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Genotoxic effects in wild rodents (*Rattus rattus* and *Mus musculus*) in an open coal mining area

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Abstract

Coal is a mixture of a variety of compounds containing mutagenic and carcinogenic polycyclic aromatic hydrocarbons. Exposure to coal is considered as an important non-cellular and cellular source of reactive oxygen species that can induce DNA damage. In addition, spontaneous combustion can occur in coal mining areas, further releasing compounds with detrimental effects on the environment. In this study the comet assay was used to investigate potential genotoxic effects of coal mining activities in peripheral blood cells of the wild rodents *Rattus rattus* and *Mus musculus*. The study was conducted in a coal mining area of the Municipio de Puerto Libertador, South West of the Departamento de Cordoba, Colombia. Animals from two areas in the coal mining zone and a control area located in the Municipio de Lorica were investigated. The results showed evidence that exposure to coal results in elevated primary DNA lesions in blood cells of rodents. Three different parameters for DNA damage were assessed, namely, DNA damage index, migration length and percentage damaged cells. All parameters showed statistically significantly higher values in mice and rats from the coal mining area in comparison to the animals from the control area. The parameter "DNA Damage Index" was found to be most sensitive and to best indicate a genotoxic hazard. Both species investigated were shown to be sensitive indicators of environmental genotoxicity caused by coal mining activities. In summary, our study constitutes the first investigation of potential genotoxic effects of open coal mining carried out in Puerto Libertador. The investigations provide a guide for measures to evaluate genotoxic hazards, thereby contributing to the development of appropriate measures and regulations for more careful operations during coal mining.

Keywords: Genotoxicity; Coal dust; Rodents; Comet assay

1. Introduction

The mining of coal is an activity with a high potential for pollution of the environment. Coal has been described as the most significant pollutant of all fossil energy sources containing important polluting compounds as sulfur dioxide and its derivatives [1]. The activities of stripping of coal liberate large quantities of pollutants into the atmosphere. In addition, ashes and products of liquefaction and combustion of coal contain polycyclic aromatic hydrocarbons (PAH), which constitute a significant risk to the environment. Several of these PAH

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exhibit well-known mutagenic and carcinogenic activity, and therefore, more rigorous control measures have been established by international organizations with regards to the presence of coal in the environment [2].

Colombia possesses the biggest natural reserves of coal in Latin America and it is the fifth biggest exporter of thermal coal in the world. The mining region of the Atlantic Coast, conformed by the departments Guajira, Cesar and Córdoba, produces 90% of the thermal coal of the country that in turn corresponds to 98% of the national coal resources [3]. The carboniferous area of the Departamento de Córdoba is located the South West, in the Municipio de Puerto Libertador, Alto San Jorge. The coal mined in the area is classified as sub-bituminous, of low quality and caloric power, with a content of total sulfur of 1.31%. Coal it is openly mined, with middle-sized mining systems, meaning that although more advance technology exists stripping of the material is conducted with a minimum degree of environmental surveillance. The operations carried out in Puerto Libertador consist of two activities: stripping (extraction of coal) and crushing (mincing of coal for transporting) which have been acknowledged to release fugitive particles into the environment [4]. For example, stripping of coal releases significant quantities of potentially toxic substances into the atmosphere where they constitute complex mixtures [5]. The exposure to a combination of compounds is considered to present a higher health risk due to potential synergistic effects of the resulting mixture [6]. Once in the environment pollutants resulting from coal mining have the potential to penetrate into water sources of the biota or into the atmosphere in significant amounts, thus presenting potential hazards for the environment and human health [7]. The pollution resulting from coal mining and the potentially genotoxic effects on organisms have been investigated using bacteria [8], earthworms [9], fish [10], plants [11], rodents [12,13] and human cells [8,14].

The comet assay is recognized as one of the most sensitive methodologies available for DNA damage detection [15], and is distinguished by being simple, fast, and effective, especially for small samples sizes and is applicable to cells from virtually any organ of eukaryotic organisms [16]. The comet assay has several advantages over other in vivo genotoxicity test methods as cytogenetic evaluations, such as the micronucleus test or the chromosome aberration assay applicable to proliferating cells only. However, there are relatively few limitations of the comet assay, very short lived primary DNA lesions, such as single-strand breaks, which may undergo rapid DNA repair could be missed when using inadequate sampling times. However, an appropriate study design should ensure that these lesions are captured at higher dose levels, at which DNA repair may be significantly slowed down or even overwhelmed [17].

The aim of the present study was to evaluate genotoxic effects of coal mining activities on two species of rodents (rat, *Rattus rattus* and mouse, *Mus musculus*) using the comet assay. The studies demonstrate that this test system is a useful tool to assess environmental genotoxicity in polluted areas and demonstrate the importance of such investigations to assess environmental hazards resulting from open coal mining.

2. Materials and methods

2.1. Sampling procedures

The study on the rodent populations was started in August 2005 and it continued until March of the 2006. Each of the field work phases consisted of a period of 15 days, during which the animals were captured and slide preparation for the comet assay was carried out. The capture of the animals was authorized by the CVS (the official Cordoba's environmental protection agency). The procedure for obtaining the different species of rodents was the capture-removal method, which means that the captured animals were not returned to their place of origin. For the capture of the animals three areas were selected, two located inside the area of coal mining in the Municipio de Puerto Libertador (Departamento de Córdoba, Colombia) and a third area located in the Municipio de Lorica (Departamento de Córdoba, Colombia) approximately to 151 km of the mining area. The later served as a control area to investigate animals that were not exposed to residuals of the mining of coal (Fig. 1). The two areas inside the coal mining area consisted of a stripping zone and crushing zone. Animals were trapped using 60 medium-size Sherman traps located in each of the three designated areas. Each group of traps was posted in the designated area between 05:00 and 06:00 pm and picked up the following day between 06:00 and 07:00 am.

No previous records on the diversity and density of wild rodents in the region were available. Therefore, the species for this study were only chosen after the captures. Three criteria were established: the distribution range (that the two species were present in both areas), populational density (sufficient individuals to ensure a meaningful sample size of each species) and sympatry. Based on these criteria and on the results of the captures, two species of wild rodents were chosen: rat (*R. rattus*) and mouse (*M. musculus*) which are species that are commonly used in genotoxicity testing, and well-known as species with peridomestic habits. The captured animals were anesthetized to facilitate the blood sampling and recording of morphometric data. Peripheral blood of the animals was obtained from tail pricks with the help of capillaries.

In parallel to the collection of the animal blood samples additional samples of human blood by finger pricks were collected and processed under the same conditions. These samples

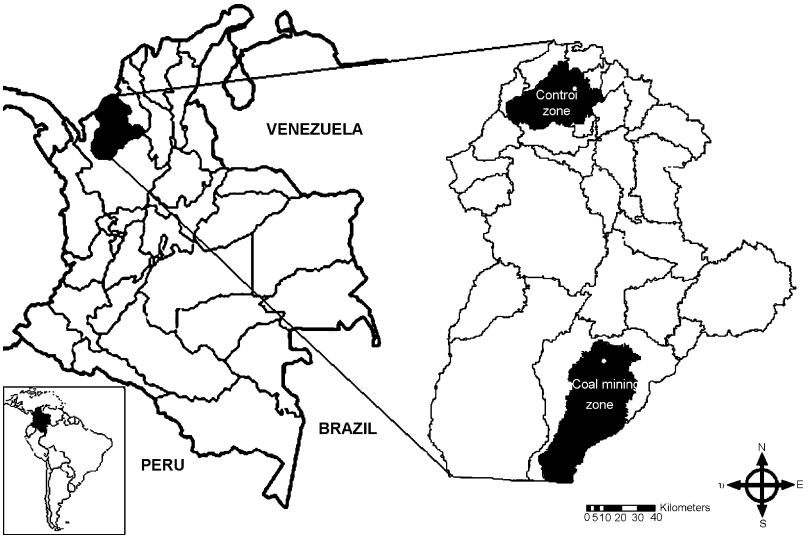

Fig. 1. Geographic localization of sampling areas: Puerto Libertador (coal mining area) and Lorica (control area).

were used as internal standards to allow for the detection of potential confounding factors that may have been caused by sample handling or transportation to the laboratory.

2.2. Comet assay

Prior to the initiation of the field study, the comet assay procedure was established in the laboratory using whole blood samples from healthy volunteers. For this purpose, samples were treated for 2 h with methyl methanesulfonate (MMS, Sigma) at 1×10^{-5} M in Hank's balanced salt solution (HBSS, Sigma). For negative control samples whole blood in HBSS was used. The samples were incubated for 2 h at 37 °C. This concentration was used to demonstrate different levels of damage and the sensitivity of the electrophoresis and test conditions used.

The comet assay was carried out according to of the original methodology (alkaline version) described by Singh et al. [18] with modifications. Additional modifications for field work were integrated [12]. For the preparation of the samples, 5 μl of whole blood were mixed with heparin and 120 μl of low melting point agarose (LMA)(Invitrogen) at 37 °C. This mixture was placed in a slide previously covered with 1.5% of normal melting point agarose (NMA) (Cambrex Bioscience Rockland) processed at 60 °C. The mixture of LMA and blood on the slide was covered with a cover slip. After solidifying of the gel on the slides, the cover slip was removed and the slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0–10.5, 1% with freshly added 1% Triton X-100 and 10% DMSO) at 4 °C. The slides remained for two weeks in this solution until transport to the laboratory where they were further processed. Direct light exposure of the samples was avoided during the whole process. The slides were removed of lysis solution and placed for 30 min close to the anode of an electrophoresis box containing alkaline buffer at 4 °C (300 mM NaOH and 1 mM EDTA, pH > 13). The electrophoresis was carried out for 30 min at 25 V and 300 mA. Afterwards the alkalinity was neutralized with 0.4 M Tris (pH 7.5) with washes of 5 min for each slide. Finally, the DNA was stained with ethidium bromide solution (2 μl/ml) and assessed using a fluorescence microscope equipped with a green filter of 540 nm.

For each sample images of 100 randomly selected cells (50 cells from each of two replicate slides) were analyzed from each animal. DNA migration length (nuclear region plus tail) were measured in microns (μm) using the Motic® Images 2000 software version 1.2. In addition, the cells were classified according to tail size into five classes ranging from undamaged (0) to maximally damaged (4) (Fig. 2), obtaining a measure of the individual damage for each animal and consequently for each analyzed group. The calculation of the damage index was car-

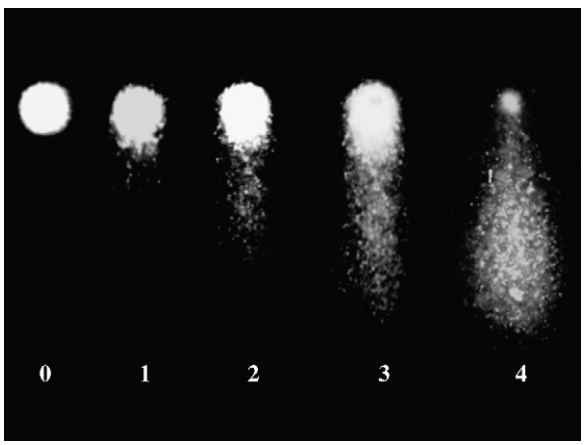

Fig. 2. Visual DNA damage classification of the nuclei in the range of 0–4.

Table 1
Average values of DNA damage index, DNA migration length and percentage damaged cells of controls and cultures treated with methyl methanesulfonate (MMS)

DNA damage parameter	Average value of four experiments [range]	
	Control (HBSS)	MMS (10^{-5})
Migration length (μm)*	21.5 [18.1–25.0]	42.7 [33.5–48.6]
Damage index*	70.3 [47–94]	134 [94–181]
Percentage damaged cells*	41.8 [34–50]	49.0 [42–61]

* Average of four independent experiments per data point, mean of 100 cells per experiment.

ried out according to the visual classification system [15]. The values for the damage index can range from 0 (100 cells class 0) up to 400 (100 cells class 4). Similarly, the frequencies of damaged cells were calculated for each one of the areas under investigation. This parameter was based on the number of cells with tail versus number of cells without tail. Statistical analysis of the data was conducted by means of the variance analysis using the statistical software SPSS (version 12.0) with a level of established significance ($\alpha = 0.05$). The statistical test used in these hypothesis tests (significance in the values of each area) was the *F*-test (Fisher).

3. Results

Prior to conducting the field study, the comet assay procedure was established in our laboratory. In order to demonstrate that a genotoxic effect can be detected by the procedure and results are reproducible, human blood samples were treated with saline solution or 10^{-5} M methyl methanesulfonate (MMS). The results of four independent experiments (average values of the four experiments and range) are shown in Table 1. The results demonstrate that a genotoxic effect was reproducibly identified in each of the independent experiments. While the parameters "DNA migration length" and "DNA Damage Index" found to sensitively demonstrate the induction of genotoxicity by MMS, the parameter "Percentage Damaged Cells" was less sensitive.

In the field study, a total of 22 rodents were investigated in the open mining areas of which 10 were trapped in the crushing zone and 12 inside the stripping zone. In total, 7 males and 5 females mice (*M. musculus*) and 5 males and 5 females rats (*R. rattus*) were investigated. In the control area five rats (two males, three females) and four mice (two males, two females) were investigated. During the field studies, additional finger-prick samples of human blood were taken in parallel to the animal samples and were used as internal controls. These controls were included into every electrophoresis run to assure acceptable levels of DNA damage. The values for DNA damage of animal samples were considered acceptable

Table 2
Average values of DNA damage parameters of mice and rats from coal mining and control areas

Species	Area	n[a]	Gender	DNA damage index[b,c]	DNA migration length (μm)[b,c]	Percentage damaged cells (%)[b,c]
M. musculus	Crushing	3	Female	197.6 ± 61.4	28.3 ± 3.2	71.0 ± 21.0
		3	Male	212 ± 42.5	29.5 ± 10.1	90.6 ± 14.4
	Stripping	2	Female	195 ± 77.7	20.4 ± 1.4	48.0 ± 7.0
		4	Male	186 ± 19.2	28.9 ± 4.2	77.2 ± 18.7
	Control	2	Female	38.0 ± 29.6	11.8 ± 1.7	16.5 ± 10.6
		2	Male	12.5 ± 3.5	14.0 ± 4.2	12.0 ± 2.8
R. rattus	Crushing	3	Female	188 ± 77.1	34.1 ± 10.6	76.0 ± 18.5
		3	Male	221 ± 56.4	31.1 ± 9.0	71.0 ± 16.0
	Stripping	2	Female	191 ± 31.8	17.0 ± 4.1	84.0 ± 5.6
		2	Male	208 ± 45.9	53.0 ± 9.7	27.2 ± 5.6
	Control	3	Female	90.6 ± 37.0	16.2 ± 5.0	40.6 ± 1.2
		2	Male	69.5 ± 19.0	16.3 ± 3.0	37.5 ± 3.5

[a] Number of animals per group.
[b] Average values of 100 cells per animal.
[c] Average value ± standard deviation.

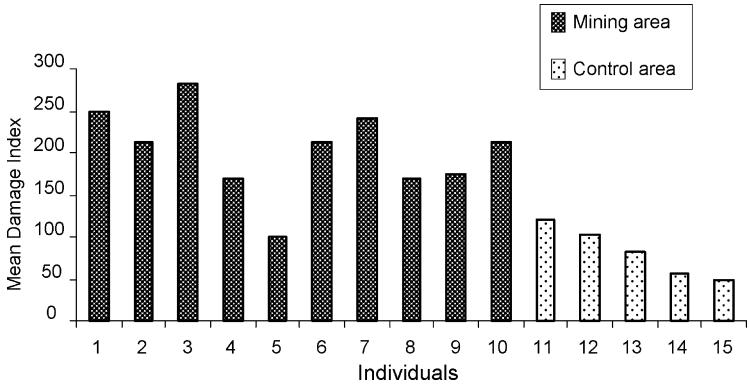

Fig. 3. Individual DNA damage index values for rats from coal mining areas and control area.

when the following values were achieved with the finger-prick samples—DNA damage index: 100 ± 59.1; DNA migration length: 17.7 ± 14.0 μm; percentage damaged cells: 49.0 ± 38.1.

Table 2 summarizes the mean values of all animals investigated from the crushing, stripping and control areas. The average values and standard deviations are presented for damage index, migration length, and the percentage of damage cells. Mice as well as rats from both coal mining areas presented clearly higher values for DNA damage compared to animals from the control area. The values obtained for the crushing and stripping areas were comparable for each parameter; however, the difference between the two mining areas was not statistically significant. When comparing the different DNA damage parameters, it is obvious that the parameter "DNA Damage Index" showed the clearest separation between animals from the mining areas and the control area. As this parameter was found the most sensitive for the indication of a genotoxic effect in rodents, values for the "DNA Damage Index" of the individual animals are presented in Figs. 3 and 4. This comparison clearly demonstrates higher levels of DNA damage in individuals of both species from the coal mining areas compared to the control area. The differences between the "DNA Damage Index" for mining and control zone were statistically significant.

It is interesting that all mice from the coal mining areas exhibited clearly higher DNA damage values when compared to the controls. As for rats, there was one individual animal from the coal mining area showing a DNA damage value which was comparable to control area values. Such a clear separation between the mining and control areas was not observed for the parameters "DNA Migration Length" or "Percentage Damaged Cells" (individual data not shown). In spite of this, difference between males and females were not significant. Fig. 5 compares the average group parameters for both species from the mining and control areas. The statistical analysis shows that all parameters for DNA damage measured in the animals from the coal mining areas were significantly elevated above the respective controls.

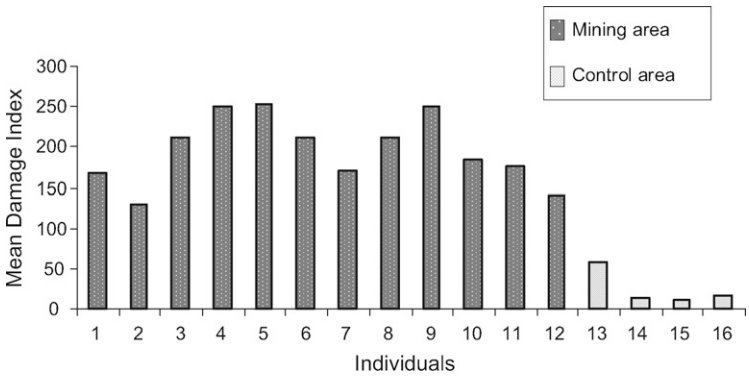

Fig. 4. Individual DNA damage index values for mice from coal mining areas and control area.

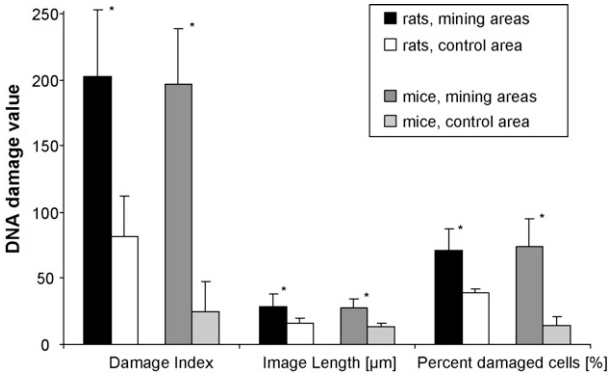

Fig. 5. Comparison of average group values and standard deviations of mice and rats from coal mining and control areas. Asterisk (*) indicates statistically significant difference of average group mean vs. control.

4. Discussion

Coal is a heterogeneous mixture of more than 50 elements, including oxides and other elements as silica, PAH, heavy metals and ash. During the processes of coal extraction, large amounts of these substances can be liberated to the atmosphere, where they constitute complex mixtures. Complex mixtures have been considered to present a significant risks for health taking into account potential synergistic effects cause by combination of individual compounds [6]. Only a few studies in mammals have been conducted to assess the environmental mutagen hazards of coal and complex mixtures that it forms with other substances [12,13,19,20]. In the present study, wild rodents were investigated for the potential genotoxicity of exposure to coal. We applied the comet assay to compare the extent of primary DNA damage in peripheral blood cells of rodents from areas subject to open coal mining activities and a control area.

The data clearly demonstrate that mice and rats originating from the coal mining area exhibited a significantly higher extent of DNA damage as assessed by length of DNA migration, damage index and percentage of damaged cells compared to animals from a non-polluted control area. The parameter "DNA Damage Index" was found to best demonstrate a difference between the control and mining area groups. A more pronounced difference in average DNA damage index was observed in mice compared to rats: while the DNA damage index in mice from the mining area was approximately 8-fold higher than that of mice from the control area, the difference between the rat populations was only 2.5-fold. This difference between the species may in part be explained by the comparatively low baseline value in mice from the control area. Although underlying mechanisms for the difference were not investigated and samples from animals under controlled conditions (e.g., laboratory studies) were not available, a potential cause for the interspecies difference may be differences in DNA repair mechanisms.

In contrast, the extent of the DNA damage index found in mice and rats from the coal mining area were comparable. This observation may point towards a difference in baseline DNA damage values in the species investigated. However, it has to be emphasized that the total number of both, rats and mice investigated from the control area is rather limited and the use of a larger number of individuals may result in more similar baseline values in both species. Besides the DNA damage index, the parameters "Percentage Damaged Cells" and "Migration Length" also showed a statistically significant difference in the average values between the groups from the mining and control areas. The parameter "Percentage Damaged Cells" is considered a simple method for the collection of comet assay data and can easily be compared between laboratories [21].

The rodent species investigated in the mining area are subject to exposure due to different mining activities, specifically, stripping and crushing of coal. The first activity includes the extraction of rocks and transportation to the crushing machines. During the crushing procedure, coal is processed into small particles in order to enable transportation. These activities are liberating great quantities of fugitive particles into the environment which contain ashes including PAHs and toxic gases [4]. During the crushing process of the coal large quantities of coal dust particles can be spread into the surrounding environment and they are deposited on the surfaces of the plants or in river beds. Results similar to ours were obtained in a biomonitoring study conducted in a carboniferous areas using wild rodent species *Ctenomys torquatus* [12]. The study investigated micronucleus and comet formation in areas of coal mining and showed elevated DNA and chromosomal lesions in peripheral blood cells of rodents. Other species have been used as well to study genotoxic and toxic effects of coal mining. Using the comet assay, increased DNA damage was observed in liver and blood cells of fish exposed to wastewaters from coal mining areas [22]. Other environmental studies include the evaluation of genotoxicity of rodents exposed to coal dust and diesel emissions [19], the evaluation of carcinogenic effects from emissions of mines and plants of coal [20], and the effects of coal ashes on the lungs of guinea pigs [13]. In addition, in vitro investigations have demonstrated hazardous effects of coal and its derivatives [23,24]. Significant inter-individual differences were observed in all populations of rats and mice of our study. While the variability in rat populations

from both areas was comparable, the inter-individual difference in mice was much higher in the polluted compared to the control area. This observation may indicate differences in the individual exposure of mice to genotoxic agents or may point towards different amounts of toxicants in the coal mining area.

The mined coal of Puerto Libertador, Córdoba, is classified as sub-bituminous and is characterized by a low content of carbon. This type is expected to undergo reactions of spontaneous combustion to a significantly higher degree as compared to other classes of coal. Indeed, upon processing of coal into finer particles by a crushing process, the exposure to ambient oxygen and the sun can result in a combustion process within the stored material. A further source of environmental pollution is the processing of coal which releases significant amounts of fine particles into the surrounding environment. The results obtained indicate the presence of a genotoxic hazard towards the rodent populations in the coal mining area, however, in this type of study is a complicated task mainly because of the relatively low levels of genotoxicants and the existence of multiple potential genotoxic pollutants often encountered as complex mixtures [16]. The interactions between these genotoxicants and the organism's DNA can lead to a variety of damage [25]. The detected DNA damage in the comet assay in rodents residing in close proximity to coal mines is thought to be attributed to coal or its by-products; however, other environmental factors (water, other genotoxicants) cannot be totally ruled out since these factors were not controlled for in the present study design.

The comparatively high average DNA damage values in samples of rodents from the coal mining area may point towards the type of DNA lesions induced in the animals. The comet assay is particularly sensitive towards direct and indirect DNA strand breakage and alkaline–labile sites in the DNA. These types of DNA damage are usually induced by most of the genotoxic agents [26], which induce breaks in the phosphodiester skeleton or between bases and sugars of the DNA resulting in abasic sites. It is known that coal mining activities liberate significant quantities of fugitive particles and toxic gases as sulfur dioxide into the environment [4]. Therefore, the large extent of DNA damage observed in the present study may be related to oxidative damage caused by reactive substances contained in the coal, such as iron and sulfur [24]. In addition, since large amounts of mutagenic compounds, such as PAHs derived from coal combustion products are liberated into the atmosphere [2], DNA adduct formation by PAH is likely an important contributing to the high extent of DNA damage observed.

In conclusion, the results obtained in our study demonstrate that coal mining activities in Puerto Libertador–Córdoba-Colombia present a genotoxic hazard to wild rodents. The two species investigated were shown to be sensitive and suitable to investigate environmental genotoxicity caused by coal mining activities.

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