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Environmental genotoxicity evaluation using a micronucleus test and frequency of chromosome aberrations in free-living small rodents

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ABSTRACT

An *in vivo* micronucleus (MN) test in peripheral erythrocytes and frequency of bone marrow cells with chromosome aberrations in free-living small rodents, chronically exposed to heavy metal pollution were used for detection the genome response to genotoxic agents in the environment. Yellow-necked mice (*Apodemus flavicollis*), common vole (*Microtus arvalis*) and East-Mediterranean (Macedonian) mice (*Mus macedonicus*) were collected in a polluted region near lead-zinc smelting factory – Asenovgrad (South Bulgaria, near Plovdiv) and in the background region of the Strandzha National Park (Southeastern Bulgaria). Mean frequencies of MN and aberrant cells in the individuals from the impact region were significantly higher compared to the mean frequencies from the same species in the background region. The comparative analysis of results confirmed that the species *Apodemus flavicollis* and *Microtus arvalis* may be suitable bioindicators for biomonitoring studies using MN test and chromosome aberrations. Obtained results demonstrated that the *in vivo* MN test may be a sensitive end-point for the detection of genotoxity that may result from the simultaneous action of several metals and may be useful as a biomarker of environmental stress in situ.

Key words: micronucleus test, chromosome aberrations, small rodents

Introduction

Human activities induce changes in ecosystems by releasing pollutants into the environment. The need to detect and estimate the impact of pollution on natural environments has led to the search for sentinel species, so-called "biomonitors" and sensitive end-points. Free-leaving small mammals are suitable organisms to monitor environmental pollution on terrestrial ecosystems *in situ*, because they are known to concentrate the pollutants presented in the ecosystem, such as pesticides, radionuclides, heavy metals and other contaminants. In addition, they are usually abundant over easily identified areas and rapidly trapped.

The micronucleus (MN) test has been utilized on wild rodents since 1978 to investigate the genetic damage induced by environmental pollution. Significant correlations between heavy metal contamination and MN have been detected in wild rodents leaving in polluted areas (Cristaldi et al., 1985; Tice et al., 1987; Ieradi et al. 1992, 1996; Müller & Streffer, 1994; Tull-Singleton et al., 1994; Evans, 1997; Degrassi et al., 1999; Tapisso et al., 2009). Previous studies using four different species of wild living rodents, house mouse (*Mus musculus domesticus*), Algerian mouse (*Mus spretus*), yellow-necked mouse (*Apodemus flavicollis*) and bank vole (*Clethrionomys glareolus*), showed that MN frequencies in peripheral blood and bone marrow erythrocytes from Giemsa stained slides were increased in animals collected in contaminated areas from three European countries, i.e. Italy, Spain and the Czech Republic (Degrassi et al., 1999; Zima et al., 1999).

The area of the lead-zinc smelting factory is one of the most polluted regions in the South Bulgaria. According to data from the Plovdiv regional inspectorate of environment and waters (2008) the contamination is present by polymetal dust emission of lead, cadmium and zinc microagregates.

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Cytogenetic markers such as chromosomal aberrations (CAs) in free-living rodents involving gross alterations of the genetic material have been regarded as a sensitive endpoint for detecting genotixic effects induced by heavy metals and toxic chemicals (Tice et al., 1987; Ieradi et al., 1992; Topashka-Ancheva et al., 2003).

Animals from areas contaminated with lead and zinc have high concentrations of these metals in the liver and kidney, which, among other effects, was correlated with increased frequencies of micronuclei and sperm abnormalities (Ieradi et al., 1992). Cadmium is a potent genotoxic agent and its effects are dependent on the exposure time, probably due to the animals' inability to eliminate this metal from their tissues (Johannesson, 2002). One of the main mechanisms contributing to the genotoxic potential of cadmium is the inhibition of DNA repair by interacting with metal-binding sites of proteins involved in this process (Hartwiq & Schwerdtle, 2002). Lead toxicity is well known and includes renal, hepatic, neurological, hematological, and reproductive adverse effects, and also gene and chromosome mutations (Johnson, 1998). Lead induces a significant increase in the number of micronuclei, sister chromatid exchange and sperm abnormalities in Pb-treated animals but, in contrast with cadmium no significant increase in the induction of micronuclei and sperm abnormalities with the duration of the experiment has been found (Tapisso et al., 2009). Induction of lipid peroxidation and reactive oxygen species has been considered as one of the direct mechanisms underlying lead-mediated DNA damage (Acharya et al., 2003).

The genotoxicity effects of zinc were reported in several *in vitro* and *in vivo* studies (Walsh et al., 1994). The results of Tapisso et al. (2009), demonstrated that zinc can induce significant increases of MN frequencies (only after 10 doses) when compared with the control groups, although the effects are smaller than in animals treated with cadmium or lead of animals treated with 5 doses of cadmium. This confirms that zinc is less genotoxic than Cd or Pb.

The aim of the present study was to evaluate the performance of *in vivo* MN assay and CAs in the early detection of genotoxic agents in the polluted areas and in the identification of species and populations at higher risk. The comparison of the results with these, obtained previously in rodent species collected in different European countries, will contribute to the identification of a model species for ecological risk assessment.

Materials and Methods

The area of study covers two regions determined by "National Biomonitoring Program of Bulgaria" (1999) as impact (polluted – Asenovgrad, lead-zinc smelting factory) and background (unpolluted – Strandzha National Park).

In 2008 the concentration of Cd in the atmosphere in the Asenovgrad region was 13.45 ng/m^3 by TLV (Threshold Limit Value) of 0.00001 ng/m^3. The yearly average concentrations of 862 μg/m^3 SO_2, (2.46 times higher than the TLV), 292.63 μg/m^3 NO_2 and 76.21 μg/m^3 dusty aerosols were recorded in the area. The Strandzha National Park in the Southeastern Bulgaria also suffers from global air pollution caused by industrial emissions in this part of Southeastern Europe. However, the yearly average concentrations of pollutants are considerably lower and there are no important local sources of industrial pollutionis and the animals are not directly exposed to environmental pollution.

A total of 49 adult animals (27 from the polluted and 22 from the background region) of three rodent species (*Apodemus flavicollis*, *Microtus arvalis* and *Mus macedonicus*) were examined for the presence of MN in the circulating erythrocytes. To avoid intraspecific differences related to age and sex, only adult specimens were examined and data are presented pooling together the sexes, since the t-test analysis did not show significant differences between female and male animals within the same group.

The peripheral blood (5 μL) was collected from the tail vein of all experimental animals, and diluted in 45 μL of phosphate-buffered saline (pH 7.0). A drop of the solution was smeared on glass slides, fixed with absolute methanol for 10 min, dried in air, and stained with acridine orange according to Hayashi et al. (1983). The slides were randomized and coded to blind score within a fluorescence microscope at a magnification of 100× with B excitation filter. For each animal, 2000 peripheral blood erythrocytes (both normochromatic and polychromatic)/slides were counted for the presence of MN. All slides being scored by one person to avoid interobserver variability.

Mean MN frequencies, expressed as number of MN per 2000 erythrocytes, and standard deviation, and were calculated for each species. Data were analyzed for normality and MN frequencies were compared between species and between sampling sites by means of the Mann–Whitney U test (α= 0.01). All differences were tested with ANOVA (Statistika 7, 2004).

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The cytogenetic investigations of small rodents' species have been done using routine cytogenetic methods for chromosome analysis. The slides were made not later than 24 hours after trapping. The slide preparations with well-spread complete metaphases were obtained from colchicine-blocked bone marrow cells from the femur according to a routine method (Macgregor & Varly, 1986). The hypotonic treatment was carried out in 0.075M KCl for 20 min at 37°C. Fixation was carried out with methanol: glacial acetic acid (3:1). Flame dried bone marrow slides were stained with 5% Giemsa solution. Fifty well spread metaphases per animal were analyzed. The chromosome aberrations were recorded according to standard recommendations (Preston et al., 1987).

The chromosome rearrangements in the investigated species, collected in the impact stations were compared with those of the background station by the G test (Sokal & Rohlf, 1995). A probability of $P<0.05$ was taken as significant in all cases.

Results

The evaluation of the MN test in peripheral erythrocytes (Figure 1) of wild rodents, chronically exposed to heavy metal pollution, showed that there were differences in sensitivity of the species selected as bioindicators.

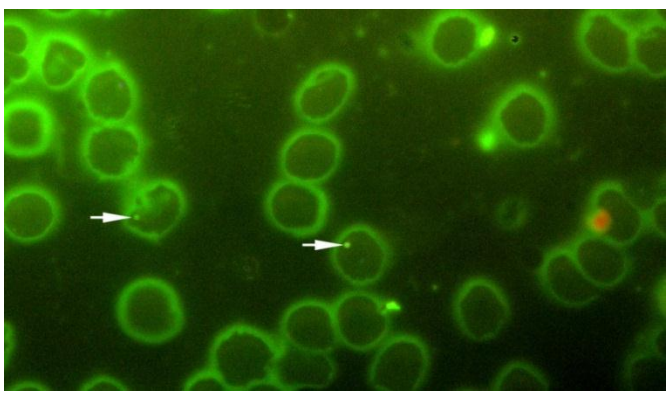

Figure 1. *Microphotograph of acridine orange-stained peripheral blood film of Apodemus flavicollis, showing micronucleated cells.*

Mean MN frequencies of *A. flavicollis*, *M. arvalis*, and *M. macedonicus* in the polluted region were 0.176%, 0.037% and 0.028% respectively (Table 1). Data analysis of MN frequencies yielded no significant differences among *M. macedonicus* and *M. arvalis* (U test $P>0.01$), but both species differed significantly from *A. flavicollis* (U test $P<0.01$). Overall, mean MN frequencies were elevated almost three-fold in *A. flavicollis* and two-fold in *M. arvalis* from the Asenovgrad region, as compared to samples from the Strandzha region (Table 1). Data analyses of the MN frequencies with respect to sampling sites (polluted vs. reference sites) yielded significant differences only in *Apodemus flavicollis* (U test $P<0.01$).

Table 1. *Frequencies of micronuclei observed in the erythrocytes of free-living rodents from unpolluted (Strandzha) and polluted (Asenovgrad) sites.*

Samples	Species	n	TMN	% MN frequencies ± SD
Asenovgrad	*A. flavicollis*	10	19	0.176 ± 0.017
	M. arvalis	12	20	0.037 ± 0.012
	M. macedonicus	5	24	0.028 ± 0.018
Strandzha	*A. flavicollis*	10	5	0.051 ± 0.011
	M. arvalis	12	3	0.016 ± 0.019

Yellow-necked wood mouse (*Apodemus flavicollis*) has a chromosome set 2n=48 (NF=46) and common vole (*Microtus arvalis*) has a chromosome set 2n=46 (NF=56). No variations from the standard karyotype of the investigated individuals were identified. The analysis of the type of chromosomal aberrations in the cells of small rodent species shows that the isochromatide breaks and pair fragments are predominant in almost all the examined individuals (Table 2, Figure 2). The number of aberrant cells increase significantly in the individuals from the impact region in comparison with these from the background region – *M. arvalis*, $G = 4.54$, $df = 1$, $P<0.05$.

However, the cells with chromosome aberrations in *A. flavicollis* were not in significantly higher frequency in comparison with unpolluted regions ($G = 1.99$, $df = 1$, $P<0.1$), but a tendency of increasing the genome instability of this species was found.

Discussion

The assessment of genotoxic effects of heavy metals compounds in the erythrocytes of the free living rodents have been conducted by determining the induction of MN. The present results show that the analysis of MN is a useful method to investigate the genetic damage induced in wild rodents living in areas neighbouring industrial settlements and, thereby, monitor environmental contamination *in situ* (Degrassi et al., 1999).

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Table 2. *Chromosome aberrations found in Apodemus flavicollis and Microtus arvalis (n – number of investigated individuals; m – male; f – female)*

Biotops	Species	Mitoses	Mitoses with aberrations	Breaks	Fragments	Exchanges	% cells with aberrations $\bar{x} \pm \delta$
Asenovgrad	*A. flavicollis* n=10 (4m+6f)	446	42	29	8	4	19.0±6.8
	M. arvalis n=12 (7m+5f)	582	59	31	2	7	11.42±1.3
Strandzha	*A. flavicollis* n=10 (2m+8f)	182	19	6	2	1	13.11±4.9
	M. arvalis n=12 (5m+7f)	294	12	4	3	2	9.73±1.9

In spite of these, the genotoxic mechanism of heavy metals remains unknown. Some metals can interact directly with DNA, while others interact primarily with proteins (Bilban, 1998).

Figure 2. *Chromosome aberrations in the bone marrow cells of Apodemus flavicollis (fragments).*

The data from cytogenetic analysis of bone marrow cells in rodents are similar to those established by other authors (Deknudt et al., 1977; Gerber et al., 1980; Degraeve, 1981; Ieradi et al., 1992; Topashka-Ancheva & Metcheva, 1999), which also determinated higher frequencies of chromosomal aberrations in the cells of wild rodents collected from regions with heavy metal pollution. The results of laboratory investigations suggest that the cytotoxic and genotoxic effects of the polymetal dust depend mainly on the chemical characteristics of the heavy metal content (Topashka-Ancheva et al., 2003). Ieradi et al. (2003) demonstrated that as a result of the synergistic interaction among the metals the polymetal dust particles from a heavy metal producing factory are more toxic than the action of each component alone.

The conventional analysis of MN frequencies and chromosome aberrations in free-living rodents has already been shown to be reliable indicators of environmental stress in several studies (Materiy & Maslova, 1978; Eckl & Riegler, 1997; Degrassi et al, 1999). The comparative analysis of results obtained in free living rodents collected in the same areas, indicated that the *Apodemus flavicollis* species may be a suitable species for biomonitoring studies using MN frequencies. This conclusion is in accordance with the statement of Degrassi et al. (1999), which suggest a higher suitability of the genus *Apodemus* in terrestrial ecogenotoxicological studies. The results obtained also confirm the higher resistance of *Mus sp.* to the induction of MN in terms of genetic make up, ecological characteristics and feeding habits of the species.

Conclusion

The present results show that the analysis of MN and the frequency of chromosome aberrations in the bone marrow cells are useful methods to investigate the genetic damage and, thereby, monitor environmental contamination *in situ*. The obtained results indicate that the MN assay in free living rodents may be a sensitive and representative end-point in ecological risk assessment in applied investigations on

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environment quality. Because natural populations in polluted environments typically are exposed to complex mixtures of pollutants, organisms from such populations do not express a simple dose-response curve with respect to any specific biomarker. Thus, the need exists to employ a battery of biomarkers in order to adequately characterize toxic effects.

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