Aquatic Toxicology 104 (2011) 291–298

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

In vivo and *in vitro* exposures for the evaluation of the genotoxic effects of lead on the Neotropical freshwater fish *Prochilodus lineatus*

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ARTICLE INFO

Article history:
Received 27 November 2010
Received in revised form 27 April 2011
Accepted 4 May 2011

Keywords:
Comet assay
DNA damage
Metallothionein
Micronucleus test
Neutral red retention assay
Lysosomal membrane stability

ABSTRACT

In the present study, *in vivo* and *in vitro* exposures were used to assess the genotoxicity of lead (Pb) to the freshwater fish *Prochilodus lineatus*. The comet assay using blood, liver and gill cells, and the occurrence of micronuclei (MN) and other erythrocytic nuclear abnormalities (ENA) were used to assess the genotoxic potential of lead *in vivo*. Metallothionein content (MT) was measured in fish liver in order to evaluate the protection of fish against Pb toxicity. Fish erythrocytes were exposed to Pb *in vitro* (1, 3 and 6 h) and the number of viable cells, DNA integrity, using the comet assay, and lysosomal membrane stability, measured by the neutral red retention assay (NRRA) were analyzed. The results of the comet assay after *in vivo* toxicity tests (6, 24 and 96 h) showed that Pb was genotoxic for all the three tissues analyzed after 96 h exposure. A significant increase in liver MT content was observed after 6 and 24 h of Pb exposure. MN frequency did not increase after Pb exposures, but the frequency of the other ENA, such as kidney-shaped nuclei, segmented nuclei and lobed nuclei, showed a significant increase after 24 and 96 h, indicating that ENA is a better biomarker for Pb exposure than MN alone after short-term exposures. The results of the comet assay performed with erythrocytes *in vitro* exposed to lead confirmed its genotoxic effect and showed that DNA damage increased with increasing exposure time. Moreover, the NRRA clearly indicated that Pb induces a destabilization of the lysosomal membrane. These results demonstrate the potential genotoxicity and cytotoxicity of lead after acute exposures.

1. Introduction

Lead (Pb) is a ubiquitous metal, dispersed throughout the environment primarily as the result of anthropogenic activities. Today, its predominant use (71%) is in batteries, mainly for vehicles, but also for electricity backup systems and industrial batteries (Skerfving and Bergdahl, 2007). Many anthropogenic sources of lead, most notably paint production and leaded gasoline, have been eliminated or strictly regulated due to lead's persistence and toxicity. Despite the reduction in its use, lead continues to enter the environment primarily by anthropogenic means, and it retains its status as a priority pollutant (USEPA, 2006). The concentration of lead in natural river water has been estimated at 5 $\mu g\,L^{-1}$, but, in aquatic environments near to steel and iron industries and lead production and processing operations much higher concentrations can be found (ATSDR, 2007). This contamination of water through anthropogenic practices is the primary cause of lead poisoning in fish (Rogers and Wood, 2004).

Metal ions are well known inducers of oxidative stress (Lushchak, 2011). Exposure of fish to metals can cause increases in highly reactive oxygen species (ROS) leading to oxidative stress, osmoregulatory dysfunctions associated with inhibition of ATPase activity and tissue damage (Atli and Canli, 2007). Some studies have reported that the toxic effects of lead on aquatic animals are associated with oxidative stress (Zhang et al., 2007). Emerging evidence suggests that lead intoxication is related to the production of reactive oxygen species (ROS) that result in DNA damage and depletion of cell antioxidant defense systems (Farmand et al., 2005; Shalan et al., 2005; El-Ashmawy et al., 2006). In addition, Hong et al. (2007) found support for the direct interaction between lead and DNA by covalent binding of Pb^{2+} to DNA.

The analysis of DNA alterations in aquatic organisms has been shown to be a highly suitable method for evaluating the genotoxic contamination of environments; exposures to low concentrations of contaminants can be detected in a wide range of species (Frenzilli et al., 2009) and various techniques for measuring genotoxicity in aquatic animals are available (Dixon et al., 2002). The single cell gel electrophoresis (SCGE) 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

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doi:10.1016/j.aquatox.2011.05.002

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). Another well-established assay that has proven useful in assessing the genotoxic effects of a wide range of compounds in fish is the evaluation of micronucleus induction (Udroiu, 2006). Micronuclei are small masses of cytoplasmic chromatin, present outside the main nucleus, which can arise from either the breakage of chromosomes or the dysfunction of the mitotic spindle apparatus (Winter et al., 2007). 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 (Costa et al., 2008).

The use of *in vitro* systems in toxicological studies has been rapidly growing in the recent years (Sandrini et al., 2009). While the *in vitro* experiments provide basic information on the nature of the tested agents and/or the cellular response (Binelli et al., 2009a), the *in vivo* experiments allow exploring the entire effect of toxicant without excluding any biochemical pathway (Binelli et al., 2009b). In the present study, *in vivo* and *in vitro* exposures were used to assess the genotoxicity and cytotoxicity of Pb to the freshwater fish *Prochilodus lineatus*. This fish species 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 as a potential bioindicator fish species (Camargo et al., 2009).

The comet assay with blood, liver and gill cells, and the occurrence of micronuclei (MN) other nuclear abnormalities (ENA) in erythrocytes were used to assess the genotoxic potential of lead *in vivo*. Metallothionein content was measured in fish liver in order to evaluate the protection of fish against lead toxicity. In another set of experiments fish erythrocytes were exposed to lead *in vitro* and the following endpoints were analyzed: number of viable cells, DNA integrity using the comet assay, and lysosomal membrane stability, measured by the neutral red retention assay (NRRA), to identify cellular stress. Lysosomal membrane stability is considered one of the most reliable of the recommended biomarkers for water quality assessment (UNEP, 1997).

2. Materials and methods

2.1. Animals

Juveniles of *P. lineatus* weighing 7.47 ± 2.13 g (mean ± SD, $n = 73$) were obtained from the hatchery of State University of Londrina. Prior to experiments, the fish were acclimated for 7 days in 300 L tanks with non-chlorinated water, constant aeration and a photoperiod of 10 h light: 14 h dark. During acclimation, the animals were fed with commercial food with 36% protein (Guabi®, BR) every 2 days, and the feeding was suspended 24 h before the beginning of the toxicity tests. The physical and chemical parameters of the water were continuously monitored ($T = 20.58 \pm 1.31$ °C; pH = 6.79 ± 0.60; DO = 7.89 ± 0.93 mg O_2 L^{-1}; conductivity = 133.4 ± 9.7 μS cm^{-1}; hardness = 60.75 ± 2.99 mg $CaCO_3$ L^{-1}).

2.2. In vivo exposures and sampling

After acclimation, groups of fish (10 per aquarium) were transferred to glass aquaria (100 L each) containing only water (negative control group or NC) or water with lead nitrate in a nominal concentration of 5 mg $Pb(NO_3)_2$ L^{-1} (lead group or Pb). This concentration was based on lead determinations in rivers of northern Paraná state (Yabe and Oliveira, 1998; Amado et al., 2006). Static toxicity tests were performed in three experimental periods (6, 24 and 96 h) and NC and Pb groups were run simultaneously for each experimental time. A group of fish injected with cyclophosphamide, a well-known genotoxic drug, was used as positive control (CP). For this, fish were anesthetized (benzocaine 0.1 g L^{-1}), weighted and injected with cyclophosphamide (0.04 mg of cyclophosphamide per g of fish) and then transferred to glass aquaria containing dechlorinated water in the same conditions as the negative control group. All toxicity tests were carried out in duplicate.

During the tests, the water was continuously monitored for temperature, dissolved oxygen, pH and conductivity using a multiparameter water quality meter (Hanna HI9828). Water hardness was determined by the EDTA titrimetric method. Water samples were collected immediately after each experimental time for lead determination, using atomic absorption spectrophotometry (AAS Perkin Elmer 700). The concentration of total Pb was determined in samples of non-filtered water and the concentration of dissolved Pb was determined in water samples passed through a 0.45 μm syringe filter; for both analyses, samples were acidified by addition of HNO_3 to a final concentration of 1%.

After each exposure period, fish were removed from the aquaria and immediately anesthetized with benzocaine (0.1 g L^{-1}) and blood samples were collected from the caudal vein using a heparinized plastic syringe. Blood was used for the determination of the frequency of micronuclei (MN) and other erythrocytic nuclear abnormalities (ENA) and a blood sub-sample (10 μL) were stored in microtubes (1.5 mL) with 700 μL of PBS (NaCl 126.6 mM, KCL 4.8 mM, $CaCl_2$ 1.5 mM, $NaHCO_3$ 3.7 mM, Na_2HPO_4 8.9 mM, NaH_2PO_4 2.9 mM) and kept on ice for the comet assay. Thereafter animals were euthanized by medullar section for gills and liver removal. Immediately after excision the gills and liver were carefully washed with PBS and gill filaments and part of the liver were transferred to microtubes for the cellular dissociation to be used in the comet assay. Another part of the liver was transferred to criotubes and kept frozen (−80 °C) until metallothionein analysis.

2.3. Cellular dissociation

The method for cellular dissociation was based on Cavalcante et al. (2008). Briefly, gill filaments and liver tissue were sectioned and pieces were transferred to microtubes, incubated for 15 min at 30 °C in 0.05% trypsin (diluted in PBS Ca^{+2} and Mg^{+2} free) and homogenized by periodic manual inversion at room temperature for tissue dissociation. After that, the solution was filtered (30 μm mesh size) into a tube containing 10% fetal calf serum to halt the enzymatic digestion. The resultant solution was centrifuged (10 min, 1000 × *g*) and the pellet was resuspended in PBS to be used in the comet assay.

2.4. In vitro exposures

A volume of 10 μL of blood collected from the caudal vein of *P. lineatus* (as described before) was mixed with 700 μL of PBS in microtubes for the *in vitro* exposures, which were performed in 24-well microplates for 1, 3 and 6 h at room temperature (25 °C). Three different treatments were used: negative control (NC), where 140 μL of PBS were added to 60 μL of blood PBS solution; lead treatment (Pb), where 140 μL of PBS with 5 mg $Pb(NO_3)_2$ L^{-1} were added to 60 μL of blood PBS solution; positive control (PC), where 140 μL of a solution of PBS with 1.5 μM methyl methanesulfonate (MMS) were added to 60 μL of blood PBS solution. The concentration of dissolved Pb was determined in samples of the lead treatment solution as described earlier (item 2.2). Blood cells submitted to *in vitro* exposures were used in the comet assay and in the neutral red retention assay.

2.5. Cell viability assay

Cell viability of the erythrocytes, gill and liver cells obtained after each *in vivo* exposure as well as of the erythrocytes, after each period of *in vitro* exposure, was determined by the Trypan blue exclusion method. For each sample, 100 cells were analyzed using a Neubauer chamber and the viability was expressed as the percentage of viable cells (white cells) in the total number of cells. At least 80% of cells should be viable to run the comet assay and the neutral red retention assay (Tice et al., 2000).

2.6. Comet assay

The Comet assay was performed using the alkaline (pH > 13) version of the assay developed by Singh et al. (1988), with the modifications detailed by Cavalcante et al. (2008). Basic steps of the assay for the three cell types were executed as follows: (a) lysis: 1 h, at 4 °C, protected from light, in a lysis buffer (2.5 MNaCl, 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}; (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.

The slides, stained with ethidium bromide (20 μg mL^{-1}) were analyzed with a Leica microscopy (DM 2500) adapted for fluorescence, equipped with a blue excitation filter (450–490 nm) and a barrier filter of 515 nm at 1000× magnification. All slides were blind-reviewed (Lee and Steinert, 2003). 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. According to Kobayashi et al. (1995), DNA damage was classified in four classes ((0) no visible damage; (1) a short tail smaller than the diameter of the nucleus; (2) a tail length 1–2 times the diameter of the nucleus; (3) a tail length >2 times the diameter of the nucleus). The score of DNA damage for 100 comets was obtained by multiplying the number of cells in each class by the damage class, and ranged from 0 (all undamaged) to 300 (all maximally damaged). Results for DNA damage in each cell type were expressed as the mean score of DNA damage for each treatment group, for each exposure period.

2.7. Frequency of micronuclei (MN) and other erythrocytic nuclear abnormalities (ENA)

The analyses of MN and ENA were performed with the erythrocytes of fish after *in vivo* exposures according to the methodology of Hooftman and Raat (1982) and Carrasco et al. (1990). Immediately after sampling blood was smeared on clean glass slides, dried overnight, fixed with methanol for 10 min and stained with Giemsa (5%). A total of 3000 erythrocytes per fish were examined under an Olympus optical microscope (1000× magnification). ENA were classified, following Pacheco and Santos (1997) into three categories: segmented nuclei (SN), lobed nuclei (LN) and kidney-shaped nuclei (KSN). The mean frequencies of MN and erythrocytic nuclear abnormalities found in each experimental group were calculated and expressed per 1000 cells (‰). The result in each group was also expressed as the mean value (‰) of the sums (MN + LN + SN + KSN) for all the individual lesions observed.

2.8. Metallothionein content (MT)

MT in the liver of fish after *in vivo* exposures was determined by the concentration of SH-groups following the method of Viarengo et al. (1997). Liver samples were homogenized (sucrose 0.5 M, PMSF 0.5 mM, β-mercaptoethanol 0.01% in Tris–HCl buffer 20 mM, pH 8.6), centrifuged (14 000 × *g*, 20 min, 4 °C) and cold absolute ethanol and chloroform were added to the supernatant. These samples were centrifuged (7000 × *g*, 10 min, 4 °C), cold ethanol was added to the supernatant and the mixture was maintained at −20 °C for 1 h. Samples were centrifuged again (7000 × *g*, 10 min, 4 °C) and the pellet was resuspended in the same buffer as used for homogenization, but with 87% ethanol and 1% chloroform. After that samples were dried at 35 °C for up to 24 h, the pellet was resuspended (0.25 M NaCl, 1 N HCl, 4 mM EDTA) and Ellman reagent (0.43 mM DTNB buffered with 0.2 M Na-phosphate, pH 8.0) was added to the sample. MT was estimated using reduced glutathione as a reference at 412 nm and expressed as nM MT mg $protein^{-1}$. Total protein levels were determined following the method of Lowry et al. (1951) using bovine serum albumin as a reference.

2.9. Neutral red retention assay (NRRA)

NRRA is based on that healthy cells could take up and retain larger quantities of the dye (neutral red) than damaged cells. The assay was carried out according to Lowe et al. (1995). A stock solution of neutral red was prepared in DMSO (0.05 mg mL^{-1}) and a working solution was prepared by diluting the stock solution in PBS (2 μL mL^{-1}).

After *in vitro* exposures sub-samples from each well were centrifuged (10 min, 1000 × *g*) and the pellet was resuspended in PBS and neutral red working solution (1:1). After 10 min aliquots of this mixture was carefully placed on microscope slides. Two slides were used for each sample. Slides were suspended on a light-proof humidity chamber and after 20-min incubation they were observed under an optical microscope. Slides were thereafter examined systematically at 20 min intervals to determine at what point in time there was evidence of dye loss from the lysosomes to the cytosol. Tests were completed when the dye loss was evident in at least 50% of the erythrocytes (Lowe et al., 1995). Between every microscope observation, slides were returned to the humidified chamber.

2.10. Statistical analysis

The results of the comet assay for both *in vivo* and *in vitro* exposures and the results of ENA frequency for *in vivo* exposures, obtained in each treatment (NC, Pb or PC) at the same experimental time, for *in vivo* (6, 24 or 96 h) and *in vitro* (1, 3 or 6 h) exposures, were compared by parametric (one-way ANOVA) or non-parametric (Kruskall–Wallis) analysis of variance. Differences were analyzed by a post hoc Student–Newman–Keuls test for all pairwise comparisons between treatments. The results of MT content determined for control and lead groups at each period of *in vivo* exposure (6, 24 and 96 h) were compared using the Student *t*-test. The results of the NRRA obtained for NC and lead groups at each *in vitro* exposure (1, 3 and 6 h) were compared using the Mann–Whitney Rank Sum Test. The decision to use parametric or non-parametric tests was based on analysis of normality and homogeneity of variance. Statistical significance was designated as $p < 0.05$.

3. Results

3.1. Water parameters and lead concentrations

Water characteristics remained stable in the *in vivo* experiments and the mean values (± DP) considering both control and lead groups were: (i) temperature: 23.02 ± 2.1 °C; (ii) pH: 7.41 ± 0.22; (iii) dissolved oxygen: 7.05 ± 0.61 mg O_2 L^{-1}; (iv) conductivity 63.55 ± 7.3 μS cm^{-1}. Hardness remained close to 60 mg $CaCO_3$ L^{-1}. No mortality was registered neither in the control nor in the lead-exposed groups of the tests.

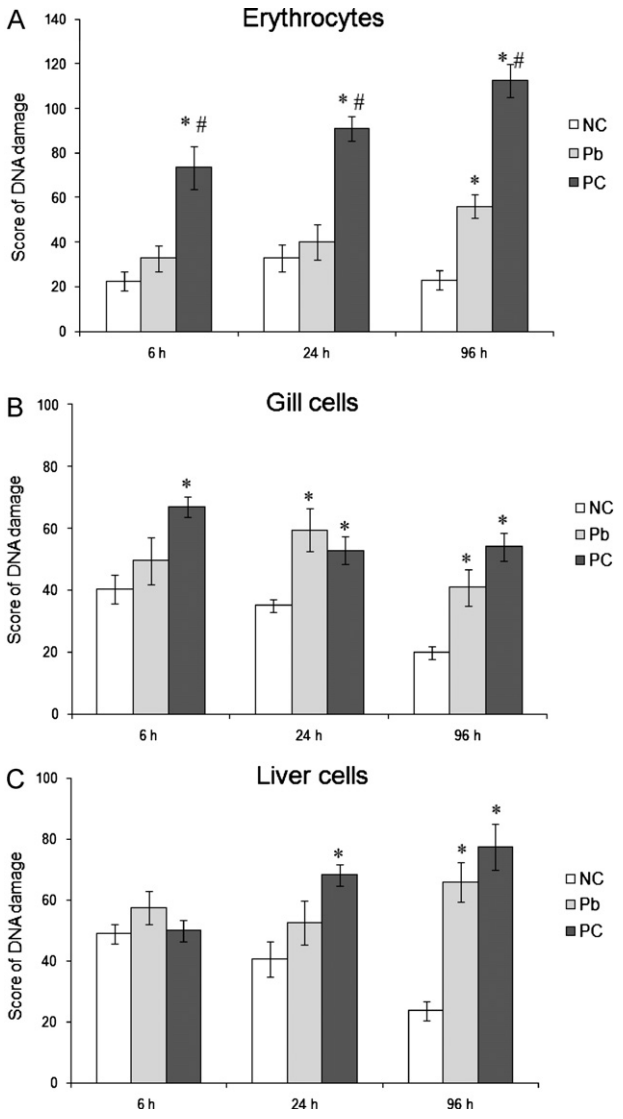

Fig. 1. Comet scores in erythrocytes (A), gill cells (B) and liver cells (C) of *Prochilodus lineatus* exposed to lead (Pb) and the respective negative (NC) and positive controls (PC), at each period of *in vivo* exposure (6, 24 and 96 h). Bars represent means and vertical lines the S.E. * indicates significant difference in relation to negative control at the same time of exposure and # indicates significant difference in relation to Pb exposed group at the same time of exposure ($p < 0.05$).

Lead concentrations in the experimental medium after the three periods of *in vivo* exposure were similar, the mean value of total lead was 2.10 ± 0.37 mg Pb L^{-1} and the mean value of dissolved lead was 0.48 ± 0.04 mg Pb L^{-1}. Lead was not detected in the water of the control groups. The concentration of lead measured in the experimental solution of the *in vitro* exposures was 0.46 mg of dissolved Pb L^{-1}. Thus, the concentrations of dissolved lead used in both types of exposures, *in vivo* and *in vitro,* were comparable.

3.2. In vivo exposures

3.2.1. Comet assay

Fish injected with cyclophosphamide (PC) showed the score of DNA damage significantly higher in relation to NC group both for blood cells at all exposure periods ($p < 0.001$) and for gill cells at all exposure periods (6 h: $p = 0.026$, 24 h: $p = 0.004$, 96 h: $p < 0.001$) (Fig. 1A and B). On the other hand, for the liver cells the score of DNA damage was significantly higher than NC group only after 24 h ($p = 0.013$) and 96 h ($p < 0.001$) of cyclophosphamide injection (Fig. 1C). Fish exposed to lead for 96 h showed a significant increase in the score of DNA damage in blood cells ($p < 0.001$) and liver cells ($p < 0.001$), when compared to respective NC (Fig. 1A and 1C). For the gill cells a significant increase in DNA damage occurred after 24 h ($p = 0.004$) and 96 h ($p < 0.001$) exposure to lead (Fig. 1B). For all the three tissues analyzed the frequency of class 1 damage was higher than class 2 and class 3 for all the three tissues analyzed.

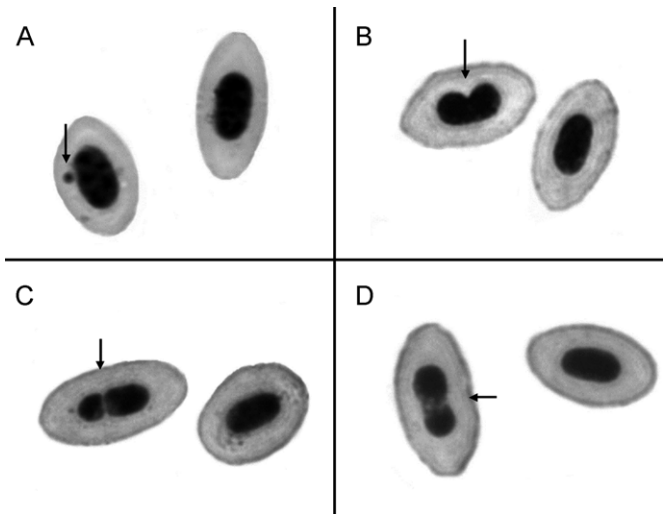

Fig. 2. Nuclear abnormalities identified in erythrocytes of *Prochilodus lineatus*: (A) micronuclei; (B) kidney-shaped nuclei; (C) segmented nuclei; (D) lobed nuclei. Arrows show ENA (1000×).

3.2.2. Frequency of MN and other ENA

The nuclear abnormalities observed in peripheral erythrocytes of *P. lineatus* were micronuclei (Fig. 2A), kidney-shaped nuclei (Fig. 2B), segmented nuclei (Fig. 2C) and lobed nuclei (Fig. 2D). Frequencies of MN and the other nuclear abnormalities (ENA) in erythrocytes of fish exposed to Pb and their respective negative and positive controls groups are shown in Table 1. MN frequencies registered in fish erythrocytes from all groups were very low, varying from 0 to 0.14 (‰). After Pb exposures, as well as for the fish injected with cyclophosphamide, MN frequencies were not significantly different from the respective negative controls at any time. The frequency of ENA in erythrocytes of fish exposed to Pb was also low and the type of nuclear abnormality most commonly detected was kidney-shaped nucleus (KSN). After 96 h exposure to lead there was a significant increase ($p = 0.004$) in the frequency of KSN in relation to NC, from 0.44 to 2.05 (‰). Also, a significant increase ($p = 0.029$) in the frequency of lobed nuclei was observed after 24 h exposure to lead. Fish from positive control group showed significant increases, in relation to NC, in the frequency of KSN after 96 h ($p = 0.004$), SN after 96 h ($p = 0.026$) and LN after 24 h ($p = 0.029$). When the frequency of MN and all the nuclear abnormalities were summed up (MN + KSN + SN + LN) and analyzed together, significant increases were observed after 24 h ($p = 0.01$) and 96 h ($p = 0.009$) both in positive control and lead groups, in relation to respective negative controls (Fig. 3).

3.2.3. Hepatic MT content

Fish exposed to lead showed a significant increase in MT content in the liver after 6 h ($p = 0.002$) and 24 h ($p = 0.007$) exposure to metal in comparison to respective controls (Fig. 4).

Table 1
Frequency of erythrocytic nuclear abnormalities: micronuclei (MN), kidney-shaped nuclei (KSN), segmented nuclei (SN) and lobed nuclei (LN), in *Prochilodus lineatus* exposed to lead (Pb) and the respective negative (NC) and positive controls (PC), at each period of *in vivo* exposure (6, 24 and 96 h). 3000 erythrocytes were examined per fish and *N* indicates the number of fish analyzed in each group.

Nuclear abnormality	Time	Group	*N*	Total number of alterations	Frequency of occurrence (‰)
MN	6 h	NC	9	0	0
		Pb	8	0	0
		PC	8	0	0
	24 h	NC	9	1	0.04 ± 0.04
		Pb	9	4	0.14 ± 0.06
		PC	7	2	0.10 ± 0.25
	96 h	NC	8	1	0.04 ± 0.04
		Pb	6	2	0.11 ± 0.07
		PC	7	3	0.14 ± 0.10
KSN	6 h	NC	9	20	0.74 ± 0.25
		Pb	8	12	0.50 ± 0.11
		PC	8	15	0.33 ± 0.25
	24 h	NC	9	1	0.04 ± 0.04
		Pb	9	15	0.33 ± 0.15
		PC	7	20	0.95 ± 0.22
	96 h	NC	8	12	0.44 ± 0.14
		Pb	6	27	1.50 ± 0.41*
		PC	7	43	2.05 ± 0.41*
SN	6 h	NC	9	4	0.15 ± 0.10
		Pb	8	4	0.17 ± 0.11
		PC	8	5	0.21 ± 0.21
	24 h	NC	9	0	0
		Pb	9	5	0.24 ± 0.15
		PC	7	4	0.19 ± 0.07
	96 h	NC	8	0	0
		Pb	6	8	0.44 ± 0.38
		PC	7	21	1.00 ± 0.31*
LN	6 h	NC	9	9	0.33 ± 0.19
		Pb	8	20	0.83 ± 0.34
		PC	8	5	0.33 ± 0.06
	24 h	NC	9	1	0.04 ± 0.04
		Pb	9	18	0.71 ± 0.30*
		PC	7	12	0.57 ± 0.14*
	96 h	NC	8	3	0.11 ± 0.08
		Pb	6	6	0.33 ± 0.17
		PC	7	1	0.05 ± 0.05

* Significantly different form respective NC ($p < 0.05$).

3.3. *In vitro exposures*

3.3.1. *Comet assay*

Cellular viability of red blood cells *in vitro* exposed to lead was 97.3%. Fish erythrocytes exposed to lead for 1, 3 and 6 h showed significant increases in the score of DNA damage in relation to respective NC ($p < 0.001$) (Fig. 5). The cells exposed to MMS (positive control) showed score of damage significantly higher than negative control and lead groups at all periods of exposure ($p < 0.001$) (Fig. 5). The score of DNA damage increased throughout the length of exposure, both to lead and to MMS. Red blood cells exposed to lead for 6 h showed score of damage significantly higher than the cells exposed to Pb for only 1 h ($p = 0.011$); the same pattern was observed for erythrocytes exposed to MMS, the scores of damage after 3 and 6 h exposure were significantly higher than after 1 h ($p < 0.001$) (Fig. 5). After 6 h of exposure to Pb the frequency of nucleoids classified as class 2 was higher than after 1 and 3 h, and it was observed nucleoid

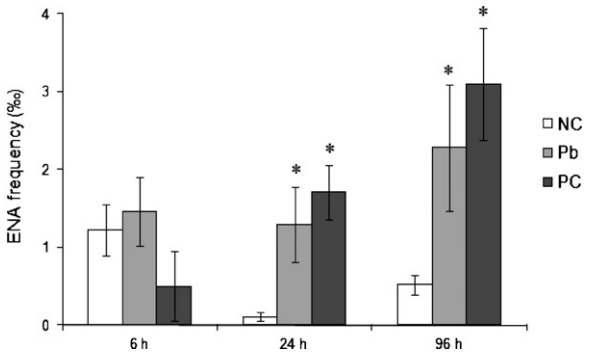

Fig. 3. Frequency of ENA (MN + KSN + SN + LN) in erythrocytes of *Prochilodus lineatus* exposed to lead (Pb) and the respective negative (NC) and positive controls (PC), at each period of *in vivo* exposure (6, 24 and 96 h). Bars represent means and vertical lines the S.E. * indicates a significant difference in relation to negative control at the same time of exposure ($p < 0.05$).

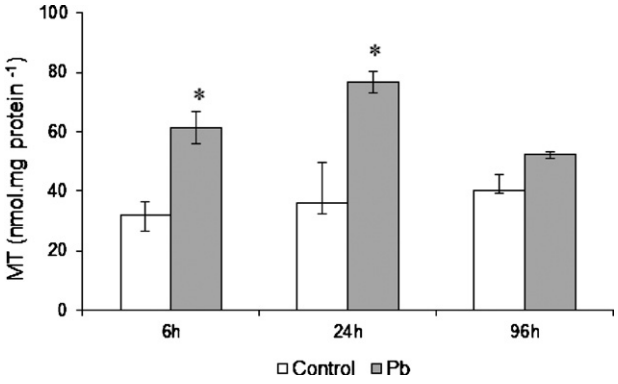

Fig. 4. Metallothionein content in the liver of *Prochilodus lineatus* exposed to lead (Pb) or only to clean water (Control), at each period of *in vivo* exposure (6, 24 and 96 h). Bars represent means and vertical lines the S.E. * indicates a significant difference in relation to control at the same time of exposure ($p < 0.05$).

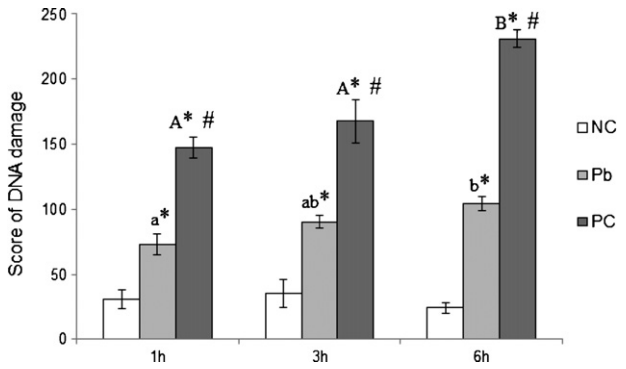

Fig. 5. Comet scores in erythrocytes of *Prochilodus lineatus* exposed to lead (Pb) and the respective negative (NC) and positive controls (PC), at each period of *in vitro* exposure (1, 3 and 6 h). Bars represent means and vertical lines the S.E. * indicates a significant difference in relation to negative control at the same time of exposure and # indicates a significant difference in relation to Pb exposed group at the same time of exposure ($p < 0.05$). Different letters indicate significant differences among mean values obtained at different exposure times for Pb groups (small case letters) and for PC groups (capital letters).

of class 3, that was not observed in the shorter exposures (1 and 3 h).

3.3.2. Stability of lysosomal membrane

The retention time (RT) of the neutral red dye observed in control assays was about 80 min (Fig. 6). Lead was able to produce a destabilization of the lysosomal membrane in a time-dependent manner. After 1 h exposure there was a non-significant decrease of 42% in the in the RT of neutral red, followed by a significant decrease of 67% ($p = 0.002$) and 75% ($p = 0.002$), after 3 and 6 h exposure, respectively, demonstrating that this metal was clearly able to induce cellular stress.

4. Discussion

In fish, a tissue often chosen to perform the comet assay is the blood, because it is easy to collect and there is no need for cellular dissociation (Kilemade et al., 2004). However, different tissues can accumulate metals to different degrees, depending on the biochemical characteristics of the metal (Suiçmez et al., 2006). Thus the choice of tissues for the comet assay should also be related to the ability of the tissue to take up and metabolize xenobiotics. In this work the choice of gill and liver cells, besides the red blood cells, was due to the fact that the gills represent the first organ which is in direct contact with water and, consequently, with the pollutants

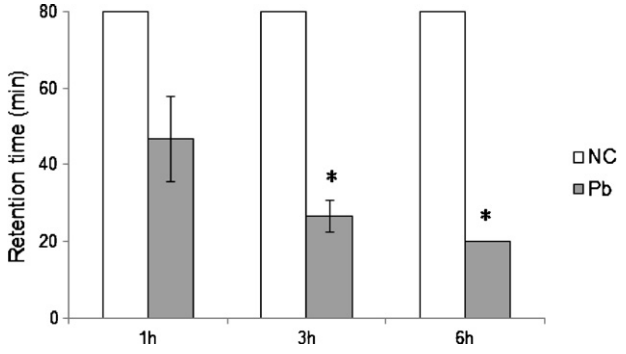

Fig. 6. Retention times of the neutral red dye for *Prochilodus lineatus* erythrocytes exposed to lead (Pb) and the respective negative (NC), at each period of *in vitro* exposure (1, 3 and 6 h). Bars represent means and vertical lines the S.E. * indicates a significant difference in relation to negative control at the same time of exposure ($p < 0.05$).

present in it, and the liver due to its role in xenobiotic metabolism and accumulation (Kilemade et al., 2004).

The comet assay has been widely used in genetic studies of toxicological biomonitoring of aquatic environments (Mitchelmore and Chipman, 1998; Lee and Steinert, 2003) and as a tool to measure the relationship between the damage in the DNA molecule and the exposure of organisms to toxic pollutants. Although the comet assay is a test widely used and appropriate for the assessment of genotoxic damage in nucleated eukaryotic cells (Sharma et al., 2007), the prerequisite for cell isolation for the application of this technique may be a limitation to its use (Mitchelmore and Chipman, 1998). In this work, despite the methodological difficulties imposed by the use of cells of organs such as gills and liver, trypan blue test showed cell viability greater than 80% for cells isolated from these two organs. However, the negative control groups for gill and liver showed relatively higher frequency of damaged nucleoids than blood, possibly due to mechanical stress during the process of cellular dissociation and to the cell resuspension protocol. The results of the comet assay after *in vivo* toxicity tests showed that lead was genotoxic for all the three tissues analyzed after 96 h exposure and the frequency of damaged nucleoid categorized as class 1 was higher than classes 2 and 3, indicating that the majority of DNA damage promoted by lead can be repaired.

The occurrence of DNA breaks in blood and liver cells only after 96 h of lead exposure may be related to the content of metallothioneins (MT). These cytosolic proteins of low molecular weight have the ability to bind to metal ions due to a large number of cysteine residues (Nordberg, 1998). Metal sequestration by MT is not a static system and an increase in the rate of MT synthesis may be determined by increased levels of metals in the body (Hogstrand and Haux, 1991). In the present study, the increased MT content after 6 and 24 h of lead exposure might have prevented metal effects on DNA. However, after 96 h of Pb exposure MT content returned to control levels and the amount of metal might have overcome the neutralizing capacity of MT and lead may have interacted with other intracellular compounds (Roesijadi, 1996).

In mitochondria, metals can bind to crucial enzymes and respiratory protein complexes uncoupling oxidative phosphorylation, leading to the generation of reactive oxygen species (ROS) (Di Giulio et al., 1995). ROS can act on the plasma membrane, causing lipid peroxidation, or directly on the DNA molecule, causing damage (Ahmad et al., 2006). Although organisms have antioxidant defenses to protect tissues against oxidative damage, if the rate of ROS production exceeds the capacity of the defense mechanisms, injury can occur in the cells and DNA (Cadet et al., 2003), inducing damage to their bases, causing breaks in the DNA strand (Reinecke and Reinecke, 2004). Wang and Jia (2009) reported that lead has the potential to induce oxidative stress and DNA damage at very low concentrations (0.2 mg Pb L^{-1}) in the testes of the frog *Rana nigromaculata*. Zhang et al. (2008) also observed that 0.5 mg Pb L^{-1} causes DNA damage in hepatopancreas cells of loach, *Misgurnusanguilli caudatus*. These data show that aquatic animals are sensitive to very low levels of Pb.

The micronucleus test for fish has proved to be a useful experimental technique for assessing the genotoxic properties of compounds present in the aquatic environment (Al-Sabti and Metcalfe, 1995). The MN appear when a whole chromosome or a chromosome fragment fails to migrate with one of the two daughter nuclei formed during mitosis and in order for MN to be detected it is necessary that the cells undergo at least one cell cycle (Udroiu, 2006). In this work MN frequency did not increase after Pb exposures. Micronucleated erythrocytes in fish occur from 1 to 5 days following exposure to cytotoxic and/or genotoxic agents (Udroiu, 2006). Thus, the appearance of micronuclei was not expected in the first 6 h of lead exposure. However, an increase in MN frequency could be observed after 24 or 96 h exposure to lead. When

carp (*Cyprinus carpio*) was exposed to 2.0 mg L^{-1} of mercury for 90 days Nepomuceno et al. (1997) did not find increased frequency of micronuclei. Cavalcante et al. (2008) also found no increase in MN in erythrocytes of *P. lineatus* exposed to a sublethal concentration of the herbicide Roundup® for 96 h. These examples illustrate the low sensitivity of the micronucleus test for fish, which in turn could be related to a very low frequency of occurrence of micronucleated cells in fish (Carrasco et al., 1990). Because of this, the occurrence of other types of ENA has been used as a tool to quantify nuclear changes.

Besides MN, several studies have described the presence of other erythrocytic nuclear abnormalities in cells of fish exposed to genotoxic substances (Carrasco et al., 1990; Çavas and Ergene-Gözükara, 2005; Baršienè et al., 2006; Ergene et al., 2007; Costa et al., 2008) but the mechanisms that originate these nuclear abnormalities are not fully understood (Ayllón and Garcia-Vazquez, 2000). These changes in nuclear morphology have been found to be originated from a genotoxic event as a result of exposure to xenobiotic contaminants (Pacheco and Santos, 1997). The results from the analysis of ENA in *P. lineatus* exposed to Pb showed a significant increase after 24 and 96 h exposure indicating that ENA is a better biomarker for Pb exposure than MN alone. Matsumoto et al. (2006) have also observed an increase in ENA frequency in tilapia, *Oreochromis niloticus*, collected from sites contaminated with chromium.

Cyclophosphamide (CP) is a well-known indirect alkylating agent widely used in many test systems (Anderson et al., 1995). In this study, the results of the comet assay showed a significant increase in the score of DNA damage in tissues of fish injected with CP in the three experimental times, except for in the liver after 6 h exposure. Thus, the efficiency of CP as a positive control varied according to the organ studied. CP exposures were good positive controls for blood and gill cells, but not so efficient for the liver cells. CP is also frequently used as positive control in the micronucleus test because it is a known clastogenic substance (Pacheco and Santos, 1997; Ayllón and Garcia-Vazquez, 2000, 2001). However, in contrast to the results of the comet test, treatment with cyclophosphamide failed to induce micronuclei in erythrocytes of *P. lineatus*. The reason for the low induction of micronuclei observed following cyclophosphamide treatment, compared with the majority of previous published data, is not known. The lack of significant induction of MN by CP was also observed by Winter et al. (2007) using erythrocytes of the fathead minnow *Pimephales promelas*. These authors suggested that the reasons for this lack of MN may be due to the low metabolic activation of CP *in vivo*. On the other hand, when all the types of nuclear abnormalities were considered together it was observed a significant increase in ENA frequency after 24 and 96 h after CP injection. Other studies have also shown an increase in the frequency of cells with ENA in fish treated with cyclophosphamide (Ayllón and Garcia-Vazquez, 2000, 2001). These authors suggest that the nuclear abnormalities, other than MN, should be considered indicators of genotoxicity, at least as indicators of clastogenic activity, once they were induced by CP.

In order to allow comparisons between the results obtained after *in vivo* and *in vitro* exposures the concentration of dissolved lead applied in both tests were very similar and the results showed that erythrocyte viability after 6 h *in vitro* exposure to 0.46 mg of dissolved Pb L^{-1} was greater than 80%. Thus, the use of this concentration is in accordance with the guidelines for the comet assay proposed by Tice et al. (2000), which recommend avoiding concentrations that decrease cell viability by more than 30%. The results of comet assay performed with erythrocytes *in vitro* exposed to lead confirmed its genotoxic effect and showed that DNA damage increased with increasing exposure time. Moreover, the neutral red retention assay clearly indicated that Pb does induce a destabilization of the lysosomal membrane, a classical parameter of cellular stress (Lowe et al., 1995). These results reinforce the idea that lead toxicity, at least in part, is driven by ROS production, since at the cellular level lysosomes are a well-known target for toxicity exerted by reactive oxygen species. A close relationship between lysosomal and antioxidant responses to pollutants has been postulated where lysosomal damage is at least in part dependent on intra- and extra-lysosomal generation of reactive oxygen species (Regoli, 2000). The membrane destabilization promotes the rupture of lysosomes, releasing a large amount of endonucleases that might act on the DNA molecule causing a disruption (Wang and Jia, 2009). According to Binelli et al. (2009c) a good correlation has been reported between the impairment of antioxidant activity, the neutral red retention time and changes in DNA integrity.

The current study brings together information based on two different systems (*in vivo* and *in vitro*) to evaluate lead genotoxicity on a freshwater Neotropical fish species and give elements to elucidate the mechanisms of lead toxicity. The comet assay performed with gill and liver cells, besides erythrocytes, showed to be an important complementary tool for detecting DNA damage. The results demonstrate the potential genotoxicity and cytotoxicity of environmental levels of lead after acute exposures. Moreover, the data indicate that the mechanisms of action to explain the genotoxicity of lead to *P. lineatus* might be related to the generation of reactive oxygen species. The use of *in vivo* and *in vitro* approaches was demonstrated as a valuable tool for understanding the effects of lead on DNA molecules.

Acknowledgments

The authors thank the Hatchery Station of State University of Londrina (EPUEL) for the supply of fish. This work is part of the Master Dissertation of V. Monteiro and was supported by the Brazilian National Higher Education Coordinating Council (CAPES). C.B.R. Martinez is research fellow from the Brazilian Council for Scientific and Technological Development (CNPq) and member of the Brazilian Institute of Aquatic Toxicology (INCT-TA, CNPq: 573949/2008-5).

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