BIOMONITORING OF GENOTOXIC RISK IN RADAR FACILITY WORKERS: COMPARISON OF THE COMET ASSAY WITH MICRONUCLEUS ASSAY AND CHROMATID BREAKAGE ASSAY

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Abstract

Genotoxic risks of occupational exposure in a radar facility were evaluated by using alkaline comet assay, micronucleus assay and chromatid breakage assay on peripheral blood leukocytes in exposed subjects and corresponding controls. Results show that occupational exposure to microwave radiation correlates with an increase of genome damage in somatic cells. The levels of DNA damage in exposed subjects determined by using alkaline comet assay were increased compared to control and showed interindividual variations. Incidence of micronuclei was also significantly increased compared to baseline control values. After short exposure of cultured lymphocytes to bleomycin, cells of occupationally exposed subjects responded with high numbers of chromatid breaks. Although the level of chromosome damage generated by bleomycin varied greatly between individuals, in exposed subjects a significantly elevated number of chromatid breaks was observed. Our results support data reported in literature indicating that microwave radiation represents a potential DNA-damaging hazard. Alkaline comet assay is confirmed as a sensitive and highly reproducible technique for detection of primary DNA damage inflicted in somatic cells. Micronucleus assay was confirmed as reliable biomarkers of effect and chromatid breakage assay as sensitive biomarker of individual cancer susceptibility. The results obtained also confirm the necessity to improve measures and to perform accurate health surveillance of individuals occupationally exposed to microwave radiation.

Introduction

The people of industrialised societies are continuously exposed to increasing levels of electromagnetic fields (EMF) emitted by various electrical installations and telecommunication systems [1]. In recent years there has been growing interest in the health effects of the electromagnetic radiation's designated extremely low frequency (ELF) and radiofrequency radiation (RFR). There is evidence that microwaves cause different biological effects depending upon field strength, frequencies, wave forms, modulation and duration of exposure. These effects were mainly attributed to microwave heating but recent reports have shown or suggested that there are nonthermal microwave effects in terms of energy required to produce various types of molecular transformations and alterations [2]. It is known that exposure to microwave radiation has different biological effects on eye, the nervous system and its function, circulatory and the reproductive system [3,4]. Reports on cytogenetic risks from microwave exposure on the induction of chromosome damage are sometimes contradictory, mainly because of different experimental conditions of *in vitro* and *in vivo* studies [5]. However, in occupationally exposed subjects increased levels of DNA damage as expressed by means of cytogenetic endpoints were observed [3, 6-8]. Increased incidence of micronuclei is also reported after *in vitro* exposure to microwave radiation on human lymphocytes [9]. It has been suggested that exposure to radiofrequency radiation may have genetic effects which predispose to the development of cancer, particularly lymphoma and leukaemia, and also birth defects such as Down's syndrome [10-11].

Biomonitoring of human populations exposed to potential mutagens / carcinogens is an early warning system for genetic diseases or cancer. It also allows detection of different risk factors at a time when control measures still could be implemented [12]. For the detection of early biological effects of DNA-damaging agents in environmental and occupational settings a wide range of methods is presently used. Among them, there are well-established cytogenetic biomarkers such as structural chromosomal aberrations (CA), micronuclei (MN) and sister chromatid exchanges (SCE) [13,14,15]. During the last decade, the comet assay or single cell gel electrophoresis (SCGE) was introduced as a useful technique for human biomonitoring studies [16,17]. Contrary to cytogenetic biomonitoring techniques that are limited to circulating lymphocytes and proliferating cell populations, the comet assay can be applied both to proliferating and non-proliferating cells. Moreover, the comet assay is a rapid, simple and

sensitive technique for measuring DNA breakage with a small number of cells and detects intercellular differences in DNA damage [18,19].

Long term occupational exposure to physical and chemical mutagens often is related with an elevated risk for DNA damage. Most exposures in an occupational setting are chronic or repeated and the induced DNA damage might be expected to be elevated above baseline levels. The individuals with even slight deficiency in any step of DNA repair can accumulate mutations, and consequently their cancer risk is elevated. The simple and inexpensive test, as chromatid breakage assay, which allows selection of persons with a defect in DNA repair, could be a useful additional marker in biomonitoring studies [20]. Susceptibility to bleomycin-induced chromatid breaks in cultured peripheral blood lymphocytes may reflect the way a person deals with carcinogenic challenges [21].

The objective of the present study was to evaluate the levels of primary DNA damage, chromosomal damage and mitotic spindle disturbances as well as the mutagen sensitivity in peripheral blood leukocytes of radar-facility workers daily exposed to microwave radiation compared to unexposed control subjects. As sensitive biomarkers the alkaline comet assay, micronucleus assay and chromatid breakage assay (bleomycin sensitivity test) were selected.

Subjects and methods

The study was carried out on 10 clinically healthy male workers, employed on radar equipment and antenna system service. They worked within a microwave field of 10 (W/cm^2 – 20 mW/cm^2 with frequency range of 1250-1350 MHz. Their average age was 50.1 yr (range 45 - 56 yrs) and mean employment duration 24.3 yrs (range 7-29 yrs). Two subjects were smokers, three of them were ex-smokers and five non-smokers. The control group consisted of 10 subjects of similar age. Three of them were smokers, two ex-smokers and five non-smokers. All subjects were questioned concerning age, their presented medical history, family history on cancer, lifestyle, occupational and extraoccupational exposure to potential mutagenic hazards. Samples of peripheral blood were collected by venipuncture into heparinized tubes in the morning. The heparinized blood was then coded and stored in the refrigerator (4^oC). The alkaline comet assay and initiation of in vitro blood cultivation for the cytogenetic endpoints and chromatid breakage assay were performed within a maximum of 2 h period after blood collection.

The alkaline comet assay was carried out under alkaline conditions, basically as described by Singh et al. [16]. Fully frosted slides were prepared by covering with 1 % and 0.6 % normal melting point (NMP) agarose (Sigma). After solidification, a layer containing the whole blood sample mixed with 0.5 % low melting point (LMP) agarose (Sigma) was put on the slide and afterwards covered with 0.5 % LMP agarose. Slides were immersed for 1 h in ice-cold freshly prepared lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, 1 % Na-sarcosinate (Sigma), pH 10) with 1 % Triton X-100 (Sigma) and 10 % dimethyl sulfoxide (Kemika). The slides were then placed on a horizontal gel-electrophoresis tank, facing the anode. The unit was filled with fresh electrophoretic buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13.0). Denaturation (20 min) and electrophoresis (20 min at 25 V or 300 mA) were performed at 4^{o}C under dim light. After electrophoresis the slides were rinsed gently three times with a neutralisation buffer (0.4 M Tris-HCl, pH 7.5) to remove excess alkali and detergents. Each slide was stained with ethidium bromide (20 μg/ml) and covered with a coverslip. A total of 100 randomly captured comets per sample were examined at 400 x magnification using an epifluorescent microscope (Zeiss) connected through a black and white camera to an image analysis system (Comet Assay II, Perceptive Instruments Ltd., U.K.). To quantify the DNA damage, tail length (TL), and tail moment (TM) were evaluated. Tail length (length of DNA migration) is related directly to the DNA fragment size and presented in micrometers. It was calculated from the centre of the cell. Tail moment was calculated as the product of the tail length and the fraction of DNA in the comet tail. All measurements were sumarrized as the mean ± SE. Measured comet parameters were evaluated by using analysis of variance and non-parametric Mann-Whitney U test. The level of statistical significance was set at $p < 0.05$.

Lymphocyte culture - For each blood sample double cultures were set up. Lymphocytes were grown for 72 h at 37 ^{o}C in F-10 medium (Sigma, USA), supplemented with 10 % foetal calf serum (Biological Industries, Israel), phytohaemagglutinin (Murex Biotech Ltd., England) and antibiotics penicillin (Crystacillin®, Pliva, Croatia) and streptomycin (Streptomycin sulfat, Krka, Slovenia).

Micronucleus assay. The micronucleus assay was performed as described by Fenech and Morley [15] with some modifications. Cytochalasin-B (Sigma, USA) at final concentration of 6 (μg /ml was added to each sample at 44 h and the cells were harvested after a further incubation of 28 h. After a brief treatment with physiological saline, cells were fixed with 3:1 mixture of methanol

and acetic acid. Permanent preparations were obtained by pipetting a few drops of the cell suspension onto clean slides. Slides were air-dried overnight at room temperature and stained with 5 % Giemsa (Sigma, USA) solution at pH 6.8 for 10 minutes. After staining, slides were washed and air-dried. Incidence of MN was evaluated by scoring of 500 binucleated cells. Total number and distribution of MN was determined. The statistical significance of differences between frequencies of MN in exposed and control subjects was tested by using the χ^2 test. The level of significance was set at $p < 0.05$.

The chromatid breakage assay - Duplicate cultures were set up for each subject. The cultures were set up by adding 0.5 mL of whole heparinized blood to RPMI 1640 medium (Euroclone), supplemented with 10 % fetal calf serum and antibiotics. After the lymphocyte cultures were incubated for 3 days at 37^{o}C, they were incubated for 5 hours with bleomycin sulfate (30 μg/ml; Nippon Kayaku Co). To arrest the cells at metaphase, 0.04 μg/ml colchicine (Sigma Chemical Co) was added to the cultures 1 hour before harvesting. The cells were harvested for chromosome analysis by standard procedures, and slides were made by the conventional air-drying technique. Metaphase spreads were stained with 5 % Giemsa solution (Merck). 200 metaphase spreads per subject were scored blindly for the presence of chromatid breaks. Chromatid breaks were recorded by following previously established criteria [21]. The mean number of breaks per cell was used as a measure for the individual susceptibility. Differences between groups with respect to mutagen sensitivity were assessed by use of Mann-Whitney U test.

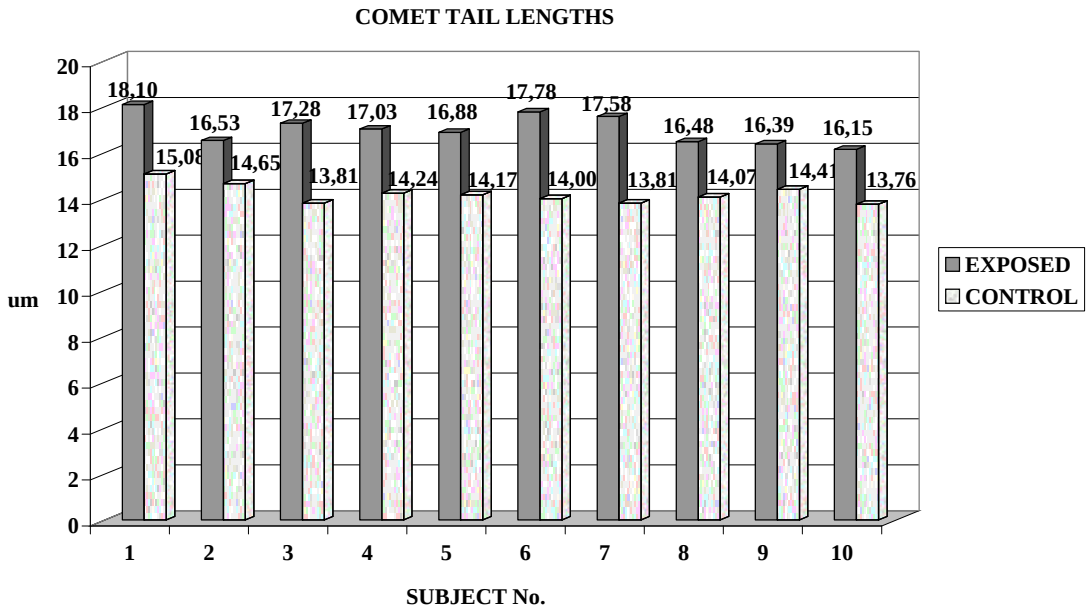
Results

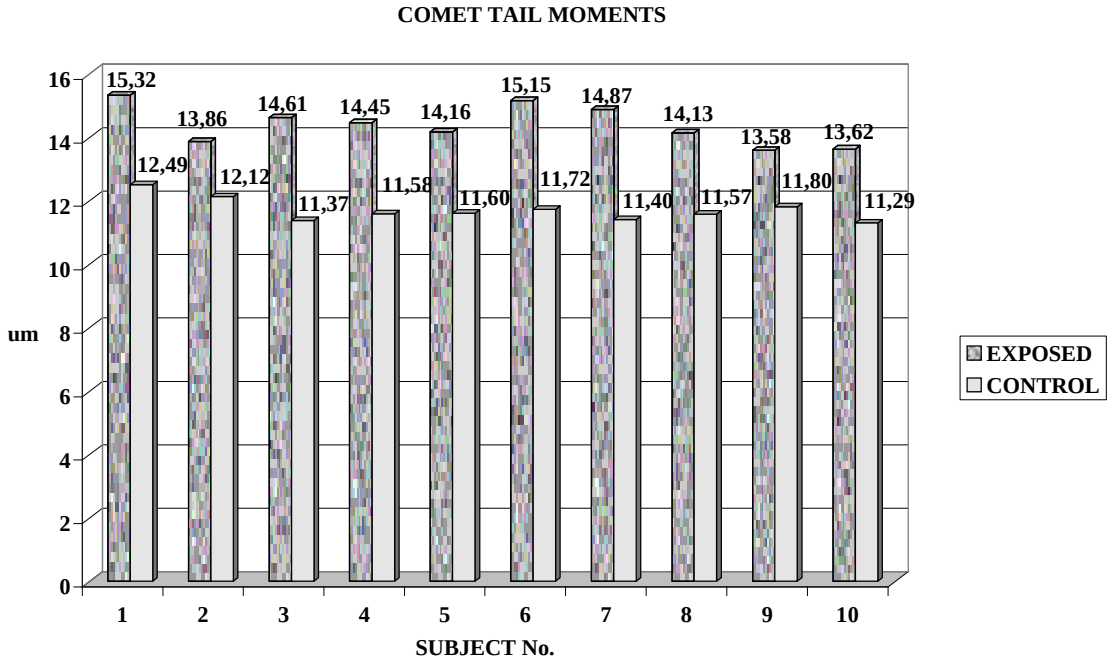
The alkaline comet assay

Results of the alkaline comet assay are shown in Figure 1. Between the exposed and control groups statistically significant differences in both comet parameters evaluated were observed. In blood samples collected from the exposed subjects significant interindividual differences in DNA migration pattern regarding both comet parameters were recorded ($p<0.05$, the analysis of variance), with a shift to the upper level of damage compared to control. For the exposed group the mean value of comet tail length was 17.06 ± 0.10 μm (range 12.18 μm - 53.85 μm), while a mean value of comet tail moment was 14.40 ± 0.09 (range 9.44 – 47.04). The highest DNA damage, as determined by means of comet tail length and tail moment was found to be in the subject No. 1. The observed DNA damage was found to be the lowest in the subject No. 10, however it was still significantly elevated compared to values of comet parameters measured

in control healthy blood donors (Figure 1). When compared occupationally exposed smokers (ex-smokers included) and non-smokers there were no significant differences regarding both comet parameters observed ($p>0.05$, Mann-Whitney *U* test). On the other hand, within the blood samples collected from control subjects significantly lower values of both comet parameters studied were recorded (Figure 1). Mean value of comet tail length was 14.20 ± 0.05 μm (range 10.90 μm – 18.95 μm), while a mean value of comet tail moment was 11.70 ± 0.05 (range 8.26 – 16.03). However, unexposed smokers (ex-smokers included) had significantly higher levels of overall DNA damage than non-smokers, as expressed by means of both comet parameters measured ($p<0.05$, Mann-Whitney *U* test). In unexposed smokers mean tail length was 14.39 ± 0.07 μm, and mean tail moment 11.93 ± 0.07, while in non-smokers mean tail length was 14.01 ± 0.06 μm, and mean tail moment 11.56 ± 0.06.

Figure 1. Results of the alkaline single cell gel electrophoresis on the peripheral blood leukocytes from 10 radar-facility workers daily exposed to microwave radiation and 10 unexposed control subjects.





Micronucleus assay

Results regarding the frequency, distribution and total number of micronuclei (MN) for the exposed and control group are reported in Table 1. In exposed group there were statistically significant increases in total number of micronuclei recorded ($p<0.05$ using (χ^2 test) compared to control. Distribution of micronuclei per 500 binucleated cells in all exposed subjects was also disturbed compared to control (Table 1).

Table 1. Distribution and total number of micronuclei (MN) in peripheral blood lymphocytes from microwave-exposed subjects and corresponding controls.

Subject		**Distribution of MN**				**Total No. of**
smoking / exposure		**0**	**1**	**2**	**3**	**MN / 500 cells**
EXPOSED GROUP						
1	**(S; 22)**	489	9	2	0	13
2	**(S; 29)**	484	15	1	0	17
3	**(ES; 28)**	478	19	3	0	25
4	**(ES; 26)**	484	15	0	1	18
5	**(ES; 26)**	482	16	2	0	20
6	**(N; 7)**	490	10	0	0	10
7	**(N; 26)**	472	13	4	1	24
8	**(N; 25)**	471	19	0	0	19
9	**(N; 29)**	489	11	0	0	11
10	**(N; 25)**	479	9	2	0	11
Mean ± SD						**16,8 ± 5,4**-
CONTROL GROUP						
1	**(S)**	495	3	1	0	5
2	**(S)**	494	6	0	0	6
3	**(S)**	495	5	0	0	5
4	**(ES)**	497	3	0	0	3
5	**(ES)**	494	5	1	0	7
6	**(N)**	496	3	1	0	5
7	**(N)**	496	4	0	0	4
8	**(N)**	496	4	0	0	4
9	**(N)**	494	6	0	0	6
10	**(N)**	497	3	0	0	3
Mean ± SD						**4,8 ± 1,3**

- $p<0.05$ compared to control (χ^2 test).

The chromatid breakage assay

The results of the chromatid breakage assay are summarised in Table 2. In occupationally exposed subjects chromatid breaks were found in 95-179 cells out of 200 metaphases scored (mean value of 123 damaged metaphases per subject). Most frequently there were 1 to 4 breaks in one damaged metaphase, frequently 5 to 7 breaks, and less frequently 8 to 11 breaks in one damaged metaphase. The multiple aberrations (>11 breaks in one cell) were seldom. The highest number of chromatid breaks scored per metaphase was 30, and it was recorded in two subjects (Nos. 2 and 4). The average number of breaks per cell (b/c) per person varied widely between exposed subjects, from a low value of 0.88 to as high as 3.66 (Table 2). The distribution of the b/c values recorded was shifted towards higher values compared to control. In control subjects chromatid breaks were found in 34-59 cells out of 200 metaphases scored (mean value of 44 damaged metaphases per subject). Most frequently there were 1 to 2 breaks in one damaged metaphase, frequently 3 to 4 breaks. The multiple aberrations were seldom. The highest number of chromatid breaks scored per metaphase was 14, and it was recorded in only one subject (No. 9). The average number of b/c per person varied widely between exposed subjects, from a low value of 0.39 to as high as 0.61 (Table 2). The differences in b/c values recorded between smokers and non-smokers in both study groups were not statistically significant.

Table 2. Distribution of results of the chromatid breakage assay on the peripheral blood lymphocytes from microwave-exposed subjects and corresponding controls.

Subject No.	**EXPOSED GROUP**			**CONTROL GROUP**		
	Metaphases with breaks (no.)*	**Mean (± SE) b/c**	**Min. – Max. b/c**	**Metaphases with breaks (no.)***	**Mean (± SE) b/c**	**Min. – Max.b/c**
1	114	1.61 ± 0.17	1-16	34	0.53 ± 0.11	1-11
2	179	3.66 ± 0.28	1-30	46	0.54 ± 0.10	1-11
3	95	0.88 ± 0.11	1-14	41	0.55 ± 0.11	1-12
4	144	2.27 ± 0.22	1-30	38	0.39 ± 0.07	1-7
5	114	1.22 ± 0.12	1-8	40	0.54 ± 0.11	1-13
6	127	1.46 ± 0.13	1-11	50	0.61 ± 0.11	1-10
7	100	0.88 ± 0.09	1-9	59	0.54 ± 0.09	1-11
8	154	2.86 ± 0.24	1-21	46	0.53 ± 0.09	1-7
9	98	0.97 ± 0.12	1-16	47	0.59 ± 0.12	1-14
10	103	1.10 ± 0.11	1-11	38	0.40 ± 0.08	1-10
Mean (± SE)	**123**	**1.69 ± 0.06 -**	**1-17**	**44**	**0.52 ± 0.03**	**1-14**

b/c – breaks per cell; *****200 metaphases per subject scored; - $p<0.05$ compared to control (Mann-Whitney *U* test)

Discussion

Almost every member of our technologically modern society is exposed to some nonionizing radiation. However, the intensity of these exposures in the general population is very low compared to exposures in occupational settings, where people may work near various sources [26]. Many epidemiological and experimental studies have been carried out to investigate the possible health hazards associated with human exposure to ELF or RF electromagnetic fields. Various studies have provided evidence suggesting that EMFs with relatively low intensity are capable of interacting with many molecular, cellular and systemic processes associated with carcinogenesis, mutagenesis and teratogenesis [1]. Biologically detrimental effect of the forementioned radiation in occupationally exposed persons has not yet been sufficiently explored and the existing data are often contradictory. Protection of the radar-exposed personnel is complicated due to the fact that there are no suitable personal dosimeters similar to those carried by subjects occupationally exposed to ionizing radiation. Therefore, using of different cytogenetic biomarkers become very important. In the present study potential genotoxic effects of the long-term occupational exposure to microwave radiation were evaluated on subjects daily employed on radar equipment and antenna system service. As sensitive biomarkers of the exposure, effect and susceptibility, the alkaline comet assay, micronucleus assay and chromatid breakage assay (bleomycin sensitivity test) were selected. For human biomonitoring of environmental and occupational exposure to mutagens and / or carcinogens different chemical and biological endpoints are used. Cytogenetic endpoints such as chromosomal aberrations, micronuclei and sister chromatid exchanges in peripheral blood lymphocytes have been extensively employed as cytogenetic biomarkers to assess genotoxic risks in the occupational settings. It has been found out that increased cytogenetic damage reflects an enhanced cancer risk [14]. During the last decade, the comet assay or single cell gel electrophoresis (SCGE) was also introduced as a sensitive and reliable biomarker in human biomonitoring studies [11,15-17].

Present study reports the results of the application of alkaline comet assay on peripheral blood leukocytes in long-term radar-exposed workers. Although the mechanism by which RFR affects DNA is not known, and the results of the reported studies on genotoxic effects of RFR exposure are often conflicting [4], our results indicate potential of RFR to induce measurable DNA-damaging effects in long-term exposed personnel. Our results indicate that the alkaline comet assay can be successfully applied to the study of microwave-induced primary DNA damage and repair in peripheral blood leukocytes of exposed subjects. By using the alkaline

comet assay in exposed subjects a lot of DNA single strand breaks and alkali-labile sites were detected. Similar results reported Lai and Singh [21] on rat brain cells after acute exposure to radiofrequency electromagnetic radiation. Their results indicate that acute exposure to RFR at an average body SAR of 1-2 W/kg caused a significant increase in both single- and double- strand DNA breaks. By using other cytogenetic endpoints it was also recorded earlier that similar occupational settings are associated with increased chromosome damage [5,6]. It should be pointed out that the type of DNA damage recorded by alkaline comet assay is rather continuously and efficiently repaired. Therefore, the measured damage level is a result of equilibrium between damage infliction and repair. It is known that DNA single strand breaks are rapidly repaired, however, other lesions, such oxidized bases may persist longer and be misrepaired to somatic mutations [15]. On the other hand, apurinic/apyrimidinic sites (alkali-labile sites) may not be readily repaired in G_0 cells and without DNA replication they may prove to be “silent” DNA lesions [22]. It is known that DNA damage accumulates with time and the repair capacity decreases during long-term occupational exposure. Therefore, when DNA damage is improperly repaired or is not repaired, different biological effects may result. Although majority of DNA lesions is repaired in a few hours and days following their infliction, a part of DNA damage induced persisted over time. It can be considered that this elevated level reflects an accumulation of non-repaired DNA damage. High level of persistent DNA damage can lead to cell death. Large numbers of cells dying can lead to serious organ failures while damaged or improperly repaired DNA may develop into cancers. Reports on the possible cancer-related effects of RFR are still controversial. However, it is possible that in complex cellular processes involved in carcinogenesis it could have co-carcinogenic effect [7]. Differences in an individual’s capability to deal with mutagenic assault can be measured using chromosomal aberration tests. In addition to measuring chromosomal aberrations in lymphocytes, the analysis of micronuclei in binucleate cytokinesis blocked lymphocytes has also been suggested for biological dosimetry. It is thought that any aberration or even a whole chromosome might become micronuclei. Our results also confirmed that enumeration of micronuclei provides a reliable index of chromosome loss from the main nuclei indicating clastogenic and aneugenic impacts of long-term occupational exposure to microwave radiation. Using the bleomycin-based mutagen sensitivity assay in present study radar-exposed and control subjects have been screened. The level of chromosome damage generated by bleomycin varies greatly between individuals. Our results indicate that observed

variability is not associated with age of the donor or duration of occupational exposure. Smokers and non-smokers were also present in the studied groups. However, similarly to age, this factor did not affect the b/c values significantly. Those results are in agreement with reports of other authors [18,19]. It seems that exposure to tobacco smoke induced resistance to bleomycin. Probably, the cells acquired a mechanism to detoxify various toxic compounds [23]. It is important to recognise that the susceptibility to DNA damage varies in different individuals. Our results have also shown that some exposed subjects exhibited significantly higher level of DNA damage as expressed by means of chromatid breakage assay. It could be assume that subjects with a high susceptibility phenotype in our study may, in fact, be heterozygotes of the some chromosomal instability syndromes. Namely, heritability of persistent chromosomal damage is well established for the severe chromosomal instability syndromes such as ataxia teleangiectasia and Fanconi anemia [19]. Avoidance of harmful occupational exposure, especially in sensitive persons may become an important factor in the prevention of cancer.

The results of present study indicate that the alkaline comet assay, as sensitive biomarker of exposure, can be successfully applied in assessment of primary DNA damage in occupationally exposed subjects. As a microdosimetric technique based on the selection of individual cells in a heterogeneous cell population on the basis of nuclear morphology and shape of comets it is suitable for *in vivo* human biomonitoring. By gathering information on clastogenic and aneugenic impacts micronucleus assay is confirmed as sensitive biomarker of effect. On the other hand, chromatid breakage assay (bleomycin sensitivity test) seems to be a useful biomarker of susceptibility in predicting individual risk of cancer. All methods employed in the present study provide powerful tools for successful biomonitoring of populations occupationally exposed to microwave radiation.

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