Evaluation of genotoxic damage in wild rodents from a polluted area in the Czech Republic

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Received 11 December 2001; Accepted 22 July 2002

A b s t r a c t . An integrated system of evaluation of genotoxic damage was employed in two rodent species (*Clethrionomys glareolus, Apodemus flavicollis*) collected in a polluted and a control site in northern Bohemia. Elevated concentrations of heavy metals (Cd, Mn, Pb, Cu, Zn) were found in soil samples from the polluted site. Significantly higher concentrations of certain non-essential heavy metals were observed in kidney and liver of the animals studied in the polluted site, however, temporal variation between years and seasons was significant. Heavy metals originating from industrial aerial pollution may be not distinctly reflected in tissue concentrations recorded in small terrestrial rodents, in contrast to soil pollution resulting from mine works and/or from road traffic. In the same animal samples, micronucleus test and sperm abnormalities assays were used to study the correlation between environmental pollution and genetic damage. Higher genotoxic damage was demonstrated in the animals collected in the polluted site. Micronuclei frequencies appeared to be more suitable markers of mutagenicity than those of abnormal sperms. *A. flavicollis* seems to be a more suitable model in studies of environmental genotoxicity than *C. glareolus*.

Key words: environmental pollution, heavy metals, rodents, central Europe

Introduction

Many investigations have been performed to reveal heavy metal contamination in mammals. Field studies have shown that high levels of heavy metals in the environment may appropriately be monitored by the assessment of their concentrations in target organs of free-living small mammals. The increased levels have usually been considered to relate to environmental pollution, and significant relationships were found between residues of metals in soil and body organs or tissues (W r e n 1986, S h o r e 1995). Bioaccumulation of heavy metals in tissues of small terrestrial mammals was studied in different areas of several countries (e.g. J o h n s o n et al. 1978, A n d r e w s et al. 1984, H u n t e r et al. 1987, 1989, S a w i c k a - K a p u s t a et al. 1987, T i c e et al. 1987, W l o s t o w s k i 1987, C o o k e

et al. 1990, Talmage & Walton 1991, Ieradi et al. 1992, 1996, Tull-Singleton et al. 1994, Šumbera et al. 2003), and several species were proposed as promising indicators to estimate the hazards of heavy metal pollution.

One of the most important sources of environmental contamination with heavy metals is the coal industry. The dust emitted contains zinc, copper, lead and cadmium, and this contamination may increase the content of the metals in the tissues of mammals inhabiting polluted areas. Among heavy metals, copper, zinc and manganese play an important role in metabolism as components or activators of enzymes and their tissue concentrations are effectively controlled over a wide range of metal intake (Roberts & Johnson 1978). Other heavy metals such as cadmium and lead are non-essential and xenobiotic and their concentrations are physiologically more poorly regulated (Nelson Beyer et al. 1996). Therefore, these elements have to be included in the range applied in the environmental pollution assessment.

Our previous results, made particularly in relation to the occurrence of supernumerary chromosomes in *A. flavicollis,* suggested the animals from the polluted area at the town of Litvínov in northern Bohemia actually experience an increased genotoxic damage (Degrassi et al. 1999, Zima et al. 1999). Heavy metals may be among xenobiotic pollutants responsible for this genotoxic damage.

Monitoring of genotoxic damage usually includes various tests, such as cytogenetic disturbances analysed in somatic cells, and/or analyses of sperm abnormalities. These endpoints are based on tests validated on laboratory mice (Mavournin et al. 1990, Salamone & Mavournin 1994). The micronucleus test in bone marrow (Heddle et al. 1983) or peripheral blood erythrocytes (MacGregor et al. 1987) is widely used as a short term assay for the detection of agents which induce chromosome aberrations in somatic cells. The sperm abnormality assay was proposed by Wyrobek & Bruce (1975) as a system, which uses the sperm morphology as an index of mutagens or carcinogens. It is particularly relevant for studying the genotoxic effects on germ cells because in this system transmissible genetic damage from one generation to another takes place. Sperm cells may be examined rapidly and in large numbers, and this is important because the sensitivity of sperm cells do not exactly parallel that of many types of somatic cells. The micronucleus test, chromosome aberrations and sperm abnormality assays have been successfully used on wild rodents to put in evidence the correlation between genetic damage and radioactive contamination (Cristaldi et al. 1985, 1990, 1991) or heavy metal exposure in free-living animals (McBee & Bickham 1988; Ieradi et al.1992, 1996, Tull-Singleton et al. 1994).

In this study, an integrated system of mutagenicity tests is proposed to evaluate the response to the damage induced by mutagens and carcinogens, produced by industrial pollution. The two mutagenicity tests (micronucleus test and sperm abnormality assay) were applied to animals collected in two areas with different degrees of pollution, located in the Czech Republic, and compared with the level of heavy metal concentration assessed in certain tissues.

Material and Methods

The field research was performed in two areas of northern Bohemia in October 1995 and July 1996. The first study site, Litvínov (13°42'E 50°37'N), was located in the Most Basin, an area

with an extensive concentration of petrochemical industries and thermal power plants. The yearly average concentrations of 68 mg/m^3 of SO_2, 69 mg/m^3 of NO_x, and 70 mg/m^3 of dusty aerosols were recorded in the area by the Czech Ecological Institute (1997). The soil samples were collected and small mammals were trapped at the beginning of the Šumný důl Valley at the edges of an old beech forest. The control site was at a village Filipov near the town Česká Kamenice (14°23'E; 50°49'N), situated within the Labe River Sandstone Protected Landscape Area. The soil samples were collected and small mammals were trapped in small woods and shrubs along a stream. The weight, sex and age were determined in all trapped animals.

Heavy metals (cadmium, manganese, lead, copper, and zinc) were analysed in soil samples from both sites, and in kidney and liver. Liver and kidney were removed and stored at minimum -20 °C prior to analysis. All instruments used in sample preparation were rinsed with metal-free deionized water. Tissues were then digested in suprapur nitric acid and hydrogen peroxide and the solution was brought to volume with deionized filtered water. Soil cores (5.5 cm in diameter, 10 cm depth) were dried at 60 °C, processed by a standard sieve, and 500 mg was homogenized and digested with HNO_3 with following addition of H_2O_2. All the samples were analysed by Sequential Inductively Coupled Plasma Spectro-Photometer. Detection limits were as follows: 0.3, 1.2, 1.5, 2, 0.9 g/l (ppb) for Mn, Pb, Cd, Cu and Zn, respectively. Transformed data (log x + 1) were analysed statistically using Student t-test for independent groups ($p<0.05$). Altogether, 27 *A. flavicollis* and 28 *C. glareolus* were examined in Filipov, and 40 *A. flavicollis* and 29 *C. glareolus* from Litvínov.

The micronucleus (MN) test was performed in bone marrow and in peripheral blood. Marrow cells were flushed from a single femur of each animal with foetal calf serum. After centrifugation, the supernatant was removed and pellet was re-suspended in a small amount of serum and smeared on a clean glass slide. The slides, air dried and fixed with absolute methanol or alcohol, were stained with May-Grunwald (Cole & Arlett 1979). Peripheral blood, taken from heart, was prepared using the same method. Altogether, 27 *A. flavicollis* and 32 *C. glareolus* were examined in Filipov, and 28 *A. flavicollis* and 30 *C. glareolus* from Litvínov. From each animal, 2000 erythrocytes were analysed in bone marrow and in peripheral blood respectively.

The sperm abnormality assay was applied to mature males (totally 11 *A. flavicollis* and 11 *C. glareolus* from Filipov; 11 *A. flavicollis* and 4 *C. glareolus* from Litvínov). The number of mature males was low because many of them were affected by testicular regression. A suspension of sperm cells, obtained from *cauda epididymis*, was filtered through 80 µm and mixed with 1% of eosin-Y solution (10:1). The air-dried smears were mounted in Permount and coded for microscopic examination. The frequency of abnormal sperm cells (ASC) was scored in 1500 sperm cells (SC) per individual. Only cells with tails were taken into consideration. In order to assess the abnormal sperm morphology, the basic criteria of the classification of abnormal sperm cells of laboratory mouse were used (Wyrobek & Bruce 1975). Data obtained were analysed with routine statistical methods, such as the analysis of variance (ANOVA), t-test and G test.

Results and Discussion

The surface soil layer in Litvínov contained higher concentrations of the heavy metals studied than that in Filipov (Table 1), and higher contents of Cd and Pb were observed in 1995 in

Table 1. Metal soil concentrations (µg/g, wet weight) in the sites studied.

Metal	Filipov		Litvínov	
	1995	1996	1995	1996
Cd	0.97	0.18	34.33	1.87
Mn	186.5	165	279	246
Pb	22.75	20	47.13	35
Cu	20	13	18.6	26
Zn	44.57	46	72	88

comparison with 1996. In data obtained for metal tissue concentrations, variation between the trapping periods, sites, tissues and species was found. No difference was found in the concentrations between the sexes. The age structure in the groups of animals from the two sites and years did not allow us to perform any exact statistical analysis. Nevertheless a slight increase of cadmium and lead was observed in adult animals in comparison to the young. In the pooled samples from the both trapping periods, significantly higher concentrations of certain metals were found in the kidney (Pb, $p<0.05$; Cu, $p<0.001$; Zn, $p<0.001$), and in liver (Cu, $p<0.001$; Zn, $p<0.001$) in *A. flavicollis* from Litvínov than from Filipov. In the kidneys of pooled samples of *C. glareolus* from Litvínov a significantly higher concentrations of cadmium ($p<0.05$) and manganese ($p<0.01$) were found than in the sample from Filipov, whereas no significant differences were observed in the liver. The comparison of metal levels between samples collected in different periods revealed marked differences between 1995 and 1996. Elevated metal concentrations of heavy metals (particularly of Cd and Pb) were found in the animals collected from Litvínov in 1995 compared to 1996 (Tables 2, 3). This difference was similar to that found in soil samples. Distinct differences between cadmium and lead contents were observed between kidney and liver in *A. flavicollis.* (Pb: $t= 2.077$, $p= 0.043$; Cd: $t=2.928$, $p= 0.005$). Significant differences between the two sites were found more frequently in *A. flavicollis* than in *C. glareolus*. In particular, a significant increase in the cadmium content was observed in the kidney of *A. flavicollis.*

Our results thus demonstrated slightly elevated accumulation of heavy metals at the polluted site in Litvínov. In this site, the maximum concentrations of 20.00 µg/g wwt of lead and of 19.42 µg/g wwt of cadmium were found in the kidney of *A. flavicollis*. The concentration of cadmium exceeds the critical value (6.0 µg/g wwt) proposed by Aughey et al. (1984). The maximum concentration of lead did not reach the critical level which is indicative for adverse health effects in mammals (Ma et al. 1991, Shore & Douben 1994, Nelson – Beyer et al. 1996). Furthermore, our data indicate that temporal variation between years and seasons may be important in the monitoring of heavy metal pollution and the bioaccumulation assessment.

Environmental industrial pollution in the Most Basin produce considerable acidity of forest soils which is known to increase solubility of many metals (Bergvist 1986). Our data indicate that this supposed factor apparently did not markedly affect tissue concentrations in small rodents (Folkeson et al. 1990). It could be supposed that the heavy metal pollution originated from industrial air pollution is not distinctly reflected in tissue concentrations recorded in small terrestrial rodents, in contrast to soil pollution resulting from mine works. Koroljeva et al. (1992) studied heavy metal concentrations in small mammals, including *C. glareolus* and *A. flavicollis* in southern Bohemia, and found rather low levels of burden in animals trapped in relatively unpolluted habitats.

Table 2. Tissue metal concentrations (wet weight, standard deviation in italics) in the samples of *C. glareolus* from Litvínov and Filipov collected in 1995 and 1996. n, sample size; µg/g, concentration. Differences between the sites indicated by * (p<0.05), ** (p<0.01), *** (p<0.001).

kidney	Filipov 1995		Litvínov 1995	
	n	µg/g	n	µg/g
Cd	5	0.35 *(0.25)*	6	1.60 *(1.57)*
Mn	7	1.34 *(1.13)*	6	3.90 *(1.43)***
Pb	11	1.71 *(2.33)*	6	2.92 *(3.23)*
Cu	12	6.41 *(4.20)*	6	10.63 *(7.59)*
Zn	12	18.42 *(19.11)*	6	26.33 *(27.16)*
	Filipov 1996		Litvínov 1996	
	n	µg/g	n	µg/g
Cd	8	0.13 *(0.13)*	13	0.36 *(0.27)*
Mn	8	0.96 *(0.77)*	17	1.82 *(0.88)*
Pb	2	0.27 *(0.18)*	14	1.76 *(0.20)*
Cu	15	3.71 *(1.02)*	20	1.54 *(2.33)*
Zn	15	18.34 *(7.61)*	20	7.33 *(8.33)*
liver	Filipov 1995		Litvínov 1995	
	n	µg/g	n	µg/g
Cd	2	0.80 *(1.12)*	6	1.82 *(1.51)*
Mn	8	2.34 *(1.25)*	6	3.06 *(1.44)*
Pb	12	0.82 *(0.27)*	6	0.93 *(0.19)*
Cu	11	4.83 *(1.53)*	6	10.55 *(7.65)**
Zn	12	18.49 *(5.21)*	7	22.60 *(2.58)*
	Filipov 1996		Litvínov 1996	
	n	µg/g	n	µg/g
Cd	11	0.12 *(0.12)*	12	0.32 *(0.48)*
Mn	16	1.61 *(0.67)*	22	1.98 *(1.23)*
Pb	1	0.13	5	0.40 *(0.26)*
Cu	16	3.89 *(1.30)*	22	3.91 *(1.43)*
Zn	16	17.96 *(5.22)*	19	15.49 *(6.73)*

The data obtained from the mutagenicity tests are presented in Table 4. The micronucleus test (Fig.1) in peripheral blood erythrocytes shows a significantly higher frequency of micronuclei in wood mice collected in Litvínov than those collected in Filipov (t=2.568, p=0.013). The frequency of micronuclei in the bone marrow cells was also higher in Litvínov, but the difference was not significant (t=1.663, p=0.102). A similar significant increase of MN frequency in peripheral blood was observed in bank voles from Litvínov in comparison with Filipov (t=2.787, p=0.007). No significant differences were observed in the bone marrow cells, however. The MN frequency observed in peripheral blood in *A. flavicollis* ($\bar{x}$=2.50±1.72) was significantly higher (t=2.997, p=0.004) than in *C. glareolus* ($\bar{x}$=1.4±1.09); the MN frequency in bone marrow was also significantly higher (t=3.248, p=0.002) in *A. flavicollis* ($\bar{x}$=2.31±1.83) than in *C. glareolus* ($\bar{x}$=0.91±0.57). The results obtained in peripheral blood micronucleus test in both species indicate possible absence of spleen function, demonstrated previously in *Mus spretus* (Tanzarella et al. 2001). Regarding the influence of the age, a small increase in the MN frequency was observed in adult wood mice in comparison with subadults , both in peripheral blood and in bone marrow.

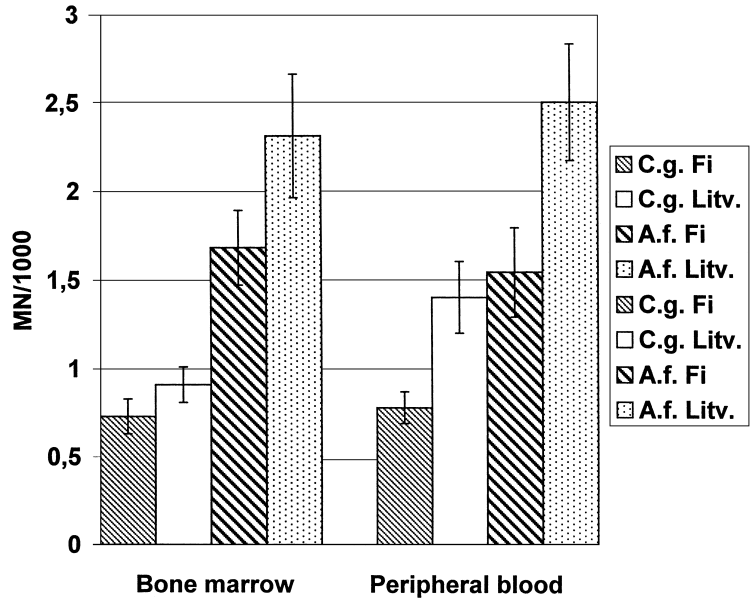

Fig. 1. Frequencies of micronuclei (MN/1000) observed in bone marrow and in peripheral blood of *A. flavicollis* (*A.f.*) and *C. glareolus (C.g.)* collected in Filipov (Fi) and Litvínov (Litv).

The results were quite similar in bank voles, but the difference was not significant. No significant differences in MN frequency were observed between males and females in both species.

The frequency of abnormal sperm cells in both species was relatively low (Fig. 2), however a higher incidence of the abnormal sperm cells was observed in wood mice from Litvínov in comparison with Filipov (t=2.0897, p=0.049), whereas the difference observed in the frequency of abnormal sperm cells between *C. glareolus* collected in Litvínov and Filipov was not significant. The level of sperm abnormalities was higher in *A. flavicollis* than in *C. glareolus* (t=2.3878, p=0.0328).

Conclusions

Environmental mutagenetic effects related to industrial and other pollution have affected more intensively the animals collected in Litvínov than those collected in Filipov at Česká Kamenice. Therefore, according to the available data, this locality can be considered heavily polluted and exposed to a higher mutagenetic impact than Filipov. This conclusion is congruent with the previous results obtained in studies of CREST-staining of micronuclei and scoring of chromosome aberrations in the same sites (D e g r a s s i et al. 1999, Z i m a et al. 1999). Most of the mutagenicity tests performed indicated a significantly higher genotoxic impact in the samples from Litvínov than from Filipov. The micronucleus and chromosome aberration frequencies seem to be more suitable markers than abnormal sperm cells frequency to investigate the genetic damage produced in animals living in areas displaying environmental contamination. In particular, the micronucleus frequency in peripheral blood has demonstrated to be a more effective biomarker for detection of chronic exposition to environmental contaminants.

Table 3. Tissue metal concentrations (wet weight, standard deviation in italics) in the samples of *A. flavicollis* from Litvínov and Filipov collected in 1995 and 1996. For explanations see Table 2.

kidney	Filipov 1995		Litvínov 1995	
	n	µg/g	n	µg/g
Cd	6	0.31 *(0.24)*	9	6.73 *(6.51)***
Mn	15	3.67 *(2.98)*	9	5.77 *(1.27)**
Pb	13	1.10 *(0.43)*	9	5.57 *(6.35)***
Cu	15	3.82 *(1.23)*	9	4.97 *(1.09)**
Zn	15	14.92 *(3.80)*	9	46.28 *(37.29)***
	Filipov 1996		Litvínov 1996	
	n	µg/g	n	µg/g
Cd	2	0.55 *(0.07)*	12	0.70 *(0.66)*
Mn	6	1.02 *(0.96)*	16	1.68 *(0.99)*
Pb	5	1.64 *(1.38)*	19	2.10 *(1.47)*
Cu	9	3.68 *(1.23)*	23	4.98 *(1.38)*
Zn	12	17.45 *(3.35)*	23	26.76 *(10.26)*
liver	Filipov 1995		Litvínov 1995	
	n	µg/g	n	µg/g
Cd	8	0.85 *(0.78)*	9	2.01 *(2.67)*
Mn	14	4.06 *(1.68)*	9	7.57 *(0.91)****
Pb	15	0.90 *(0.60)*	9	4.40 *(6.42)**
Cu	15	3.18 *(0.88)*	9	12.35 *(6.37)****
Zn	15	15.89 *(4.09)*	9	33.94 *(15.01)****
	Filipov 1996		Litvínov 1996	
	n	µg/g	n	µg/g
Cd	12	0.23 *(0.30)*	27	0.41 *(0.78)*
Mn	12	1.44 *(0.32)*	31	2.53 *(1.50)*
Pb	12	0.64 *(0.38)*	14	0.80 *(0.91)*
Cu	12	3.38 *(1.62)*	31	4.77 *(2.17)*
Zn	12	22.49 *(13.46)*	31	31.90 *(10.63)*

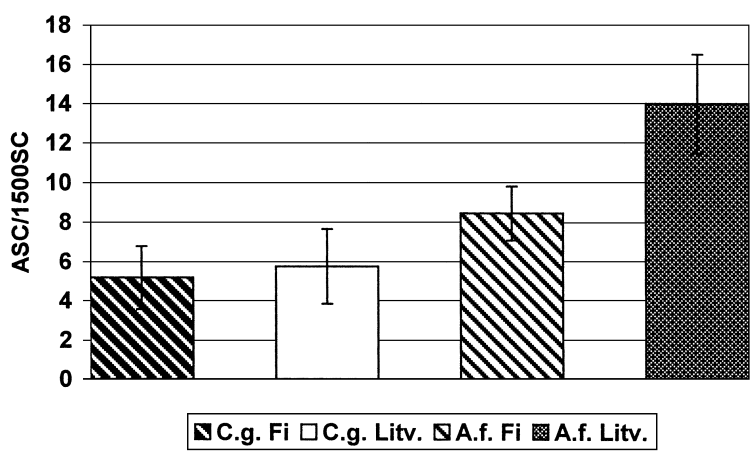

Fig. 2. Frequencies of abnormal sperm cells observed (ASC/1500 SC) in *cauda epididymys* of *A. flavicollis (A.f.)* and *C. glareolus (C.g.)* collected in Filipov (Fi) and Litvínov (Litv).

Table 4. Results of the tests of mutagenetic effects in samples from Litvínov and Filipov. MN/1000 = micronuclei per 1000 cells, ASC/1500 = abnormal sperm cells per 1500 cells, n = number of animals, $\bar{x}$ = mean, SD = standard deviation.

	site	n	MN/1000 peripheral blood		MN/1000 bone marrow			ASC/1500	
			$\bar{x}$	SD	$\bar{x}$	SD		$\bar{x}$	SD
A. flavicollis	Filipov	27	1.54	*1.32*	1.68	*1.10*	11	8.45	*4.56*
	Litvínov	28	2.50*	*1.72*	2.31	*1.83*	11	14.0*	*8.37*
C. glareolus	Filipov	32	0.78	*0.50*	0.73	*0.56*	11	5.18	*5.28*
	Litvínov	30	1.40*	*1.09*	0.91	*0.57*	4	5.75	*3.86*

The differences between the polluted and control sites were mostly more pronounced in *A. flavicollis* compared to *C. glareolus*. Therefore, the former species appeared a more suitable indicator of environmental pollution and genotoxic damage in the area under study. Similar conclusion was proposed by Abramsson-Zetterberg et al. (1997). Nevertheless, bank voles collected in ^{137}Cs contaminated sites showed an increased micronuclei frequency (Cristaldi et al. 1991).

The comparison of the results obtained in two different regions will allow an evaluation of the local situation as well as the achievement of the generalized conclusions. In fact, this research provides fundamental information on the selection of appropriate biomarkers of mutagens and carcinogens in the environment and may contribute significantly to the basic knowledge of industrial pollution.

Acknowledgements

We would like to thank Mgr. Milan Šťovíček, director of the SCHOLA HUMANITAS, Litvínov, for enabling us to use the laboratory and housing facilities. In particular, we would like to thank Mariangela Coriandri and Alessandra Sartoretti for their support in the field and laboratory work. A part of this work was supported by the Italian National Research Council (n. 96.00084.CT04 – CNR, Italy). Work in the Czech Republic was partly supported by the Grant Agency of the Czech Republic (no. 206/97/0850) and the Ministry of Education, Youth and Sports of the Czech Republic (no. VS 97102).

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