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DNA Damage Detected by Comet Assay in *Apodemus flavicollis* (Melchior, 1834) from Strandzha Natural Park

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Abstract: Genotoxicity monitoring using the Comet assay on peripheral blood leukocytes was performed. Blood samples of yellow-necked wood mouse (*Apodemus flavicollis*) from different protected areas of the SNP (Lopushna, Silkosia and Sredoka) in the autumn of 2010 and 2011 were collected. An increase in the Comet assay parameters in the analysed individuals of yellow-necked mouse from Sredoka protected area was established. Those results indicate that there was genetic damage in some mice populations and a chronic contamination was still present.

Key words: Comet assay, genotixicity, zoomonitoring, *Apodemus flavicollis*

Introduction

Strandzha Nature Park is the biggest protected area in Bulgaria and belongs to the localities with the most representative examples of biodiversity of the small mammals' fauna in Bulgaria.

Rodents are routinely used as bioindicators of environmental contamination, partly due to their ubiquitous distribution, high relative abundance, r-type reproductive strategy, ease of trapping and handling, limited home range size and close association with the soil. Mice from the genus *Apodemus* have been shown to be relevant pollution bioindicators (Berry 1975; Abramsson-Zetterberg *et al.* 1997; Stopka, Mcdonald 1999; Atanasov *et al.* 2000; Adrian *et al.* 2002; Damek-Poprawa, Sawicka-Kapusta, 2003; Ieardi *et al.* 2003; Metcheva *et al.* 1999, 2001; Topashka-Ancheva *et al.* 2003). The consequences of such exposure on the biology of animals have been assessed using cytogenetic signs, such as: micronuclei frequencies (Abramsson-Zetterberg *et al.* 1997; Ieardi *et al.* 2003), chromosomal aberrations frequencies (Metcheva *et al*. 2001; Topashka-Ancheva *et al.* 2003; Velickovic, 2004), and sperm abnormalities (Ieardi *et al.* 2003). The species of the genus *Apodemus* are the most common members of small mammal communities in Bulgaria (Peshev *et al*. 2004). All maintained traits make them useful mammalian models for environmental monitoring (Ninova *et al.* 2004; Metcheva *et al*. 2001).

In recent years, the Comet assay, also known as a ''single cell gel'' (SCG) electrophoresis assay, has become an important tool for assessing DNA damage in exposed animal populations. This is the method of choice for population-based studies of environmental exposure to air pollutants, metals, pesticides, radiation, and other xenobiotics. The Comet assay provides a rapid, visual method for quantitatively assessing DNA damage in single cells and is established as a valuable tool in the investigations of fundamental DNA damage and DNA repair mechanisms as well as biomonitoring in wild animal and plant populations. (Fairbairn *et al.* 1995; Visvardis *et al.* 1997, 2000).

The aim of the present study is to evaluate the possible overall mutagenic potential of the environ-

ment in the SNP protected areas via genotoxic analyses of a zoomonitor rodent species.

Material and Methods

The area of study covers locations inside the SNP: Lopushna (LOP), Silkosia (SIL) and Sredoka Reserves (SRD) (Fig.1) and an impact region (IR) in the area of the Asenovgrad lead-zinc smelting factory as a negative control. According to data from the Plovdiv regional inspectorate of environment and waters (2008) the contamination in the impact region is present by polymetal dust emission of lead, cadmium and zinc microagragates.

The yellow-necked mouse is a nonprotected rodent, which attained high population densities, that lives as a predominant small mammal species in the park and it is highly suitable as an ''*in situ*'' pollution zoomonitor. A total of 28 individuals of *A. flavicollis* from SNP, 18 collected in October 2010 and 10 in October 2011 were analysed. The 15 mice from the IR were caught in October 2011. The individual home range of *A. flavicollis* obtained by the inclusive boundary strip method varied from 100 to 2300 m^2 (Vukicevic-Radic *et al*. 2006). In according with these data we assume that due to its usual home range the animals which were collected on each location were of different generations. Therefore, as the limits of each trapping area were within the territory of the above-mentioned home range, we believe that the collected mice were spent their lifetimes inside the areas of trapping.

The animals were killed by cervical dislocation and prepared immediately for analysis. Blood was collected in heparin by cardiac puncture, placed in vials with DMSO (0.1%).

The Comet assay (CA) was applied on peripheral blood leukocytes as described by Singh *et al*. (1988), with some modifications. Stained nucleotides were scored visually using a fluorescence microscope equipped with a camera. Fifty comets on each slide, coded and blindly scored, were acquired using an image analysis system to obtain 100 images for each mouse examined. To quantify the induced DNA damage, we considered two parameters: the tail moment (TM) and the percentage of DNA in the tail. TM is a measure of the migrated DNA in the tail multiplied by the tail length (Olive et al., 1990). In both the tables and the figures, TM values are expressed in arbitrary units ±SD. DNA % in the tail is expressed as the proportion of DNA in the tail vs. the total DNA in the Comet.

Analysis of variance (ANOVA) was applied and a t-test for independent groups was used to calculate the level of significance for differences in the mean value of TM and % of DNA in the tail of mice collected in single sites. The level of significance was established at $P \leq 0.05$. All analyses were carried out using the GraphPad Prism 5 package.

Results

The DNA damages in investigated mice were evaluated on the basis of apoptotic cells, which appearing as a Comet cells (fig.2). The mean values (± SD) of TM and % DNA in the tail of *A. flavicollis* collected in the four sampling sites are presented in Table 1. In 2010 samplings no significant differences among the TM and the DNA% values of the mice from the three protected areas in the SNP were found (F= 2.24, P=14.1; F=1.72, P=0.21 respectively). The TM and DNA% values of the mice from the IR were significantly higher than those in the LOP and SIL mice (Table 1). On the contrary, these parameters between SRD and IR mice show no significant differences (TM: P= 0.14; DNA%: P=0.14). Mice sampled at

Table 1. Mean values of TM and % of DNA in mice collected in four different sites

Site	N	October 2010		N	October 2011	
		TM ± SD	% DNA in the tail ± SD		TM ± SD	% DNA in the tai ± SD
LOP	5	8.45 ± 6.03[a]	7.48 ± 4.22[c]	3	12.67 ± 8.15	14.5 ± 8.11
SIL	6	11.23 ± 8.67[b]	10.15 ± 7.05[d]	4	17.5 ± 12.10	18.8 ± 19.31
SRD	7	18.95 ± 10.84	17.05 ± 12.88	3	19.96 ±13.47	22.24 ± 17. 99
IR	11	30.28 ± 19.78	29.8 ± 19.31	15	38.13 ± 26.79	32.8 ± 19.34

Note: Statistically significant differences between investigated protected areas using t-test: a) IR>LOP P=0.022; b) IR>SIL P=0.015; c) IR>LOP P=0.025;d) IR>SIL P= 0.031.

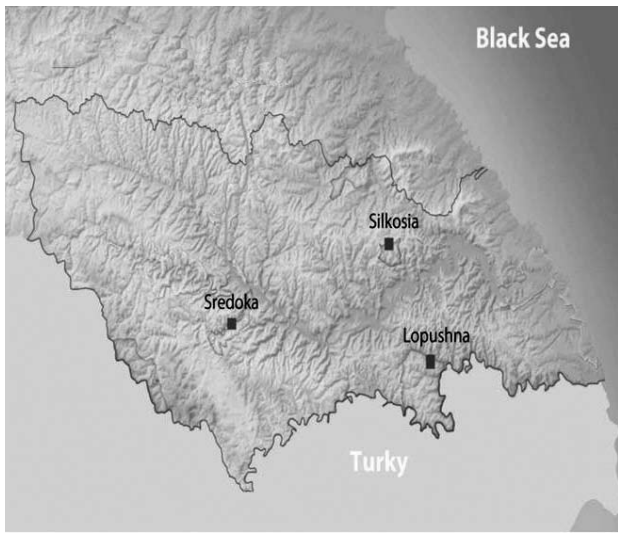

Fig. 1. Sampling sites inside the Strandzha Nature Park.

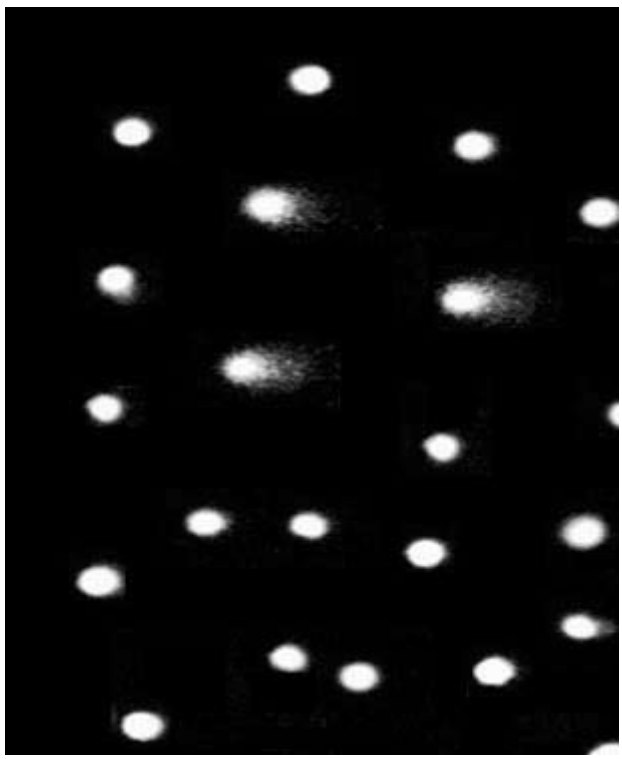

Fig.2. Comet images with different levels of DNA damage.

SRD appear as those showing higher genotoxic damage as a whole, as compared with those animals from Sites LOP and SIL. The data analyzes for the 2011samples shows other tendency. There are no significant differences between the investigated parameters among the mice from the protected areas (TM: F=0.31, P=0.74; DNA%: F=0.17, P=0.85). Also, no significant differences were found in parameters values among mice from all investigated sites (TM: F= 1.23, P=0.32; DNA%: F= 1.82, P=0.75) in 2011 samplings. Comparing the data from mice collected in the different years TM values and DNA percentages were significantly higher (F=36.10, P= 0.002) in 2011 samplings.

Discussion

In the present study there was significantly greater DNA damage score in the SRD protected area than in the other investigated areas in the SNP in 2010. On the other hand the animals from the 2011 samplings had significantly more DNA damage than the animals sampled in 2010. This indicates that the background contamination in the SNP is not an accidental occurrence. The area of SNP may suffer from global air pollution caused by industrial emissions in this part of the country. In our opinion, the result indicates that a chronic contamination is still present and the significantly increase of TM and % DNA values between the 2010 and 2011 samplings may reflects the enlargement of the industrial activeness (renewed mining industry in the SNP areas, air contamination of the closely located refinery near Burgas) and automobile use. Different toxicants find its way into plants and animals beings through the consumption of water and food, and the breathing of air.

The results obtained in this study are in accordance with results presented by Chassovnikarova *et al.* (2010) and Michailova *et al*. (2010), which investigated other genetically end points (micronucleus frequencies and chromosomal aberrations) for evaluation of the genome response of *Apodemus flavicollis* to environmental stress agents in the region of Asenovgrad lead-zinc smelting factory. In both studies genome instability was found, realized by many structure chromosome rearrangements and increase of micronucleus frequencies. The comparative analysis of these results and the present study indicates that the *Apodemus flavicollis* is a suitable species for zoomonitoring and confirms the suitability of the applied Comet assay as a low cost and effective indicator of genotoxicity, and hence suitable marker of potential environmental stress.

The Comet assay allows DNA damage to be detected in individual cells after acute and/or chronic exposure (Singh *et al*. 1988; Tice *et al.* 1991, 2000; Festa *et al*. 2003; Scheirs *et al.* 2009). In the present study, the single cells gel electrophoresis was sufficiently sensitive to detect naturally occurring levels of genotoxic agents in the investigated protected areas.

The result suggests that the mutagenicity level in natural populations, living in protected areas, should be controlled and wild mice could be used as key organisms in pollution monitoring and environmental conservation in protected areas. Successive monitoring should be carried out to evaluate a long-term persistence of genotoxic agents inside the SNP.

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References

Abramsson-Zetterberg L., J. Grawe, G. Zetterberg 1997. Spontaneous and radiation –induced micronuclei in erythrocytes from four species of wild rodents: a comparison with CBA mice. – *Mutation research/Genetic Toxicology and Environmental Mutagenesis,* **393**: 55-71.

Adrian M., M. S. Johnson, J. A. Cooke 2002. Lead within ecosystems on metalliferous mine tailings in Wales and Ireland. Sci. Total Environ. **299:** 177-190.

Atanassov N., T.Chassovnikarova, G. Markov 2000. Zoomonitoring in Regional background station "Strndzha": Cytogenetic characteristic of *Apodemus flavicollis* Melch. – In: Procc. "75 years forestry education in Bulgaria", Sofia, 227-230.

Berry R. J. 1975. On the nature of the genetical distance and island races of *Apodemus sylvaticus*. – *Journal of Zoology*, **176**: 293- 296.

Chassovnikarova Ts., N. Atanassov, V. Kalaydzhieva, H. Dimitrov 2010. Micronucleus test from free living rodents as a biomarker for environmental stress in situ. – *Biotechnology& Biotechnological Equipment,* **24:** 390-396.

Damek-Poprawa M., K. Sawicka-Kapusta 2003. Damage to the liver, kidney, and testis with reference to burden of heavy metals in yellow-necked mice from areas around steelworks and zinc smelters in Poland, *Toxicology,* **186:** 1–10.

Festa F., M. Cristaldi, L. A. Ieradi, S. Moreno, R. Cozzi 2003. The Comet assay for the detection of DNA damage in *Mus spretus* from Doñana National Park. – *Environmental Research*, **94** (1): 54-61.

Ieardi, L.A., J.Zima, F. Allegra 2003 Evaluation of genotoxic damage in wild rodents from a polluted area in Czech Republic. – *Folia Zoologica,* **52:** 57-66.

Metcheva R., M.Topashka-Ancheva 1999. Investigations on the bioaccumulation and distribution of heavy metals and chromosome patterns in the monitor species yellow-necked wood mice (*Apodemus flavicollis*) from Rila National park. – *Contrib. Zoogeogr. Ecol. East. Mediterr. Region,* suppl. **1**: 31-36.

Metcheva R., S.Teodorova and M.Topashka-Ancheva 2001. A comparative analysis of the heavy metal and toxic elements loading indicated by small mammals in different Bulgarian regions. – *Acta Zoologica Bulgarica* **53:** 61-80.

Michailova P., N. Atanassov, J. Ilkova, T. Chassovnikarova, M. Duran, E. Karadurmus 2010. Genome response of model invertebrates and vertebrates species to stress agents in the environment. – *Biotechnology& Biotechnological Equipment*, **24:** 675-679.

Ninova P., T. Chasovnikarova, G. Luvchieva, N. Atanassov, G.Russev 2004. Comparisson of the ability of sisiter chromatid exchange test and comet assay to detect environmental mutagens. – *Proc. bulg.acad. sci*., **57 (**7): 109-112.

Olive, P. L., J. P. Banath, R. E. Durand 1990. Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells using the ''comet'' assay. – *Radiation Research*, **122**: 86-94.

Peshev Ts., D.Peshev and V. Popov 2004. Fauna Bulgarica, 27 (Mammalia), Edition Academica "Marin Drinov", Sofia. (In Bulgarian with English summary).

Scheirs A., An de Coen, A. Covaci, J. Beernaert, V. Kayawe, M.Caturla, H. de Wolf, P. Baert, P. Van Oostveldt, R. Verhagen, Ronny Blust, W.de Coen 2009. Genotoxicity in wood mice (*Apodemus sylvaticus*) along a pollution gradient: Exposure-, age-, and gender-related effects. – *Environmental Toxicology and Chemistry*, 25(8): 2154-2162,

Singh N. P., M. T. McCoy, R. R Tice and E. L.Schneider 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. – *Experimental Cell Research*, **175:** 184-191.

Stopka P., D. W. Macdonald 1999. The market effect in the wood mouse, *Apodemus sylvaticus*: selling information on reproductive status. – *Ethology* **105**: 969-982.

Sumner A. T. 1972. A simple technique for demonstrating centromeric heterochromatin. – *Experimental Cell Research,* **75**: 304-306.

Tice R. R., P. W.Andrews, O. Hirai and N. P. Singh 1991. The single cell gel (SCG) assay: an electrophoretic technique for the detection of DNA damage in individual cells. – In: C. R. Witmer, R. R. Snyder, D. J. Jollow, G. F. Kalf, J. J. Kocsis and I. G. Sipes (Eds.): Biological Reactive Intermediates. IV. Molecular and Cellular Effects and Their Impact on Human Health, Plenum Press, New York, 157-164.

Tice R. R., E. Agurell, D. Anderson, B. Burlinson, A. Hartmann, H. Kobayashi, Y. Miyamae, E. Rojas, J.C. Ryu, Y.F. Sasaki 2000. Single cell gel/Comet Assay: guidelines for in vitro and in vivo genetic toxicology testing. – *Environment Molecular Mutagenesis*, **35:** 206-21.

Topashka-Ancheva M., R. Metcheva, S. Teodorova 2003. A comparative analysis of the heavy metal loading of small mammals in different regions of Bulgaria II: Chromosomal aberrations and blood pathology. – *Ecotoxicology and Environmental Safety* **54** (2): 176-187.

Visvardis, E. E., A. M. Tassiou, S. M. Piperakis 1997. Study of DNA damage induction and repair capacity of fresh and criopreserved lymphocytes exposed to H2O2 and g-radiation with the alkaline comet assay. – *Mutation Research*, **383**: 71-80.

Visvardis, E. E., K. S. Haveles, T. A. Patarias, L. H. Margaritis, V. Sophianopoulou, E.G. Sideris 2000. Diversity of peripheral blood mononuclear cells as revealed by a novel multiple microgel ''Comet assay''.– *Environmental Molecular Mutagenesis*, **36**: 32–39.

Vukicevic-Radic O., R. Matic, D. kataranovski, S. Stamenkovic 2006. Spatial organization and home range of *Apodemus sylvaticus* and *A. agrarius* on Mt. Avala, Serbia. – *Acta Zoologica Academiae Scientiarum Hungaricae,* **52** (1): 81-96.