Journal of Toxicology and Environmental Health, Part A
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/uteh20

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Carla Costa [a b], Susana Silva [a], Joana Neves [a], Patrícia Coelho [a b], Solange Costa [a b], Blanca Laffon [c], John Snawder [d] & João Paulo Teixeira [a b]

[a] National Institute of Health, Environmental Health Department, Porto, Portugal

[b] ICETA, Institute of Agricultural and Agri-foodstuffs Sciences and Technologies, University of Porto, Porto, Portugal

[c] Toxicology Unit, Department of Psychobiology, University of A Coruña, Edifício de Servicios Centrales de Investigación, A Coruña, Spain

[d] Biomonitoring and Health Assessment Branch, Division of Appplied Research and Technology, National Institute for Occupational Safety and Health, U.S. Centers for Disease Control and Prevention, Robert A. Taft Laboratories, Cincinnati, Ohio, USA

Available online: 27 Jun 2011

To cite this article: Carla Costa, Susana Silva, Joana Neves, Patrícia Coelho, Solange Costa, Blanca Laffon, John Snawder & João Paulo Teixeira (2011): Micronucleus Frequencies in Lymphocytes and Reticulocytes in a Pesticide-Exposed Population in Portugal, Journal of Toxicology and Environmental Health, Part A, 74:15-16, 960-970

To link to this article: http://dx.doi.org/10.1080/15287394.2011.582024

Journal of Toxicology and Environmental Health, Part A, 74:960–970, 2011

ISSN: 1528-7394 print / 1087-2620 online
DOI: 10.1080/15287394.2011.582024

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MICRONUCLEUS FREQUENCIES IN LYMPHOCYTES AND RETICULOCYTES IN A PESTICIDE-EXPOSED POPULATION IN PORTUGAL

Carla Costa[1,2], Susana Silva[1], Joana Neves[1], Patrícia Coelho[1,2], Solange Costa[1,2], Blanca Laffon[3], John Snawder[4], João Paulo Teixeira[1,2]

[1]National Institute of Health, Environmental Health Department, Porto, Portugal
[2]ICETA, Institute of Agricultural and Agri-foodstuffs Sciences and Technologies, University of Porto, Porto, Portugal
[3]Toxicology Unit, Department of Psychobiology, University of A Coruña, Edifício de Servicios Centrales de Investigación, A Coruña, Spain
[4]Biomonitoring and Health Assessment Branch, Division of Appplied Research and Technology, National Institute for Occupational Safety and Health, U.S. Centers for Disease Control and Prevention, Robert A. Taft Laboratories, Cincinnati, Ohio, USA

A wide range of chemical products known to be acutely toxic is currently used in the agricultural sector, including numerous pesticides with different compositions. Nevertheless, the effects in human health as result of chronic exposure to low levels are not yet completely understood. The methodology for determination of micronuclei (MN) in lymphocytes (CBMN) is well established, and accumulating data demonstrated a correlation to enhanced risk of cancer development. However, analysis of MN in reticulocytes (MN-RET) in humans is a recent tool on human biomonitoring. The aim of this study was to examine the influence of pesticide exposure on MN-RET and CBMN frequencies. In total, 177 individuals were studied (93 controls and 84 exposed). All individuals included in the exposed group were exposed regularly to various chemicals. Both MN-RET and CBMN were significantly higher in the exposed subjects compared to controls. The CBMN frequencies were quantitatively higher in females than males, especially within the exposed group. Smoking habits exerted no marked influence on the frequency of the biomarkers studied. A significant and positive correlation was found between both indicators. Within the exposed group, data showed that there was a significant correlation between MN-RET and recent exposure (exposure in the previous 10 d) that is not found when considering CBMN. It is conceivable that due to the short life span of reticulocytes, MN-RET were found to be more reliable to characterize recent genetic damage as opposed to CBMN.

Pesticides are chemicals used to eliminate certain species that in specific circumstances are considered a plague. Although they are mainly used in agriculture, these compounds are also be applied in household settings or to control disease vectors (public health) (EUROSTAT 2001). These compounds are known to exert adverse effects on human health and ecosystems (Infante-Rivard and Weichenthal 2007; Knopper and Lean 2004). The lack of target specificity that generally characterizes these chemicals is responsible for these potential negative effects (Keifer 2000). Pesticide persistence, along with continuous use throughout the year, makes this a matter of concern. Understanding all the possible effects

This investigation was supported by the Portuguese Foundation for Science and Technology (FCT) under grants SFRH/BD/37190/2007 and PTDC/SAU-ESA/102367/2008.
Address correspondence to Carla Costa, National Institute of Health, Environmental Health Department; Rua Alexandre Herculano, 321, 4000-055 Porto, Portugal. E-mail: cstcosta@gmail.com

of pesticides on human health at different levels of exposure and finding adequate biomarkers to characterize exposure effects are essential. Among other effects on human health (Ritter et al. 2006), genotoxic potential of pesticides has been widely studied and evidence accumulated throughout the years (Knopper and Lean 2004). Pesticide exposure was reported to increase DNA damage observable by number of micronuclei (MN), chromosomal aberrations (CA), sister chromatid exchange (SCE), and comet assay in human lymphocytes (Costa et al. 2008).

There is a significant amount of evidence that MN frequency in peripheral blood lymphocytes is predictive of cancer risk (Bonassi et al. 2007). Genetic damage expressed as MN in lymphocytes may originate from chromosome fragments or whole chromosome loss (Fenech 2006). Micronuclei may also be detected in other types of cells, such as buccal epithelial, nasal, and urothelial cells. However, data in different types of cells are not usually available as authors tend to choose a specific surrogate tissue.

As knowledge increases on the genotoxic potential of chemicals, new validated biomarkers are necessary to provide information on mechanisms and determinants variables. Detecting micronuclei in bone-marrow cells has long been an established technique as a routine screening test to characterize cytogenetic damage in experimental animals. Analysis of MN in erythrocytes was also possible as these animals do not remove micronucleated erythrocytes from circulation and therefore damaged cells accumulate in blood. Application of this assay to humans was limited due to spleen removal of MN erythrocytes. Schlegel et al. (1986) found it useful to characterize damage due to chemotherapy drugs treatment in splenectomized individuals. Subsequently, Dertinger at al. (2007) suggested that analysis of immature reticulocytes may constitute the basis for application of this method to human samples. In fact, by restricting analysis to CD71+ cells it was possible to characterize damage in non-splenectomized subjects. In addition, Offer et al. (2005) using this same indicator found increased damage in smokers compared to non-smokers.

Peripheral blood lymphocyte micronuclei (CBMN) have long been used to characterize genetic damage in pesticide exposed populations (Pastor et al. 2002a; 2002b; Costa et al. 2007). However, MN in reticulocytes (MN-RET) are a relatively recent endpoint not yet widely used to access DNA damage in humans. The aim of the present study was to characterize the potential genotoxic damage of pesticides (multiple exposures) by measuring MN-RET and CBMN frequencies. Influence of possible confounders and exposure determinants was also examined.

MATERIALS AND METHODS

Study Population and Sample Collection

The study group consisted of 84 pesticide-exposed farmers (42 males and 42 females) and 93 controls (39 males and 54 females), all volunteers. Exposed individuals were contacted through farmers associations that integrated farmers working within Oporto district, Portugal, occupationally exposed to pesticides. Four months of pesticide exposure was considered the cutoff point for inclusion in exposed group. The control group was comprised of individuals working in administrative offices in the same district and presenting demographics and lifestyle similar to the exposed group and with unknown exposure to possible mutagens. All individuals included in the study were Caucasians. All subjects were fully informed about the procedures and objectives of this study and each of them signed an informed consent prior to the study. Ethical approval for this study was obtained from the institutional ethical board of the Portuguese National Institute of Health.

Each subject completed a detailed questionnaire to determine confounding factors such as x-ray exposure and medication. Subjects in the exposed group also gave information concerning work practices such as usage of personal protective equipment (PPE)

TABLE 1. Characteristics of the Studied Groups

Group	Exposed	Control
Number of subjects	84	93
Age (yr)[a]	40 ± 12 (18–63)	39 ± 13 (18–68)
Gender		
Males	42 (50%)	39 (42%)
Females	42 (50%)	54 (58%)
Years of employment[a]	23.0 ± 16.1 (0.3–52)	
Smoking habits		
Smokers	4 (5%)	12 (13%)
Nonsmokers	80 (95%)	81 (87%)

[a]Mean ± SD (range).

and years of employment. Characteristics of the studied groups are presented in Table 1.

Not all the individuals included in the exposed group were pesticide applicators (55 pesticide applicators vs. 29 not pesticide applicators); nevertheless, all were exposed to these compounds either by preparing the mixtures for application, providing assistance during applications, or providing assistance during maintenance activities. All individuals included in the exposed group dealt regularly with a wide variety of chemicals and therefore it is considered a scenario of multiple exposures. Detailed information regarding the last contact with pesticides was collected. In regard to work environment, out of the 84 subjects included in the exposed group, only 5 worked exclusively in greenhouses; 13 worked exclusively in open fields and the remaining divided their time between both environments ($n = 66$). Blood samples were obtained by venipuncture into standard heparinized vacutainers (BD Vacutainer) and collected over an 11-mo period (April 2008–February 2009). Since this may be considered a bias factor as pesticide application is more frequent in spring and summer, this was considered in result analysis. The majority of exposed individuals were in contact with pesticides a few days before sample collection (a list of pesticide compounds reported by exposed groups is presented in Table 2). All samples were coded and analyzed under blind conditions. Blood cultures for cytogenetic analysis were conducted few hours after sampling.

MN-RET Analysis by Flow Cytometry

Chemicals and Other Reagents Heparin was obtained from B Braun (catalog number 3644502; 5000 UI/ml), methanol p.a. from Panreac (CAS number 67-56-1), and Hanks balanced salt solution (HBSS) from MediaTech, Inc. Propidium iodide (PI; CAS number 25535-16-4), phosphate-buffered saline (PBS) tablets, RNase A, and fetal bovine serum (FBS) were all purchased from Sigma Aldrich. Antibodies anti-CD71-FITC (clone YDJ1.2.2; FITC: fluorescein isothiocyanate), anti-CD61-PE (clone SZ21; PE: phycoerythrin), and anti-CD45-PC5 (clone J.33; PC5: phycoerythrine-cyanine 5) were all Beckman Coulter products. All the remaining reagents used for quality control, such as Flow Check, Flow Set, CytoComp Cells, and CytoComp reagent kit, were also obtained from Beckman Coulter and employed as described in instructions for use.

Sample Preparation Whole blood was diluted by adding 500 μl of blood to 2.5 ml heparin diluent solution (500 USP units heparin/ml PBS). Diluted whole blood (810 μl) was fixed in 9 ml methanol (previously in the freezer at −80°C), and samples were kept at −80°C until analysis. Transportation of samples from Portugal to the United States was held to 2 d to prevent large temperature variations.

Samples were prepared for analysis as described by Dertinger et al. (2003; 2007) with some modifications. Briefly, on the day of analysis, 2 ml fixed sample was washed with 12 ml HBSS. Samples were centrifuged at 800 × g for 4 min and supernatant was decanted. Cells were resuspended using a micropipette and kept on ice until addition of RNase/antibody solution. To each tube, the following were added: 100 μl RNase/antibody solution (solution contained 4 μl RNase 1mg/ml, 5 μl anti-CD61-PE and anti-CD45-PC5, 10 μl anti-CD71-FITC, and 80 μl HBSS). Samples were incubated for 30 min at 4°C and then 20 min at 37°C followed by 10 min at room temperature (during incubation period, samples were always kept in the dark). After incubation, samples were washed with 5 ml

TABLE 2. List of Pesticides Reported as Used by Exposed Subjects and Their Classification Regarding Carcinogenicity (U.S. EPA) and Acute Hazard (WHO)

Pesticide	Compound	Chemical class	U.S. EPA	WHO
Fungicides	Mancozeb	Dithiocarbamate	B2	U
	Azoxystrobin	Strobin	Not likely	U
	Folpet	Thiophthalimide	B2	U
	Propineb	Dithiocarbamate		U
	Cymoxanil	Unclassified	Inadq. data	III
	Mefenoxam	Xylylalanine	Not likely	
	Fosetyl-Al	Unclassified	Not likely	
	Tolylfluanid	Sulfonamides	Likely	U
	Benalaxyl	Xylylalanine		U
	Penconazole	Azole		U
	Carbendazim	Benzimidazole	C	U
	Propamocarb	Other carbamate	Not likely	U
	Fluazinam	2,6-Dinitroaniline	Suggestive	
	Fenhexamid	Anilide	Not likely	U
Insecticides	Chlorpyrifos	Organophosphorus	E	II
	Thiamethoxam	Unclassified	Not likely	
	Cyhalothrin, lambda	Pyrethroid	D	II
	Dimethoate	Organophosphorus	C	II
	Methiocarb	*N*-Methyl carbamate	D	IB
	Diazinon	Organophosphorus	Not likely	II
	Buprofezin	Unclassified	Suggestive	U
	Cypermethrin, alpha	Pyrethroid		II
	Deltamethrin	Pyrethroid	Not likely	II
Herbicides	Paraquat dichloride	Bipyridylium	E	II
	Oxyfluorfen	Diphenyl ether	C	U
Aphicides	Pirimicarb	carbamate	Likely	II

Note. WHO hazard classification: IB, highly hazardous; II, moderately hazardous; III, slightly hazardous; U, unlikely to pose an acute hazard in normal use. U.S. EPA classification: B2, probable human carcinogen with sufficient evidence from animal studies but inadequate evidence or no data from epidemiological studies; C, possible human carcinogen; D, not classifiable as to human carcinogenicity; E, evidence of noncarcinogenicity for humans.

HBSS (1% FBS) and centrifuged again for 4 min at 800 × g. Supernatant was discarded and 1.4 ml propidium iodide (PI) solution (1.25 μg/ml) was added to the pellet constituting the high-density sample (HD) A low-density sample (LD) was also prepared by diluting 200 μl HD sample in 400 μl PI.

Analysis by Flow Cytometry Flow cytometric analysis was performed on a Coulter Epics XL-MCL (Beckman Coulter, Inc.). All experiment sessions began with quality control procedures. These included verification of flow cytometer´s optical alignment and fluid system (flow check), standardization of light scatter and fluorescence parameters (flow set), and adjustments of color compensation settings to multicolor analysis (CytoComp cells and CytoComp reagent kit).

HD and LD samples were analyzed using different protocols although with similar histograms and interpretations. The LD protocol was set to acquire data with a forward scatter higher than 100 (discriminator value), and the HD protocol had an FL1 threshold in order to acquire only CD71+ events. Since the time of analysis of HD samples was long (approximately 20 min), analysis was paused at 10 min to vortex the sample preventing loss of events by deposition. Both protocols gated single cells in the first histogram. The following histograms excluded cells that were not red blood cells (platelets and white blood cells). The LD protocol was set to acquire 1×10^6 events and HD 20,000 events. Data obtained provided necessary information to determine RET and MN-RET frequencies (relative frequencies) for each sample.

Determination of Micronuclei in Peripheral Blood Lymphocytes (CBMN)

Aliquots of 0.5 ml heparinized whole blood were cultured and treated as described elsewhere (Costa et al. 2006). To determine the total number of MN in binucleated cells (MNL), in total 1000 binucleated cells with well-preserved cytoplasm (500 per replicate) were scored for each subject. MN were scored blindly by the same reader and identified according to the criteria of Caria et al.(1995) using 500× magnification.

Statistical Analysis

Distribution of variables obtained in this work was compared with the normal distribution by means of the Kolmogorov–Smirnov goodness-of-fit test. Fisher´s exact test for homogeneity was applied to assess the relationship between categorical variables. All studied variables departed significantly from normality and therefore nonparametric tests were applied to the data. Associations between two variables were analyzed by Spearman´s correlation. Mann–Whitney and Kruskal–Wallis tests were employed to search for associations between dependent and independent variables. The level of significance considered was $p < .05$. All analyses were conducted using the SPSS for Windows statistical package, version 16.0.0.

RESULTS

Demographic features of studied groups are presented in Table 1. Groups were similar in regard to age. Although relative frequencies related to gender and smoking habits vary from one group to another, these differences are not statistically significant.

Results obtained in each group are reported in Table 3. In regard to MN-RET analysis, the number of analyzed cells was 1×10^6 in low-density samples (as instrumentally set), but in high-density samples, the number was much lower than the initially established value; the mean number of analyzed cells was approximately 4000, in contrast to 20,000 instrumentally set. This difference is probably due to poor preservation of samples between collection and analysis, due to either temperature fluctuations or inadequacy of absolute methanol for long storage period. Since it is possible that differences in number of evaluated cells in different groups lead to bias in observed results, it was of interest to determine whether there was a statistically significant difference between groups in number of cells counted. Results demonstrated that differences were not significant and therefore comparisons of results are valid. Reticulocytes frequencies were found to be similar between pesticide-exposed individuals and controls.

Regarding MN-RET and MNL, pesticide-exposed individuals presented a statistically significant increase in the frequency of this indicator compared to controls (Figure 1, a and b). In order to highlight possible smoking habits-related differences, data are expressed separately for smokers and nonsmokers in Table 4. Smokers presented numerically higher MN-RET values than nonsmokers only in the exposed group, but these differences are not statistically significant. In all the remaining comparisons, nonsmokers presented increased frequency of damage. However, it is important to highlight the low number of smokers included in both the control and the exposed

TABLE 3. RET, MN-RET, and MNL Frequencies

Group	Exposed	*n*	Control	*n*
RET (%)	0.05 ± 0.01 (0.01–0.26)	83	0.04 ± 0 (0–0.18)	93
MN-RET (%)	1.14 ± 0.09 (0.03–4.63)*	82	0.47 ± 0.04 (0.08–1.79)	93
MNL (‰)	6.76 ± 0.47 (0–19)*	83	2.66 ± 0.22 (0–11)	93

Note. Values are mean ± SE (range). Asterisk indicates significantly different from control ($p < .05$), Mann–Whitney test.

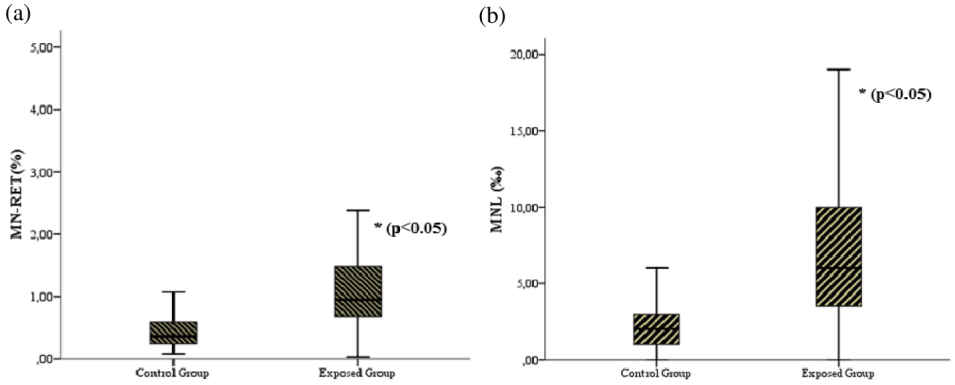

FIGURE 1. Boxplots showing frequency distribution of (a) MN-RET (%) and (b) MNL (‰) in both studied populations (control and exposed). Asterisk indicates significantly different from control group ($p < .05$), Mann–Whitney test (color figure available online).

TABLE 4. MN-RET and MNL Frequencies According to Smoking Habits

Group	Exposed	*n*	Control	*n*
MN-RET (%)				
Smokers	1.26 ± 0.35 (0.36–1.99)	4	0.43 ± 0.07 (0.13–0.88)	12
Nonsmokers	1.13 ± 0.09 (0.03–4.63)	78	0.48 ± 0.04 (0.08–1.79)	81
MNL (‰)				
Smokers	4.00 ± 1.87 (0–9)	4	1.42 ± 0.26 (0–3)	12
Nonsmokers	6.90 ± 0.48 (1–19)	79	2.84 ± 0.25 (0–11)	81

Note. Values are mean ± SE (range).

TABLE 5. MN-RET and MNL Frequencies According to Gender

Group	Exposed	*n*	Control	*n*
MN-RET (%)				
Males	1.08 ± 0.10 (0.07–3.16)	41	0.46 ± 0.04 (0.09–1.07)	39
Females	1.20 ± 0.15 (0.03–4.63)	41	0.48 ± 0.06 (0.08–1.79)	54
MNL (‰)				
Males	6.05 ± 0.52 (0–12)	42	2.44 ± 0.29 (0–8)	39
Females	7.42 ± 0.77 (1–19)	42	2.81 ± 0.32 (0–11)	54

Note. Values are mean ± SE (range).

group. A quantitative rise in frequency damage was found in females for both indicators (Table 5). Nevertheless, none of these differences are significant. Age was also studied as a possible confounder, but no trend was found between genetic damage and this variable (Spearman´s correlation).

With respect to exposure variables, the possible influence of time of exposure, work environment, timing of sample collection (considering both time since last exposure and season of the year), and differences between applicators and nonapplicators was studied. Time of exposure (as years of activity) exerted no marked influence on frequencies of MN-RET or MNL (Spearman´s correlation). Work environment did not seem to influence observed genetic damage (data not shown). In regard to timing of sampling collection (Figure 2), it was found that both MN-RET and MNL frequencies were higher in samples that were collected during spring and summer compared to those collected in autumn and winter (within the exposed group); this difference was statistically significant for MN-RET frequency. For time since last exposure, the study did not find any significant correlation between genetic damage and last day of exposure. However, if

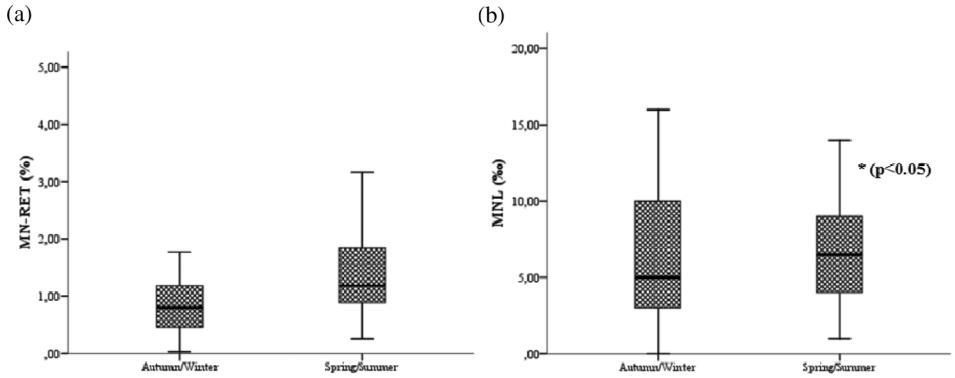

FIGURE 2. Boxplots showing frequency distribution of (a) MN-RET (%) and (b) MNL (‰) of samples collected at different seasons (within exposed group). Asterisk indicates significantly different from control group ($p < .05$), Mann–Whitney test.

only individuals exposed to pesticides in the previous 10 d were considered, a negative, significant correlation was found between day since the last exposure and MN-RET frequency. Applicators and nonapplicators presented similar frequencies with both studied endpoints and therefore no bias was attributed to this variable. A significant, positive correlation was found between the two studied endpoints, MNL and MN-RET.

DISCUSSION AND CONCLUSIONS

Although an increased incidence of hematopoietic cancers was reported in pesticide-exposed subjects (Mehri et al. 2007), no study was developed to understand the possible DNA damage that these compounds induce in this system. Analysis of MN in RET was mainly used to understand potential effects of compounds but in a controlled exposure environment in mice. Application of this technique in humans became possible by restricting analysis to young RET (CD71+) (Dertinger et al. 2002). In addition, study of MN-RET by flow cytometry significantly increased the sensitivity of the method, as a significantly higher number of cells can be examined (Kissling et al. 2007).

Analysis revealed that samples were poorly preserved due to either temperature fluctuations or inadequacy of methanol for long-term preservation. Temperature fluctuations that are not prevented by methanol are probably on the basis of CD71 marker loss, explaining the inability to examine 20000 CD71+ cells as initially established.

The loss of CD71 marker may explain the observed frequency of RET in samples. Bessman (1990) reported normal RET frequencies around 1% being considered, as normal ranges from 0.4% to 3%. Dertinger et al. (2007) found a frequency of RET-CD71+ (younger reticulocytes) of 0.10% in healthy adult subjects. Our samples presented a much lower frequency of reticulocytes in studied groups, confirming that preservation was not adequate to maintain the CD71 marker in the cell surface. Comparison of frequencies obtained in both groups showed that controls presented lower numerical values than the exposed group.

Increase of RET values are an indication of enhanced production of red blood cells that might be lost by bleeding or destruction and were reported after chemical exposure to few compounds such as nickel (Ni) and lead (Pb) (Ni may be present in fungicides, while Pb may be found in herbicides, insecticides, or rodenticides) (Das et al. 2008; Stoleski et al. 2008). In this study, exposed subjects were not in contact with pesticides containing either Ni or Pb and presented RET similar to control individuals.

The loss of CD71 marker suggested a lower number of RET-CD71+ in samples and therefore when analyzing HD samples the established number of cells to examine (20,000) was generally not attained. Previous studies showed that the number of analyzed cells is

an important factor concerning assay sensitivity (Kissling et al. 2007). In view of this, in this study sensitivity was lost with the fall of RET-CD71+ present on the sample. In addition, and because there is a consistent negative correlation between the number of examined cells by flow cytometry and the outcome frequency (Kissling et al. 2007), it was important to understand whether the number of analyzed cells in each group was significantly different. Experimental results demonstrated that it was valid to compare the exposed with the controls, as differences regarding number of examined cells in HD samples were not significant between groups.

Despite the low number of analyzed RET-CD71+ in samples, a significant increase was found in exposed samples compared to controls. Probably due to the low number of observed cells, frequencies are similar to those obtained using microscopy (MacGregor et al. 1997) compared to flow cytometry (Offer et al. 2005).

Concerning the remaining studied variables, only two presented statistically significant differences: timing of sampling, and time since last exposure. Timing of sampling proved to be important as individuals analyzed in the spring/summer period presented higher MN-RET frequencies than the ones participating in the autumn/winter period. This is probably related to exposure frequency, as major use of pesticides is carried out during spring and summer.

MN-RET constitute an indicator of recent damage. Reticulocytes have a short life span—hours to a few days (MacGregor et al. 1997)—and therefore a negative correlation between MN frequency and time since last exposure was an expected result. In fact, MN-RET is an endpoint that seems to have no memory of early damage (Stopper et al. 2005). Elevated frequencies of this endpoint are only found while there is still an effect of the xenobiotic in cells and that is why one only finds a significant, negative correlation between MN-RET and time since last exposure when considering recent exposure (in the previous 10 d).

MNL frequencies were also found to be significantly elevated in pesticide-exposed individuals. This result is in agreement with other investigations (Márquez et al. 2005; Bhalli et al. 2006; Costa et al. 2006; Sailaja et al. 2006; Bolognesi et al. 2009; Ergene et al. 2007). Frequencies of MNL were not significantly influenced by any of the other variables studied. Gender is widely described as a demographic factor influencing observed DNA damage assessed by means of MN frequency (Bonassi et al. 1995). Results obtained in this study showed numerical increased DNA damage in females. This rise in DNA damage frequency was previously reported by Fenech et al. (1994) and is generally attributed to aneuploidogenic events preferentially involving the X chromosome. Tucker et al. (1996) suggested that the X chromosome lagging behind during anaphase is the inactive one, but Surralles et al. (1996) found contradicting results. In addition, X chromosome is also overrepresented in men who have only one copy of this chromosome, an active one (Norppa and Falck 2003). Evidence indicates that it is the location of this chromosome during metaphase and anaphase (Zijno et al. 1996) or kinetochore defects (Tucker et al. 1996b) as the basis for X chromosome loss. This association was also observed in pesticide-exposed populations (Bolognesi et al. 2002; 2004; El-Khatib and Hammam 2003; Grover et al. 2003; Costa et al. 2006).

Endpoints studied herein offer different information since human reticulocytes and lymphocytes present divergent life spans. CBMN is not only carried out in cells with a higher life span, as the technique enables the expression of accumulated DNA damage to express itself in one in vitro cell division. In fact, several investigators found a significant correlation between MN frequency in lymphocytes and years of exposure to pesticides (Bhalli et al. 2006; Sailaja et al. 2006). However, the results presented here did not show a significant correlation between time of exposure and MNL frequencies. The lack of correlation between these two variables may be related to work restraints, as younger individuals are

the ones exposed more frequently to pesticide compounds. Another possible explanation for the absence of correlation between time of exposure and MNL frequencies refers to differences in DNA repair mechanisms, death of highly damaged cells (apoptosis), and decreased quantity of damaged cells, as new and undamaged cells are generated through normal turnover of cell populations (Garaj-Vrhovac and Zeljezic 2001). Again, the lack of correlation between MN-RET and years of exposure is probably a consequence of the short life span of these cells.

Correlation between MN-RET and MNL was found to be significant. Stopper et al. (2005) studied the effects of radioiodine therapy and demonstrated a significant correlation between increase in MNL and in MN-RET induced by the last treatment (an acute exposure situation).

In conclusion, MN-RET frequency was found to be influenced by pesticide exposure. This appears to be a reliable endpoint to study recent exposure. Additional studies with an increased number of analyzed cells may demonstrate enhanced sensitivity and possibly provide more definitive conclusions on exposure determinants. Use of CBMN, although more laborious, remains a reliable and adequate biomarker to assess pesticide genetic damage.

REFERENCES

Bessman, J. D. 1990. Reticulocytes. In *Clinical methods: The history, physical and laboratory examinations*, ed. Walker, H. K. W. D. Hall, and J. W. Hurst, 735–738. Stoneham: Butterworths.

Bhalli, J. A., Q. M. Khan, A. M. Khalid, and A. Nasim. 2006. Cytogenetic analysis of Pakistani individuals occupationally exposed to pesticides in a pesticide production industry. *Mutagenesis* 21: 143–48.

Bolognesi, C., G. Carrasquilla, S. Volpi, K. R. Solomon, and E. J. P. Marshall. 2009. Biomonitoring of genotoxic risk in agricultural workers from five Colombian regions: Association to occupational exposure to glyphosate. *J Toxicol. Environ. Health A* 72: 986–97.

Bolognesi, C., E. Landini, E. Perrone, and P. Roggieri. 2004. Cytogenetic biomonitoring of a floriculturist in Italy: micronucleus analysis by fluorescence in situ hybridization (FISH) with all-chromosome centromeric probe. *Mutat. Res*. 557: 109–17.

Bolognesi, C., E. Perrone, and E. Landini. 2002. Micronucleus monitoring of a floriculturist population from western Liguria, Italy. *Mutagenesis* 17: 391–97.

Bonassi, S., C. Bolognesi, A. Abbondandolo, R. Barale, P. Bigatti, L. Camurri, L. Dalpra, M. D. Ferrari, A. Forni, C. Lando, P. Padovani, R. Pasquini, M. Stella, and R. Puntoni. 1995. Influence of sex on cytogenetic end points: Evidence from a large human sample and review of the literature. *Cancer Epidemiol. Biomarkers Prev*. 4: 671–79.

Bonassi, S., A. Znaor, M. Ceppi, C. Lando, W. P. Chang, N. Holland, M. Kirsch-Volders, E. Zeiger, S. Ban, R. Barale, M. P. Bigatti, C. Bolognesi, A. Cebulska-Wasilewska, E. Fabianova, A. Fucic, L. Hagmar, G. Joksic, A. Martelli, L. Migliore, E. Mirkova, M. R. Scarfi, A. Zijno, H. Norppa, and M. Fenech. 2007. An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. *Carcinogenesis (Oxford)* 28: 625–31.

Caria, H., T. Chaveca, A. Laires, and J. Rueff. 1995. Genotoxicity of quercetin in the micronucleus assay in mouse bone marrow erythrocytes, human lymphocytes, V79 vell line and identification of kinetochore-containing (CREST staining) micronuclei in human lymphocytes. *Mutat. Res*. 343: 85–94.

Costa, C., S. Silva, P. Coelho, J. Roma-Torres, J. Teixeira, and O. Mayan. 2007. Micronucleus analysis in a Portuguese population exposed to pesticides: Preliminary survey. *Int. J. Environ. Health* 210: 415–18.

Costa, C., J. P. Teixeira, and O. Mayan. 2008. Chapter III: Pesticides as genetic damage inducers. In *Progress in DNA damage research*, ed. S. Miura and S. Nakano, 111–63. New York: Nova Science.

Costa, C., J. P. Teixeira, S. Silva, J. Roma-Torres, P. Coelho, J. Gaspar, M. Alves, B. Laffon, J. Rueff, and O. Mayan. 2006. Cytogenetic and molecular biomonitoring of a Portuguese population exposed to pesticides. *Mutagenesis* 21: 343–50.

Das, K. K., S. N. Das, and S. A. Dhundasi. 2008. Nickel, its adverse health effects & oxidative stress. *Indian J. Med. Res*. 128: 412–25.

Dertinger, S. D., Y. Chen, R. K. Miller, K. J. Brewer, T. Smudzin, D. K. Torous, N. E. Hall, K. A. Olvany, F. G. Murante, and C. R. Tometsko. 2003. Micronucleated CD71-positive reticulocytes: A blood-based endpoint of cytogenetic damage in humans. *Mutat. Res*. 542: 77–87.

Dertinger, S. D., R. K. Miller, K. Brewer, T. Smudzin, D. K. Torous, D. J. Roberts, S. L. Avlasevich, S. M. Bryce, S. Sugunan, and Y. Chenc. 2007. Automated human blood micronucleated reticulocyte measurements for rapid assessment of chromosomal damage. *Mutat. Res*. 626: 111–19.

Dertinger, S. D., D. K. Torous, N. E. Hall, F. G. Murante, S. E. Gleason, R. K. Miller, and C. R. Tometsko. 2002. Enumeration of micronucleated CD71-positive human reticulocytes with a single-laser flow cytometer. *Mutat. Res*. 515: 3–14.

El-Khatib, H., and F. Hammam. 2003. Cytogenetic biomonitoring of Egyptian workers exposed to pesticides: Micronuclei analysis in peripheral blood lymphocytes. In *Human monitoring for genetic effects*, ed. A. Cebulska-Wasilewska, W. W. Au, and R. J. Srám, 142–150. NATO Science Series. Amsterdam: IOS Press.

Ergene, S., A. Çelik, T. Çavas, and F. Kaya. 2007. Genotoxic biomonitoring study of population residing in pesticide contaminated regions in Göksu Delta: Micronucleus, chromosomal aberrations and sister chromatid exchanges. *Environ. Int*. 33: 877–85.

EUROSTAT. 2001. *Environment pressure indicators for the EU: Data 1985–98*, ed. European Commission. Statistical Office of the European Commission: Theme 8: Environment and Energy. Luxembourg: Office for Official Publications of the European Communities.

Fenech, M., S. Neville, and J. Rinaldi. 1994. Sex is an important variable affecting spontaneous micronucleus frequency in cytokinesis-blocked lymphocytes. *Mutat. Res. Environ. Mutagen. Related Subj*. 313: 203–7.

Fenech, M. 2006. Cytokinesis-block micronucleus assay evolves into a "cytome" assay of chromosomal instability, mitotic dysfunction and cell death. *Mutat. Res*. 600: 58–66.

Garaj-Vrhovac, V., and D. Zeljezic. 2001. Cytogenetic monitoring of Croatian population occupationally exposed to a complex mixture of pesticides. *Toxicology* 165: 153–62.

Grover, P., K. Danadevi, M. Mahboob, R. Rozati, B. S. Banu, and M. Rahman. 2003. Evaluation of genetic damage in workers employed in pesticide production utilizing the Comet assay. *Mutagenesis* 18: 201–5.

Infante-Rivard, C., and Weichenthal, S. 2007. Pesticides and childhood cancer: An update of Zahm and Ward's 1998 review. *J. Toxicol. Environ. Health B* 10: 81–99.

Keifer, M. C. 2000. Effectiveness of interventions in reducing pesticide overexposure and poisonings. *Am. J. Prev. Med*. 18: 80–89.

Kissling, G. E., S. D. Dertinger, M. Hayashi, and J. T. MacGregor. 2007. Sensitivity of the erythrocyte micronucleus assay: Dependence on number of cells scored and inter-animal variability. *Mutat. Res*. 634: 235–40.

Knopper, L. D., and D. R. S. Lean. 2004. Carcinogenic and genotoxic potential of turf pesticides commonly used on golf courses. *J. Toxicol. Environ. Health B* 7: 267–79.

Kyrtopoulos, S. 2007. State of validation of biomarkers of carcinogen exposure and early effects and their aplicability to molecular epidemiology. In *Environmental cancer risk, Nutrition and individual susceptibility*, Leicester: ECNIS.

Márquez, C., C. Villalobos, S. Poblete, E. Villalobos, M. García, and S. Duk. 2005. Cytogenetic damage in female Chilean agricultural workers exposed to mixtures of pesticides. *Environ. Mol. Mutagen*. 45: 1–7.

Merhi M., H. Raynal, E. Cahuzac, F. Vinson, J. P. Cravedi, and L. Gamet-Payrastre. 2007. Occupational exposure to pesticides and risk of hematopoietic cancers: Meta-analysis of case–control studies. *Cancer Causes Control* 18: 1209–26.

Norppa, H., and G. C. M.Falck. 2003. What do human micronuclei contain? *Mutagenesis* 18: 221–33.

Offer, T., E. Ho, M. G. Traber, R. S. Bruno, F. A. Kuypers, and B. N. Ames. 2005. A simple assay for frequency of chromosome breaks and loss (micronuclei) by flow cytometry of human reticulocytes. *FASEB J*. 19: 485–87.

Pastor, S., A. Creus, N. Xamena, C. Siffel, and R. Marcos. 2002a. Occupational exposure to pesticides and cytogenetic damage: Results of a Hungarian population study using the micronucleus assay in lymphocytes and buccal cells. *Environ. Mol. Mutagen*. 40: 101–9.

Pastor, S., L. Lucero, S. Gutiérrez, R. Durban, C. Gomez, T. Parron, A.Creus, and R. Marcos. 2002b. A follow-up study on micronucleus frequency in Spanish agricultural workers exposed to pesticides. *Mutagenesis* 17: 79–82.

Ritter, L., N. C. I. Goushleff, T. Arbuckle, D. Cole, and M. Raizenne. 2006. Addressing the linkage between exposure to pesticides and human health effects: Research trends and priorities for research. *J. Toxicol. Environ. Health B* 9: 441–56.

Sailaja, N., M. Chandrasekhar, P. V. Rekhadevi, M. Mahboob, M. F. Rahman, S. B. Vuyyuri, K. Danadevi, S. A. Hussain, and P. Grover. 2006. Genotoxic evaluation of workers employed in pesticide production. *Mutat. Res*. 609: 74–80.

Schlegel, R., J. T. MacGregor, and R. B. Everson. 1986. Assessment of cytogenetic damage by quantitation of micronuclei in human peripheral blood erythrocytes. *Cancer Res*. 46: 3717–21.

Stoleski S., J. Karadzinka-Bislimovskai, E. Stikova, S. Risteka-Kuci, D. Mijakoski, and J. Minov. 2008. Adverse effects in workers exposed to inorganic lead. *Arch. Hig. Rada. Toksikol*. 59: 19–29.

Stopper, H., K. Hempel, C. Reiners, S. Vershenya, R. Lorenz, V. Vukicevic, A. Heidland, and J. Grawe. 2005. Pilot study for comparison of reticulocyte-micronulei with lymphocyte-micronuclei in human biomonitoring. *Toxicol. Lett*. 156:351–60.

Surrallés, J., P. Jeppesen, H. Morrison, and A. Natarajan. 1996. Analysis of loss of inactive X chromosome in interphase cells. *Am. J. Hum. Genet*. 59: 1091–96.

Tucker, J., J. Nath, and J. C. Hando. 1996. Activation status of the X chromosome in human micronucleated lymphocytes. *Hum. Genet*. 97: 471–475.

Zijno, A., P. Leopardi, F. Marcon, and R. Crebelli. 1996. Sex chromosome loss and non-disjunction in women: Analysis of chromosomal segregation in binucleated lymphocytes. *Chromosoma (Berlin)* 104: 461–67.