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Original scientific paper

Micronucleus frequencies in peripheral blood lymphocytes in a Serbian human population exposed to pesticides

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SUMMARY

Micronucleus (MN) is a biomarker widely used in biomonitoring studies for determining the genetic risk associated with exposure to pesticides. The purpose of this study was to assess damage to the genetic material of workers occupationally exposed to pesticides as detected in micronucleus tests. The research included 119 subjects divided into three groups: a control group of 39 subjects, a group of 40 subjects exposed to pesticides as producers and a group of 40 pesticide applicators in the field. A Mann-Whitney U-test displayed statistically significant differences between the parameter means of all variables, and the control group. Significant differences were observed between males involved in pesticide production and application for the parameter MN4, then between non-smoking producers and applicators regarding parameters MN2, MN3, MN4 and NB, as well as between the control and applicator groups for parameter MN2, and between producers and applicators for parameter MN3. Spearman's correlation test showed a positive correlation between the frequency of micronuclei and age of respondents, as well as their smoking habits. A statistically significant difference in relation to cytogenetic parameters was detected between the respondents working in pesticide production and those working in the field. The results suggest that applicators in the field do not use adequate personal protective equipment. Regular biological monitoring of workers exposed to pesticides is required.

Keywords: Pesticides; Occupational hazards; Micronucleus test; Serbia

INTRODUCTION

Pesticides constitute a heterogeneous category of chemicals specifically designed to control pests and diseases. Their application is still the most effective and widely accepted means of plant protection, which has significantly contributed to enhancing agricultural productivity and crop yield (Bolognesi, 2003). At present there are more than 1000 chemicals classified as pesticides (WHO, 2004) and about 890 active ingredients in 20.700 pesticide products (Tomlin, 2009). A perfect pesticide should be toxic only to a particular target organism, totally biodegradable to CO_2 and H_2O, and should not leave intermediate compounds in the environment. Unfortunately, this is rarely the case and our widespread use of pesticides in modern agriculture has caused increasing concern. The main problems in real agricultural systems arising from the use of pesticides are their toxicity to non-target organisms and the environment (Ros et al., 2006; Radivojević et al., 2008, 2012). Pesticides are primarily released into the environment through their widespread use in agriculture and public health. Despite the benefits of their use, many of these chemicals may pose potential hazard to man and the environment. Using these agricultural chemicals without proper protection may lead to alteration of the genetic material and potential development of some types of tumors (Javed et al., 2006). Biomonitoring studies using somatic cells have been extensively conducted to evaluate the possible genotoxic risk of a defined exposure and some indicators, such as chromosomal aberrations, have been shown to be relevant biomarkers for further cancer incidence (Hagmar et al., 1994, 2004). In addition, the use of appropriate biomarkers in these biomonitoring studies can provide tools useful for explaining the mechanisms of action of such exposure.

A certain number of field studies have been carried out that revealed an association between occupational exposure to pesticides and chromosomal aberrations as a factor increasing cancer risk (Rupa et al., 1988; Bolognesi et al., 1993; Garaj-Vrhovac & Zeljezdić, 2002). In biomonitoring studies, micronucleus assays in peripheral lymphocytes have been increasingly used as a useful technique to screen chromosomal aberrations and detect clastogenic and aneuploidogenic agents (Sorsa et al., 1992; Fenech, 1993). Human groups that are occupationally exposed to pesticides on a direct and almost daily basis stand genotoxic risks. Many biomonitoring studies have evaluated cytogenetic effects on pesticide exposed workers from different countries (Pasastor et al., 2002). However, there are only a few reports on health impact of chronic occupational exposure to pesticides in developing countries. People in developing countries stand a higher risk from chronic exposure to such chemicals because of their poor working conditions and potential hazards connected with pesticide manufacturing and application (Baker et al., 1978).

The present study focused on cytogenetic monitoring of a Serbian population occupationally exposed to a complex mixture of pesticides.

MATERIAL AND METHODS

Our cytogenetic monitoring included three study groups. The exposed groups were composed of 40 (E1) individuals working in different units of pesticide production (pesticide formulation units for liquid and solid products) and 40 (E2) individuals involved

Table 1. Characteristics of the population studied

Unexposed - Control					
		Age		Smoking habit	
	n	Mean±SD	Median; range	Smokers	Non-smokers
Men	20	36.7±7.64	(37; 23–50)	10	10
Women	19	41.16±7.25	(43; 26–56)	4	15
Producers					
		Age		Smoking habit	
	n	Mean±SD	Median; range	Smokers	Non-smokers
Men	24	40.46±8.02	(41; 25–54)	10	14
Women	16	40.44±6.94	(40.5; 30–58)	8	8
Applicators					
		Age		Smoking habit	
	n	Mean±SD	Median; range	Smokers	Non-smokers
Men	19	38.53±7.32	(37; 27–53)	12	7
Women	21	37.48±8.57	(38; 22–52)	15	6
∑	119			59	60

in application of pesticides in the field. During the production process all subjects were simultaneously exposed to a complex mixture of pesticides and coformulants (inerts) (Table 1).

The study included a control group of 39 subjects. Control individuals were not occupationally exposed to any particular chemical agent.

Table 2. Duration of pesticide exposure

Applicators and producers		
	Pesticide exposure (years)	n
Men	0–15	28
	16–30	15
Women	0–15	22
	16–30	15
Σ		80

Tables 1 and 2 show characteristics of the study groups regarding their gender (male or female), age, duration of occupational exposure to pesticides (DOE in years), smoking habits (smoker or non-smoker). Genetic damage was detected by micronucleus test (CBMN – cytokinesis-block micronucleus).

All participants were subjected to cytogenetic research using the CBMN test at the Center for Radiological Protection of the Serbian Institute of Occupational Health "Dr Dragomir Karajovic". Organization of this cytogenetic research, communication with subjects, blood sampling, laboratory procedures in handling blood samples, as well as data processing, were conducted in accordance with ethical principles and guidelines set for biomonitoring of human population (Albertini et al., 1996).

Venous blood was taken from each subject using heparinized syringes, stored in ice and brought to the laboratory for analysis. The subjects underwent minimal invasive vein blood drainage from the cubital vein by venipuncture.

The participants filled out a questionnaire in which the type of working activity, duration of exposure to pesticides, type of pesticides handled, personal protective equipment, etc., were recorded.

A standard protocol for conventional cytogenetic analysis was used in the CBMN test (IAEA, 2001). Forty-four hours after initiation, Cytochalasin – B (Sigma Aldrich) at a final concentration of 3 μg/ml solution in DMSO (Sigma Aldrich) was added to the cultures, and cultivation continued for another 24 hour. The cultures were then treated with 0.9% NaCl solution, followed by a cold hypotonic solution (0.075M KCl) to lyse red blood cells. The supernatant was removed and replaced with a fixative consisting of methanol:acetic acid (3:1) with 1% formaldehyde. After incubation for 20 min at room temperature, the cells were washed with four changes of fixative without formaldehyde. The cells were then re-suspended gently and the suspension dropped onto clean glass slides and allowed to dry. The cells were stained using 10% Giemsa in distilled water. The slides were analyzed by light microscopy (Olympus BX-51, magnification 1000x). Binuclear cells were analyzed according to a standard protocol and the criteria prescribed by the HUMN (Human MicroNucleus) international collaborative project (Bonassi et al., 2001).

One thousand binucleate cells per subject and per dose were analyzed and the total number of MN found and their distribution recorded. Parameters describing the distribution of micronuclei were defined as the number of binuclear cells with 1-4 nuclei.

The following inclusion criteria were used: micronuclei (MN), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) in peripheral blood samples.

Statistical analysis

In addition to descriptive statistics, appropriate non-parametric statistical methods were used: Mann-Whitney U-test, Pearson's X^2 test and Spearman rank test (non-parametric correlation test) on the whole body of samples (control vs. exposed) and the exposed group only. The software used for data analyses was STATISTICA (StatSoft, Tulsa, OK) and SPSS version 10.0 (SPSS Inc., Chicago, IL).

RESULTS

Here we report the results of cytogenetic monitoring of 80 occupationally exposed workers and 39 matched controls using the cytokinesis-block micronucleus (CBMN) method. Tables 1 and 2 show the main characteristics of the population studied.

The Mann-Whitney U-test indicated statistically significant differences between the average values of all examined variables in comparison to the control group.

The results of MN tests for the entire population (exposed and control), women and men separately, and smokers and non-smokers, are shown in Table 3. In the control group of subjects, the average was 8.44±3.35 MN (median 8 MN), while the span of individual values varied from 3 to 18 MN per 1000 binuclear cells. Employees in pesticide production companies (E1) had an average 14.55±5.48 MN (median 14.5), and the span of individual values for these subjects was from 4 to 29 MN, while field applicators (E2) had an average 16.31±4.79 MN (median 16), and the span of individual values was from 8 to 31.

The Mann-Whitney U-test detected statistically significant differences between men in production and application for the parameter MN4 (p = 0.004678), as well as non-smokers for the parameters MN2 (p = 0.000108), MN3 (p = 0.000005), MN4 (p = 0.000005) and NB (p = 0.014306). Also, statistically significant differences were recorded within the whole population between control and applicator subjects for the parameter MN2 (p = 0.046419), as well as between the control and producers and applicators for the parameter MN3 (p = 0.026532). The results of MN tests for the entire population (exposed and control), women and men separately, and smokers and non-smokers, are shown in Table 3.

Table 3. Results of MN tests for the studied population

	Unexposed - Control		Producers		Applicators	
Parameter	Mean±SD	Median; range	Mean±SD	Median; range	Mean±SD	Median; range
			Entire population			
MN	8.44±3.35	8; 3–18	14.55±5.48	14.5; 4–29	16.31±4.79	16; 8–31
MN1	7.23±2.66	7; 3–14	10.58±3.13	10; 4–18	12.13±2.81	12; 4–18
MN2	0.56±0.75	0; 0–2 **a***	1.28±1.15	1; 0–6	1.10±1.03	1; 0–5 **b**
MN3	0.03±0.16	0; 0–1	0.43±0.21	0; 0–3 **a**	0.45±0.75	0; 0–3 **b**
MN4	0.00±0.00	0; 0–0	0.05±0.02	0; 0–1	0.20±0.46	0; 0–2
NB	0.51±0.97	0; 0–4	2.68±1.95	3; 0–7	2.58±1.30	3; 0–5
NPB	0.08±0.27	0; 0–1	0.48±0.32	0; 0–3	0.53±0.82	0; 0–3
			Women			
MN	9.21±3.36	9; 4–18	14.5±5.62	16; 4–26	16.90±4.57	16; 10–26
MN1	7.63±2.75	8; 4–14	10.88±3.36	10.5; 4–18	13.14±2.63	12; 9–18
MN2	0.79±0.85	1; 0–2	1.00±1.26	0.5; 0–4	1.00±0.89	1; 0–2
MN3	0.00±0.00	0; 0–0	0.5±0.45	0; 0–2	0.33±0.58	0; 0–2
MN4	0.00±0.00	0; 0–0	0.06±0.05	0; 0–0	0.19±0.40	0; 0 –1
NB	0.58±1.07	0; 0–4	2.25±1.81	2; 0–7	2.71±1.23	3; 0–5
NPB	0.16±0.37	0; 0–1	0.38±0.21	0; 0–3	0.62±0.97	0; 0–3
			Men			
MN	7.7±3.25	8; 3–13	14.58±5.50	14; 5–29	15.74±4.96	15; 8–31
MN1	6.85±2.57	7; 3–11	10.38±3.02	10; 5–17	11.00±2.62	11; 4–16
MN2	0.35±0.59	0; 0–2	1.46±1.26	1; 0–6	1.21±1.18	1; 0–5
MN3	0.05±0.22	0; 0–1	0.37±0.21	0; 0–3	0.53±0.40	0; 0–3
MN4	0.00±0.00	0; 0–0	0.04±0.02	0; 0–1 **a**	0.21±0.53	0; 0–2 **b**
NB	0.45±0.88	0; 0–3	0.04±0.02	0; 0–1	0.21±0.53	0; 0–2
NPB	0.00±0.00	0; 0–0	2.96±2.03	3; 0–7	2.42±1.39	3; 0–5
			Smokers			
MN	9.21±3.91	8.5; 3 –18	15.33±5.52	14; 4–28	16.52±5.37	15; 8–31
MN1	7.86±3.06	8; 3–14	10.89±3.53	10.5; 4–18	11.70±2.85	12; 4–18
MN2	0.57±0.76	0; 0–2	1.39±1.29	1; 0–4	1.15±1.17	1; 0–5
MN3	0.07±0.27	0; 0–1	0.56±0.92	0; 0–3	0.52±0.85	0; 0–3
MN4	0.00±0.00	0; 0–0	0.00±0.00	0; 0–0	0.26±0.53	0; 0–2
NB	0.5±0.76	0; 0–2	2.78±2.29	2; 0–7	2.54±1.39	2.5; 0–5
NPB	0.00±0.00	0; 0–0	0.34±0.26	0; 0–3	0.52±0.89	0; 0–3
			Non–smokers			
MN	8.00±2.99	8; 3–13	13.91±5.49	15; 5–29	13.91±5.49	15; 5–29
MN1	6.88±2.40	6; 3–11	10.32±2.82	10; 5–15	10.32±2.82	10; 5–15
MN2	0.56±0.77	0; 0–2	1.18±1.59	0.5; 0–6 **a**	1.18±1.59	0.5; 0–6 **b**
MN3	0.00±0.00	0; 0–0	0.32±0.48	0; 0–1 **a**	0.32±0.48	0; 0–1 **b**
MN4	0.00±0.00	0; 0–0	0.09±0.03	0; 0–1 **a**	0.09±0.29	0; 0–1 **b**
NB	0.52±1.08	0; 0–4	2.59±1.68	3; 0 –6 **a**	2.59±1.68	3; 0–6 **b**
NPB	0.12±0.33	0; 0 –1	0.59±0.85	0; 0–1	0.60±0.85	0; 0–3

*- Mann-Whithey U-test; a, b - significant differences (p<0.05)

The performed correlation analysis showed statistically positive correlations between subject age and total number of MN, NB and NPB in women (smokers and non-smokers) in control (R=0.23078), as well as in women applicators (R=0.50850), and women in production (R=0.20656). Also, a statistically positive correlation was noticed between the age and total number of MN, NB and NPB in men (smokers and non-smokers) in control (R= 0.50200), as well as in men involved in pesticide application (R=0.55918) and production (R=0.19119) (Figure 1).

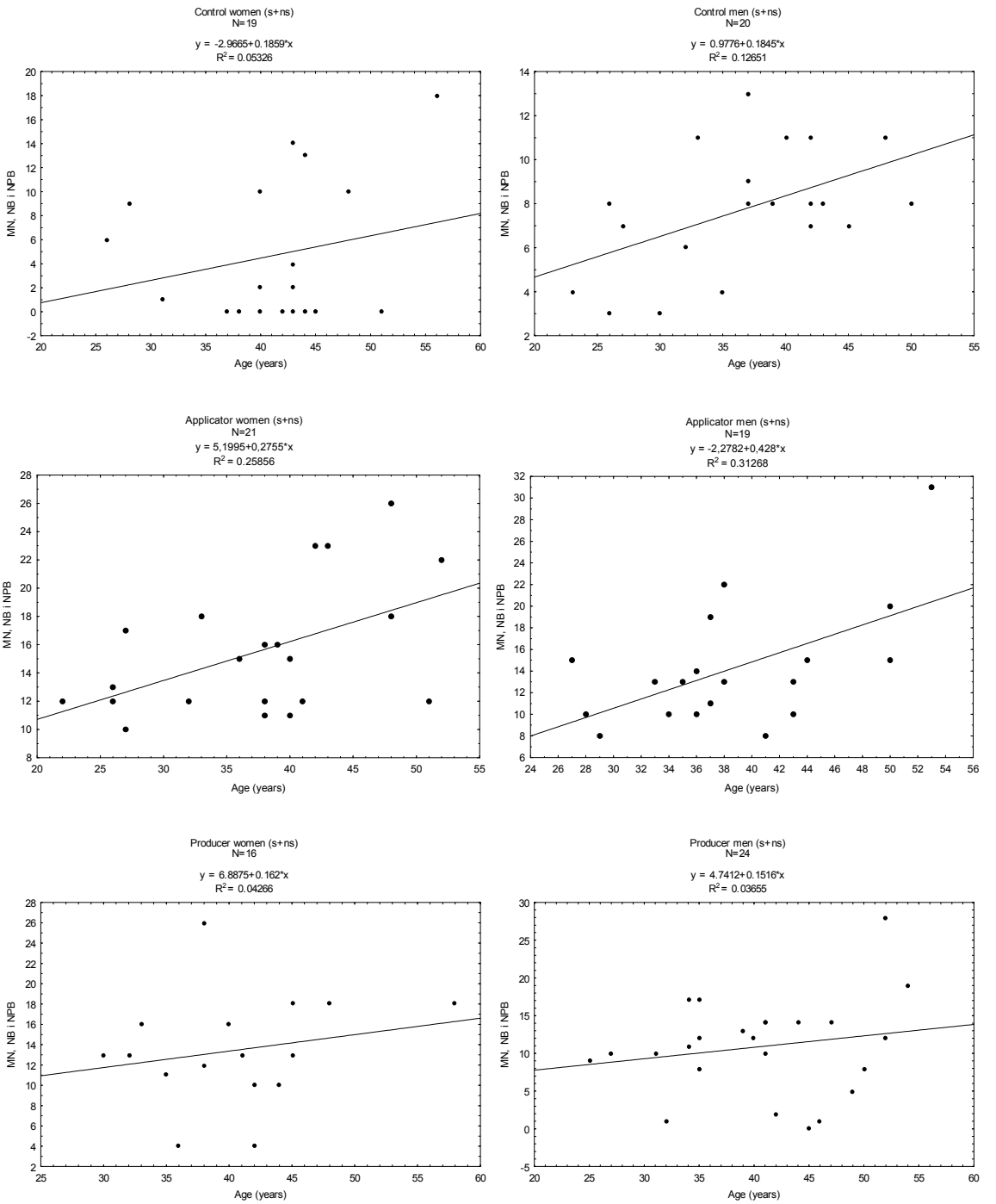

Figure 1. Correlations of MN, NB, NPB and age with gender (smokers and non-smokers)

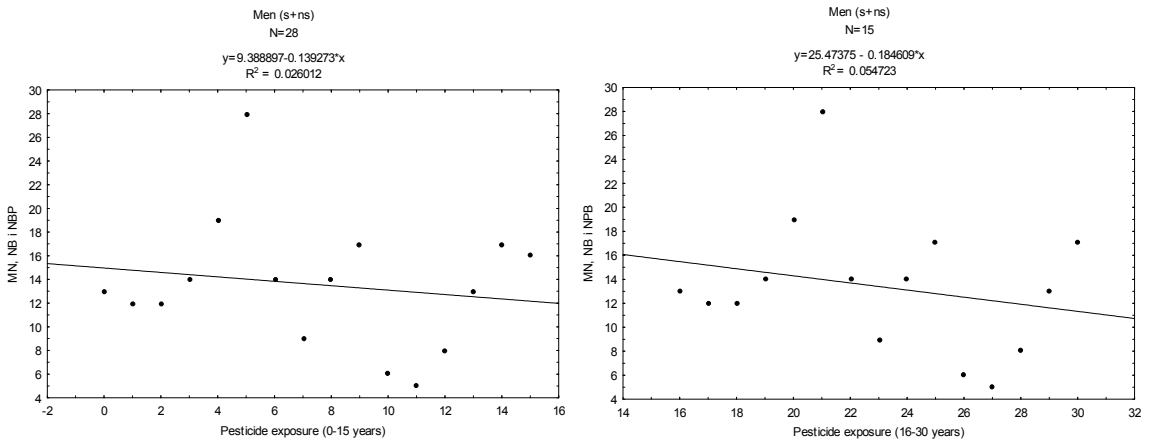

Figure 2. Correlations of MN, NB, NPB and pesticide exposure with gender (smokers and non-smokers)

Correlation analysis based on Figure 2 revealed a statistically positive correlation between the exposed work period of 0-15 years (R=0.16128), and slightly higher for the exposed work period of 16-30 years (R=0.23393), and the total number of MN, NB and NPB in men (smokers and non-smokers) producers and applicators.

Based on the performed Mann-Whitney U-test there were no statistically significant differences between the exposed work periods of 0-15 years and 16-30 years for the parameters MN, MN1, MN2, MN3, MN4, NB and NPB in men (smokers and non-smokers) producers and applicators.

DISCUSSION

The human population screened in this research, consisting of non-exposed subjects in the control group (C) and two groups of professionals exposed to pesticides (E1 and E2), was subjected to cytogenetic research aimed to detect changes in their genetic material and determine possible differences between the exposed and non-exposed subjects. An analysis of our results revealed a statistically significant difference between the control and exposed groups based on the examined parameters. The analyzed MN parameters were examined in relation to gender, age and habit of cigarette consumption as factors that, according to literature data, can influence their basal incidence (Hagmar et al., 1994; Bonassi et al., 2003).

Knowing the mechanism and cause of spontaneous occurrence of micronuclei (MN) is very important for correct interpretation of results of a MN test, especially in cases when the test is being applied in cytogenetic biomonitoring of professionally exposed populations. Since MN are indicators of damaged DNA, all processes which directly or indirectly cause such damage are also causing increase in MN counts. Oxidative processes in cells and organisms are especially important, as well as gene polymorphisms and various mutations which may result in genome instability.

Our results showed that the average values of the parameters indicating genetic damage detected in MN tests were higher in the exposed groups of subjects than in control subjects. The average was 8.44±3.35 MN (median 8 MN) in the non-exposed control, while the employees in pesticide production (E1) had an average of 14.55±5.48 MN (median 14.5), and field workers (E2) 16.31±4.79 MN (median 16).

The span of individual values in the group of applicators was 8-31, and it was the higher one considering both exposed groups. Subjects with the highest number of MN were involved in pesticide application, which many studies (Bolognesi et al., 2003) had earlier identified as the point of highest exposure. Besides, blood for the cytogenetic analyses was taken at the moment of most intensive pesticide application when, as literature data show, the highest incidence of genetic damage is expected (Da Silva Augusto et al., 1997; Bhalli et al., 2006).

The distribution of MN shows an arrangement of micronuclei in binuclear lymphocytes and it is indicative of the size of damage to the genetic material found in micronuclei (Fenech et al., 2011). Researchers studying the parameters of distribution on human material *in vitro* claim that multiple MN can be caused by aneugenic, as well as clastogenic agents, while the ratio of binuclear lymphocytes with more than two MN and total number of analyzed binuclei

grows with increasing concentrations of the applied chemicals (Matsuoka et al., 1998). Nuclear buds are characterized by the same morphology as MN, but the difference is that they are connected to the main nucleus by stalks of variable width, depending on the stage of bud development (Fenech et al., 2011). They represent a process of elimination of amplified DNA, the complex of DNA reparation and probably too many chromosomes from aneuploidy cells. Their existence is related to clastogenic, as well as aneugenic genotoxic agents (Fenech, 2002), so these data are crucial in explaining this study.

In our work, we found an increased frequency of MN in exposed subjects (men and women), compared to the control group. The presented data is consistent with other studies researching similar problems (Shaham et al ., 2001; Costa et al., 2006).

As specific parameters of the micronucleus test NB and NPB are indicators of a process of gene amplification, i.e. chromosomal rearrangements occurring as a result of the activity of genotoxic agents. Furthermore, the results of this study confirm other research studies isolating the group of exposed examinees with statistically significantly higher values of NPB parameters than control groups (Bolognesi, 2003).

A positive correlation between the frequency of MN and other parameters of the MN test and the age of examinees has also been noted in many studies. This phenomenon is usually explained by the fact that during the process of aging, primary DNA damage occurs, as well as an increased frequency of chromosomal aberrations, and aneuploidies become accumulated both at the cellular level and the level of entire organism (Bonassi et al., 1995; Mateuca et al., 2006).

The results obtained in correlation analysis are consistent with other studies dedicated to research of the influence of joined factors on the incidence of chromosome damage emphasizing that an increased number of micronuclei in women can be connected to increased tendency of the X chromosome to be included in micronucleus in comparison to other chromosomes and the fact that women, unlike men, have two X chromosomes (Tucker et al., 2011).

Analysis of our results relating to the age category was based on literature data that ascribed increased incidence of genetic damage to age and a combination of factors, including: a) cumulative effect of acquired mutations of genes involved in reparation DNA molecules, separation of chromosomes and control point of the cell cycle, and b) numerical and structural aberrations of chromosomes caused by exposure to endogenous toxins, inadequate diet and other mutagenic factors of the environment (Fenech et al., 2011).

It is important to stress that our studies have shown that years of being exposed to pesticides may influence the frequency and distribution of MN, which has also been confirmed by many other research studies (Migliore et al., 1991; Garaj-Vrhovac & Zeljezdić, 2002; Jovicic et al., 2010).

Our analysis of the observed parameters in relation to smoking habit was based on literature data showing that, although the influence of cigarettes on MN frequency may be different and often conflicting, the final conclusion is that a statistically significant increase in damage should be expected in persons smoking more than 30 cigarettes a day. Collection of mandatory and detailed data on smoking habits, as well as the number of cigarettes smoked during each biomonitoring study, has been recommended because a simple comparison of smokers and non-smokers may lead to invalid results (Bonassi et al., 2003).

The research of various authors has revealed that some pesticides have genotoxic properties (Grover et al., 2003, Jovičić et al., 2013). Occupational exposure to such xenobiotics may result in covalent binding of their molecules to DNA, which leads to chromosome alterations and may be an initial event in the process of chemical genotoxic carcinogenesis.

Cytogenetic damage in individuals occupationally exposed to pesticides has received the attention of investigators in several countries but no definitive conclusions have yet been made. Reviews on this matter (Bolognesi, 2003; Bull et al., 2006) suggest that most studies have detected increases in biomonitoring indices of genotoxicity after pesticide application.

Bonner et al. (2010) reported that smokers occupationally exposed to pesticides exhibited significantly more total chromosomal aberrations than non-smokers who had no contact with pesticides. Some studies have also shown higher levels of cytogenetic biomarkers in smokers than non-smokers (Carbonell et al., 1990). Bhalli et al. (2006) reported that smoking had an additive effect on the frequency of BNMN (binucleated lymphocytes with micronucleus) and MNL (micronuclei in binucleated lymphocytes). Some other confounding factors, such as smoking habit and exposure time, were also analyzed (Bhalli et al., 2006). Thus, smoking had an additive effect on the frequency of BNMN and MNL, as more BNMN and MNL were found in the lymphocytes of smokers than non-smokers. On the other hand, micronuclei analyses conducted by Lucero et al. (2000) and Pasastor et al. (2002)

had shown that smoking habits caused no significant elevation in chromosomal aberrations. All results of our study indicate that the level of cytogenetic damage was significantly affected by the subjects' exposure to pesticides. They also indicate a need for regular biomonitoring of persons occupationally exposed to various mixtures of pesticides.

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Učestalost mikronukleusa u limfocitima periferne krvi kod radnika srpske populacije izloženih pesticidima

REZIME

Mikronukleus test (MN) se koristi u biomonitoring studijama za praćenje genetičkih efekata kod ljudi izloženih pesticidima. Cilj rada je bio da se, metodom mikronukleus testa, utvrdi oštećenje genetičkog materijala kod ispitanika koji su profesionalno izloženi pesticidima. Istraživanja su obuhvatila 119 ispitanika koji su podeljeni u tri grupe: u kontrolnoj grupi bilo je 39 ispitanika, u grupi izloženoj pesticidima u procesu proizvodnje (proizvođači) bilo je 40 ispitanika i 40 ispitanika koji rade u primeni na terenu. Mann-Whitney U-test je pokazao statistički značajnu razliku između srednjih vrednosti parametara svih ispitivanih varijabli u odnosu na kontrolnu grupu. Statistički značajne razlike konstatovane su između muškaraca u proizvodnji i primeni za parametar MN4, potom između nepušača u proizvodnji i primeni za parametere MN2, MN3, MN4 i NB, kao i u okviru cele posmatrane populacije između kontrolnih i radnika u proizvodnji za parametar MN2, i između proizvodnje i primene za parametar MN3. Spearman-ov test korelacije je pokazao pozitivnu korelaciju između učestalosti mikronukleusa i starosne dobi ispitanika, kao i navika vezanih za pušenje. Utvrđene su i statistički značajne razlike između ispitanika koji rade u proizvodnji i ispitanika koji rade u primeni u odnosu na praćene citogenetičke parametre. Svi dobijeni rezultati upućuju na zaključak da radnici koji rade na terenu ne koriste adekvatna sredstva lične zaštitite. Takođe, rezultati pokazuju da postoji potreba za kontinuiranim biomonitoringom radnika koji su u kontaktu sa pesticidima.

Ključne reči: Pesticidi; profesionalno izlaganje; mikronukleus test; Srbija