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ScienceDirect
Environmental Pollution 153 (2008) 590–593
ENVIRONMENTAL POLLUTION
www.elsevier.com/locate/envpol

Genotoxicity detected in wild mice living in a highly polluted wetland area in south western Spain

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Received 12 June 2007; received in revised form 3 September 2007; accepted 9 September 2007

We have found an increased genotoxic damage in wild mice in a highly polluted area from industry, mining and agriculture in SW Spain, as assessed by the Comet assay.

Abstract

A field study was carried out in the south of the Iberian Peninsula in an industrial area in the neighbourhood of Huelva city, SW Spain, and in a natural area (Doñana National Park) for comparison, to estimate the genetic risk induced by environmental pollution in wild mice. Genotoxic effects in a sentinel organism, the Algerian mice (*Mus spretus*) free living in the industrial area were compared with animals of the same species living in the natural protected area. The single cell gel electrophoresis, or Comet assay, was performed as a genotoxicity test in peripheral blood of mice. Our results clearly show that mice free living in the contaminated area bear a high burden of genetic damage as compared with control individuals. The results suggest that the assessing of genotoxicity levels by the Comet assay in wild mice can be used as a valuable test in pollution monitoring and environmental conservation.

Keywords: Rodents; Biotests; Genotoxicity; DNA damage; Pollution; Comet assay

1. Introduction

The environmental impacts of human industrial and agricultural activities are gaining increasing international attention and concern. The purpose of this study was to evaluate the environmental risk posed by a large chemical industrial settlement located near Huelva city, in the southwest of the Iberian Peninsula. Contamination with heavy metals from mining activity reaching the area in river water, as well as from intensive agricultural activity (mainly strawberry) was also an important component to add to the industrial focus.

When it comes to monitoring for genotoxicity in the environment, an issue of paramount importance is the proper selection of representative organisms as sentinels, as well as the performance of sensitive and reliable tests such as those designed for the evaluation of DNA damage. Regardless of the particular features of the contamination event as a whole, it is also very important that the assay(s) of choice has been fully validated by laboratories worldwide and can be used to monitor virtually any potentially endangered wild species. In earlier reports we selected as sentinel organisms the white stork (*Ciconia ciconia*) as well as the black kite (*Milvus migrans*), as appropriate bird species for evaluating the potential genotoxic effects in the wake of the toxic accidental spill of mining waste on wildlife of the Doñana area (Pastor et al., 2001a,b, 2004). The biotest applied as an indicator of DNA damage was the alkaline single cell gel electrophoresis (SCGE), or Comet assay (Singh et al., 1988), performed on peripheral blood lymphocytes from young birds sampled in the contaminated area and compared with reference animals with positive results as to an increase in genotoxic damage. Earlier studies carried out using small mammals have also demonstrated

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0269-7491/$ - see front matter
doi:10.1016/j.envpol.2007.09.008

that they are able to accumulate a wide spectrum of pollutants present in the ecosystem (Talmage and Walton, 1991). Some significant correlations between pesticides (McBee and Bickham, 1988), radioactivity (Cristaldi et al., 1991), or heavy metal contamination (Tull-Singleton et al., 1994; Ieradi et al., 1996) and genetic damage in free living rodents have been reported.

According to the above considerations, in the present investigation we have chosen the Algerian mouse, *Mus spretus* (Rodentia, Muridae), because this non-protected species typically inhabits marshlands, follows an r-type reproductive strategy, and attains high population densities (Camacho and Moreno, 1989). These traits may make this rodent a useful mammalian model in environmental biomonitoring. Samples collected in both the industrial settlement and a protected area were assessed by means of a rapid method of the SCGE or Comet assay (Daza et al., 2004) to determine the potential DNA damage induced by chemical mutagens.

2. Materials and methods

2.1. Collection sites and sampling procedure

The study was carried out in a marshland (''Estero Domingo Rubio'') nearby a large industrial settlement close to Huelva city and in a protected area, the ''Laguna de Santa Olalla'', inside the Doñana Biological Reserve in Doñana National Park for comparison (Fig. 1). The industrial settlement is a large petro-chemical complex located in the estuary formed by the mouths of the Odiel and Tinto rivers. It includes some chalcopyrite transformation plants and several phosphate fertilizer factories which use phosphorite as a primary material.

Inside the studied marshland, three areas were selected for sampling, according to the differences as to the possible main source of contamination. The main impact in Site no 1, on the western verge of ''Estero Domingo Rubio'', is expected to be due to potentially toxic heavy metals discharged from mining activity to the River Tinto (see Fig. 1). Sampling Site no 2, while also receiving an impact from the above, as a result of water entry in the marshland during high tide, is more exposed than no 1 to possible accidental leaks from the large petro-chemical settlement as well as to organic biocides and fertilizers profusely used in intensive agriculture (strawberry production). This latter is the main source of contamination in sampling Site no 3, located near the eastern border of the marshland studied. The natural area, Doñana Biological Reserve (''Laguna Santa Olalla'') is a marshland area located around the mouth of the Guadalquivir River.

In total, 166 Algerian mice were caught using live traps (Sherman type) from November 2006 to March 2007. Sex and weight were determined in all animals. Controls: 18 mice, 12 males (M) and 6 females (F); Site 1: 77 mice, 36 M and 41 F; Site 2: 48 mice, 24 M and 24 F; Site 3: 23 mice, 9 M and 14 F.

2.2. Blood sampling

Blood collection from mice was carried out as follows. Briefly, blood samples (0.5 mL) were collected from mice by cardiac puncture, placed in vials (Soria Greiner, Spain) with EDTA as an anticoagulant, and transported in coolers to the laboratory on the day of collection.

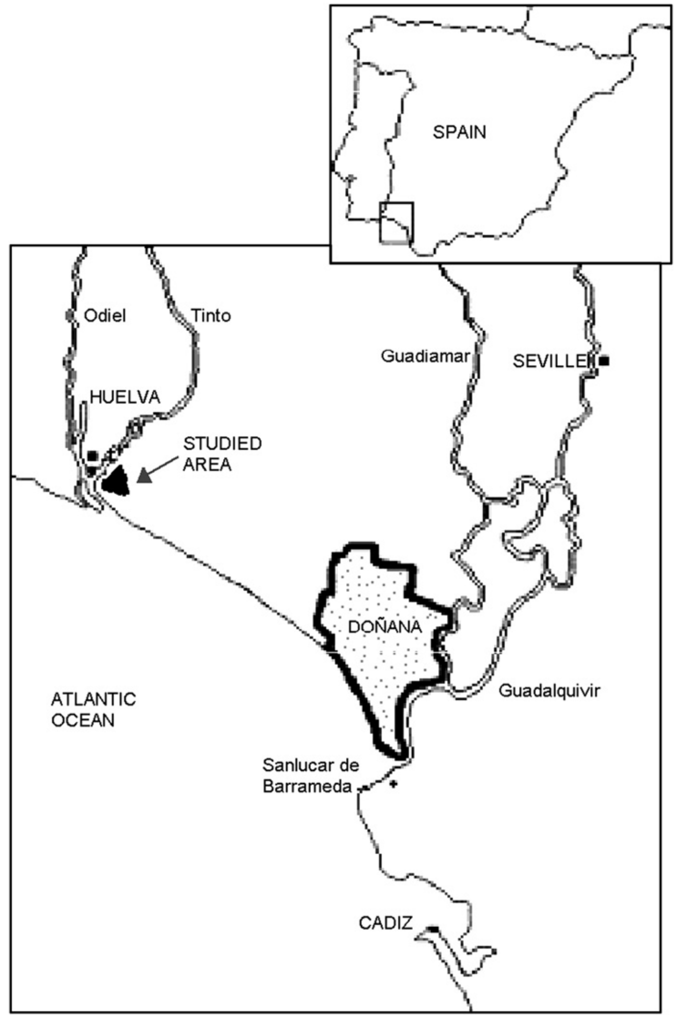

Fig. 1. Map of the studied highly polluted area (outskirts of Huelva, SW Spain) and control site (Doñana National Park).

2.3. Alkaline DNA-Comet assay

2.3.1. Slide preparation

Regular slides were coated with a 1% solution of standard agarose in distilled water, by immersing vertically for 2 s and air-drying to solidify the agarose. Once the slides were dry and totally transparent, they were kept at 4 °C and used up to 1 month after preparation.

We employed a modification (Daza et al., 2004) of the protocols described by Singh et al. (1988) and Fairbairn et al. (1995). Only 50–100 µL of cell solution was embedded in a 0.7% low melting agarose solution in PBS and immediately pipetted onto the coated slides and a coverslip was placed on. The slides were incubated at 4 °C for 10 min. Coverslips were removed and a third (low melting) agarose layer was added, together with new coverslips. They were again incubated at 4 °C for 10 min. Coverslips were finally removed and the cells were lysed by incubating for 1 h at 4 °C in the dark in a lysis solution containing: 10 mM Tris–HCl, 2.5 M NaCl, 100 mM Na_2-EDTA, 0.25 M NaOH, 1% (v/v) Triton X-100 and 10% (v/v) DMSO, pH 12.0.

2.3.2. Electrophoresis

In order to unwind the DNA, the slides were incubated for 20 min in electrophoretic buffer containing 1 mM Na_2-EDTA and 300 mM NaOH, pH 12.8. Electrophoresis was carried out at 1 V/cm for 20 min. After neutralization with 3×5 min washes of 0.4 M Tris–HCl pH 7.5 to remove alkali and detergent, cells were stained with the fluorochrome 4′,6-diamidino-2-phenylindole (DAPI) in Vectashield (mounting medium for fluorescence H-1000, Vector Laboratories).

2.3.3. Slide scoring

Cells were analysed using the Comet score program (an online free Comet scoring software). Two parameters were estimated for each Comet: (1) DNA damage (percentage migrated DNA in the tail), and (2) tail moment (tail length × tail intensity or percent migrated DNA). Five different classes of DNA damage were established. Class 1 corresponded to cells showing DNA damage between 0 and 20%, class 2 were cells with DNA damage between 20 and 40%; class 3 were cells with DNA damage between 40 and 60%; class 4 were cells with DNA damage between 60 and 80%; and class 5 were cells with DNA damage between 80 and 100% (highest damage).

For each experimental point at least 492 Comets were measured. Student's *t*-test was used for statistical evaluation.

3. Results and discussion

Since, in most cases, the general population is exposed to many potentially dangerous agents simultaneously, predicting health and environmental risk is a very complicated problem, because a mixture of genotoxic chemicals may undergo a variety of interactions, which can affect the transport, metabolism or molecular binding of the components. Thus, the biological effects of complex environmental mixtures, especially genotoxicity, seem to be very informative for risk assessment.

In environmental monitoring, when it comes to the selection of biotests for the assessment of genotoxic damage, there is a wide agreement in the validity and accuracy of the SCG electrophoresis or Comet assay (Fairbairn et al., 1995) as a highly sensitive, relatively inexpensive and reproducible method. Alkaline SCG DNA electrophoresis is especially sensitive in detecting DNA single strand breaks and alkali-labile damage in individual cells (Singh et al., 1988; Gedik et al., 1992). We made use of the Comet assay for the analysis of DNA damage in peripheral blood lymphocytes from birds living in the Doñana National Park sampled after the toxic spill of mine waste in April, 1998 (Pastor et al., 2001a,b, 2004) and our results showed a significantly increased level of genotoxic damage as compared with reference animals sampled in non-polluted locations.

In the present study we have made use of a rapid method applicable to G0 mammalian lymphocytes, which do not need to be isolated with the potentially cytotoxic drug Ficoll, as previously described by Daza et al. (2004). As can be seen in Table 1 and Fig. 2, DNA damage assessed by the Comet assay in mice sampled in all of the three highly contaminated sites along the marshland "Estero Domingo Rubio" (Huelva, Spain) was statistically significantly higher (Student's *t*-test) than that observed in control reference animals living in the Doñana National Park, an environmentally protected area. Significant statistical differences were as well observed among

Table 1
Genotoxic damage in the Algerian mice (*Mus spretus*) as assessed by the Comet assay

Sampling site	Number of mice	Male/female	Number of cells	DNA damage (%)	Tail moment
Control	18	12/6	492	4.39 ± 0.27	0.37 ± 0.06
Site 1	77	36/41	3936	7.75 ± 0.15*	2.02 ± 0.18*
Site 2	48	24/24	1871	7.82 ± 0.23*	1.38 ± 0.18*
Site 3	23	9/14	682	5.65 ± 0.30*	1.75 ± 0.31*

*Significant differences ($p < 0.01$; Student's *t*-test) respect to control values. Average DNA damage (%) and tail moment values for the different sampling sites.

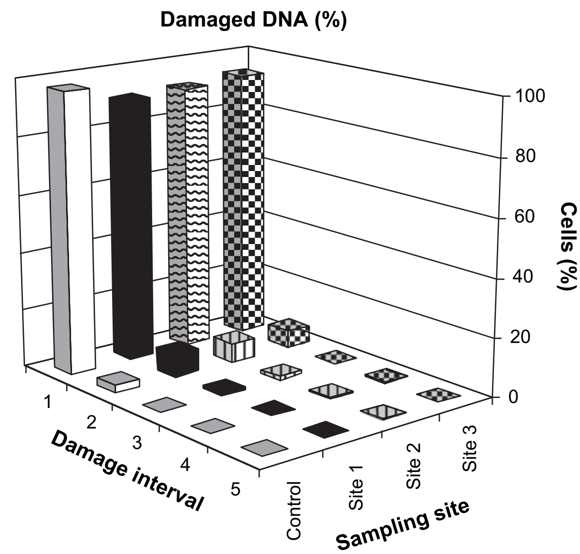

Fig. 2. DNA damage (Comet assay) in wild mice (*Mus spretus*) from different sampling sites. Intervals 1−5 are established according to increased DNA damage.

the particular sampling sites (1, 2, and 3) as to the parameters scored (percent of damaged DNA in the Comet tail, and tail moment of the Comets). Considering both DNA damage and mainly Comet tail values, mice sampled at Site 1 appear as those showing higher genotoxic damage as a whole, as compared with those animals from Sites 2 and 3 (Table 1 and Fig. 2). In our opinion, these differences in genotoxic damage can be the result of differences in exposure to some particular classes of mutagens, as we will discuss later.

The importance of using small mammals as monitors of environmental contaminants has been emphasized in the recent years (Talmage and Walton, 1991). Concerning this, factors worth considering are the type of contaminants under study as well as the appropriateness of the endpoints selected. The micronucleus test in the Algerian mouse (*M. spretus*) has been used previously to monitor genotoxic damage in both the Doñana National Park after the environmental disaster of 1998 (mining waste spill) (Tanzarella et al., 2001) and in the neighbourhood of the highly contaminated industrial settlement of Huelva (same area as in the present report) (Ieradi et al., 1998). Also, the Comet assay has been performed in *M. spretus* for the detection of DNA damage in the wake of the mining spill in Doñana (Festa et al., 2003). Taken as a whole, these biomonitoring studies have clearly shown that wild mice are excellent sentinel mammalian organisms for the detection of environmental contaminants.

Since this study using the Comet assay for the direct measurement of DNA damage in individual cells has been carried out in a population of mice living in a highly contaminated area under the influence of a variety of environmental mutagens from mining, industry and agriculture, at present we cannot conclude that the increased level of genotoxic damage is exclusively attributable to any of them in particular, but we can hypothesize as to a possible explanation for the differences observed among the different sites sampled in our study (Table 1 and Fig. 2). The industrial settlement near the area of study is a large petro-chemical complex that includes some chalcopyrite transformation and phosphate fertilizer plants which use radioactive phosphorite as a primary material. In this context, it is worth mentioning the existence of phosphogypsum deposits (an important source of phosphates, sulfates and arsenic, among others) located in the area of the Tinto Estuary. Besides, biocides and fertilizers from agriculture in the surrounding fields, and a high concentrations of heavy metals from mining activity reaching the area in rivers Tinto and Odiel extremely acidic waters (pH lower than 3) may synergistically add to the industrial contamination.

The above notwithstanding, although an important contribution of the different pollutants cannot be dismissed, in our opinion, according to our previous investigation in Doñana National Park after the ecological disaster of 1998 (Pastor et al., 2001a,b, 2004) a major influence of heavy metals can be considered as a working hypothesis. There exists an unquestionable body of evidence about the deleterious effects of heavy ions, such as Fe, As, Pb, Cd, Cu, Zn, Tl and Hg (LeBlanc and Bain, 1997; Fan et al., 1995). A description of how metal pollution occurs in the Tinto–Odiel estuary has been recently reported (Grande et al., 2003). In this study, an important presence of As, Cu and Zn has been demonstrated. As mentioned above, the highest level of genotoxic damage has been shown for sampling Site no 1, more in direct contact with the acidic waters rich in heavy metals in the Tinto–Odiel estuary than Sites 2 and 3, although these latter are also expected to receive the heavy metals impact as water flows to them during high tide.

In our opinion, apart from the importance of the present results for environmental monitoring, it is worth mentioning that the Algerian mouse (*M. spretus*) is included in the diet of at least 14 predator species, among them reptilian, avian and mammalian (Kufner, 1986). This latter represents a danger of transferring pollutants throughout the trophic web (Ieradi et al., 1998) and points to a serious impact in the environment as a whole.

Acknowledgements

We are grateful to Professors Juan López-Barea and Emilio Galán Huertos for their constant support. This work was supported by a Grant from Junta de Andalucía, Spain (Proyecto de Excelencia 2005/RNM-523).

References

Camacho, J., Moreno, S., 1989. Datos sobre la distribución espacial de micromamíferos en el Parque Nacional de Doñana. Doñana Acta Vertebrata 16 (2), 239–245.

Cristaldi, M., Ieradi, L.A., Mascanzoni, D., Mattei, T., 1991. Environmental impact of the Chernobyl accident: mutagenesis in bank voles from Sweden. International Journal of Radiation Biology 59, 31–40.

Daza, P., Torreblanca, J., Moreno, F.J., 2004. The comet assay differentiates efficiently and rapidly between genotoxins and cytotoxins in quiescent cells. Cell Biology International 28, 497–502.

Fairbairn, D.W., Olive, P.L., ÓNeill, K.L., 1995. The comet assay: a comprehensive review. Mutation Research 339, 37–59.

Fan, A., Howd, R., Davis, B., 1995. Risk assessment of environmental chemicals. Annual Review of Pharmacology and Toxicology 35, 341–368.

Festa, F., Cristaldi, M., Ieradi, L.A., Moreno, S., Cozzi, R., 2003. The comet assay for the detection of DNA damage in *Mus spretus* from Doñana National Park. Environmental Research 91, 54–61.

Gedik, C.M., Ewen, S.W.B., Collins, A.R., 1992. Single cell gel electrophoresis applied to the analysis of UV-C damage and its repair in human cells. International Journal of Radiation Biology 62, 313–320.

Grande, J.A., Borrego, J., Morales, J., De la Torre, M.L., 2003. A description of how metal pollution occurs in the Tinto–Odiel rias (Huelva-Spain) through the application of cluster analysis. Marine Pollution Bulletin 46, 475–480.

Ieradi, L.A., Cristaldi, M., Mascanzoni, D., Cardarelli, E., Grossi, R., Campanella, L., 1996. Genetic damage in urban mice exposed to traffic pollution. Environmental Pollution 92, 323–328.

Ieradi, L.A., Moreno, S., Bolívar, J.P., Cappai, A., Di Benedetto, A., Cristaldi, M., 1998. Free-living rodents as bioindicators of genetic risk in natural protected areas. Environmental Pollution 102, 265–268.

Kufner, M.B., 1986. Tamaño, actividad, densidad relativa y preferencia de habitat de los pequeños y medianos mamíferos de Doñana, como factores condicionantes de su tasa de predación. Ph.D. thesis, Universidad Autónoma de Madrid, Madrid.

LeBlanc, G.A., Bain, L.J., 1997. Chronic toxicity of environmental contaminants: sentinels and biomarkers. Environmental Health Perspectives 105, 65–80.

McBee, K., Bickham, J.W., 1988. Petrol-chemical related DNA damage in wild rodents detected by flow cytometry. Bulletin of Environmental Contamination and Toxicology 40, 343–349.

Pastor, N., López-Lázaro, M., Tella, J.L., Baos, R., Hiraldo, F., Cortés, F., 2001a. Assessment of genotoxic damage by the comet assay in white storks (*Ciconia ciconia*) after the Doñana ecological disaster. Mutagenesis 16, 219–223.

Pastor, N., López-Lázaro, M., Tella, J.L., Baos, R., Forrero, M.G., Hiraldo, F., Cortés, F., 2001b. b. DNA damage in birds alter the mining waste spill in southwestern Spain: a comet assay evaluation. Journal of Environmental Pathology, Toxicology and Oncology 20, 317–324.

Pastor, N., Baos, R., López-Lázaro, M., Jovani, R., Tella, J.L., Hajji, N., Hiraldo, F., Cortés, F., 2004. A 4 year follow-up analysis of genotoxic damage in birds of the Doñana area (south west Spain) in the wake of the 1998 mining waste spill. Mutagenesis 19, 61–65.

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

Talmage, S.S., Walton, B.T., 1991. Small mammals as monitors of environmental contaminants. Reviews of Environmental Contamination and Toxicology 119, 47–108.

Tanzarella, C., Degrassi, F., Cristaldi, M., Moreno, S., Lascialfari, A., Chiuchiarelli, G., Ieradi, A., 2001. Genotoxic damage in free-living Algerian mouse (*Mus spretus*) after the Coto Doñana ecological disaster. Environmental Pollution 115, 43–48.

Tull-Singleton, S., Kimball, S., McBee, K., 1994. Correlative analysis of heavy metal bioconcentration and genetic damage in white-footed mice (*Peromyscus leucopus*) from hazardous waste site. Bulletin of Environmental Contamination and Toxicology 52, 667–672.