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Genotoxic effects in blood cells of *Mus musculus* and *Iguana iguana* living near coal mining areas in Colombia

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ABSTRACT

Coal is a mixture of chemicals with the capacity of promoting biochemical changes that may lead to DNA damage. In this study, the comet assay in peripheral blood cells, and the micronucleus test in blood smears were used to evaluate potential genotoxic effects derived from exposure to coal mining activities on wild populations of *Mus musculus* and *Iguana iguana*. Four locations from Colombia were evaluated: La Loma and La Jagua de Ibirico, two municipalities located near coal mining fields at the Department of Cesar; and Valledupar and Arjona, cities used as reference sites, both localized at least 100 and 200 km far from the mines, respectively. Compared to Valledupar and Arjona, animals collected in close proximity to coal mining areas showed highest percentages of DNA damage for both species, evidencing that living around coal mining fields may result in an increase of DNA lesions in blood cells of rodents and reptiles. The results for micronucleus test were conflicting. Mice from Arjona had greater number of cells with micronucleus than those from the other studied locations, probably as a result of infection found by blood parasites. In summary, it was demonstrated that animals living around coal mining areas have a greater chance of having DNA damage, as measured by the comet assay, than those from sites far from the coal dust source.

1. Introduction

Coal is a key mineral used as an energy source for many countries (Bian et al., 2010). It is a complex mixture of chemicals that is not always homogeneous and depends on the source and rank of the coal (Donbak et al., 2005). It is made of carbon, hydrogen, oxygen, nitrogen, inorganic minerals, quartz, trace metals, and other contaminants (Castranova and Vallyathan, 2000). Many of the coal components are mutagenic and carcinogenic (da Silva et al., 2000a); for instance, quartz could be a prominent risk factor for lung cancer in coal miners (Borm and Tran, 2002), and the International Agency for Research on Cancer (IARC) classified it into IARC's Group 1 (carcinogenic to humans), due to sufficient evidence for carcinogenicity in experimental animals and in humans (IARC, 2010). Heavy metals such as arsenic, cadmium, as well as, polycyclic aromatic hydrocarbons (PAHs), present in coal, are also well recognized carcinogens (da Silva et al., 2000a, 2000b).

Coal dust extracts have also been reported to be cytotoxic and mutagenic in mammalian systems (Ulker et al., 2008). Oxidative DNA damage was observed to be significantly higher in lymphocytes of retired coal miners than in controls (Shins et al., 1995). Besides, there was an increase in the frequencies of sister chromatid exchanges, chromosomal aberrations, and micronucleus in underground coal miners, indicating the genetic damage due to coal dust exposure (Donbak et al., 2005).

Studies of genetic toxicology have given rise to a number of testing procedures, designed to assess the effects of substance exposure on genetic mechanisms and the consequent risk to organisms, including humans (Krishna and Hayashi, 2000). One of the tools aimed to evaluate genotoxic effects in the biota is the neutral comet assay, an electrophoresis-based method that is able to detect the actual single-strand (SS) and double-strand (DS) breaks in individual cells (Collins et al., 2008; Tice et al., 2000). This test has shown to be a simple, fast, and effective assay to detect DNA damage (Mitchelmore and Chipman, 1998). It has been already used to assess environmental genotoxicity in coal mining areas (León et al., 2007) and for the detection of early biological effects of DNA damaging agents (Sasaki et al., 1998) such as radiation, chemotherapeutic agents, and reactive oxygen radicals, in both environmental and occupational settings (Fairbairn et al., 1995; Moller et al., 2000). Another toxicity test widely used to measure genotoxicity is the micronucleus assay (MN) in peripheral blood. This assay, when performed appropriately, detects both clastogenicity (chromosome breakage) and aneugenicity (chromosome lagging due to dysfunction of mitotic apparatus) (Krishna and Hayashi, 2000).

Colombia is the country with the largest coal reserves in Latin America. It has potential resources of 16,992 million tons (UPME, 2007), being the sixth largest exporter of coal in the world. Most

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coal reserves and mines (95%) are located in the Departments of La Guajira and Cesar (UPME, 2005). There are several coal mines operated by different companies in the Department of Cesar, and the most important are located in the towns of La Loma and La Jagua de Ibirico. In 2004, La Loma produced 38%, and La Jagua de Ibirico about 10% of the total Colombian Atlantic Coast exports (UPME, 2006b). For 2005, the coal mine in La Loma (Cesar) had the second largest coal production in Colombia, which was estimated at 16.5 million tons (UPME, 2006a).

The goal of this research was to evaluate the potential genotoxic damage in peripheral blood cells of *Mus musculus* (mice) and *Iguana iguana* (iguana) living near coal mining polluted areas at the Department of Cesar, Colombia, using the neutral comet assay and the micronucleus test in blood smears.

2. Materials and methods

2.1. Study area

This study was performed between August 2010 and April 2011 in different areas of the Departments of Cesar and Bolivar, at the north of Colombia. Two towns located next to the most important coal mines in the Department of Cesar were evaluated: La Jagua de Ibirico (10°31′19″ N, 73°18′56″ W) and La Loma (10°23′58″ N, 75°30′9″ W). The municipality of La Jagua de Ibirico is located in the central region of the department, at the foothills of the Colombian Andean Eastern Mountain Range. Its land area is 728.9 km^2 with an average altitude of 150 m.a.s.l. and an estimated population of 21,386 inhabitants (DANE, 2005). The village of La Loma, located 28.74 km far from La Jagua de Ibirico, has the second largest coal mine in the country.

Mice were also collected from two different reference sites: The urban area of Valledupar (10°28′06″ N, 73°15′04″ W), the capital of the Department of Cesar, approximately 100 km far from the coal mining area (Fig. 1), and Arjona (10°15′17″ N, 75°20′32″ W), a small town at the Department of Bolivar, at least 200 km far from the mines. The city of Valledupar covers an area of 4493 km^2, with 169 m.a.s.l., and an expected population of 413,301 habitants in 2011 (DANE, 2009). Its average annual temperature is 28.4 °C, with highs and lows of 22 °C and 34 °C, respectively (Ortega-Montero, 2007). The topography of the municipality of Arjona is slightly undulating, with elevations not exceeding 200 m.a.s.l, and a calculated population of 67,325 inhabitants (DANE, 2009). The specimens of *I. iguana* were captured only in 2011, in rural areas of La Loma and near to the natural reserve Los Besotes, at the north of Valledupar (10°34′26″N, 73°16′19″W).

2.2. Sampling methods

The specimen collection procedure was conducted with permission of CORPOCESAR, the environmental protection agency for the Department of Cesar (Resolution No. 739, dated June 22, 2010). Characteristics of collected specimens are shown in Table 1. *M. musculus* specimens were collected by a capture-removal method using Sherman live-capture traps (8×9×23 cm; Sherman Traps, Inc., Tallahassee, FL). These were placed inside houses between 04:00 and 06:00 pm and picked up the following day between 06:00 and 07:30 am. Iguanas were captured during early morning hours with the help of local farmers.

The rodents were processed in the field according to established biosafety guidelines (Mills et al., 1995). Mice were treated intraperitoneally

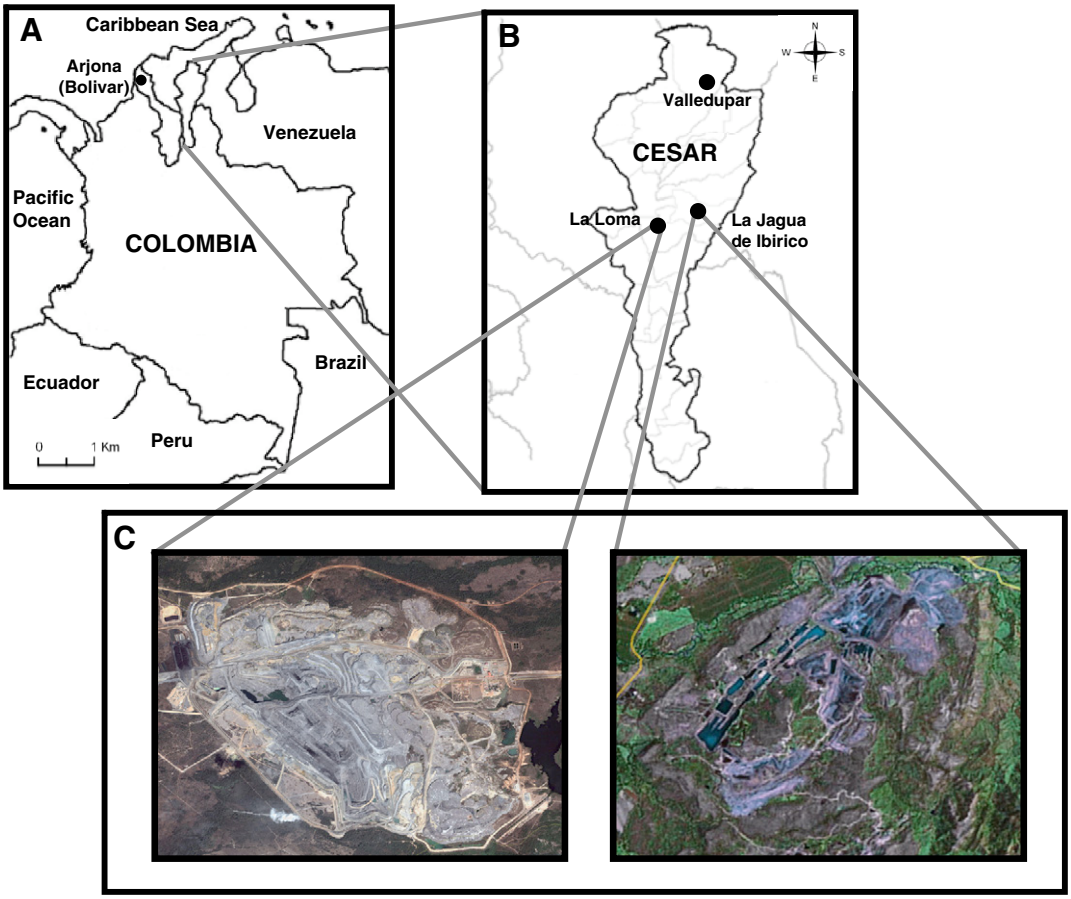

Fig. 1. Map of Colombia showing the geographic localization of sampling areas. A. Arjona (Department of Bolívar). B. La Loma. La Jagua and Valledupar (Department of Cesar). C. Satellite images of the coal mines.

Table 1
Characteristics of collected specimens from sampling sites.

Study area		*Mus musculus*						*Iguana iguana*		
		2010			2011			2011		
		Male	Female	Total	Male	Female	Total	Male	Female	Total
Arjona	Juvenile	1	1	2	1	3	4	*N.A.	N.A.	N.A.
	Adult	9	2	11	4	4	8	N.A.	N.A.	N.A.
	Total	10	3	13	5	7	12	N.A.	N.A.	N.A.
Valledupar	Juvenile	2	1	3	0	4	4	2	7	9
	Adult	4	9	13	8	5	13	1	0	1
	Total	6	10	16	8	9	17	3	7	10
La Jagua	Juvenile	3	0	3	3	2	5	N.A.	N.A.	N.A.
	Adult	3	2	5	2	2	4	N.A.	N.A.	N.A.
	Total	6	2	8	5	4	9	N.A.	N.A.	N.A.
La Loma	Juvenile	3	0	3	2	3	5	3	2	5
	Adult	3	5	8	5	8	13	4	2	6
	Total	6	5	11	7	11	18	7	4	11
Total	28	20	48	25	31	56	10	11	21	

* N.A. not available.

with sodium thiopental and iguanas with ketamine until a level of deep anesthesia was attained. Blood samples were obtained from the caudal vena cava and collected in tubes containing 3.8% sodium citrate, stored at 4 °C and protected from light until processed. All samples were analyzed within 24 hours.

2.3. Comet assay

The neutral comet assay was performed following the method proposed by Trevigen (Trevigen; Gaithersburg, MD) with some modi cations (Yang et al., 2011). Blood samples were combined with 0.5% low melting agarose in a 1:12.5 and 1:75 (v/v) ratio for mice and iguanas, respectively. Then, 10 μL of the cell suspension were dispensed into two-well Comet Slide™ (Trevigen; Gaithersburg, MD). After gel solidi cation, slides were submerged in pre-cooled lysis solution (2.5 M NaCl, 10 mM Tris base, 1% sodium dodecyl sulfate, 10% dimethyl sulfoxide, and 1% Triton X-100) at 4 °C for 1 h. Once lysed and rinsed, slides were equilibrated in prechilled neutral electrophoresis buffer (1.0 M Tris base, 3.0 M sodium acetate) for 30 min at 4 °C, and then electrophoresed at 300 mA for 60 min in darkness. Slides were then immersed in DNA precipitation solution (1.5 M ammonium acetate in absolute ethanol) for 30 min at room temperature, followed by immersion in 70% ethanol for 30 min at room temperature. Once dried, slides were stained for 30 min with SYBR Green and observed under uorescence microscope equipped with a green lter of 540 nm.

At least, images of 1000 randomly selected cells from the two wells were analyzed using a Nikon uorescence microscope. Based on tail intensity and length, DNA damage was quanti ed by visual classi cation of nucleoids into ve comet classes, from undamaged (class 0), to maximum DNA damage (class 4) (Fig. 2), resulting in a single DNA damage score for each specimen (Collins, 2004). Besides,

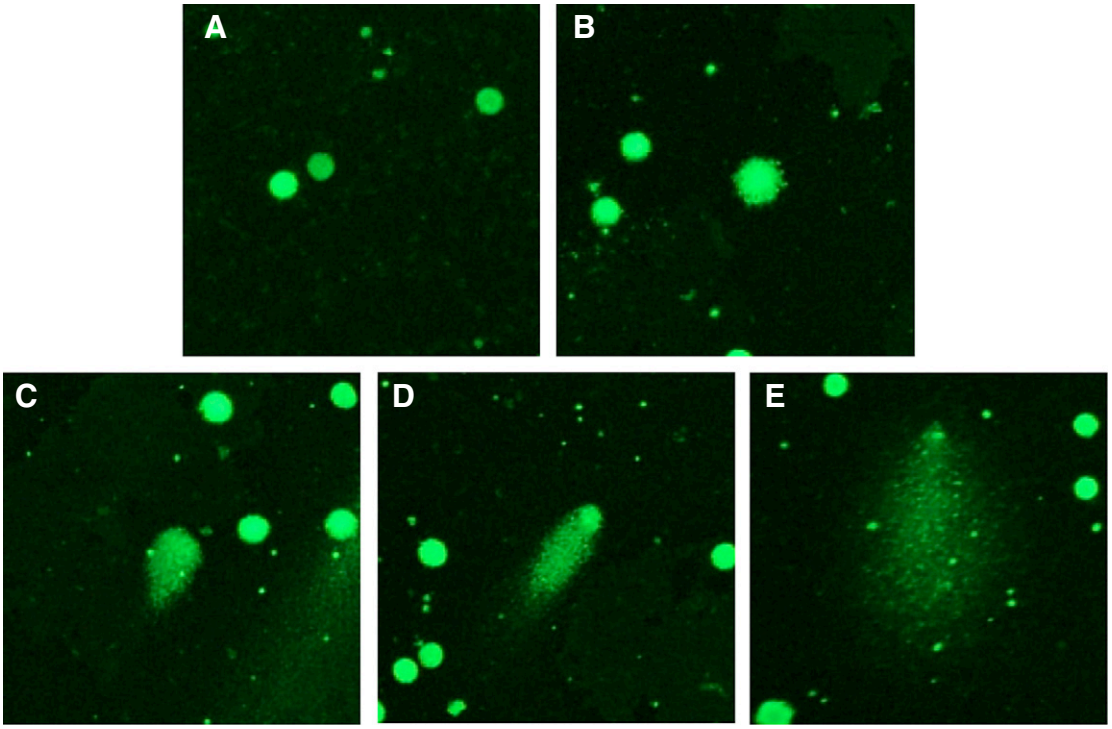

Fig. 2. Classi cation of blood cells in the comet assay. A. Class 0. no visible DNA migration from the nucleoid. B. Class 1. minimal DNA migration with an intact nucleoid. C. Class 2. moderate DNA migration with reduction in the size of the nucleoid. D. Class 3. extensive DNA migration with only a remaining pinpoint nucleoid. E. Class 4. complete migration of DNA into a comet tail with no visible nucleoid.

the total score expressed as a genetic damage indicator (GDI) was calculated multiplying the percentage of nucleoids in each class by the corresponding factor (Azqueta et al., 2009), according to the formula:

$$GDI = [(\%\ \text{nucleoids class } 0) \times 0] + [(\%\ \text{nucleoids class } 1) \times 1] + [(\%\ \text{nucleoids class } 2) \times 2] + [(\%\ \text{nucleoids class } 3) \times 3] + [(\%\ \text{nucleoids class } 4) \times 4].$$

In order to improve the reading of the expression of the DNA damage extent (Çavaş and Könen, 2007; Palus et al., 1999), the subtotal frequency of nucleoids (F_{2+3+4}) with medium, high, and complete damaged DNA was calculated as the sum of cells in classes 2, 3, and 4.

2.4. Micronucleus in peripheral blood smears

Blood smears were made on clean glass slides and air-dried. At least ve blood smears were prepared per animal. The slides were stained using Wright-Giemsa stains (Schlegel and MacGregor, 1982), and the mean frequency of erythrocytes with MN in peripheral blood smears was scored by random analysis of ve slides, examining 10 elds per slide, with approximately 2500 cells per slide.

2.5. Data analysis

Kruskal–Wallis test was used to compare means between sampling sites, followed by the Dunn's Multiple Comparisons test. A *P*-value <0.05 was set as statistically signi cant.

3. Results

DNA damage was assessed in peripheral blood cells of mice and iguanas collected near coal mining areas and from reference sites, at the north of Colombia.

3.1. Comet assay

The results of the comet assay performed in blood samples from mice collected during 2010 and 2011 are shown in Table 2. In 2010, mice from La Loma had the highest GDI and the highest F_{2+3+4} for both male and female groups. Compared to Arjona, the extent of DNA migration in peripheral blood leukocytes from male animals, measured as GDI and F_{2+3+4}, was statistically greater in La Jagua de Ibirico. This difference was maintained when data for both sexes were combined (Table 2). When independent data for each sex in animals from La Jagua de Ibirico were compared to those from Valledupar, no statistical differences were observed. However, signi cant differences were detected between these two sampling sites when data were pooled.

In 2011, GDI data for each sex group from Arjona were signi cantly different than those collected from both mining areas. This was not observed when Valledupar was compared to these places. Regarding La Jagua, statistical differences were found only when GDI pooled data were compared with Valledupar. During this year, similar results were obtained for F_{2+3+4}, although Arjona presented signi cant differences also with Valledupar (Table 2).

The results obtained for comet assay in blood cells of *I. iguana* are shown in Table 3. GDI values obtained for male and female specimens captured in La Loma were statistically different when compared to those from Valledupar. Values for F_{2+3+4} presented differences with Valledupar only for females (Table 3). When males and females were pooled, results for both GDI and F_{2+3+4} were statistically different when comparing both sampling sites.

3.2. Micronucleus test in blood smears

Some images of micronuclei observed in blood samples from examined mice and iguanas are shown in Fig. 3. However, the results of MN in blood smears showed that mice captured in areas affected by coal mining (La Jagua de Ibirico and La Loma) had percentages of MN similar to those obtained from Valledupar, for both sampling years and sexes (Table 4). It was also noted the presence of protozoan parasites in smears of some animals from Arjona.

The results of MN in peripheral blood smears of *I. iguana* specimens are also presented in Table 4. It is observed that blood cells from iguanas collected from a site near a coal mine (La Loma) have a tendency to present greater values for MN (Table 4) that those from individuals not impacted by this activity (Valledupar). However, this difference was not statistically signi cant.

4. Discussion

The results obtained for *M. musculus* and *I. iguana* showed a clear relationship between their habitats and the frequency of DNA-damaged blood cells. Mice (Table 2) and iguanas (Table 3) living near coal mining operations, such as in La Loma and La Jagua de Ibirico, exhibited a signi cantly greater extent of DNA damage, measured as GDI and F_{2+3+4}, compared to specimens from reference sites (Fig. 4). It is interesting to mention that regarding these biomarkers, the obtained pattern for males and females is similar to that seem when data are pooled. In other words, the extent of the damage is independent from the sex of the animal. To the best of our knowledge, this is the rst report on DNA damage assessed by the comet assay in

Table 2
GDI and subtotal frequency of nucleoids from *M. musculus* blood scored for DNA damage based on tail intensity and length.

Year	Capture area	Sex	N	GDI ± SD	F_{2+3+4} ± SD
2010	Arjona	Male	10	1.06 ± 0.46	0.09 ± 0.11
		Female	3	1.11 ± 0.67	0.06 ± 0.07
		Total	13	1.07 ± 0.48	0.09 ± 0.10
	Valledupar	Male	6	1.62 ± 1.04	0.38 ± 0.32
		Female	10	2.56 ± 2.06	0.38 ± 0.40
		Total	16	2.20 ± 1.77	0.35 ± 0.36
	La Jagua de Ibirico	Male	6	22.78 ± 11.09*	5.27 ± 2.94*
		Female	2	29.40 ± 9.92	11.27 ± 2.52
		Total	8	24.43 ± 10.55*,**	6.77 ± 3.85*,**
	La Loma	Male	6	45.15 ± 22.05*,**	10.55 ± 6.63*,**
		Female	5	50.89 ± 53.49*,**	18.60 ± 26.30*,**
		Total	11	47.76 ± 37.37*,**	13.03 ± 17.45*,**
2011	Arjona	Male	5	0.37 ± 0.38	0.03 ± 0.05
		Female	7	0.26 ± 0.21	0.04 ± 0.06
		Total	12	0.31 ± 0.28	0.04 ± 0.05
	Valledupar	Male	8	3.39 ± 3.07	0.85 ± 0.79
		Female	9	2.27 ± 1.71	0.76 ± 0.65*
		Total	17	2.80 ± 2.43	0.81 ± 0.69*
	La Jagua de Ibirico	Male	5	16.23 ± 5.62*	1.64 ± 0.56*
		Female	4	17.96 ± 7.94*	1.86 ± 1.72*
		Total	9	17.00 ± 6.34*,**	1.74 ± 1.13*
	La Loma	Male	7	6.38 ± 4.37*	1.43 ± 1.81*
		Female	11	5.35 ± 6.06*	1.15 ± 1.62*
		Total	18	5.75 ± 5.35*	1.26 ± 1.65*

* Signi cantly different ($P<0.05$) when compared to Arjona. ** Signi cantly different ($P<0.05$) when compared to Valledupar.

Table 3
GDI and subtotal frequency of nucleoids from *I. iguana* blood scored for DNA damage based on tail intensity and length.

Capture area	Sex	N	GDI ± SD	F_{2+3+4} ± SD
Valledupar	Male	3	0.46 ± 0.14	0.03 ± 0.03
	Female	7	0.55 ± 0.28	0.02 ± 0.04
	Total	10	0.53 ± 0.24	0.02 ± 0.04
La Loma	Male	7	15.39 ± 8.80**	0.80 ± 0.68
	Female	4	27.34 ± 12.58**	0.68 ± 0.33**
	Total	11	19.73 ± 11.42**	0.76 ± 0.56**

** Signi cantly different ($P<0.05$) when compared to Valledupar.

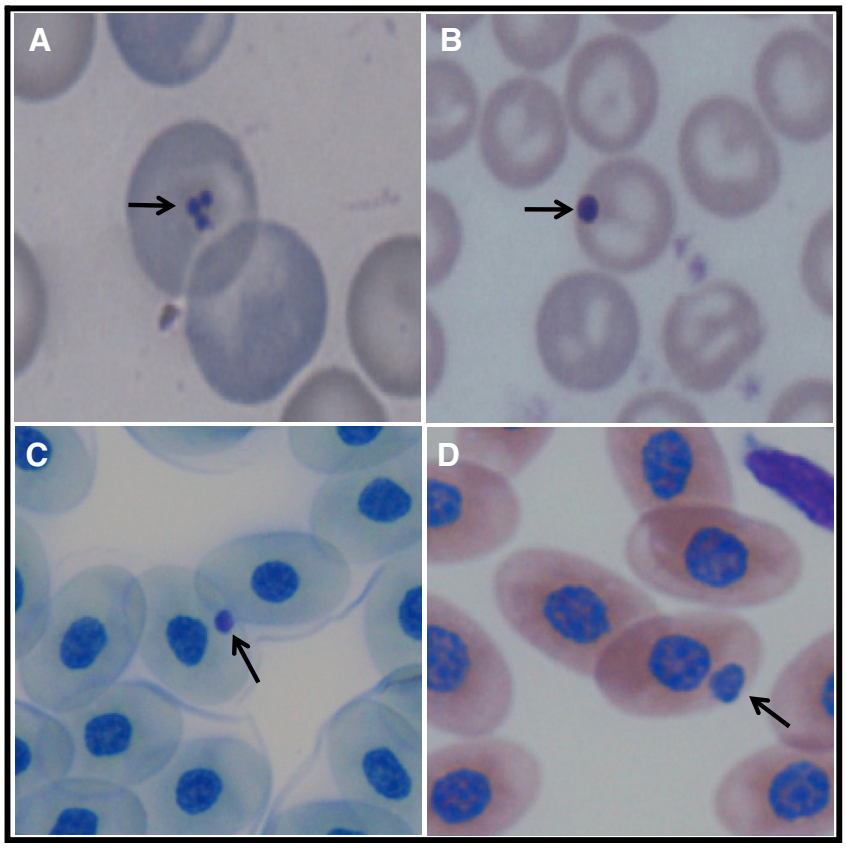

Fig. 3. Images of micronuclei observed in *Mus musculus* (A. B) and *Iguana iguana* (C. D) red blood cells. The micronuclei are shown by an arrow.

Table 4
Percentage of micronuclei found in *M. musculus* and *I. iguana* blood cells.

Species	Year	Capture area	Sex	N	%MN ± SD
Mus musculus	2010	Arjona	Male	10	0.19 ± 0.07
			Female	3	0.38 ± 0.49
			Total	13	0.23 ± 0.23
		Valledupar	Male	6	0.07 ± 0.03*
			Female	10	0.06 ± 0.02
			Total	16	0.07 ± 0.03*
		La Jagua de Ibirico	Male	6	0.07 ± 0.03*
			Female	2	0.09 ± 0.01
			Total	8	0.08 ± 0.03
		La Loma	Male	6	0.06 ± 0.02*
			Female	5	0.06 ± 0.02
			Total	11	0.06 ± 0.02*
	2011	Arjona	Male	5	0.13 ± 0.07
			Female	7	0.19 ± 0.49
			Total	12	0.16 ± 0.12
		Valledupar	Male	8	0.05 ± 0.03*
			Female	9	0.03 ± 0.04*
			Total	17	0.04 ± 0.04*
		La Jagua de Ibirico	Male	5	0.03 ± 0.02*
			Female	4	0.03 ± 0.03
			Total	9	0.03 ± 0.02*
		La Loma	Male	7	0.06 ± 0.05*
			Female	11	0.03 ± 0.03*
			Total	18	0.05 ± 0.04*
Iguana iguana	2011	Valledupar	Male	3	0.01 ± 0.02
			Female	7	0.02 ± 0.05
			Total	10	0.02 ± 0.04
		La Loma	Male	7	0.10 ± 0.11
			Female	4	0.03 ± 0.03
			Total	11	0.07 ± 0.09

* Signi cantly different ($P<0.05$) when compared to Arjona.

wild iguanas. Therefore, the information collected in this study, is a useful baseline for future research.

The presence of DNA damage in cells from the biota of coal mining areas is not surprising. Coal open-cast mining extraction is an activity considered an important source of pollutants (Bian et al., 2010). Coal itself has been described as the most signi cant pollutant of all fossil fuels, containing a heterogeneous mixture of contaminants (Leffa et al., 2010). Moreover, airborne coal particles as well as coal tailings are rich in potentially toxic hydrocarbons and genotoxic metals, among other contaminants (Celik et al., 2007), that ultimately may lead to profound changes in cells, tissues, populations, and ecosystems (Leffa et al., 2010).

Some of the DNA damaging agents can be breathed and therefore increase the risks of health hazards (NIOSH, 1995). Coal ash residues as well as dust from polluted areas by coal mining operations have been shown to contain signi cant amounts of heavy metals (Bai et al., 2004; Mugica et al., 2003; O'Shea, 2001; Tiwary, 2001), some of which have been considered to possess carcinogenic properties, including nickel, chromium, mercury, and cadmium. DNA lesions attributable to metal exposure are known to be driven by mechanisms involving the generation of highly reactive oxygen species (Hartwig, 2000), and interference with DNA repair processes (Kasprzak, 1991). However, the molecular interactions that lead to tumor formation after exposure to metal mixtures remain poorly understood.

Contrasting GDI and F_{2+3+4} data from mice collected in 2011 with those obtained in 2010, it is possible to observe differences in the registered percentages for each one of the comet classes, being 2010 the year with greater DNA damage (Table 2).

It is interesting that in 2011, the sampling sites close to mine showed statistical signi cance only with Arjona. These results seem to be due to the wide distance between Arjona and sampling sites of the Department of Cesar. The values generated for GDI and F_{2+3+4} show that, after the rainy season occurred in 2011, the sampling

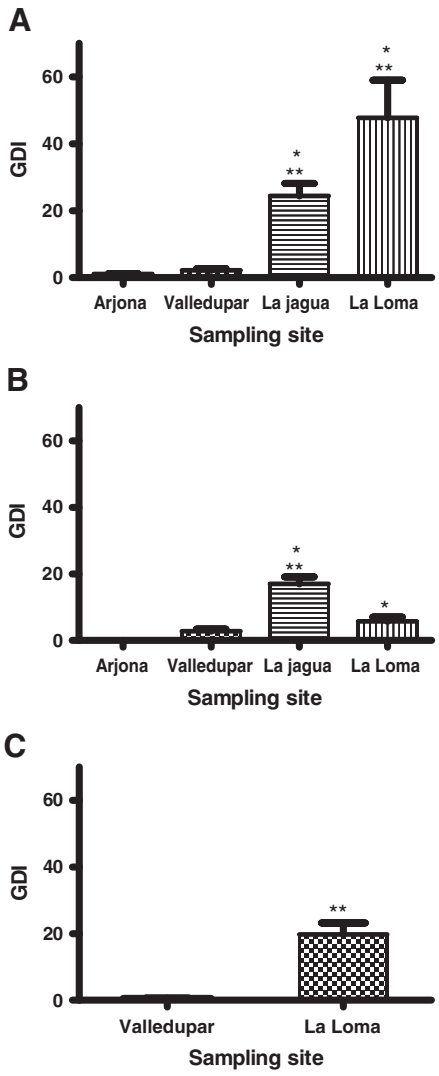

Fig. 4. Bar chart showing the GDI scores of *Mus musculus* blood cells for 2010 (A). and 2011 (B); and *Iguana iguana* (C). *. Signi cantly different (P<0.05) when compared to Arjona. **. Signi cantly different (P<0.05) when compared to Valledupar.

sites from the Department of Cesar behave similarly to each other when compared to Arjona. This observation allows us to suggest that samples from Arjona are a reliable control baseline group in this study.

Some authors have reported higher levels of DNA damage in summer than in winter (Verschaeve et al., 2007; Moller et al., 2002), which may indicate a direct relationship between DNA damage and increased intensity sunlight (Verschaeve et al., 2007), as well as greater exposure to PAHs (Moller et al., 2002), as a result of the phototoxicity of PAHs, which have been listed as photomutagenic (Yan et al., 2004). The last two months of 2010 and the rst months of 2011, periods used for specimen collections, were particularly abundant in heavy rains that triggered deadly oods and landslides; the heaviest rainfall since records began in 1969 (World Meteorological Organization, 2010). Water droplets can capture coal dust, and this is an ef cient method to decrease respirable coal dust. In fact, water spray has become a strategy for longwall mining machines during underground mining operations (Kilau, 1993). Given the strong rainy season that took place in late 2010 and early 2011 in Colombia, the concentrations of genotoxic compounds could have decreased as a result of heavy precipitations.

Regarding MN data for mice and iguanas (Table 4), it may be stated that blood smear was not as sensitive as the comet assay to detect genotoxic effects, as no difference was detected among the coal polluted sampling sites and Valledupar (Fig. 5). The greater MN average found in blood cells of mice collected in Arjona (control site) could had been the result of protozoa parasite infections, as it has been previously reported (Dertinger et al., 2000). These negative results observed by MN assay in blood smear for both species, agree with data from other authors, which did not detect a signi cant increase of MN in mice exposed to coal dust (Ong et al., 1985; da Silva et al., 2000a). Unde ned factors may also in uence the sensitivity of this method (Speit and Schmid, 2006). Besides, the frequency of micronuclei in reticulocytes may be low due to the ef ciency by which the spleen sequesters and destroys these aberrant cells (Dertinger et al., 2003). Some authors suggested that the *in vivo* comet assay can compensate the limitations of the micronucleus assay in blood smear (Sasaki et al., 1998).

5. Conclusion

Biota residing near coal mining activities in La Loma and La Jagua de Ibirico, Cesar, Colombia, presents greater risk of DNA damage in blood cells than those living far from these sites. Moreover, *M. musculus* and *I. iguana* showed to be sensitive and suitable organisms to investigate environmental genotoxicity derived from coal mining activities.

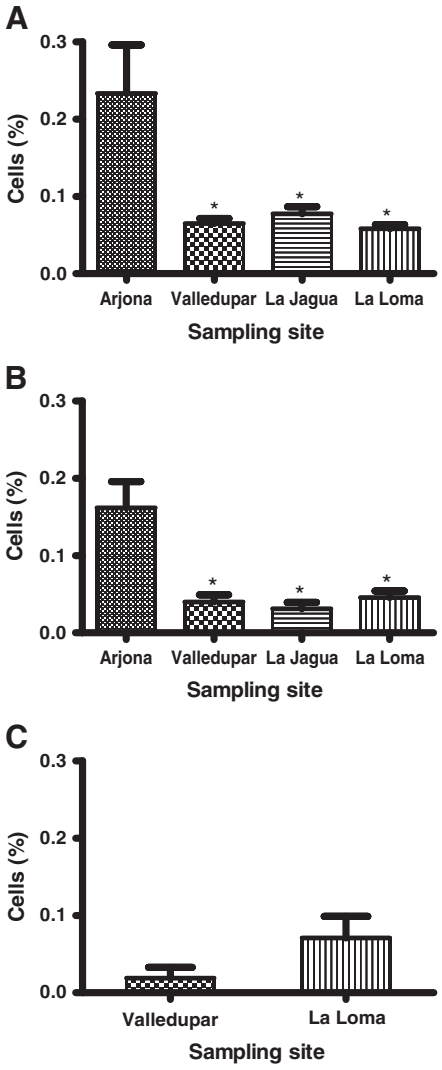

Fig. 5. Percentage of blood cells with micronucleus found in *Mus musculus* in 2010 (A) and 2011 (B); and *Iguana iguana* (C). *. Signi cantly different (P<0.05) when compared to Arjona. **. Signi cantly different (P<0.05) when compared to Valledupar.

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