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Genotoxic evaluation of different doses of inorganic lead (PbII) in *Hoplias malabaricus*

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Abstract Different genetic biomarkers have been used to evaluate the pollution effects of mutagenic agents such as metals and also a great variety of chemicals delivered on the environment by human activities. This way, the aim of the present report was to evaluate the effects of inorganic lead in fishes through the frequency of piscine micronuclei and nuclear morphological alterations in peripheral cells, chromosomal aberration frequency and comet assays in blood and kidney cells. Specimens of *Hoplias malabaricus* received different doses of lead by intra-peritoneal injections at time of 96 h. There was not a significant difference between control and treated groups for the piscine micronucleus and chromosomal aberration assays. In the comet assays there was a significant difference between control and contaminated groups. However, a significant difference between the applied doses was not observed. The results obtained with the comet assays also show that blood presented a higher sensibility than the kidney tissue, possibly due to the acute contamination. Although the results showed the genotoxic potential of lead at the 21 and 63 μg Pb^{2+}/g doses for both tissues, the lowest dose is considered more appropriate for future bioassays.

Keywords Lead · Micronucleus test · Comet assay · Chromosomal aberration

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Introduction

The impact of toxic materials on the integrity and functioning of the DNA has been investigated in many organisms under different conditions (McCarthy and Shugart 1990). The use of biomarkers as a biological response measured in the affected organisms is very important to simplify and lower costs of biological monitoring, especially in aquatic environments. These biomarkers consist in adducts in the DNA, chromosomal aberrations, DNA breakage and micronuclei frequency and other nuclear abnormalities (Bombail et al. 2001). Fishes are one of the most indicated organisms for the monitoring of aquatic environments (Van Der Oost et al. 2003), however there are few toxicity studies conducted with

South American fresh water fishes at the moment (Akaishi et al. 2004; Rabitto et al. 2005). The spilling of industrial residues without the proper treatment in these environments may cause several damages since organisms are capable of absorbing metals through skin, gills or feeding (Tao et al. 1999). Lead, a very toxic metal, is widely used in several industrial processes such as clothe tinge, varnish, pesticide, explosives, batteries and painting manufacturing (Johnson 1998) and could be responsible for death or sub lethal changes in reproduction, growth and behavior of the fishes (Burdena et al. 1998).

The evaluation of nuclear abnormalities and micronuclei are some of the largely used assays for the investigation of genotoxic effects of environmental pollutants in fishes (Al-Sabti 1986). The formation of chromosomal aberrations, either numeric or structural, represents the majority of the damages caused by physical, chemical and biological agents on the genetic material. Most damages are not detectable.

The comet assay is usually performed with blood cells due to easy access and also the composition of 97% of nucleated erythrocytes (Mitchelmore and Chipman 1998), but other tissues have also been tested because the genotoxic effects of contaminants may be, many times, tissue-specific. The most investigated tissues, besides blood, are liver (the main organ of the metabolism), gills (due to the continual contact with the aqueous phase) and kidney (the hemopoietic tissue in fishes) (Belpaeme et al. 1998).

The present report presents contamination results by intra-peritoneal injection of inorganic lead (Pb^{2+}) in different doses using the specie *Hoplias malabaricus* (traíra). The genotoxic evaluation was performed with the piscine micronucleus test, chromosomal aberrations and comet assay in blood and kidney tissue.

Material and methods

Experimental design

The specimens of neotropical fish traíra (*H. malabaricus*: 71.97 ± 28 g) were collected in the PESCOPAR Pisciculture (Curitiba, Brazil). Prior to the experiment, fish were acclimated to experimental conditions for 30 days (one fish for each 30 L aquarium in dechlorinated tap water, temperature 21 ± 2°C, 12 h:12 h photoperiod) and no feeding. Eighteen animals were divided into six groups with three individuals each: (1) a control (individuals 1, 2 and 3), (2) positive control, i.e., a control of induction of damages in DNA (individuals 4 to 6) and the others were contaminated with $Pb(NO_3)_2$ solution at the concentrations of seven (individuals 7 to 9) (3), 21 (individuals 10 to 12) (4), 63 (individuals 13 to 15) (5) and 100 μg Pb^{2+}/g of body weight (individuals 16 to 18) (6). The fishes were contaminated through intra-peritoneal injection and sacrificed 96 h after the injection. The negative control group received a distilled water injection also 96 h before sacrifice, and the positive control group received a 30% hydrogen peroxide injection 1 h before sacrifice.

For $Pb(NO_3)_2$ (Pb^{2+}, CAS No. 10099-74-8) the dose administered was calculated based on a concentration factor of 3,000 g L^{-1}, proposed for secondary consumers and dietary contamination (Vighi 1981), while the average total lead concentration in the Ribeira river, Registro, state of São Paulo, Brazil, was 27.88 μg L^{-1} between 1978 and 1997, as measured by the local water company (CETESB). The concentration permitted by Brazilian Legislation, CONAMA Resolution No. 20 (BRASIL 1986) is 30.00 μg L^{-1}.

Each individual was anesthetized with 0.02% MS222 (ethyl-ester-3-aminobenzoic acid, Sigma). Peripheral blood (Fenocchio and Bertollo 1988) and kidney tissue were collected.

Piscine micronucleus

The Piscine Micronucleus Test (MNT) was performed through the analysis of 2,000 peripheral red blood cells per fish according to the technique described by Heddle (1973) and Schmid (1975). The frequency of micronuclei and nuclear morphologic alterations were observed according to Carrasco et al. (1990), summed and named as MNT.

Chromosomal aberration frequency

The mitotic metaphases were obtained through the indirect method with short time solid tissue culture (Fenocchio et al. 1991). The frequency of chromosomal aberrations was observed through the analysis of 50 metaphases of each individual.

Comet assay

The comet assay with peripheral blood (erythrocytes; CAE) was performed according to Speit and Hartmann (1999), with some modifications according to Ferraro et al. (2004) and Cestari et al. (2004). The kidney tissue cells (CAK), used for the comet assay, was homogenized (homogenizer Potter type at 1500 rpm) in Saccharose Tris-HCl buffer solution (pH 8.6). The volume of additional buffer was four times the collected tissue volume. From the final solution, 50 μL was mixed with 120 μL of low-melting-point agarose (0.5%). For the CAE, 10 μl aliquot was taken from each diluted sample and mixed with 120 μL of low-melting-point agarose. The suspension was spread on slides previously coated with normal agarose layer. The slides placed in lyses solution (lysis stock solution: NaCl, 2.5M; EDTA, 100 mM; Tris, 10 mM; NaOH, 0.8%; *N*-lauryl-sarcocinate, 1%; lysis working solution: 1 ml Triton X100; 10 ml DMSO; and 89 ml of lysis stock solution), for 24 h at 4°C. In the following step, the slides were subjected to electrophoresis, where they were first immersed in a NaOH and 200 mM EDTA, pH >13. The slides were allowed to stand for 20 min, to effect DNA denaturation, and then electrophoreses at 300 mA and 25 V. After neutralization in 0.4 M Tris, pH 7.5 and fixation in ethanol for 10 min, slides were stained with 20 μL of 10 μL/mL ethidium bromide. Comets were scored using a Zeiss epifluorescence microscope. For each fish, 100 nucleoids were analyzed (Kobayashi et al. 1995), utilizing the visual classification based on the migration of DNA fragments from the nucleus of class 0 (no visible damage), class 1 (little damage), class 2 (medium damage), class 3 (extensive damage) and 4 (maximally damaged). The score was calculated by multiplying the number of nuclei found in a class times the class number.

Statistical analysis

The evaluation of micronucleus frequency and other nuclear morphological abnormalities, as well as the frequency of chromosomal aberrations and comet assays for each tissue separately, comparing negative and positive control groups and the contaminated ones groups through the Kruskal–Wallis tests. Wilcoxon test was used for the comparison between comet assays with erythrocyte and kidney tissue. Results with $p < 0.05$ were considered statistically significant.

The results are presented in mean ± standard derivation (Table 1) and also in medians and quartiles (Q1–Q3) in Table 1. When the data is not symmetrical, this is the 'average' way that provides a better idea of any general tendency in the data. The median is at the middle of an ordered (ranked) data set and is a useful measure for ordinal variables. Strictly, the mean only makes sense for interval and ratio scales of measurement.

Results

In the piscine micronucleus assay there were no micronuclei, only nuclear morphological alterations (Fig. 1a) and after the analysis of 50 metaphases of each specimen of *H. malabaricus*, some structural alterations were found, such as gaps (Fig. 1b). However, the results, after statistical analysis, were not significant for both tests ($p = 0.6755$ in micronucleus test e $p = 0.8294$ in structural alterations in metaphases; Table 1 and Fig. 2).

The comet assays of erythrocytes and kidney cells performed with 30% hydrogen peroxide application 1 h before sacrifice, initially proposed as positive controls, showed higher damages than the negative control groups for both cell types. However, the differences were not statistically significant for neither of the analyzed tissues ($p = 0.3153$ for blood and $p = 0.2733$ for kidney cells; Table 1).

We also verified that inorganic lead damages both cell types, because the results of control fishes were statistically different than treated fishes, either for blood ($p = 0.019$ between nega-

Table 1 Nuclear Abnormalities (NA), structural alterations in metaphases (SA) and Scores of comet assay in erythrocytes (CAE) and kidney cells (CAK)

Treatment	Indiv.	NA	SA	CAE	CAK
Negative control	1	14	3	47	55
	2	23	3	59	31
	3	18	1	23	33
μ± ds		18,33 ± 4,5	2,33 ± 1,1	43 ± 18,3	39,67 ± 13,3
Medians/ Q1–Q3		18/16–20,5 [a]	3/2–3 [b]	47/35–53 [c]	33/32–44 [f]
Positive control	4	41	2	116	57
	5	61	2	167	89
	6	7	4	211	56
μ± ds		36,33 ± 27,3	2,67 ± 1,1	164,67 ± 47,5	67,33 ± 18,8
Medians/Q1–Q3		41/24–51[a]	2/2–3 [b]	167/141,5–189 [c]	57/56,5–73 [f]
7 μg Pb^{2+} g^{-1}	7	15	2	235	73
	8	34	5	169	72
	9	24	1	211	78
μ± ds		24,33 ± 9,5	2,67 ± 2,1	205 ± 33,4	74,33 ± 3,2
Medians/Q1–Q3		24/19,5–29 [a]	2/1,5–3,5 [b]	211/190–223 [c,d]	73/72,5–75,5 [f,g]
21 μg Pb^{2+} g^{-1}	10	34	2	290	130
	11	46	3	279	129
	12	22	5	234	112
μ± ds		34 ± 12	3,3 ± 1,5	267,67 ± 29,7	123,67 ± 10,1
Medians/Q1–Q3		34/28–40 [a]	3/2,5–4 [b]	279/256,5–284,5 [d]	129/120,5–129,5 [g]
63 μg Pb^{2+} g^{-1}	13	52	2	211	116
	14	75	3	260	97
	15	13	1	212	98
μ± ds		46,67 ± 31,3	2,0 ± 1,0	227,67 ± 28	103,67 ± 10,7[g]
Medians/Q1–Q3		52/32,5–63,5 [a]	2/1,5–2,5 [b]	212/211,5–236 [d]	98/97,5–107 [g]
[(+)]100 μg Pb^{2+} g^{-1}	16	49	3	195	127
	17	77	1	264	115
	18	n.a.	n.a.	n.a.	n.a.
μ± ds		63 ± 19,8	2,0 ± 1,4	229,5 ± 48,8	121 ± 8,5
Medians/Q1–Q3		63/56–70[+]	2/1,5–2,5[+]	229,5/212,2–246,7[+]	121/118–124[+]

[(+)]not statistically analyzed: the animals contaminated with the highest dose (100μg $Pb^{2+}.g^{-1}$) were not statistically analyzed because the group had only two individuals, since one of them died during the experiment
Indiv = Individuals;
NA = Nuclear Abnormalities;
SA = Structural Alterations in metaphases;
CAE = Comet Assay Erythrocytes;
CAK = Comet Assay Kidney Cells

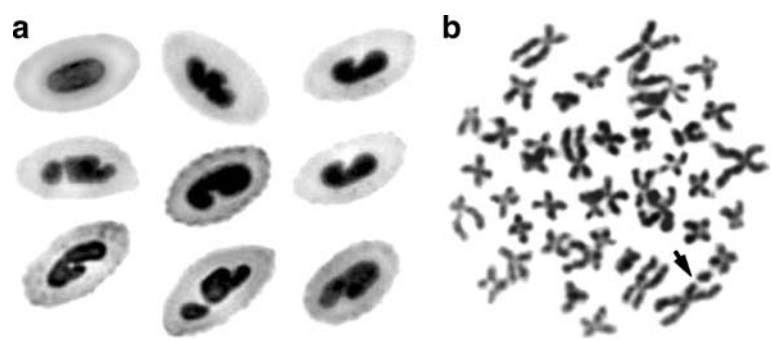

Fig. 1 (a) Normal cell and cells with nuclear morphological abnormalities and (b) Metaphase of *H. malabaricus* (2n = 42) contaminated with inorganic lead (Pb^{2+}). The arrow indicates a gap

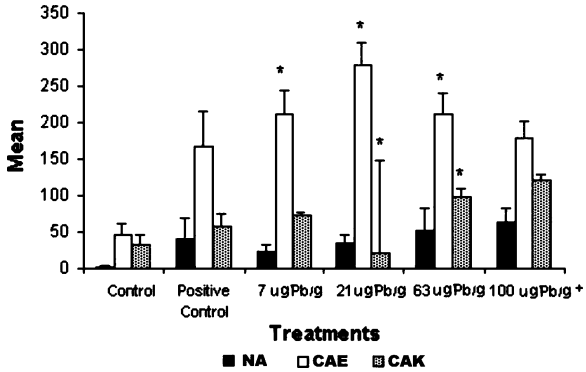

Fig. 2 Comparisons between exposed treatments. Specimens of *H. malabaricus* (control, positive control, 7 μg Pb^{2+}/g, 21 μg Pb^{2+}/g, 63 μg Pb^{2+}/g and 100 μgPb^{2+}/g) in tests NA (nuclear alterations), CAE (comet assay with erythrocytes) and CAK (comet assay with kidney cells). * $p < 0.05$ statistically significant compared to the control group

tive control and 21 μg Pb^{2+}/g and 0.0225 between negative control e 63 μg Pb^{2+}/g) and kidney tissue ($p = 0.0014$ between negative control and 21 μg Pb^{2+}/g and $p = 0.0106$ between negative control and 63 μg Pb^{2+}/g; Table 1). There was only one exception in cells at the lowest dose (7 μg Pb^{2+}/g; $p = 0.0769$ for blood and $p = 0.1769$ for kidney cells; Table 1 and Fig. 2).

The comet assay also showed that there was not a significant difference between the lead doses used in treated groups. For blood, between the doses 7 and 21 μg Pb^{2+}/g, p was 0.2012; between 7 and 63 μg Pb^{2+}/g, $p = 0.6481$; and between 21 and 63 μg Pb^{2+}/g, $p = 0.4113$. The same way for the kidney tissue, between 7 and 21 μg Pb^{2+}/g, p was 0.0769; between 7 and 63 μg Pb^{2+}/g, $p = 0.2353$; and for 21 and 63 μg Pb^{2+}/g, $p = 0.5228$ (Table 1 and Fig. 2).

There was a significant difference between the responses observed in the results of comet assay performed with circulating cells and kidney cells through the Wilcoxon test for paired samples ($p = 0.0005$). Blood presented a higher response to lead indicating that this tissue is more sensitive than the kidney tissue.

Discussion

Lead is one of the most commonly used metals in industry and its toxicity is important partly due to its persistence in the environment (Ogunseitan et al. 2000; Gurer-Orhan et al. 2004). Over the past two decades in Brazil, data assessing lead exposure showed a wide distribution and toxic effects of this metal (Costa et al. 2007).

Traira, *H. malabaricus* (Bloch) (Characiformes, Erythrinidae) is a freshwater carnivorous fish widely distributed in South America. This species is an interesting biological model for experimental study of dietary exposure to contaminants due to its voracious behavior, its ability to adapt to experimental conditions, and its food chain position. *H. malabaricus*, in addition, have great tolerances to food deprivation, surviving for periods of up to 180 days without reduction in metabolic rates (oxygen uptake; Rios et al. 2005).

Micronucleus are whole or partial chromosomes which have not been incorporated into the daughter nucleus during cell division and which appear as small round dark stained structures, otherwise identical in appearance to the cell nucleus (Bombail et al. 2001) and various authors (Hooftman and de Raat 1982; Hose et al. 1987) have suggested that variations in the shape of nucleus could represent an alternative approach for detecting genotoxicity. In spite of the fact that a correlation between nuclear abnormalities and genotoxic effects has not yet been established, preliminary observations strongly suggest that such morphological alterations could be a manifestation of the effects of xenobiotics (Ferraro et al. 2004). The result observed in the piscine micronucleus test might have occurred due to the low number of fishes analyzed, what could lead to an error on the final response of the nuclear alteration frequency, mainly because this method is less sensitive than the comet assay (Kim and Hyun 2006). Besides, the piscine micronucleus assay may lack sensibility, since it does not detect the mitotic disjunctions if they do not provoke chromosomal loss in the anaphases neither chromosomal aberrations caused by rearrangement, such as translocations or inversions if these changes do not originate acentric fragments (Metcalfe 1989).

The occurrence of chromosomal aberrations depends on intensity or concentration of the mutagenic agents as well as the studied cell type and the moment of the cellular cycle which have occurred the exposure (Obe et al. 2002). In the

present report, after the analysis of 50 metaphases of each specimen of *H. malabaricus*, some little structural alterations were found, such as gaps. However, the results, after statistical analysis, were not significant. This way, the absence of chromosomal aberrations may be related to the short exposure time (96 h), not enough to cause chromosomal damages in the proliferating cells.

Ferraro et al. (2004), working with sub-chronic trophic contamination (13 trophic doses in a period of 60 days) at the concentration of 21 μg Pb^{2+}/g in *H. malabaricus*, verified an increase in the frequency of nuclear alterations, chromosomal aberrations and also a significant increase of tailed nucleoids in the erythrocytes of fishes treated with Pb^{2+}, showing again that extended exposures to contaminants made of lead are capable of originating damages in the genetic material of fishes. Besides, Cipriano et al. (2004), studying the frequency of chromosomal aberrations in *Astyanax sp* (lambaris) exposed to TBT (tributyltin) at the doses of 0.3 mg/kg for 19 and 37 days, observed different chromosomal anomalies such as breakage of one or two chromatids and acentric fragments.

Cytogenetic markers such as chromosomal aberrations, micronuclei and sister chromatid exchanges are among the most extensively used markers of early biological effects of DNA damaging agents. During the last few years, the single cell gel electrophoresis (SCGE) or comet assay was introduced as a useful technique for biomonitoring studies. While biomonitoring studies employing cytogenetic techniques are limited to circulating lymphocytes and involve proliferating cell populations, the comet assay can be applied to proliferating and non-proliferating cells and cells of those tissues which are the first sites of contact with mutagenic/carcinogenic substances. The difference between effects in comet assay and cytogenetic tests is basically due to variations in the type of DNA alterations that the test systems detect: the comet assay detects repairable DNA lesions or alkali-labile sites while cytogenetic tests (MN and CA) detect fixed mutations which persist at least one mitotic cycle (Kassie et al. 2000).

Several studies show that the comet assay is really capable of detecting DNA damages caused by different classes of mutagenic contaminants in fishes, attributing high sensibility to the test. We may indicate Pandrangi et al. (1995), who showed an increase of the DNA damage in erythrocytes of bullheads (*Ameiurus nebulosus*) and carp (*Cyprinus carpio*) after exposure to cyclophosphamide and Devaux et al. (1997), who also verified an increase on the length of the nucleoid tail after the exposure of *Onchorynchus mykiss* hepatocytes to benzopyrene and hydrogen peroxide. Thus, the comet assay is an important tool for monitoring studies because it shows the genotoxicity of the exposure. The answer may, of course, depend on the experimental conditions of the specie, cell type, mutagenic agent and exposure time (Belpaeme et al. 1998).

The comet assays of erythrocytes and kidney cells performed with 30% hydrogen peroxide application 1 hour before sacrifice were not statistically significant for neither of the analyzed tissues ($p = 0.1936$ for blood and $p = 0.2211$ for kidney cells). This result was partially expected because, despite the high concentration of the agent, the exposure time was very low, considered not enough to cause damages on the DNA of the analyzed cells.

We also verified that inorganic lead damages both cell types, because the results of control fishes were statistically different than treated fishes, either blood ($p = 0.01$) and kidney tissue ($p = 0.01$; Table 1). There was one exception in kidney cells at the lowest dose (7 μg Pb^{2+}/g) ($p = 0.14$) probably due to the short time exposure (96 h) and also the low quantity of the contaminant.

While micronucleated erythrocytes from the hemopoietical organs reflect a genotoxic damage which occurred during a time equivalent to the cell cycle, those from the peripheral circulation reflect events that occurred in a time equal to the lifespan of the circulating erythrocytes. Therefore, the application of the micronucleus test on peripheral blood samples is particularly indicated for conditions of chronic exposure. The assay needs that the cell population undergoes mitosis, so the duration of the cell cycle should be known (Udroiu 2006). Processes of erythrocyte formation in the fish hemopoietic tissue are submitted to

pronounced seasonal fluctuations. No general regularities have been revealed. The obtained data are rather species-specific (Soldatov 2005).

Although the literature shows that this metal is capable of causing damages in the proteins of DNA structural maintenance or in those involved with repair mechanisms (Merian 1991; Pain 1995; Hartwig et al. 2002), our results in the piscine micronucleus assay and evaluation of chromosomal aberration frequency did not indicate contamination, different than the results in the comet assays, which showed differences between control and contaminated groups for blood and kidney cells.

According to Boelsterli (2002), lead induces DNA synthesis in a number of cells. Evidence indicates that inorganic lead interferes with cellular signal transduction pathways, in particular with members of the calcium-dependent protein kinase C (PKC) family. These kinases play an important role in cell proliferation. The mechanism is not entirely clear, but evidence suggests that Pb^{2+} causes activation of PKC-mediated pathways by facilitating the translocation of PKC from the cytosol to the plasma membrane (perhaps by mimicking Ca^{2+}). This kinase activation greatly stimulates cell cycle progression.

The whole S phase (DNA replication) of the cell cycle in fresh water fishes lasts near 14 h (Almeida Toledo et al. 1988). Thus, we believe that after 96 h of Pb^{2+} exposure the decrease of damage in blood cells in the highest doses, even not statistically significant (the dose 21 μg Pb^{2+}/g caused a little bit more damage to the blood cells than the dose 63 μg Pb^{2+}/g), was due to the induction of the cell cycle by Pb^{2}. The highest Pb^{2} doses could have renewed blood cells more rapidly causing, thus, lower damages.

There was a significant difference between the responses observed in the results of comet assay performed with circulating cells and kidney cells. Blood presented a higher response to lead indicating that this tissue is more sensitive than the kidney tissue, probably due to the acute contamination performed which had immediate responses first detected in the erythrocytes.

Biomonitoring studies with a combination of cytogenetic tests and comet assay enable comparison of the relative sensitivity of the two test systems and may also give a clue about the fraction of DNA damage detected in the comet assay that will lead to fixed mutations (Kassie et al. 2000). However, it is important to remember that the comet assay performed with peripheral blood is the most indicated method to analyze the acute contamination by inorganic lead because it is able to detect significant differences between control and treated groups even in very low doses (7 μg Pb^{2+}/g). This results show that the comet assay, in circulating cells, detects the mutagenic activity of Pb^{2+} even in low doses and for a short contamination period. Despite the low size of our samples for the experiments, the results may already show that low doses of Pb^{2+} cause alterations in the fish DNA as well as point the best method to detect this genotoxicity (comet assay with blood). However, more studies are needed to verify the genotoxic damages of Pb^{2+} for extended time periods.

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