicals may provide a good opportunity to study their adverse health consequences. Widespread use of pesticides in agriculture represents a threatnot only to the environment but also to the human populations exposedto them [18, 19].

A monitoring study to evaluate DNA damage in exfoliated cells from the buccal mucosa of a group of male workers exposed to pesticides by using biomarkers of genotoxicity is necessary in order to prevent cancer

development. The main objective of this study was to detect whether the exposure to pesticides induces an increase in the levels of cytogenetic damage in the cells from the buccal mucosa of agricultural workers fromPiauí. The study was carried out simultaneously in workers exposed to pesticides and a control group. In this study, batteries of hematological study and enzyme analysis were used to evaluate a complete range of toxic damage in agricultural workers from both the exposed groups and those from the control group (data not shown). In the present study, we wanted to assess whether prolonged exposure to complex mixtures of pesticides leads to an increase in cytogenetic damage (Table 03). In relation to exfoliated cells from the buccal mucosa, the population exposed to pesticides showed a higher MN frequency, attaining significant differences ($P < 0.01$). No statistical difference was observed in frequency of micronucleus in relation to haematological and enzyme analysis ($P > 0.05$).

Considering the time exposure, a significant increase ($P < 0.05$) in number of MN was measured only within the group with longer duration of exposure (10 years) in comparison with the control group (Table 03). An increase in the cytogenetic effects, such as MN increased frequency, might be caused because of individual genetic characteristics or cumulative damage of the genotoxic components induced by the complex mixture of pesticides. The majority of the studies on cytogenetic biomarkers in pesticide-exposed workers have indicated some dose-dependent effects, along with the increasing duration or intensity of exposure [19, 20, and 17] showed no correlation between various biomarkers in the evaluation of genotoxic damage in a group of workers exposure to petroleum. Chromosomal damage, as a result of inefficient or incorrect DNA repair, is expressed during the cell division and represents an index of accumulated genotoxic effects [21].

Results obtained from this study indicate that there was a significant ($P < 0.01$) increase in the MN of the group exposed to pesticides as compared to the control group. Increase in MN frequencies in the group of agricultural workers exposed to agrotoxics was well reported in lymphocytes or buccal cells [22]. Other workers have also confirmed the induction of cytogenetic damage after exposure to pesticides indicating lack of chromosomal damage related to pesticide exposure [17, 18].

While interpreting the results, smoking habits was kept in consideration to describe any possible interference of tobacco, but no correlation ($P > 0.05$) was found in relation to the increase of MN and habits (smoking/drinking), age, adoption of protective measures (Table 04), or in relation to the exposed workers (1 and 10 years) in relation to the control group. No association was found between tobacco smoking and evaluation of genotoxicity in a group of workers from a petroleum refinery aromatics plant [20]. The results obtained are also consistent with other studies which evaluated genetic damage in humans after exposure to anesthetics gases in Indian hospitals. Likewise, no association was found between years of exposure, smoking, age, gender, alcohol consumption and higher levels of genetic damage as assessed by the chromosomal aberration ($P > 0.05$) [17,19,23]. The influence of smoking in cytogenetic damage (MN) in relation to the time exposure (Table 03) was observed within the exposed group. The lack of association observed between MN frequencies and tobacco exposure was also observed by other authors [17, 22].

Duration of exposure (years of employment) has been positively correlated with cytogenetic damage. Apparently, clastogenic effects seem to be cumulative for continuous exposure to pesticide mixtures [19]. It indicated that the protection measures did not have obvious influence on the genotoxic effects of vincristine [10]. However, no good correlation between years of exposure and six parameters was observed ($P > 0.05$). Age did not show any positive correlation with the increase in MN. Such increase in MN can only be attributed to duration of exposure.

Increased number of MN in agricultural workers exposed to pesticides for 10 years was observed (Table 04). The clastogenic effects seem to be cumulative for continuous exposure to pesticide mixtures. People chronically exposed are more susceptible to the clastogenic action of pesticides [19]. Association between years of employment and MN frequency has been demonstrated in farmer populations as a result of a continuous exposure to a complex mixture of pesticides [24]. Duration of exposure (years of employment) has been positively correlated with cytogenetic damage. Apparently, clastogenic effects seem to be cumulative for continuous exposure to pesticide mixtures [19]. It indicated that the protection measure did not have obvious influence on the genotoxic effects of vincristine. The protection measures did not have obvious influence in the cytogenetic damage of workers exposed to pesticides. The similar situation appeared in our previous investigation of cytogenetic effect in workers occupationally exposed to vincristine [10].

Organophosphate pesticides are cholinesterase inhibitors and are extensively used in agriculture. Cholinesterase inhibition is an indirect indicator used to monitor organophosphates exposure [25]. They can also cause hepatotoxicity [26]. Studies on organochloride insecticides in animals and humans have detected hepatotoxicity at high and low doses, which might be linked to hepatic enzyme induction [15].

Acetylcholinesterase activity in blood can be biomarkers in environmental and occupational organophosphrous. Studies have demonstrated that the exposure to pesticides causes the decrease of 25% in the level of enzyme activity [27]. The inhibition of acetylcholinesterases by organophosphorous compounds is well known, resulting in the accumulation of endogenenous acetylcholine responsible for toxicity in the nervous system.

Measurements of acetylcholinesterase activity in red blood cells have routinely been performed to survey exposure to environmental organophosphates that caused the decrease in ACHE activity [9]. In this study, we discovered that there was not a good correlation (P>0.05) between MN and haematologics parameters and enzyme analysis in workers. However, the induction by pesticides of enzymes implicated in the activation of ubiquitous exogenous and endogenous genotoxic compounds that could increase their genotoxicity [27]. There are few papers indicating that occupational exposure to this ACHE inhibiting insecticide might be connected to increased risk of developing non-Hodgkin's lymphoma and lung cancer [28].

Pesticides constitute a heterogeneous category of chemicals specifically designed for the control of pests, weeds or plant diseases. Pesticides have been considered potential chemical mutagens: experimental data revealed that various agrochemical ingredients possess mutagenic properties inducing mutations, chromosomal alterations or DNA damage. Biological monitoring provides a useful tool to estimate the genetic risk deriving from an integrated exposure to a complex mixture of chemicals. Studies available in scientific literature have essentially focused on cytogenetic end-points to evaluate the potential genotoxicity of pesticides in occupationally exposed populations, including pesticide manufacturing workers, pesticide applicators, floriculturists and farm workers. A positive association between occupational exposure to complex pesticide mixtures and the presence of chromosomal aberrations (CA), sister-chromatid exchanges (SCE) and micronuclei (MN) has been detected in the majority of the studies, although a number of these failed to detect cytogenetic damage [19,30,31].

The results showed that the cytogenetic markers of workers exposed to pesticides significantly increased when compared with those of the control group. These cytogenetic changes might originate in the cumulative effect of the many chemical compounds that are present in the mixture of pesticides. However, the cause of this genetic damage cannot be attributed to only a single genotoxic agent due to the mixed constitution of pesticides. Biological monitoring of exposure to pesticides has a great importance to the evaluation of DNA damage in agricultural workers. The micronucleus test carried out in exfoliated cells from the buccal mucosa of agricultural workers provides a feasible approach to monitor the effects of occupational genotoxic agents in humans.

In conclusion, the increase in MN frequencies in agricultural workers from Piauí indicates that the genetic damage caused by exposure to pesticides suggests potential health implications. A comprehensive assessment of the real toxic and genotoxic potential for pesticides in agricultural workers from Piauí, Brazil is needed. The use of biomarkers establishes a connection between a certain exposure to pesticides contaminants and the increased risks to the health of individuals and populations. Biomarkers are used in the evaluation of environmental and occupational risks, as well as in the search of information about human health for future interventions.

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