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Effects of chronic exposure to coal in wild rodents (*Ctenomys torquatus*) evaluated by multiple methods and tissues

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Abstract

Rio Grande do Sul (RS) coal is low quality and typically obtained by strip mining. In a recent study concerning 2 years of biomonitoring in coal regions, we demonstrated the genotoxicity of coal and related products on blood cells of native rodents, from RS, Brazil. With the goal of studying the variations in the effects of RS coal on different tissues of the same rodent, we utilized, besides the single cell gel (SCG) and micronucleus (MN) assay on blood, histological analyses and SCG assay of bone marrow, spleen, kidney, liver and lung cells, and MN assay of bone marrow and spleen cells. In addition, to identify agents that can potentially influence the results, concentrations of several heavy metals were analyzed in livers and in soil, and the total concentration of hydrocarbons in the soil was determined. Rodents exposed to coal were captured at two different sites, Butiá and Candiota, in RS. Reference animals were obtained from Pelotas, where there is no coal mining. This report provides chemical and biological data from coal regions, indicating the possible association between Zn, Ni, Pb and hydrocarbons in the induction of DNA damage (e.g. single strand-breaks and alkali-labile sites) determined by the alkaline SCG assay in cells from *Ctenomys torquatus*. The results of the present SCG study indicate that coal and by-products not only induce DNA damage in blood cells, but also in other tissue cells, mainly liver, kidney and lung. Neither the MN assay nor histopathological observations showed significant differences; these analyses may be useful under circumstances where genotoxicity is higher. In conclusion we believe that the in vivo genotoxicity of coal can be biomonitored by the SCG assay, and our studies suggest that wild rodents, such as *C. torquatus* are useful for monitoring genotoxic damage by both methods, the SCG assay and the MN test.

Keywords: Coal; Comet assay; *Ctenomys torquatus*; Environmental monitoring; Genotoxicity; Micronucleus assay

1. Introduction

In a recent 2-year biomonitoring study in coal regions [1], we showed the genotoxicity of coal and related products on *Ctenomys torquatus*, using blood cells, from Rio Grande do Sul (RS), in southern Brazil. This fossorial rodent occupies northern Uruguay and southern Brazil [2], and its geographic distribution in RS almost coincides with the distribution of coal reserves [3]. Therefore, they were utilized as sentinel organisms for coal hazards.

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In recent years, a great deal of effort has focused on the development of techniques that could be used to monitor the health of organisms chronically exposed to pollutants [1,4,5]. Monitoring the environment by means of biological test systems, so-called biomonitors and biomarkers, provides promising ways to identify hazards to human health and the environment [6]. Currently, it is difficult to monitor environmental pollution because it is mostly identified by chemical observations and analytical techniques or by epidemiologic investigations. Furthermore, organisms in nature are not always exposed to a single compound, but to complex mixtures of compounds. Thus, analytical techniques fail to provide insight into biological hazards. Similarly, epidemiological studies also fail, mainly due to the inaccurately defined exposure conditions and the long time elapsed before the onset of some diseases [7].

Another approach to the evaluation of the possible consequences of hazardous waste pollution involves the assessment of genotoxic and cytotoxic damages, and other detrimental effects on sentinel organisms. Besides aquatic organisms [8] and plants [9,10], mammalian species [7] living in close proximity to man have been used as sentinel organisms.

An optimal method to detect genotoxic damage in sentinel organisms should be able to detect many classes of damage in a variety of cell types from a range of organisms, provide data concerning the individual cell, and be sensitive and rapid. The most commonly used method to evaluate levels of damage involves scoring chromosomal aberrations, micronuclei, and/or sister chromatid exchanges in proliferating cell populations.

Recently, the alkaline single-cell gel electrophoresis (SCG) assay was suggested as a useful technique for environmental biomonitoring [1,7]. This technique is a rapid, sensitive procedure to quantify DNA lesions in mammalian cells [11,12].

In our first investigation concerning the genotoxicity of RS coal, we utilized both the SCG and micronucleus (MN) assay, detecting for DNA damage in blood cells of *C. torquatus*. The goal of the present study was to investigate differences in the effect of RS coal on multiple tissues of the same rodent; for this purpose, we utilized, besides the same tests in blood cells, histological analyses, SCG assays of bone marrow, spleen, kidney, liver and lung cells, and MN assays of bone marrow and spleen cells. In addition, concentrations of heavy metals were analyzed in livers and soil, and total concentration of hydrocarbons was determined in soil. These analyses were conducted to identify agents that could potentially influence the results.

RS coal is low quality and is typically obtained by strip mining. Coal is a mixture of a variety of chemicals, especially hydrocarbons, which can give rise to polycyclic aromatic hydrocarbon (PAH). Many PAH compound produce mutagenic and carcinogenic effects [13–15]. Some of PAHs, such as benz[a]anthracene, chrysene, benzo[a]pyrene, indeno[1,2,3-c,d]pyrene and benzo[g,h,i]perylene, are found in the RS coal [16]. Besides hydrocarbons, this type of coal contains high concentrations of various heavy metals. Copper (Cu), lead (Pb), cadmium (Cd), nickel (Ni), vanadium (V) and zinc (Zn) are related to coal or activities, such as mining and burning fossil fuels [4,17,18]. There is insufficient evidence regarding its carcinogenicity in humans [17], although heavy metals, either alone or by enhancing the effect of other agents, are a potentially mutagenic class of environmental pollutants [19].

2. Materials and methods

2.1. Animal sampling

C. torquatus (Octodontidae-Rodentia) exposed to coal were captured at two different sites in RS: (a) Butiá (30°S 51°W), a region about 5 km from a strip coal mine and about 35 km from the Charqueadas-Jacuí coal power plant: 10 individuals — one male (subadult) and nine females (one juvenile, three subadult, and five adults), and (b) Candiota (31°S 54°W), close to a strip coal mine, <1 km, and beside the Presidente Médici coal power plant: eight individuals — one male (adult) and seven females (one juvenile, five subadults, and one adult). Reference animals for external control were obtained from Pelotas (31°S 52°W), a region where there is no coal mining: 10 individuals-three males (two subadults and one adult) and seven females (five subadults and two adults).

Oneida Victor (number zero) traps with a rubber cover for fossorials rodents were set where fresh earth mounds were located, one trapping per site. The

C. torquatus age groups were determined according to the Wilks [20] method.

All the work with the animals in this study was performed with permission from IBAMA (the Federal Brazilian Institute of the Environment).

2.2. Micronucleus assay

The MN assay was performed according to guidelines and recommendations [21,22]. Whole blood, bone marrow and spleen smears were prepared on slides, air dried and stained with a mix of 60 ml Giemsa (Merck), 30 ml May Grünwald Giemsa (Merck) and 10 ml phosphate buffer (pH 5.8) for 5 min. Afterwards, the slides were rinsed with phosphate buffer and air-dried. At least two smears were prepared from each animal for each tissue. The slides were coded for 'blind' analysis.

The micronucleated cells was analyzed in 1000 polychromatic erythrocytes (PCE) and 4000 normochromatic erythrocytes (NCE) per animal for blood smears, and 1000 PCEs and 2000 NCEs per animal for bone marrow and spleen. The frequency of micronucleated cells by sites, observed among 1000 cells (‰) analyzed, was calculated. Bartlett–Box test was used to evaluate the variances homogeneity of each site group. The statistical significance of the mean percentage of PCE and NCE with MN, test groups (Butiá and Candiota) in relation to the control (Pelotas), was determined using the two-tailed Student's *t*-test. A difference of $P < 0.05$ was considered statistically significant.

2.3. Comet assay

The alkaline Comet assay, was performed as described by Singh et al. [11] and Speit and Hartmann [23], as modified in Silva et al. [5] for field work.

Human blood was collected on the same day and under the same conditions as the samples from *C. torquatus*. This was considered an internal control for damage caused by manipulations, delay or transport to the laboratory.

Cells from different tissues were obtained according to the method described by Tice [24], where whole blood was utilized, and for bone marrow perfuse of femur with 2 ml of cold Hanks Balanced Salt Solution (HBSS) (INLAB) containing 20 mM Disodium EDTA (Vetec) was made. Small pieces of each organ were placed in two ml of cold HBSS containing 20 mM EDTA and 10% of dimethylsufoxide (DMSO) (Sigma), and minced into fine pieces.

Cells isolated from tissues (5–10 μl) were embedded in 95 μl of 0.75% low melting point agarose (Gibco BRL). The mixture (cell/agarose) was added to a fully frosted microscope slide coated with a layer of 300 μl of normal melting agarose (1%) (Gibco BRL). After solidification, the slides were placed in lysis buffer (2.5 M NaCl (Vetec), 100 mM EDTA and 10 mM Tris (Pharmacia Biotech), pH 10.0–10.5, with freshly added 1% Triton X-100 (Sigma) and 10% DMSO) for a minimum of 1 h up to 2 weeks. All procedures were developed in the field until lysis. The slides were kept in lysing solution, packed in a box with ice and transported to the laboratory. Subsequently, the slides were incubated in freshly made alkaline buffer (300 mM NaOH (Vetec) and 1 mM EDTA, pH 12.6) for 30 min. The DNA was electrophoresed for 30 min at 300 mA s and 25 V (0.90 V/cm), and then the alkali was neutralized with 0.4 M Tris (pH 7.5). Finally, the DNA was stained with ethidium bromide (2 μg/ml) (Sigma).

To demonstrate the electrophoresis conditions and sensitivity, negative and positive controls from human blood in the laboratory were used for each electrophoresis treatment. For positive control, 200 μl of whole blood was mixed with 50 μl of methyl methanesulfonate-Sigma (MMS) solutions at the following final concentrations: (a) 8×10^{-5} and (b) 4×10^{-5} M. This mix was incubated for 2 h at 37°C. The two concentrations were used to demonstrate different levels of damage and assay sensitivity, and to prevent false-negative results. The result of each electrophoresis was considered only if the negative and positive controls demonstrated negative and positive results, respectively.

Using a fluorescence microscope equipped with a BP546/12 nm excitation filter and a 590 nm barrier filter, images of 50 randomly selected cells (25 cells from each of two replicate slides) were analyzed from each animal. Comet image lengths (nuclear region + tail) were measured in arbitrary units (with a calibrated scale in the ocular). One unit was approximately 5 μm at 200× magnification.

Cells were also rated visually into five classes according to tail size, from undamaged-0, to maximally

damaged-4, resulting in a single DNA damage score for each animal, and consequently for each group studied. Thus, the damage index (DI) of the group can range from 0 (completely undamaged — 50 cells × 0) to 200 (with maximum damage — 50 cells × 4) [5,25].

Bartlett–Box test was used to evaluate the variances homogeneity of each site group. The damage frequency (%), was calculated based on number of cells with tail versus those without. The statistical evaluation was performed using the two-tailed Student's *t*-test. A difference of $P < 0.05$ was considered statistically significant.

2.4. Histopathological features

A complete autopsy was performed on all animals. Lung, spleen, liver, and kidney were fixed by immersion in 10% formalin. All tissue samples were embedded in paraffin, sectioned, and stained with haematoxylin and eosin. The slides were coded for 'blind' analysis, and were analyzed using an optical microscope by a trained pathologist.

Congestion, anthracosis, fibrosis, chronic inflammation, emphysema, mitosis, hemorrhage and acute inflammation were observed for the lung; congestion, chronic inflammation, mitosis and calcification for the kidney; congestion, chronic inflammation, steatosis and mitosis for the liver; and congestion, follicle hyperplasia and mitosis for the spleen. All tissue observations were also ranked as absent, tenuous, moderate or severe.

2.5. Chemical analysis

All chemical analyses were performed by the Ecology Center, Federal University of Rio Grande do Sul.

The soil was obtained from *C. torquatus* burrows, which were dry at 70°C during 24 h, and a sample of 0.5 g from each place was utilized. Small pieces of rodents liver (0.25 g, dry wt.), six from Pelotas, seven from Butiá, and six from Candiota, were utilized.

The concentrations of heavy metals, Cu, Pb, Cd, Ni, V and Zn, in the soil and liver of *C. torquatus* were determined by atomic absorption spectrophotometric analysis. Nitric acid (60%) was used for the digestion (MDS 2000 Microwave Digestion System) [26].

The total hydrocarbon concentrations in the soil were measured using gravimetric analysis after solvent extraction (hexane) [27].

3. Results

Table 1 shows the total number and pooled (mean per 1000 cells (‰) and standard deviation) of micronucleated peripheral blood, bone marrow and spleen cells of *C. torquatus* from Pelotas, Butiá and Candiota. The Bartlett's test indicates that the variances were essentially equal. The different tissue types from the three sites presented little variability. Candiota shows a higher number and mean of cells with micronuclei from all three tissues analyzed, but is not significant as compared with Pelotas (control). The analysis of the mean frequency of micronucleated cells using the Student's *t*-test indicated a significant increase only in PCE with MN ($P < 0.0001$) in the spleen of rodents from Butiá. The analysis of the difference between mean frequency of micronucleated cells by gender and by age group using the Student's *t*-test were not significant.

Comet assay data shown in Table 2 are the means of damage index, comet image length and damage frequency in internal control (human blood), and *C. torquatus* from Pelotas, Butiá, and Candiota for each tissue. None of the blood samples from internal control showed a positive response. Negative (DI = 0–5) and positive controls (DI = 150–200) for electrophoresis demonstrated negative and positive results, respectively. The Bartlett's test indicated homogeneity of variances of each site group. The comet image length for the two test groups, Butiá and Candiota, did not present significant differences as compared with Pelotas. However, the damage index and the frequency of damaged cells in peripheral blood, lung, bone marrow, and liver from Candiota rodents, are significantly higher than in Pelotas. The damage index and frequency of damage in kidneys from Butiá rodents are significantly lower than in Pelotas; the data are very similar in the other evaluations. The individual distribution and range of responses of DI are summarized in Fig. 1, where it can be observed that, in general, Candiota shows a higher DI than Pelotas and Butiá, and that the kidney and liver were the most highly affected tissues. The analysis of the difference between mean

Table 1
Total number and pooled (mean frequency (‰) and standard deviation) of micronucleated cells in peripheral blood, bone marrow and spleen of *Ctenomys torquatus* from Pelotas, Butiá, and Candiota[a]

Site	N[b]	Animal identification	Peripheral blood		Bone marrow		Spleen	
			mPCE[c]	mNCE[d]	mPCE[c]	mNCE[d]	mPCE[c]	mNCE[d]
Pelotas	10	J226	0	1	2	1	0	0
		J227	0	0	2	1	0	1
		J129	1	0	1	0	1	0
		J189	0	0	1	1	2	1
		J228	0	0	2	1	1	1
		J140	0	0	3	0	1	0
		J229	1	0	0	1	0	0
		J230	0	0	1	0	0	0
		J231	0	0	0	0	0	0
		J232	0	0	2	1	1	0
		Pooled	0.2±0.4	0.1±0.3	1.4±1.0	0.6±0.5	0.6±0.7	0.3±0.5
Butiá	10	J199	0	0	3	2	3	2
		J200	3	0	0	1	4	1
		J201	0	0	2	1	4	1
		J202	0	0	0	0	2	1
		J203	0	0	1	0	2	1
		J131	0	0	0	0	3	0
		J170	1	0	1	0	4	1
		J204	0	0	0	1	1	1
		J205	1	0	1	0	2	0
		J206	0	1	1	0	3	0
		Pooled	0.5±1.0	0.1±0.3	0.9±1.0	0.5±0.7	2.8±1.0*	0.8±0.6
Candiota	8	J218	1	1	3	2	0	0
		J219	1	0	1	1	2	1
		J220	0	0	3	0	2	1
		J221	1	0	1	1	2	1
		J222	0	0	3	0	2	1
		J223	1	0	2	1	0	0
		J224	0	0	1	1	3	1
		J225	1	0	2	1	0	0
		Pooled	0.6±0.5	0.1±0.3	2.0±0.9	0.9±0.6	1.1±1.2	0.5±0.5

[a] The number of cells scored for micronuclei was 1000 PCEs and 4000 NCEs per rodent for blood, and 1000 PCEs and 2000 NCEs per rodent for bone marrow and spleen.
[b] Number of rodents analyzed.
[c] Polychromatic erythrocytes with micronuclei.
[d] Normochromatic erythrocytes with micronuclei.
* $P < 0.05$/Student's *t*-test, in relation to Pelotas group (control).

frequency of damage index by gender and by age group using the Student's *t*-test were not significant.

Fig. 2 shows the extent of DNA damage, analyzed using a SCG assay (classes 0–4). More than 10% of lung, kidney and liver cells from Pelotas and Butiá were damaged, and classes 3 and 4 were the most frequent among the damaged cells. Class 4 was observed as above 10% for kidneys and livers from Pelotas, and for kidneys from Butiá. All tissue types for Candiota show more than 10% of 'comets'. The frequency of damage classes was different for each tissue type, with a frequency higher than 10% of class 4 for lung, kidney and liver.

Table 3 summarizes all histopathological findings for the lung, kidney, liver and spleen of *C. torquatus* from each site. Pelotas, Butiá and Candiota show

Table 2
Mean (M ± S.D.) of comet image length (IL), damage index (DI) in arbitrary units and damage frequency (DF) of cells from internal control (human blood; $N = 15$) and from peripheral blood, lung, spleen, kidney, bone marrow and liver of *Ctenomys torquatus* from Pelotas ($N = 10$), Butiá ($N = 10$) and Candiota ($N = 8$)[a]

Sites	Comet anlayze	Internal control	Tissues					
			Blood	Lung	Spleen	Kidney	Bone marrow	Liver
Pelotas	IL	23±4	26±5	32±17	29±16	47±26	26±10	37±21
	DI	1±1	5±2	24±9	16±8	71±29	8±5	44±16
	DF	1±1	7±4	17±7	11±6	48±15	7±4	31±8
Butiá	IL	25±5	25±5	31±17	28±12	35±18	25±10	34±20
	DI	2±1	3±2	23±20	14±7	43±27*	9±7	34±16
	DF	2±2	3±3	15±11	10±5	26±15**	6±5	23±9
Candiota	IL	24±8	29±11	37±24	30±18	44±26	32±21	43±25
	DI	1±1	19±9***	42±20*	23±8	65±17	27±11***	63±11**
	DF	1±1	27±11***	32±11**	17±7	54±10	22±11**	46±12**

[a] Measures were calculated for 50 cells per rodent. *N*, number of individuals analyzed.
* $P<0.05$.
** $P<0.01$.
*** $P<0.001$, in relation to Pelotas/Student's *t*-test.

similar histopathological findings for all organs analyzed. No distinctive macroscopic differences were observed in any organs examined, although Pelotas demonstrated slight increases in damage.

In soil samples from Butiá and Candiota, higher concentrations of heavy metals (Zn, Ni, Pb, Cd, V and Cu) were observed, compared to the control area, Pelotas (Pelotas < Butiá < Candiota) (Table 4). Heavy metal concentrations in the liver for the three sites were similar, with slight differences. Differences were observed when our results were compared with the minimum contaminant levels considered toxic by Freedman [28] and SETAC [29] for soil and liver, respectively. Concentrations of Cd, V and Cu in the soil and liver samples for all three sites were less than the minimum contaminant level considered by Freedman and SETAC. Concentrations of Zn for soil samples and livers from the three sites are toxic according to Freedman and SETAC, but coal regions showed the highest levels. For Ni, concentrations in the liver from Pelotas and Candiota show toxic levels, where Candiota demonstrated the highest level. The concentration of Pb in the livers from three sites was also toxic, and for this metal Pelotas showed higher level than coal regions.

Concerning hydrocarbons, a soil sample from Candiota (220 ppm) shows a higher total concentration than Butiá (160 ppm) and Pelotas (140 ppm) (Pelotas < Butiá < Candiota).

4. Discussion

Some studies have used the SCG assay as a method to detect genotoxicity in rodent organs, although few reports which employed mechanical methods for solid tissue dissociation were successful in their comet assay [30]. In order to detect organ-specific DNA damage caused by chemicals, the comet assay protocol should be simple enough to allow the simultaneous measurement of various organs.

The results of the present SCG study indicate that coal and by-products not only induce DNA damage in blood cells, but are at least representative of genotoxic damage to the whole organism; for instance, in the Candiota group which was the most affected, the blood cells showed the most significant increase. The MN assay was not as sensitive, showing a significant result only for Butiá spleen PCE. The negative result observed for Candiota as compared with Pelotas by MN assay, agrees with data from Ong et al. [31], which did not detect a significant increase of MN in mice exposed to coal dust.

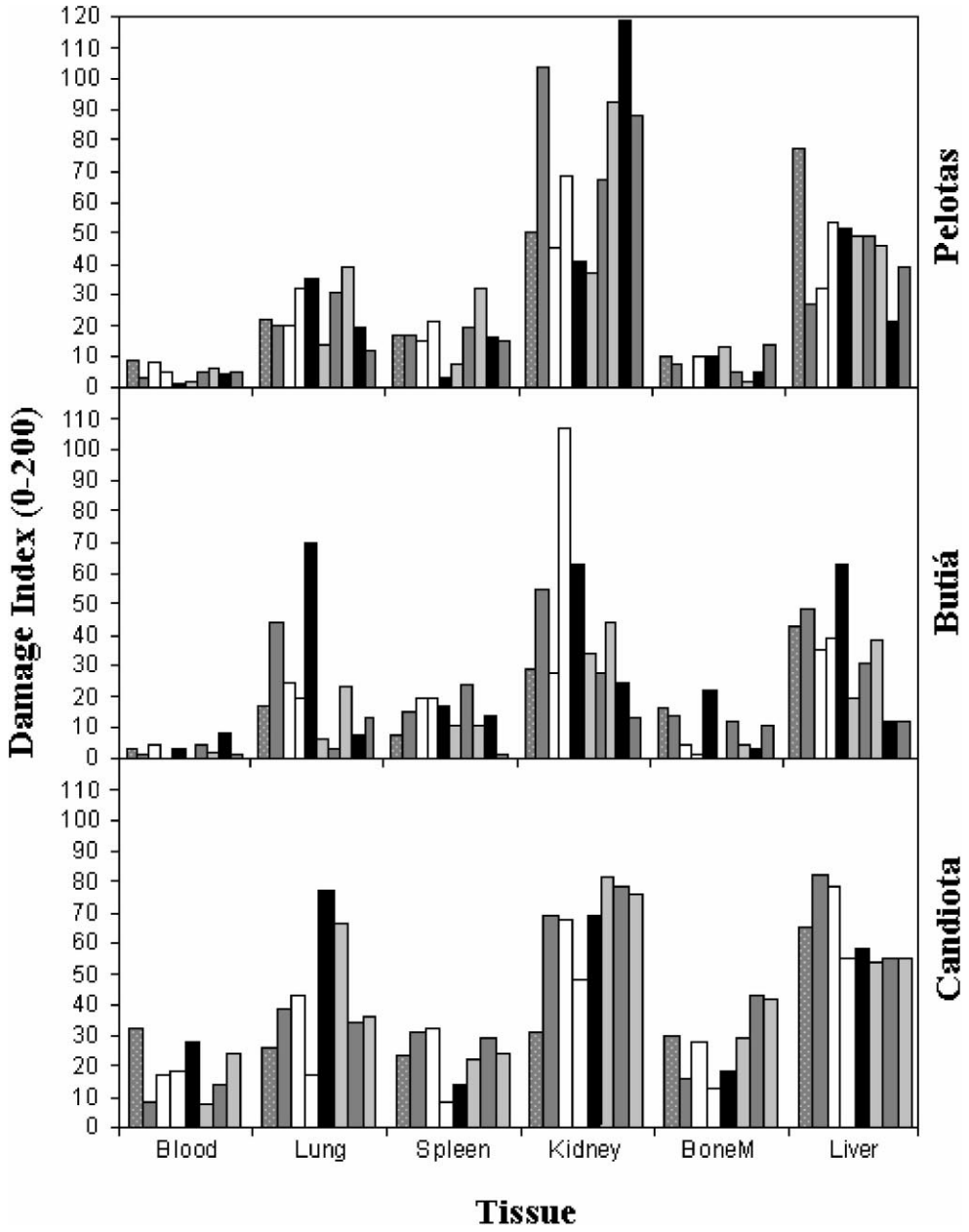

Fig. 1. Individual distribution and range of responses of damage index in blood, lung, spleen, kidney, bone marrow (BoneM) and liver cells of *Ctenomys torquatus* from Pelotas, Butiá and Candiota.

In vivo genotoxicity is usually evaluated by the MN assay, which is generally limited to hematopoietic cells and may not detect carcinogens that target organs other than bone marrow cells [30,32]. In our studies, peripheral blood cells, bone marrow cells and spleen cells of *C. torquatus* from test regions (Butiá and Candiota) did not show a significant difference as compared to the control (Pelotas), except for Butiá spleen PCE. Although not significant, all three types of tissue from Candiota have shown a higher mean

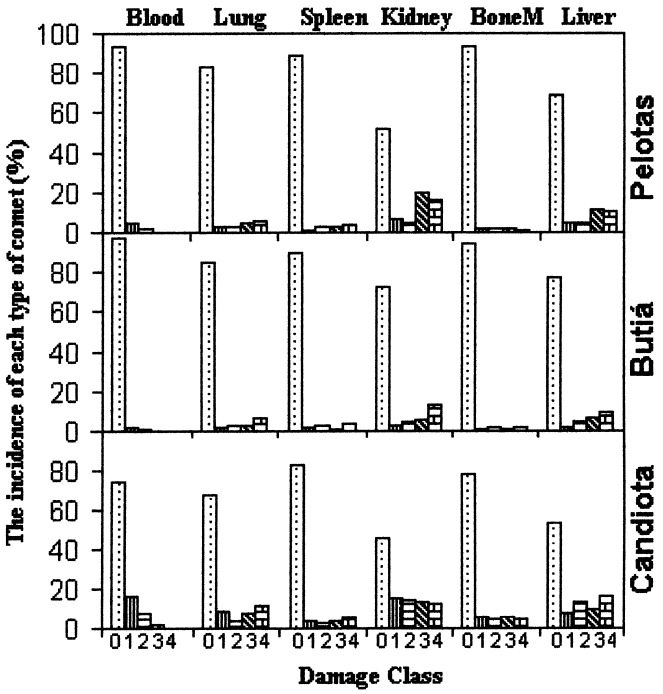

Fig. 2. The distribution of damage classes (0-undamaged to 4-maximum damage) of cells from blood, liver, kidney, lung, spleen and bone marrow of *Ctenomys torquatus* from Pelotas, Butiá and Candiota. Frequency was calculated for 50 cells per rodent.

value of cells with MN than the other sites. This result at Candiota reinforces our previous findings; the animals from Candiota show increased damage in blood cells, most likely caused by coal and by-products [1]. These results are consistent with the recently emerging data from Japan that suggest that peripheral blood may indeed be a suitable monitor for in vivo genotoxicity [33]. Although the sensitivity of the MN assay for the spleen is comparable to that of bone marrow [34,35], we observed negative results for bone marrow and positive for Butiá spleen PCE. The same significant result was not detected for Candiota, the site most exposed to coal, and also no relationship to SCG, chemical and histopathological results was detected in MN results from Butiá. As can be seen in Table 1, the PCE presented in all times higher frequency of micronucleated cells than the NCE. It can be due to a partial removal of the micronucleated cells, as by spleen, or their preferential loss and/or dead.

Histopathological analysis shows similar results for Pelotas, Butiá and Candiota, without signs of pre-carcinogenic lesions. This kind of lesion, in the case of chronic exposure, takes some time to appear, and *C. torquatus* has a life of approximately three years. Thus, histopathological analysis, such as the MN test, could be useful under circumstances where genotoxicity is higher.

The alkaline SCG assay shows a significant increase of DNA damage for peripheral blood, bone marrow, lung and liver cells of animals from Candiota. Peripheral blood and bone marrow cells showed a greater difference in relation to Pelotas cells than other tissues. This observation demonstrates the sensibility of these two tissues in relation to coal and by-products, besides validating our biomonitoring results obtained only from blood samples. Similarly, human and bovine peripheral lymphocytes show chromosomic aberrations when exposed to coal and by-products [36,37,38]. The main advantage of working only with peripheral blood is the fact that the animals are not killed. Although lung and liver cells from Candiota rodents demonstrated a significant increase of DNA damage, the animals from Pelotas show average damage frequencies above 10% for these tissues, which

Table 3
Histopathologic characteristics from absent-0 to severe-3, observed for lung, kidney, liver and spleen of *Ctenomys torquatus* from Pelotas, Butiá and Candiota[a]

Tissue	Diagnosis	Pelotas				Butiá				Candiota			
		0	1	2	3	0	1	2	3	0	1	2	3
Lung	Congestion	0	0	3	7	0	2	6	2	0	0	5	3
	Anthracosis	2	6	2	0	6	4	0	0	5	2	1	0
	Fibrosis	7	3	0	0	8	2	0	0	5	3	0	0
	Chronic inflammation	0	8	2	0	0	8	2	0	1	5	2	0
	Emphysema	0	8	2	0	0	5	5	0	1	5	2	0
	Mitosis	3	7	0	0	7	3	0	0	5	2	1	0
	Hemorrhage	4	6	0	0	10	0	0	0	7	1	0	0
	Acute inflammation	8	2	0	0	8	2	0	0	7	1	0	0
Kidney	Congestion	0	7	3	0	0	9	1	0	0	7	1	0
	Chronic inflammation	1	9	0	0	4	6	0	0	3	5	0	0
	Mitosis	8	2	0	0	10	0	0	0	8	0	0	0
	Calcification	7	1	1	1	8	1	0	1	3	0	4	1
Liver	Congestion	0	6	4	0	0	10	0	0	0	6	2	0
	Chronic inflammation	7	3	0	0	10	0	0	0	8	0	0	0
	Steatosis	2	5	3	0	7	3	0	0	3	4	1	0
	Mitosis	10	0	0	0	10	0	0	0	8	0	0	0
Spleen	Congestion	0	8	2	0	1	3	5	1	1	4	3	0
	Follicle hyperplasia	1	4	5	0	3	6	1	0	3	5	0	0
	Mitosis	5	2	3	0	4	4	2	0	6	2	0	0

[a] Ten rodents were analyzed for Pelotas and Butiá, and eight for Candiota.

Table 4
Heavy metal concentrations (ppm) in livers samples of *Ctenomys torquatus* and soil from Pelotas, Butiá and Candiota

Sites	Sample	Heavy metals concentrations (ppm)[a]					
		Zn	Ni	Pb	Cd	V	Cu
Pelotas	Soil	187.2	ND[d]	25.2	ND[d]	3.2	2.0
	Liver[b]	121.3±41.4	4.0±3.1	2.9±2.5	0.4±0.2	0.7±0.3	7.8±5.4
Butiá	Soil	199.0	4.4	34.6	0.1	8.6	3.6
	Liver[c]	121.3±49.0	1.1±1.1	2.8±3.7	0.3±0.4	0.5±0.1	4.7±5.1
Candiota	Soil	210.0	6.1	28.2	0.1	10.3	17.0
	Liver[b]	128.2±45.6	8.4±6.3	1.1±0.7	0.3±0.2	1.0±0.6	5.1±5.9
Toxic	Soil[e]	>90	>50	>35	>0.35	>90	>30
Levels	Liver[f]	>100	>2	>1	>1.5	–[g]	>30

[a] Measured in mg/kg of tissue (dry wt.).
[b] Mean ± S.D., $n = 6$.
[c] Mean ± S.D., $n = 7$.
[d] Not detected.
[e] By Freedman [28].
[f] By SETAC [29], from mammals livers.
[g] Not cited by SETAC (1996).

is not desirable for a negative control. Possibly the data on spleen and kidney cells from Candiota did not present statistical significance due to the high results from Pelotas.

Although Pelotas was selected as the negative control site for coal exposure; this region is more industrialized than Butiá and Candiota. The residual emission of particulate material from fuel burning is 307 t/year for Pelotas, 2 t/year for Butiá and 8506 t/year for Candiota [39]. The high emission values observed for Candiota are caused mainly by the Presidente Médici coal power plant.

Thus, Pelotas rodents could be affected by environmental contamination caused by genotoxic agents, resulting from emissions and waste products of industrial plants and from the use of chemicals in agriculture. Animals reared in contaminated environments are directly exposed to these contaminants, but herbivores, such as the rodents utilized in this study, may be exposed via contamined food. These high levels of DNA damage in tissues from Pelotas (kidney, liver, spleen and lung) could be associated with concentrations of lead, nickel and zinc detected above toxic levels in the samples from this site. Lead and nickel damage mainly kidneys and liver, and zinc and nickel damage the lung [17]. However, the mechanism of metal-induced carcinogens is still unknown [17], but one possible pathway may involve the production of DNA damage [40]. Rojas et al. [40] suggest that metals induces DNA single strand breaks. Although this kind of damage is soon repaired, in chronic exposures it can represent a form of continuing DNA damage.

The possibility that Pb is genotoxic is by no means clear; its genotoxicity can be mediated through indirect mechanisms, such as the inhibition of DNA repair [41]. Thus, lead alone could not induce a significant increase of MN and chromosomal aberrations in the peripheral blood and bone marrow of rats [41]. Similarly, we did not observe much damage in the blood and bone marrow cells from Pelotas and Butiá, despite the high concentration of Pb observed at these two sites. The high concentration of Pb in Pelotas agrees with the data found by Garcia-Fernandez et al. [42], that demonstrated a higher concentration of Pb for birds from more industrialized areas.

Saplakoglu et al. [43], using the SCG assay, observed that Ni creates single-strand breaks in lung cells when administered alone to rats. As reported in the same study, no DNA strand breakage was evident in the liver and kidney. Pelotas and Candiota show concentrations of Ni above toxic levels, and the lungs of animals collected at these sites show a high frequency of damaged cells.

Although the Zn concentration in the liver samples and soil is above toxic levels, it occurs similarly at all three sites. Furthermore, Zn is relatively nontoxic to mammals [44].

Kidney and liver were the main damaged tissues in all three groups. These observations agree with the fact that the liver and kidney are the principal sites of mixed-function oxygenases, which metabolize a variety of organic compounds to more polar by-products that can either react with macromolecules or be eliminated. Higher levels of organic contaminants, such as PAHs, have been associated with higher enzymatic activity in these tissues [45].

Beside the natural occurrence of hydrocarbons with coal, they can have an anthropogenic origin, for instance from automobiles, aircraft, refineries and other industries [28]. Garbage collectors who work in the street, and are exposed to motor vehicle exhaust gas, present PAHs in the blood cells [46]. Thus, we observed hydrocarbons in the soils of all three regions, but mainly from Candiota, because of the association of hydrocarbons with coal. In addition, benzo[a]pyrene detected in RS coal, induces damages in DNA detected by SCG assay, mainly in the liver and lung [30,32,35,47].

Liver, lung and kidney samples from Candiota show 10% class 4 cells. The occurrence of comets with no heads (class 4) and with nearly all the DNA in the tail has been described as the most obvious aspect of apoptotic cells in the comet assay, and apoptotic DNA fragmentation is characterized by the generation of DNA double strand breaks [12,48]. These observations probably indicate a cytotoxic effect of coal and related products for these tissues that is not directly related to genotoxicity [48]. Pelotas shows the same for liver and kidney, its cytotoxic effect can not be related to coal and by-products, but could be due to industrial wastes. The results for kidneys were unexpected, since the control samples from Pelotas, presented damage data similar to the samples from Candiota, which is the place with the highest toxicity index in all outcomes. Consequently, from some standpoints, the rodents from Pelotas are inappropriate controls.

One of the difficulties that faced investigators has been to identify the individual compounds that may be responsible for possible adverse effects associated with exposure to environmental agents. Samples obtained from environmental sources are complex mixtures of organic and inorganic compounds, consisting of thousands of individual components [49]. This study provided chemical and biological data from coal regions, indicating the association between Zn, Ni, Pb and hydrocarbons which induced DNA damage, both single-strand break and alkali-labile sites, evaluated by alkaline SCG assay in cells from the wild rodent *C. torquatus*.

Since few cells are required in the SCG assay, genotoxicity can be detected using a small portion of each organ obtained at autopsy prior to fixation. Thus, alkaline SCG assay can be combined with general rodent toxicity tests. In addition, we should note that the alkaline SCG or comet assay makes it easier to perform the in vivo genotoxicity evaluation of different target organs, showing the heterogeneity of tissue response. In the present study, we observed that there is an association between increases damage, mainly to the liver, lung and kidney, and coal production.

In conclusion, we believe that the in vivo genotoxicity of coal can be biomonitored by the SCG assay, and our experiments suggest that *C. torquatus* is very useful for monitoring genotoxic damage by both the SCG assay and the MN test.

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