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Assessment of genotoxic damage by the comet assay in white storks (*Ciconia ciconia*) after the Doñana Ecological Disaster

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Single cell gel electrophoresis, the so-called 'Comet' assay, was performed as a genotoxicity test in white storks sampled in an area heavily contaminated after the ecological disaster in south western Spain. This disaster occurred as a consequence of a massive toxic spillage of acid waste rich in heavy metals that impacted on the Doñana National Park. The importance of this protected area as a breeding and wintering site for many endangered bird species makes this analysis of DNA damage of special interest. Our results clearly show that white storks born in the contaminated area 1 year after the toxic spill bear a high burden of genetic damage as compared with control individuals. The possible implications for future survival as well as reproductive rate are discussed.

Introduction

Monitoring for genotoxicity in the environment requires the selection of representative organisms as sentinels, as well as the development of suitable and sensitive assays, such as those for the assessment of DNA damage. It is also very important that the selected assays have been widely validated by different laboratories and can be used to monitor virtually any species, regardless of the particularities of the contamination episode.

The Doñana Ecological Disaster, a toxic spill of acid waste rich in heavy metals from the processing of pyrite ore, on April 25, 1998 is considered to be one of the worst environmental disasters to have happened in western Europe, threatening the Doñana National Park (Spain), one of the most important wildlife sites in Europe, particularly as a breeding and wintering site for many bird species, including endangered ones.

The waste entered ecologically sensitive areas surrounding the marsh-lands of the park, causing sustained pH decreases and resulting in massive metal contamination. In addition to the high presence of heavy metals, such as arsenic, cadmium, lead and zinc, in the contaminating sludge waste, the acid conditions facilitated the solubilization of these metals, leading to water concentrations lethal to aquatic wildlife (Pain *et al.*, 1998).

Since many heavy metals are well known to be genotoxic, mutagenic, and even carcinogenic (LeBlanc and Bain, 1997; Yager and Ostrosky-Wegman, 1997), we investigated the possible level of genotoxic damage in white storks (*Ciconia ciconia)* living in this area. The alkaline single cell gel (SCG) electrophoresis, or 'Comet' assay (Singh *et al.*, 1988) was performed on peripheral blood lymphocytes from 50 young birds sampled in the contaminated area and compared with reference animals. The white stork is an appropriate species for evaluating the potential effects of the toxic spill on wildlife of the Doñana area, since it forages in marshlands preying on a variety of invertebrate and vertebrate species (Negro *et al.*, 2000). Thus, this species occupies a high position in the aquatic trophic chain, and this is one of the more important factors for explaining the accumulation of heavy metals in wetland birds in the area around Doñana within a few months after the spill (Benito *et al.*, 1999; Hernández *et al.*, 1999).

The suitability of the Comet assay for genotoxic analysis has been demonstrated in different systems such as trout (Belpaeme *et al.*, 1996), onion roots (Navarrete *et al.*, 1997), mussels (Wilson *et al.*, 1998) and different tissues of mice (Miyamae *et al.*, 1998).

In our study, the analysis of individual cells (comets) from storks living in Doñana National Park indicates a definitive increase in genotoxic damage, as shown by a significant increase in DNA migration, compared with that observed for control animals sampled in non-contaminated areas. These observations confirm the alkaline Comet assay as a sensitive and reliable endpoint for the detection of environmental genotoxicants. The possible present, as well as future, implications for the follow-up of endangered species are discussed.

Materials and methods

Collection sites and birds sampled

Fieldwork was conducted in June 1999 in the Dehesa de Abajo, where the largest colony of white storks in the Western Palearctic is sited (circa 300 pairs) (Tella and Hiraldo, unpublished data). This colony is less than 1 km from the area covered by the toxic spill of April 1998 (Figure 1). The biggest chick was sampled in each of 50 nests some days prior to fledgling. Additionally, 25 chicks were sampled in five sites far from the contaminated area (Figure 1) and used as controls. Age and sex of birds are factors that explained the accumulation of some metals in birds of Doñana a few months after the spill (Benito *et al.*, 1999; Hernández *et al.*, 1999). Therefore, the young white storks were sexed using the cellular fraction of the blood as a source of DNA and primers 2945F, cfR and 3224R, and aged according to a regression equation of age on the length of the wing (Negro *et al.*, 2000). Detailed information for each individual is provided in Table I.

Blood sampling and lymphocyte preparation

Blood samples (1 ml) were collected from the brachial vein, placed in vials (Soria Greiner, Spain) with edetic acid (EDTA) as an anticoagulant, and transported in coolers to the laboratory on the day of collection. Lymphocytes were isolated using a lymphocyte separation medium (Ficoll-Paque, Seromed), centrifuged in a density gradient (2000 r.p.m., 30 min) and washed in phosphate-buffered saline (PBS). Cell concentrations were adjusted to approximately 10^5 cells/ml PBS.

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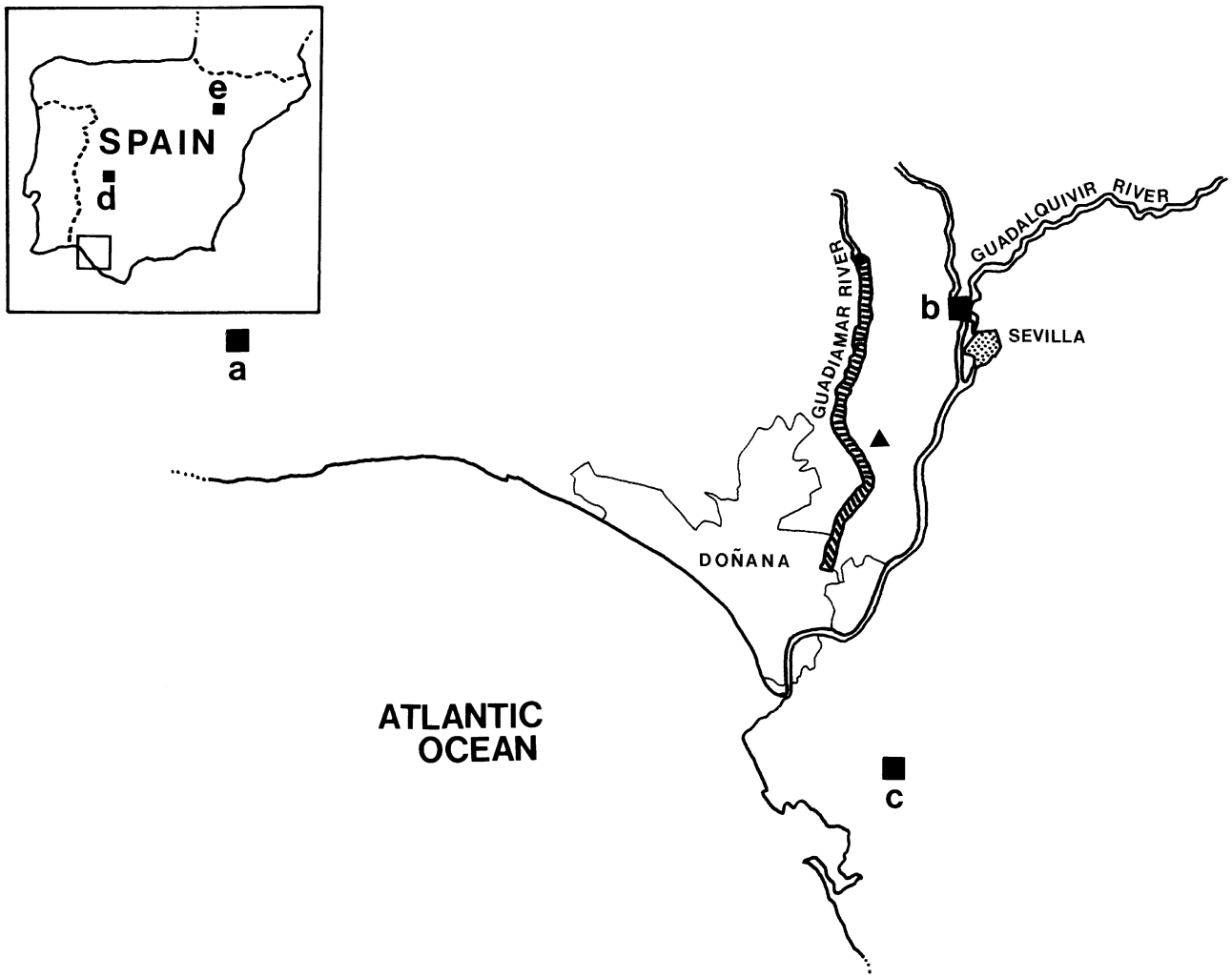

Fig. 1. Map of Doñana National Park and its surrounding area, with the location of sampling points where blood was collected from white storks nesting downstream of the contaminated Guadiamar river (▲) as well as in control locations (■, named a, b, c, d and e).

Comet assay

Slide preparation

The assay was basically performed according to the original protocols of Singh *et al.* (1988). Briefly, the standard slides were immersed vertically in 1% normal melting agarose (NMA) at 55°C and left vertically to allow the agarose to solidify. The slides were then kept at 4°C until use.

Approximately 10 000 cells were mixed with 85 µl low melting agarose (LMA; 0.7% in PBS) (FMC) at 37°C and the cell suspension was rapidly pipetted onto the first agarose layer, spread using a coverslip and kept at 4°C for 8 min for the LMA to solidify. The coverslips were then removed and a third layer of 100 µl 0.7% LMA at 37°C was added, covered with a coverslip and again allowed to solidify at 4°C for 8 min. After the top layer of agarose had solidified, the slides were immersed in a chilled lysing solution made up of 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl, 1% sodium sarcosinate, pH 10, with 1% Triton X-100 and 10% DMSO added just before use. They were kept at 4°C in the dark for at least 1 h to lyse the cells and to allow DNA unfolding.

Electrophoresis

The slides were removed from the lysing solution, drained and placed on a horizontal gel electrophoresis unit, side by side. The tank was filled with fresh chilled alkaline solution (1 mM Na_2 EDTA, 170 mM NaOH) at 4°C and pH 12.8, in order to detect double- and single-strand breaks as well as alkali-labile sites (Fairbain *et al.*, 1995). Before electrophoresis, the slides were left in the solution for 20 min to allow for the unwinding of DNA. Electrophoresis was carried out at low temperature (4°C) for 20 min at 1.6 V/cm and 300 mA. In order to prevent additional DNA damage, all the steps described above were conducted under yellow light or in the dark.

After electrophoresis, slides were gently washed in a neutralization buffer (0.4 M Tris–HCl, pH 7.5) to remove alkali and detergent, and stained with 50 µl 5 µg/ml DAPI in Vectashield (mounting medium for fluorescence H-1000, Vector Laboratories, CA, USA).

Slide scoring

Images of 50 randomly selected lymphocytes were analysed from each sampled bird. The slides were examined at 200× magnification using a 20× objective on a fluorescence microscope (OLYMPUS Vanox AHBT3), excitation filter of 550 nm and barrier filter of 590 nm. Measurements were made by image analysis CASys software (Synoptics, UK), and the parameter chosen was the tail moment (tail length×tail intensity or percent migrated DNA) (Olive *et al.*, 1990). The average

Table I. Locality, sex, age (in days) and mean tail moment of the nestling white storks sampled in this study

Locality[a]	Sex[b]	Age	Mean TM	Min TM	Max TM
p	M	55	0.141	0.003	0.490
p	M	52	0.911	0.001	2.890
p	F	62	0.433	0.003	2.187
p	F	52	0.290	0.013	1.647
p	M	59	0.596	0.015	2.727
p	F	53	0.416	0.013	1.633
p	M	55	0.436	0.003	2.696
p	M	53	0.153	0.001	1.115
p	M	51	0.108	0.003	0.277
p	F	50	0.138	0.002	1.335
p	M	61	0.161	0.002	1.688
p	M	61	0.067	0.001	0.358
p	M	52	0.251	0.019	1.386
p	M	43	0.271	0.005	1.521
p	M	52	0.125	0.005	1.064
p	F	44	0.124	0.004	0.899
p	M	49	0.283	0.004	1.6001
p	M	52	0.191	0.003	1.656
p	M	51	0.137	0.004	0.45
p	F	56	0.877	0.004	3.18
p	M	50	0.338	0.010	1.554
p	M	57	0.272	0.026	1.254
p	M	58	0.079	0.002	0.558
p	F	53	1.079	0.013	5.183
p	M	57	0.147	0.012	0.960
p	F	58	0.471	0.004	1.732
p	F	56	0.426	0.007	1.603
p	M	49	0.448	0.002	5.582
p	M	56	1.153	0.079	6.017
p	M	60	0.302	0.008	1.220
p	M	58	0.180	0.004	1.331
p	F	46	0.284	0.001	4.208
p	F	55	0.440	0.014	3.901
p	F	58	0.058	0.021	0.148
p	F	60	0.177	0.003	0.865
p	F	58	0.415	0.028	1.747
p	M	58	0.359	0.014	1.818
p	M	55	0.236	0.002	1.151
p	F	47	0.117	0.002	0.610
p	F	56	0.186	0.014	1.057
p	M	56	0.224	0.009	1.617
p	M	52	0.357	0.004	2.771
p	M	54	0.220	0.007	0.924
p	F	54	0.083	0.003	0.526
p	M	59	0.410	0.017	2.985
p	F	53	0.266	0.007	2.138
p	F	54	0.618	0.008	1.620
p	M	56	0.289	0.013	1.928
p	F	49	0.266	0.004	1.733
p	F	52	0.238	0.002	2.491
a	M	60	0.071	0.029	0.096
a	M	58	0.042	0.002	0.096
b	M	55	0.044	0.00	0.099
c	F	42	0.019	0.00	0.124
c	F	56	0.007	0.00	0.077
c	F	63	0.024	0.001	0.083
c	F	31	0.030	0.006	0.140
c	M	42	0.019	0.001	0.069
d	F	56	0.027	0.00	0.092
d	F	42	0.044	0.00	0.095
d	M	46	0.043	0.001	0.096
d	F	41	0.039	0.00	0.095
d	F	38	0.058	0.005	0.097
d	F	42	0.049	0.002	0.099
d	M	43	0.037	0.00	0.098
d	F	54	0.051	0.00	0.099
d	M	56	0.036	0.00	0.097
d	F	44	0.048	0.001	0.099
d	M	39	0.031	0.00	0.095
d	F	51	0.029	0.00	0.081
d	M	59	0.013	0.00	0.078
d	F	53	0.016	0.00	0.097
d	F	52	0.018	0.00	0.098
e	M	70	0.059	0.00	0.377
e	M	68	0.016	0.00	0.098

Table I Continued.

Table I. Continued

TM, tail moment.
[a]p, polluted area; a–e, control areas; see Figure 1.
[b]M, male; F, female.

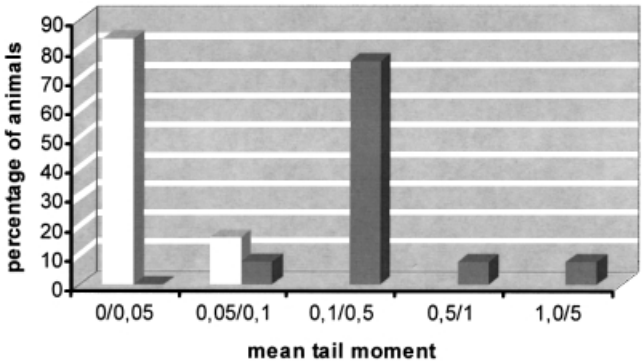

Fig. 2. Distribution of the percentage of animals showing different tail moments (comet assay) observed in lymphocytes from white storks located in the area contaminated after the mining waste toxic spill (dark) as compared with control animals. As can be seen, mean tail moments of the birds from the polluted area was much higher than those from controls ($P < 0.0001$; Student's *t*-test).

tail moment of the 50 cells from each bird was used for statistical purposes. Individual average tail moments were log-transformed to attain normality, thus allowing the application of parametric statistics. Then differences between the control and the exposed animals were tested for significance using the Student's *t*-test. Additionally, ANCOVA analysis was performed with age and sex of the birds as a covariate and a fixed factor, respectively, and the origin of birds (contaminated area versus controls) as the main random effect. All tests are two-tailed, and statistical significance is considered at $P < 0.05$.

Results

The large number of small chromosomes that make up their karyotype has made many animals (mainly fish, reptilia and birds) unsuitable for certain cytogenetic techniques, such as those assessing chromosomal aberrations or sister chromatid exchanges. In addition to overcoming this difficulty, the main advantage of the Comet assay stems from its independence from cell division (a pre-requisite for cytogenetic techniques) as well as the possibility of measuring DNA damage in individual cells.

Fifty blood samples from storks collected in the neighborhood of the Doñana National Park 14 months after the mine waste spillage as well as 25 blood samples from reference control animals were examined using the alkaline SCG assay and fluorescence image analysis. Figure 2 shows the distribution of the mean tail moments of white storks in different intervals, according to their origin. Most of the birds sampled near to the Doñana National Park show tail moments belonging to the interval 0.1–0.5. This value is clearly higher than that

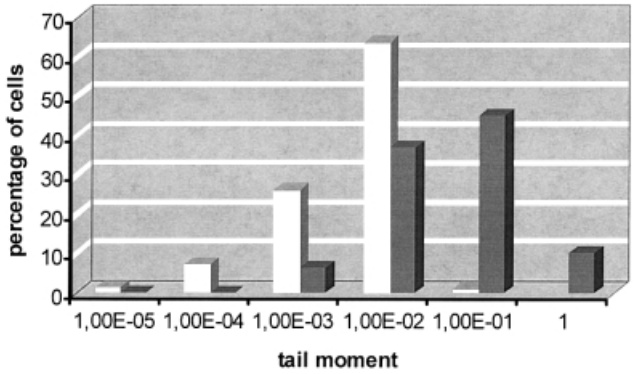

Fig. 3. Distribution of cells in the different intervals of values of tail moments for birds sampled in the polluted area (dark) as well as for those from control non-polluted locations. A statistically significant difference ($P < 0.0001$; Student's *t*-test) is observed.

observed in the control animals, where the highest tail moments were located in the interval 0–0.05, i.e. about 10–20-fold lower than that observed at the contaminated area. Therefore mean tail moments of birds sampled in the contaminated area were much higher (mean ± SD = 0.48 ± 0.81, $n = 50$) than those from control birds (0.035 ± 0.016, $n = 25$), the difference being highly significant ($P < 0.0001$; Student's *t*-test). This difference was not masked by potential age and/or sex effects, as shown by ANCOVA analysis (age: $P = 0.76$; sex: $P = 0.49$) where the difference between the contaminated area and control sites is also significant ($P < 0.001$).

The apparent genotoxicity in the animals from the vicinity of Doñana National Park is also evident when the distribution of cells of the contaminated animals in the different intervals of values of tail moments is compared with those of the birds sampled in the non-polluted area (Figure 3). As can be seen, the distribution of the tail moments of individual cells observed in exposed animals is clearly displaced towards higher values in comparison with the reference animals ($P < 0.0001$; Student's *t*-test).

Discussion

In the recent years, a number of laboratories have reported on the SCG or Comet assay (Fairbairn *et al.*, 1995) as a highly sensitive, relatively inexpensive and reproducible method to assess genotoxic damage in interphase cells for environmental monitoring.

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). With different modifications depending on the cells chosen, this methodology has been recently used in the detection of DNA damage after in situ exposure to water borne genotoxicants in erythrocytes of fish (Pandrangi *et al.*, 1995; Belpaeme *et al.*, 1996), as well as of amphibians (Ralph *et al.*, 1996; Ralph and Petras, 1997).

We have made use of the Comet assay for the analysis of DNA damage in peripheral blood lymphocytes from storks living in the Doñana National Park sampled after a toxic spill of mine waste (April, 1998). Our results seem to indicate that, as compared with reference animals sampled in non-contaminated locations, the exposed storks show a significantly increased level of genotoxic damage. This conclusion is based on either the distribution of individual birds according to intervals of tail moments of the comets (a parameter that measures DNA damage) or by comparing the DNA damage observed in cells from storks sampled in the surroundings of Doñana National Park with the corresponding distribution for lymphocytes from reference animals.

Since this is the first study carried out of a population of birds from Doñana National Park or its neighbouring area using the Comet assay for the direct measurement of DNA damage in individual cells, we cannot conclude that the increased level of genotoxic damage is exclusively attributable to the accidental toxic waste spill of April 1998. Intensive agriculture in the surrounding area, mostly rice, results in the use of plaguicides, which may have contributed to the present situation. However, residues of organochlorine pesticides and polychlorinate biphenyls appeared to be low in eggs of white storks sampled in Doñana, in both 1985–86 (Hernández *et al.*, 1988) and 1998 (Jiménez *et al.*, 1999). Therefore, the observed damage of DNA in white storks seems unlikely to be provoked by these organic pollutants. On the other hand, contamination by heavy metals was relatively low in Doñana before the toxic spill, whereas after that several bird species, including white storks, have been found to be contaminated by heavy metals and arsenic (Benito *et al.*, 1999; Hernández *et al.*, 1999).

An unquestionable body of evidence exists concerning the deleterious effects of heavy ions, such as Fe, As, Pb, Cd, Cu, Zn, Tl and Hg (Fan *et al.*, 1995; LeBlanc and Bain, 1997), and in our opinion, it seems certain that heavy metals have played an important role in the high level of genotoxic damage observed by us in storks from Doñana following the contamination. Additionally, a significant amount of aromatic amines arrived at the Doñana area with the toxic waste spill (Alzaga *et al.*, 1999). Because of the strong genotoxicity of aryl amines (IARC, 1982; Gold *et al.*, 1989) and the potential synergistic interactions between different chemical species (Yang, 1998), we cannot discount the fact that these aromatic amines may also have contributed to the DNA damage detected in this study. Anyway, taking into account our observations of a high level of genetic damage that might result in such deleterious effects as a decline in reproduction, increased mortality, etc., in the population of birds living in this important wildlife area, it becomes absolutely necessary to develop a follow-up monitoring to assess DNA damage in a wider spectrum of wild species, and whether or not there is a recovery of genome integrity after the palliative measures taken by the Spanish authorities.

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