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Genotoxic Effects of Aluminum on the Neotropical Fish *Prochilodus lineatus*

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Abstract Applying an integrated approach using the Comet, micronucleus (MN), and random amplified polymorphic DNA (RAPD) assays, occurrence of erythrocytic nuclear abnormalities (ENAs) and the liver activity of antioxidants enzymes (catalase and glutathione-*S*-transferase (GST)) was carried out to evaluate the effects of acute (6, 24, and 96 h) and subchronic (15 days) exposures to aluminum on fish *Prochilodus lineatus*. The Comet assay showed that fish erythrocytes exhibited significantly higher DNA damage after 6 and 96 h of Al exposure. MN frequencies were very low and did not increase significantly after Al exposures, while ENAs frequency increased significantly after all exposure periods. RAPD profiles obtained with DNA from fish fins collected before the toxicity tests were compared to the profiles with DNA from gills and liver of the same fish sampled after Al exposures. Alterations in RAPD profiles, including appearance and disappearance of bands, after 6 h, 24 h, and 15 days of Al exposure were detected. Fish exposed to Al for 6 and 24 h also showed significant increases in GST and catalase activities. These results indicated that Al exposure was genotoxic to *P. lineatus*, inducing DNA damage in peripheral erythrocytes. The induction of antioxidant enzymes might be an indication that Al causes oxidative damage to DNA, while the very low frequency of MN suggests that Al does not produce clastogenic or aneugenic effects. Genotoxic effects after 15 days of Al exposure was revealed only by RAPD, showing that this assay represents a sensitive method to detect genotoxic damage, occasionally not detected by other genotoxic tests used in toxicological genetics studies.

Keywords Aquatic environment · Genotoxicity · Comet assay · RAPD · Micronucleus · Oxidative stress

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1 Introduction

Increased concentrations of aqueous aluminum represent a major environmental problem due to the relationship between aluminum toxicity and fish (Alstad et al. 2005). This metal becomes more soluble and, for this reason, potentially more toxic to freshwater fish as pH decreases below 6.0 and is recognized as the main toxicant killing fish in acidified waters (Gensemer and Playle 1999). Although the genotoxic effects of aluminum for aquatic

invertebrates (Ternjej et al. 2009) as well as for humans (Crebelli et al. 2002; Lankoff et al. 2006; Lima et al. 2007) have been demonstrated, information regarding the potential genotoxicity of this metal on fish is still lacking.

In general, metal genotoxicity seems to be linked to the formation of reactive oxygen species (Soto-Reyes et al. 2005). Oxidative stress is established whenever the rate of reactive oxygen species (ROS) production exceeds the rate of its decomposition by antioxidant defenses and repair systems, leading to the oxidation of key cell components like proteins, fatty acids, and DNA (Sies 1993). The oxidation of DNA by ROS can produce strand breaks, which represent a major class of oxidative damage to DNA under oxidative stress, and a number of different modified DNA bases (Cadet et al. 1997). Aluminum is recognized as a pro-oxidant agent promoting biological oxidation both in vitro and in vivo (Exley 2004). The pathology of laboratory animals that have been experimentally intoxicated with aluminum invariably shows many indices of oxidative stress, including changes in the levels of antioxidant enzymes and the occurrence of oxidative lesions (Zatta et al. 2002).

Different approaches have been developed for the assessment of DNA strand breakage (Mitchelmore and Chipman 1998). The single-cell gel electrophoresis or Comet assay can detect DNA strand breaks at the individual cell level under alkaline conditions (Singh et al. 1988). This is a highly sensitive and rapid method that can be performed on almost any eukaryotic cell, requiring small amounts of tissue (Jha 2008). The Comet assay has been extensively used as a non-specific measure of genotoxic damage in fish (Mitchelmore and Chipman 1998; Dhawan et al. 2009). According to these authors, several of these studies have demonstrated the usefulness of this assay in fish as a model for monitoring genotoxicity of aquatic habitats using these indicator animals.

Another well-established assay that has been proven useful in assessing the genotoxic effects of a wide range of compounds in fish is the evaluation of micronucleus induction (Udroiu 2006). The analysis of erythrocytic nuclear abnormalities (ENAs), a variant of the standard micronucleus test, has also been widely used in fish toxicology (Ergene et al. 2007). In this assay, a number of alterations in red blood cell nuclei that may lead to their fragmentation and/or to micronucleus formation are recorded instead of counting the micronuclei themselves.

In the last decade, random amplified polymorphic DNA (RAPD) assay, a simple, fast, sensitive, and straightforward PCR-based method (Williams et al. 1990), has been used to detect genotoxic-induced DNA damage and mutations in different organisms, including fish (Savva 2000; Atienzar and Jha 2006; Swaileh et al. 2008). This method involves the amplification of random segments of genomic DNA using short and arbitrary primers which interact with the genomic template at sites where there is some homology (Williams et al. 1990). Although RAPD assay provides just qualitative results, given that the nature and extent of DNA alterations can only be speculated (Atienzar et al. 2002), this method complements other well-established techniques in genotoxicity (Atienzar and Jha 2006).

In this context, the present study aimed at evaluating the genotoxicity of aluminum in a native fish species and the relative sensitivity of RAPD assay compared to other commonly used assays. In order to achieve these objectives, an integrated approach using quantitative (Comet, micronucleus, and ENAs) and qualitative (RAPD) genotoxic assays associated with biochemical assays to measure antioxidant enzymes activities were used. The fish *Prochilodus lineatus* was chosen because it is representative of the Neotropical fish fauna, commonly found in rivers of the south and southeast regions of Brazil, and considered bioindicator fish species (Simonato et al. 2006).

2 Material and Methods

2.1 Animals

Juveniles of *P. lineatus* (n=89) weighing 13.6±7.3 g (mean ± SD) were obtained from the hatchery station of State University of Londrina. Animals were acclimated to laboratory conditions for 7 days in a 600-L tank with dechlorinated water (T = 21.8±1.1°C, pH 7.9±0.3; hardness, 52±2.8 mg L^{-1} $CaCO_3$), with constant aeration (DO, 8.1±1.4 mg O_2 L^{-1}) and a 14/10-h light/dark photoperiod. During this period, fish were fed each 48 h with commercial pellet food containing 36% proteins (Guabi®, BR). Animals were not fed 24 h before and during the toxicity tests.

2.2 Toxicity Tests and Fish Sampling

Acute (6, 24, and 96 h) static toxicity tests and subchronic (15 days) semi-static toxicity tests were performed in 100-L glass aquaria containing six fish each, with continuously aerated dechlorinated water. For each experimental interval, one group of fish was exposed to Al in acid water (pH 5.2) simultaneously with a negative control group (NC), exposed only to water with neutral pH. The pH was adjusted with HCl (10%) and was monitored every 6 h. Aluminum was added to the water as $Al_2(SO_4)_3$ reagent grade at a nominal concentration of 1 mg Al L^{-1}. All toxicity tests were carried out in duplicate. In the subchronic test, the water was renewed every 5 days and fish were terminally sampled after 15 days of exposure. An acid only treatment was not included in this study as it has been already established that increases in dissolved Al occur together with decreased pH (Monette et al. 2008). For the RADP and Comet assays, a positive control group, consisting of fish injected with the clastogenic agent cyclophosphamide (20 mg kg^{-1}; Sigma, CAS no. 64-86-8), was terminally sampled 24 h after treatment.

During the tests, water was continuously monitored for temperature, dissolved oxygen, pH, and conductivity. Samples of water collected immediately after each experimental period were analyzed for Al concentration using atomic absorption spectrophotometry. The concentration of total Al was determined in samples of non-filtered water, and the concentration of dissolved Al was determined in filtered water samples (0.45 μm); for both analyses, samples were acidified with HNO_3. Immediately after removal from the aquaria, fish were anesthetized with benzocaine (0.1 g L^{-1}), and blood samples were taken from the caudal vein into heparinized plastic syringes. Subsequently, animals were killed by cervical section and the gills and liver immediately removed.

2.3 Comet Assay

Immediately after blood sampling, a small amount of blood (10 μL) was diluted in 700 μL of phosphate-buffered saline (126.6 mM NaCl, 4.8 mM KCl, 1.5 mM $CaCl_2$; 3.7 mM $NaHCO_3$; 8.9 mM Na_2HPO_4; 2.9 mM NaH_2PO_4) and kept in ice until the start of the Comet assay. The alkaline Comet assay was performed on erythrocyte cells as described by Singh et al. (1988), with some modifications (Cavalcante et al. 2008). The main steps of the assay were: (a) lysis: 1 h, at 4°C, protected from light, in a lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1 mL Triton X-100, pH 10.0); (b) DNA unwinding: 30 min, in the dark, in an electrophoresis buffer (0.3 N NaOH, 1 mM EDTA, pH>13); (c) electrophoresis: 20 min, 300 mA, 25 V, 1 V cm^{-1}; and (d) neutralization: three washes for 5 min each in buffer (0.4 M Tris, pH 7.5). Slides were fixed with absolute ethanol for 10 min and kept under refrigeration until cytological analyses.

Slides stained with ethidium bromide (20 μg mL^{-1}) were analyzed under a Nikon fluorescence microscope (×1,000 magnification) fitted with a 515- to 560-nm excitation filter and a 590-nm barrier filter. All slides were independently coded, and blind analyses were performed by the same analyzer. The extent of DNA damage was quantified by the length of DNA migration, which was visually determined in 100 randomly selected and non-overlapping cells per fish. DNA damage was classified in four classes based on the Comet tail length (0: no detectable damage; 1: minimum damage; 2: medium damage; 3: maximum damage) and each Comet assigned a value of 0 to 3 according to its class; the total score will be between 0 and 300 "arbitrary units" (Kumaravel et al. 2009). Results for DNA damage in erythrocytes were expressed as the mean number of damaged nucleoids (sum of classes 1, 2, and 3) and the mean Comet score for each treatment group (NC or Al) for each exposure period and also for PC.

2.4 Micronucleus Test and ENAs

The micronucleus test was performed with fish erythrocytes according to Hoftman and de Raat (1982) and the analysis of erythrocytic nuclear abnormalities according to Çavas and Ergene-Gözükara (2005). Immediately after sampling, blood was smeared on clean glass slides, dried overnight, fixed with methanol for 10 min, and stained with Giemsa (5%). Three thousand erythrocytes per fish were examined under an Olympus optical microscope (×1,000 magnification). The mean frequencies of micronucleus (MN) and the other ENAs found in each experimental group were calculated and expressed per 1,000 cells (‰). ENAs were classified, following Pacheco and Santos (1997), into three

categories: segmented nuclei, lobed nuclei, and kidney-shaped nuclei. MN and ENA frequencies were analyzed in erythrocytes of fish exposed to the toxicity tests for 24 h or longer, taking into consideration that the estimated time for the detection of MN in circulating blood erythrocytes ranges between the second and third days after a clastogenic treatment (Udroiu 2006).

2.5 Random Amplified Polymorphic DNA

In order to compare RAPD profiles before and after toxicity tests, genomic DNA was isolated from the adipose fin, gills, and liver of each fish. Before the start of the acclimation period, fish were anesthetized with benzocaine and the adipose fin was removed and used as the source of DNA prior to the tests. A numbered small plastic ring was tight up around the caudal peduncle for fish identification.

Following each exposure period, animals had their gills and liver removed for DNA extraction. The choice of liver and gill tissues for RAPD analysis was based on the detoxification function of liver, which makes this organ a frequent target to oxidative damage, and the direct contact of gills with the environment and consequently with the xenobiotic present in the water.

DNA from adipose fin, gills, and liver of fish was extracted according to Almeida et al. (2001). DNA concentration was determined in a DyNA Quant 200 fluorometer (Hoefer) using the dye Hoechst 33258 (Sigma). All isolates were used immediately or stored at −20°C. DNA amplifications were performed as described by Sofia et al. (2006), with minor modifications. Final reaction volumes were 15 μL and contained 15–25 ng of template DNA, 250 μM dNTP (Pharmacia Biotech), 0.3 μM of ten-nucleotide primer (Operon Technologies, Alameda, CA, USA), 3.3 mM of $MgCl_2$, and 1 U of *Taq* DNA polymerase (Biotools) in the reaction buffer supplied. Thirty decamer oligonucleotides (from kits OPAC, OPX, and OPW) were used as random primers in RAPD screening. Control reactions were run with all components except genomic DNA. DNA amplifications were carried out in a thermal cycler (MJ Research PTC-100), and the amplification protocol consisted of 4 min at 92°C followed by 40 cycles of 40 s at 92°C, 1.5 min at 40°C, and 2 min at 72°C. The last round of amplification was followed by an additional extension at 72°C for 5 min.

Samples of 15 μL of amplification products were assayed by electrophoresis on 1.4% agarose gels with TBE buffer (0.89 mM Tris, 0.89 mM boric acid, 2 mM EDTA, pH 8.3) diluted 1:20 (*v*/*v*), run at 3 V cm^{-1}, and stained with ethidium bromide. Agarose gel images were documented under UV light using the Kodak Electrophoresis Documentation and Analysis System.

Comparative analyses were based on RAPD profiles obtained from different organs of each fish; fin, gill, and liver RAPD products, from the same fish, were placed side by side on the same gel. Thus, RAPD markers were determined by direct comparison of the amplified DNA electrophoretic profiles, and each sample was scored for the presence or absence of amplification products (binary variable). Aiming to obtain a high reliability of the data, only gain or loss of RAPD amplicons were considered in the analysis. RAPD profiles obtained with DNA extracted from fish fins collected before toxicity tests were compared to RAPD profiles with DNA extracted from gills and liver of the same fish collected after experimental exposures. Thus, the differences in RAPD profiles detected for each individual can be attributed to the direct or indirect effects of the contaminants (Atienzar and Jha 2006). The molecular weight of RAPD products were based on a 100-bp DNA ladder (Invitrogen) applied to the gel. When some alteration in RAPD profiles was detected, three new amplifications were carried out, in different days, to check the consistency of results.

2.6 Biochemical Assays

After removal, liver samples were stored frozen at −80°C until biochemical assays. For catalase and glutathione-*S*-transferase (GST) assays, liver samples were homogenized in 10 volumes of ice-cold 0.1 M K phosphate buffer, pH 7.0, and centrifuged for 20 min at 4°C and 15,000×*g*. The supernatant was used for enzyme assays. Catalase activity was determined by measuring the rate of decomposition of H_2O_2 in a spectrophotometer at 240 nm (Beutler 1975). GST activity was determined by enzymatic conjugation of reduced glutathione with 1-chloro-2,4-dinitrobenzene in a spectrophotometer at 340 nm (Habig et al. 1974). Catalase (CAT) activity was expressed as micromoles per minute per milligram liver per protein and GST activity as nanomoles per minute per milligram liver per protein. The concentrations of protein in liver samples were

determined according to Lowry et al. (1951) using bovine albumin as standard.

2.7 Statistical Analysis

Differences between the results obtained for the aluminum-exposed group and negative control group, at each exposure time (6 h, 24 h, 96 h, and 15 days), were analyzed by Student's *t* test. Values of $p<0.05$ were considered significant. Statistical analyses were performed using Sigma Stat 3.5.

3 Results

3.1 Water Chemistry

The overall quality of the water remained stable throughout the experiments considering all exposure periods. The mean values (±SD) for NC and Al groups were, respectively, temperature (°C): 22.3±1.7 and 22.3±1.6; dissolved oxygen (mg O_2 L^{-1}): 6.8± 1.9 and 6.7±1.6; and conductivity (μS cm^{-1}): 92.7± 15.4 and 121.4±21.3. The mean pH value for NC was 7.7±0.5, and in Al-exposed groups, pH values ranged from 5.04 to 5.36; the mean value was 5.20±0.13.

The concentrations of aluminum were very similar among Al groups in different exposure periods. The mean values (±SD, $N=5$) of total Al concentration in the water of experimental groups (Al) were 438± 36 μg Al L^{-1}, and the concentration of dissolved aluminum was 196±28.7 μg Al L^{-1}.

3.2 Comet Assay

Fish erythrocytes exhibited higher DNA damage after 6 and 96 h of Al exposure, as demonstrated by the significant increases in the number of damaged nucleoids and Comet scores in relation to respective NC (Table 1 and Fig. 1). Fish injected with cyclophosphamide (PC) showed a significant increase in both in the number of damaged nucleoids and Comet scores in relation to negative controls of all experimental periods (Table 1).

3.3 Micronucleus Test and ENAs

Frequencies of MN and nuclear abnormalities (ENAs) in peripheral fish erythrocytes from groups of fish exposed

Table 1 Frequency of nucleoids observed in each Comet class (0, 1, 2, 3) and the number of damaged nucleoids (mean ± SE) in erythrocytes of *P. lineatus* exposed to 196 μg L^{-1} of dissolved Al and the respective negative controls (NC), taking into account the total number of fish (N) analyzed for each experimental period (6 h, 24 h, 96 h, and 15 days)

Period of exposure	Group	*N*	Comet classes				No. of damaged nucleoids per fish (mean ± SE)
			0	1	2	3	
6 h	NC	12	74.3	25.3	0.3	0	25.7±2.4
	Al	7	48.6	48.7	2.7	0	51.4±6.7*
24 h	NC	10	81.6	18.4	0	0	18.4±4.2
	Al	6	68.7	27.8	3.0	0.5	31.3±11.2
96 h	NC	9	76.7	21.3	1.9	0.1	23.3±6.3
	Al	6	62.8	32.0	3.5	1.7	37.2±15.4*
15 days	NC	5	63.2	35.6	1.2	0	36.8±8.8
	Al	6	73.8	23.5	2.7	0	26.2±5.9
24 h	PC	7	11.3	63.0	23.1	2.6	88.7±3.8**

Positive control (PC) was analyzed only after 24 h of cyclophosphamide injection. One hundred nucleoids were analyzed per fish

*$p<0.05$ (significantly different from respective negative control); **$p<0.05$ (significantly different from all negative controls)

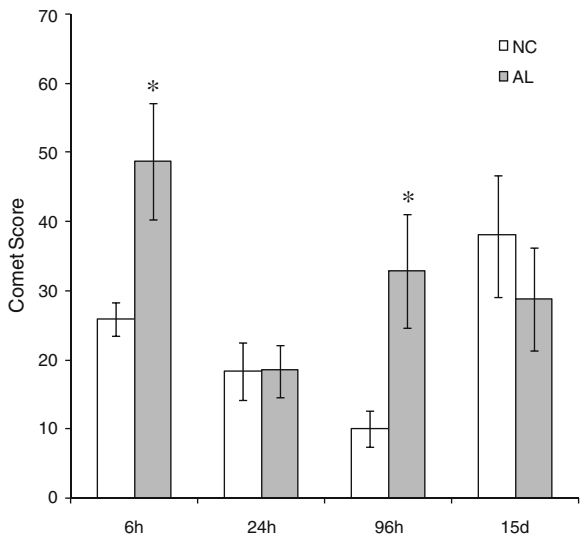

Fig. 1 Comet scores in erythrocytes of *P. lineatus* exposed to 196 μg L^{-1} of dissolved aluminum (*Al*) and the respective negative control (*NC*) for each experimental period (6 h, 24 h, 96 h, and 15 days). One hundred nucleoids were analyzed per fish. *Bars* represent means and *vertical lines* the SE. *Significantly different from respective NC ($p<0.05$)

to Al and their respective negative controls groups are shown in Table 2. In contrast to Comet results, MN frequencies determined in fish erythrocytes after Al acute and subchronic exposures were not significantly different from the respective negative controls.

On the other hand, fish exposed to Al in all experimental periods showed a significant increase in ENAs frequency in comparison to respective NC. Among the three types of nuclear abnormalities observed in *P. lineatus* erythrocytes, the most commonly detected, both in NC as in Al groups, were kidney-shaped nuclei, followed by segmented nuclei and lobed nuclei.

3.4 RAPD Analysis

From the 30 primers screened for RAPD analysis, nine (OPW-4, OPW-6, OPW-7, OPW-8, OPW-9, OPW-11, OPX-6, OPX-1, and OPC-2) that produced clear and reproducible RAPD profiles were selected. A RAPD electrophoretic profile for one of the selected primers is shown in Fig. 2 where the repeatability of RAPD band patterns produced by different tissues (fin, gill, and liver) from the same fish is clearly noticeable.

The nine primers used in the amplifications reactions produced a similar number of bands in fish of NC and Al groups in each exposure period: 260 bands (6 h), 257 (24 h), 219 (96 h), 251 (15 days), as well as in animals of PC exposed to cyclophosphamide for 24 h (273 bands). From the nine primers used, five (OPW-4, OPW-7, OPW-8, OPW-9, and OPX-6) showed alterations in RAPD profiles after fish exposure to Al and one (OPC-2) showed variation in RAPD bands in fish exposed to cyclophosphamide (Table 3). In fish exposed to Al for 6 and 24 h, the RAPD profiles obtained from both gill and liver showed a similar pattern of appearance and

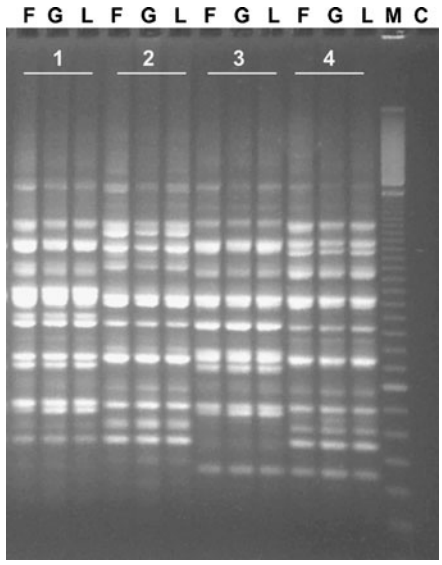

Fig. 2 RAPD profiles, amplified with primer OPW-11, from genomic DNA of four fish exposed to Al for 24 h. *Lanes F* Fin, *G* gill, *L* liver, where *F lanes* represent DNA before Al exposure and *G* and *L lanes* correspond to DNA after Al exposure. *Column M* Molecular weight marker (100 bp, Invitrogen); *Column C* control

Table 2 Frequencies of MN and other nuclear abnormalities (ENA) in erythrocytes of *P. lineatus* exposed to 196 μg L^{-1} of dissolved Al and the respective NC, taking into account the total number of fish (*N*) analyzed for each experimental period (24 h, 96 h, and 15 days)

Time	Group	N	MN frequency (‰)	ENA frequency (‰)
24 h	NC	8	0	1.44±0.44
	Al	10	0.17±0.11	4.30±0.83*
96 h	NC	9	0.09±0.09	2.73±0.86
	Al	12	0.27±0.14	5.00±1.12*
15 days	NC	5	0	3.25±0.22
	Al	9	0.22±0.15	6.75±1.15*

Three thousand erythrocytes were analyzed per fish and results are shown as mean ± SE

*$p<0.05$ (significantly different from respective negative control)

Table 3 Alterations in RAPD band patterns found among amplifications products from adipose-fin (F)—representing genomic DNA pre-Al exposure—and gill (G) and liver (L)—corresponding to genomic DNA post-Al exposure

Al exposure	Primer	F	G	L	MW (bp)
6 h	OPW8	+	−	−	139
	OPX6	−	+	+	887
24 h	OPW4	+	−	−	768
	OPW7	−	+	+	513
15 d	OPW9	−	−	+	470
	OPW7	+	−	+	1,167

The column MW indicates the molecular weight (bp) of RAPD band. The symbol + (plus) indicates the band's presence, and − (minus) indicates the band's absence

disappearance of RAPD bands. After 6 h of exposure to Al, the appearance of new band OPX6-887 bp and the disappearance of fragment OPW8-139 bp were observed. Also, after 24 h of exposure, the appearance of new band OPW7-513 bp and the disappearance of fragment OPW4-768 bp were observed (Table 3 and Fig. 3a). Figure 3b shows the repeatability of band 513, detected with primer OPW-7 after 24 h of exposure to Al, in other two different RAPD amplifications. After 96 h of exposure to Al, fish did not show any alteration in RAPD profiles. On the other hand, animals exposed to Al for 15 days showed different changes in gills and liver, while a given band appeared only in the liver (OPW9-470 bp); another band disappeared only in the gills (OPW7-1167 bp).

3.5 Biochemical Assays

Fish exposed to Al showed a significant increase in hepatic activity of GST and catalase after 6 and 24 h of exposure when compared with the respective negative control groups (Fig. 4).

4 Discussion

Most of the current literature concerning Al effects on fish mainly comes from morphological and physiological studies (Wilson et al. 1994), while information about potential genotoxic effects on this vertebrate group is still scarce. Since Al has been recognized as an oxidative stress promoter and genotoxic agent for human cells (Lankoff et al. 2006), in this study, Al was evaluated for induction of DNA damage and oxidative stress in the neotropical fish *P. lineatus* using a combination of different methodologies. The concentration of dissolved Al and the pH value used in this study have already been reported in surface waters in Brazil (Lara et al. 2001; Flues et al. 2002) due to

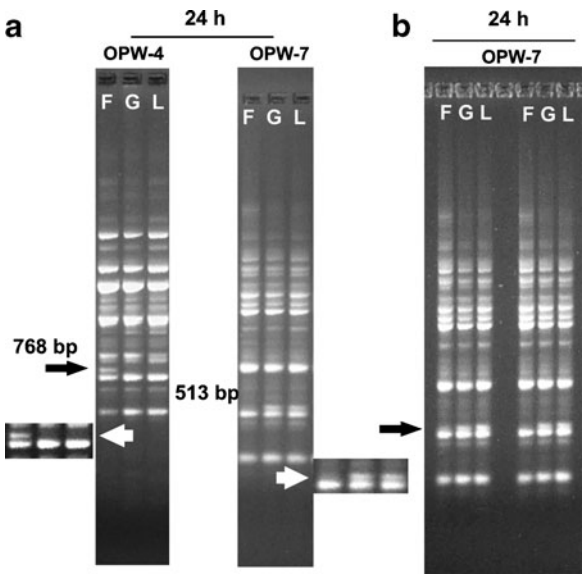

Fig. 3 RAPD profiles from genomic DNA of *P. lineatus*, amplified with primers OPW-4 and OPW-7, after contact for 24 h with water (*F*) and 196 μg L^{-1} of dissolved aluminum (*Al*, *G* and *L*), respectively. *F* (fin) *lanes* represent DNA before treatment, while *G* (gill) and *L* (liver) correspond to DNA fish after Al exposure. **a** Amplification products with primers OPW-4 and OPW-7 showing, respectively, a RAPD band disappearance (768 bp) and appearance (513 bp) both in the gill and liver of fish exposed to aluminum. The *white arrows* indicate in detail the RAPD products that showed variation. **b** Two RAPD profiles obtained with primer OPW-7 showing the reproducibility of the results

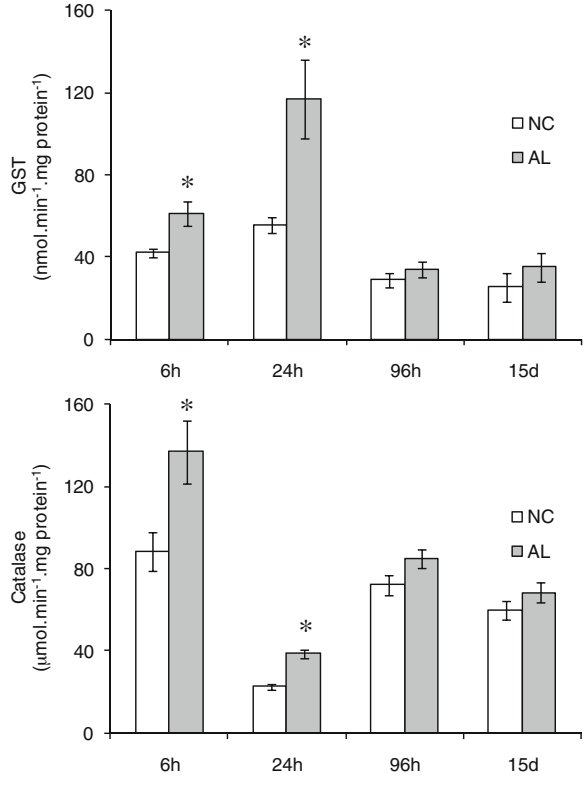

Fig. 4 Hepatic activity of glutathione-*S*-transferase (*GST*) and catalase of *P. lineatus* exposed to 196 μg L^{-1} of dissolved aluminum (*Al*) or only water (*NC*) for different periods (6 h, 24 h, 96 h, and 15 days). *Bars* represent means and *vertical lines* the SE. *Significantly different from respective NC ($p<0.05$)

natural causes or because of anthropogenic emissions. The concentration of 200 μg L^{-1} of dissolved Al corresponds to the maximal concentration allowed by the Brazilian guidelines for freshwater.

Comet assay has been proven to be an efficient tool to detect the effects of potentially genotoxic substances on DNA from different aquatic organisms (Dhawan et al. 2009; Frenzilli et al. 2009). In the present study, the Comet assay revealed a significant increase in DNA damage in erythrocytes of fish exposed to Al for 6 h; after 24 h of Al exposure, DNA damage diminished, returning to the mean score found in the respective control group. Another transient increase in DNA damage was detected by the Comet assay after 96 h of Al exposure, but after 15 days of exposure, DNA scores returned to the mean value determined for the respective control group. It is known that breaks detected by Comet assay can be transiently present when cells are able to repair lesions via base or nucleotide excision (Amado et al. 2006). Thus, these results suggest that DNA damages detected through Comet assay after 6 and 96 h of exposure to Al were efficiently repaired.

The time needed to reach a peak induction of micronuclei in peripheral blood varies greatly among teleosts; normally, micronucleated erythrocytes in fish occur at 1 to 5 days after exposure, but it is possible to visualize micronuclei at 2 to 3 days (Udroiu 2006). In the current study, the absence of micronuclei induction at any experimental time indicates that DNA damages detected by RAPD and Comet assays were possibly neither clastogenic nor aneugenic.

Despite the lack of MN induction, fish exposed to Al in all experimental periods showed a significant increase in ENA frequency. The mechanisms underlying the formation of these abnormalities have not been fully explained. However, some studies indicate that ENAs are induced in response to exposure to genotoxic agents and may complement MN scoring in routine genotoxicity surveys (Çavas and Ergene-Gözükara 2005). Some types of ENA might be resultant of chromosomal aberrations, making the cells unviable, and the presence of such nuclear abnormality leads to the induction of cell death process (Leme et al. 2008). Taken together, our results suggest that Al exposures produced genotoxic effects in *P. lineatus* and that fish respond both by repairing the errors of the damaged genome and also by cell death due to cytotoxicity and/or apoptosis, consequently avoiding permanent damage, as shown by MN data.

A number of genetic damage have been attributed to changes in RAPD profiles, including DNA adducts, breakage, rearrangements, and point mutations, which could result in either the disappearance or appearance of new amplicons (Atienzar and Jha 2006). The changes in RAPD band patterns occurred coincidently in gills and liver of fish exposed to aluminum for 6 and 24 h and might reflect the genotoxic effect of Al on liver and gill cells. The fact that no change was detected in the RAPD profiles of both organs after 96 h of exposure to Al would be indicative that DNA damage in these organs was repaired, taking into account that the DNA repair system in fish cells can be effective within 24 h after contact with the contaminant (Deventer 1996). In longer periods of exposure, such as during 15 days, cells of gills and liver could undergo new DNA lesions, which would overcome the capacity of the enzymes responsible for the repair of these damages, leading to the disappearance of some RAPD bands as well the appearance of new bands. However, presently, we cannot prove this supposition, and further studies are necessary to better understand this finding.

In the present study, the antioxidant enzyme catalase showed an increased activity after 6 and 24 h exposure to Al. This enzyme is essential to promote the degradation of H_2O_2, a precursor of hydroxyl radical which induces DNA damage (Halliwell and Gutteridge 1999). Actually, the protective role of CAT on DNA has been demonstrated in different studies (Cemeli et al. 2009). In this context, the possibility may be considered that without CAT induction, after longer Al exposures, *P. lineatus* was unable to reduce the levels of hydroxyl radical promoters, resulting in DNA damage subsequent to 96 h of Al exposure. The decreased capability of the cells to neutralize ROS can be correlated with enhanced genotoxic damage (Nigro et al. 2002). The relation between genotoxic damage and ROS generation was also suggested by Tran et al. (2007) working on mussels exposed to Hg. These authors state that a possible mechanism of Hg-induced DNA damage in mussels involves an Hg-induced elevation of hydrogen peroxide levels.

As observed for CAT activity, GST liver activity also presented a significant increase after the first two experimental periods (6 and 24 h) and a return to

basal levels after 96 h and 15 days of Al exposure. Thus, it seems that the increased catalase and GST activity in the first two experimental periods helped prevent DNA damages caused by Al exposure. However, further studies are necessary to elucidate the role of oxidative stress on DNA damage in Al-induced toxicity. Comparing the efficacy of RAPD and Comet assays in detecting DNA damages in *P. lineatus* exposed to aluminum, it was possible to notice that the RAPD technique indicated DNA damages in three times of exposure (6 h, 24 h, and 15 days), while the Comet test detected damages that occurred only after 6 and 96 h of exposure to contaminant. These results could be attributed to the intrinsic differences between both methods since changes in RAPD band patterns reflect a wide range of DNA damage (Atienzar and Jha 2006), while Comet assay mainly detects DNA double and single breaks, alkali-labile sites, and cross-linking and DNA adducts (Singh et al. 1988; Jha 2008). In addition, the occurrence of genotoxic effects of Al after 15 days exposure, as revealed by RAPD technique, indicates that DNA damages were not repaired as it could appear considering only the Comet and MN results. On the contrary, these findings show the importance of different methodologies to obtain confident results in genotoxic studies.

5 Conclusion

The current study points out relevant results of the toxicity of Al in acid water to a neotropical fish species, showing that Al is genotoxic to *P. lineatus*. This study demonstrates that acute and subchronic exposures to 200 μg L^{-1} of dissolved Al induced DNA strand breaks in peripheral erythrocytes. Besides, Al exposures promoted nuclear abnormalities in fish erythrocytes and the induction of antioxidant enzymes. This study also showed that RAPD assay can represent a sensitive method to detect genotoxic damage occasionally not detected by other alternative genotoxic tests commonly used in toxicological genetics studies.

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