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Research Article

Genetic damage induced by trophic doses of lead in the neotropical fish *Hoplias malabaricus* (Characiformes, Erythrinidae) as revealed by the comet assay and chromosomal aberrations

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Abstract

The effects of clastogenic or mutagenic agents have rarely been studied in neotropical fish species exposed to contaminated water. In this study, the genetic damage caused by lead in the widely distributed South American fish, *Hoplias malabaricus,* was assessed using the comet (SCGE) assay and by testing for chromosomal aberrations. Eighteen specimens were acclimatized to laboratory conditions and then chronically exposed to contaminated food by feeding prey (*Cyprinus* sp.) injected intraperitoneally with doses of inorganic lead adjusted to give a contamination level of 21 μg of $Pb^{2+}.g^{-1}$ net weight of *H. malabaricus*. Three fish were sampled for chromosomal analysis after four doses (18 days) and another three after eight doses (41 days) of lead and the results then compared with three untreated controls kept under lead-free conditions. An additional six treated fish and three controls were sampled for the comet assay after 13 doses (64 days). Exposure to lead significantly increased the frequency of chromosomal aberrations and the frequency of tailed cell nuclei, the latter indicating DNA damage. These results show that *H. malabaricus* is a useful biological model for screening the clastogenic effects of lead and possibly other xenobiotics. The genetic damage seen here illustrates the need to investigate the potential effects of heavy metals on fish species in South America.

Key words: chromosomal aberrations, comet assay, genotoxicity, *Hoplias malabaricus*, lead, mutagenesis.

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Introduction

The pollution of freshwater systems has long been considered a serious problem in Europe and North America (Heath, 1995). In contrast, there have been few studies of the effects of heavy metal and organic pollutants on aquatic organisms in neotropical ecosystems. The presence of heavy metals in industrial and municipal wastewater and in mine tailings has generated concern about the potential mutagenic and carcinogenic actions of these substances in aquatic organisms. In numerous aquatic ecosystems in Brazil, native species are often directly threatened by their exposure to water-borne agents or, indirectly, through a contaminated food chain (Oliveira Ribeiro *et al*., 2000).

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The potential for intoxication by lead and its organometallic derivatives in aquatic ecosystems, atmospheric particles, and soil has increased as a result of industrial activities (Pain, 1995). Investigations of the toxic effects of chemical pollutants on fish cells have demonstrated the importance of cytogenetic aberrations as an important indicator for environmental monitoring and assessment. Studies in laboratory animals have shown that exposure to lead at levels 10 mg $Pb^{2+}.mL^{-1}$ of blood leads to chromosomal aberrations (tetraploidy, mitotic anomalies, chromatid breaks), and these effects may be related to inteference with the mechanisms of replication, transcription and DNA repair (Goyer and Moore, 1974). Part of this effect on chro-

mosomes may be related to the ability of lead to form bridges with structural proteins that can adversely affect the degree of chromosomal condensation

Fish are good indicators for assessing the genotoxic and mutagenic effects of xenobiotics and physical agents (Al-Sabti, 1986). Sister chromatid exchange (SCE) tests have been applied to various fish species (Kligerman, 1979; Vigfusson *et al*., 1983) and the clastogenic effects of carcinogenic-mutagenic chemicals on kidney cells of *Cyprinius carpio* have been described (Al-Sabti, 1986). The comet assay is useful for evaluating genetic alterations and has been used as a rapid method to monitor genotoxicity in bullheads and carp (Monteith and Vanstone, 1995) and to detect the effects of carcinogens, such as aflatoxins, in trout and channel catfish (Abd-Allah *et al.*, 1999).

The use of neotropical fish to study the mutagenic effects of chemicals is still relatively uncommon (Matsumoto and Cólus, 2000). *Hoplias malabaricus* is an important predatory freshwater fish widely distributed throughout South America (Lopez and Fenocchio, 1994) and easily adapts to laboratory conditions. Since this species has only a few (2n = 40 or 2n = 42) biarmed, relatively large chromosomes, it is easy to handle in chromosomal studies. In this study, we used the comet (SCGE) assay and a test for chromosomal aberrations to assess the genetic damage caused by lead fed to *H. malabaricus*. To our knowledge, this is the first report to examine chromosomal damage *in vivo* following long-term trophic exposure of this species to inorganic lead.

Material and Methods

Experimental design

Mature *H. malabaricus* (average weight = 87.4 ±8.9 g) were collected from the Canguiri fish farm at the Federal University of Paraná (Curitiba, PR, Brazil). Before being used, the fish were acclimatized individually for 20 days in an aquarium containing well-aerated, dechlorinated water at 21 °C and a constant photoperiod (12 h light/dark cycle). Food (young specimens of *Cyprinius* sp. 5 days) was provided *ad libitum*. Twelve *H. malabaricus* were divided into three groups (A, B and D) for treatment with lead and six other fish were used as controls (groups C and E). For intoxication with lead, the food items were injected intraperitoneally with a solution of $Pb(NO_3)_2$. The volume of solution injected into the prey fish was adjusted to give a normalized ingested dose of 21 μg $Pb^{2+}.g^{-1}$ wet weight of *H. malabaricus*. The control group was fed fish injected with distilled water. Groups A and B were exposed to lead for 18 and 41 days, respectively, and the fish then used for chromosomal aberration analysis. Group D fish were exposed for a longer time (64 days, 13 doses) and the genetic effects were evaluated using the comet (SCGE) assay. In this case, group E served as the controls. Fish from each group were anesthetized with 0.02% MS222 (SIGMA (ethyl-ester.3. aminobenzoic acid). Kidney and blood samples were used for the chromosomal aberrations test and for the comet assay, respectively.

Chromosomal preparations

Mitotic chromosomes were obtained from short-term cell kidney cultures, as described elsewhere for fish cytogenetics (Fenocchio *et al*., 1991). Air-dried chromosome slides were stained with 5% Giemsa in phosphate buffer (pH 6.8). The frequency of chromosomal aberrations was scored by counting approximately 100 metaphase plates per individual. The different types of chromosomal aberrations, *i.e.*, gaps, breaks, and fragments, were grouped into a single category: altered metaphase plates.

Comet assay

This assay was done essentially as described by Speit and Hartmann (1999) using blood cells collected via the caudal vein into a 5 mL heparinized syringe. Initially, an aliquot of blood (10 μl) was diluted in 1 mL of fetal calf serum. The slides for microscopy were prepared using 10 μL of this cell suspension and 120 μL of low melting point agarose at 37 °C followed by incubation in lysis solution (1 mL of Triton X-100, 10 mL of DMSO and 89 mL of stock lysis solution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris, -adjust to pH 10 with ~0.8% NaOH-, 10 mM Na-lauroyl sarcosine]) for 1 h in the dark at 8 °C.

After lysis, the slides were placed in buffer (0.3 N NaOH, 1 mM EDTA, pH > 13) for 20 min to unravel the DNA. Electrophoresis was done for 20 min at 25 V and 300 mA, after which the slides were neutralized for 15 min in 0.4 M Tris, fixed in absolute ethanol for 10 min and stained with ethidium bromide (0.02 μg/mL).

Comet formation was observed with a Nikon epifluorescence microscope (filter B-3A; excitation: λ = 420-490 nm; emission: λ = 520 nm) at a magnification of 400X. One hundred cells were analyzed per sample (treated and controls) and were visually classified (Kobayashi *et al.,* 1995) based on the migration of the fragments (tail size) (class 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, and 3 - a tail length >two times the diameter of the nucleus). The total score 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).

Statistical analysis

The mean frequencies of the chromosomal alterations in fish exposed to lead for 18 (A) and 41 (B) days and in the control group (C) were compared by one-way analysis of variance (ANOVA), with a value of $p \leq 0.05$ indicating significance. The comet assay data were scored as classes based on the damage seen and were analyzed by the χ^2 test

in which the total number of comets in each treatment was compared with the controls.

Results and Discussion

Table 1 shows the frequency of chromosomal aberrations in metaphase plates of *H. malabaricus*. Because of the low frequencies observed, and in order to facilitate comparisons, all chromosomal alterations in each group were pooled into a single category.

Control fish showed almost no chromosomal abnormalities (1.1%) when compared to those fed lead-contaminated food for 18 or 41 days (25.6% and 19.1% of altered plates, respectively). Despite the low number of specimens used, these differences were significant ($p < 0.05$) because of the low variability among plates. Both treated groups showed an increase in several types of chromosomal abnormalities, including chromatid gaps and breaks, chromosomal fragmentation, chromatin decondensation and pericentric inversions (Table 1, < 0.05). Chromatid breaks were the predominant chromosomal aberrations after treatment.

Table 1 - Frequency of chromosomal aberrations in metaphase plates of *Hoplias malabaricus* treated with trophic doses of Pb^{2+}.

	Normal metaphase plates	Altered metaphase plates	Altered plates (%)
Control (C)	202 ±4.6	2.3 ±1.7	1.1
Treatment A (18 days)	122 ±38.8	42 ±14.6*	25.6
Treatment B (41 days)	135.6 ±39.1	31.6 ±9.1*+	19.1

The frequencies were calculated based on 1800 counts for each group and are the mean ± SD for three fish per group. *$p < 0.05$ compared to the control group and +$p < 0.05$ compared to treatment A.

There was a quantitative relationship between the occurrence of chromosomal abnormalities and the ingestion of lead. This metal can alter the genetic material of fish (Al-Sabti, 1985) following bioaccumulation in fish organs (not assessed here). The mutagenic or clastogenic activities of lead are related to disturbances in enzyme regulation that probably affect the replication, translation and repair of genetic material (Goyer and Moore, 1974).

An unexpected significant difference (Table 1 $p < 0.05$) was observed between groups A and B, with a greater number of altered metaphase plates after 18 days compared to 41 days. This difference could be related to the activation of appropriate repair mechanisms after a few weeks of exposure, and could counteract the undesired effects of lead on cells. However, this divergence could also reflect the low number of replicates used. A similar finding has been reported using the micronucleus (MN) test. However, in this case, the lower frequency of MN seen in the longest exposed groups was more probably related to the inhibitory effect of lead on the cell cycle than to the induction of cell repair mechanisms (Al-Sabti and Hardig, 1990; Poongothai *et al*., 1996). Thus, lead may affect both chromosomes and key enzymes involved in metabolic changes in the cell.

To examine the effects of a long-term exposure to lead, fish (group D) were exposed to 13 doses (64 days) and analyzed using the comet assay. Table 2 shows that lead significantly increased the frequency of tailed nuclei in treated fish, thus indicating lead-induced DNA damage. The effects were classified into four classes of damage

Table 2 - DNA damage in *Hoplias malabaricus* whole blood cells after 64 days of trophic and subchronic exposure to lead.

	Number of cells		Classes				Scores	$\chi 2$
	Analyzed	Tailed cells	0	1	2	3		
Control (group E)								
NC	100	16	84	16	0	0	16	
NC	100	6	94	5	0	1	8	
NC	100	23	77	22	1	0	24	
Total	300	45	255	43	1	1	X = 16	
Treated (group D)								
Pb	100	17	83	16	0	1	19	
Pb	100	66	34	50	16	0	82	
Pb	100	58	42	48	8	2	70	
Pb	100	15	85	13	2	0	17	
Pb	100	56	44	42	10	4	74	
Pb	100	54	46	54	0	0	54	
Total	600	266	334	223	36	7	X = 52.67*	74.804

X = mean; Pb = lead; NC = negative control.
*$p < 0.0001$ compared to negative control.

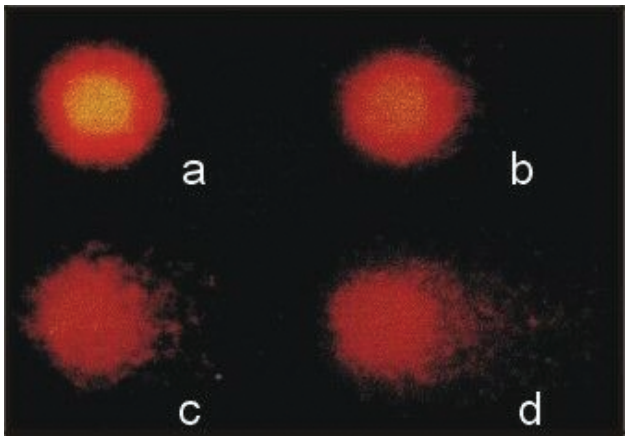

Figure 1 - Effect of long-term trophic exposure to inorganic lead on the appearance of comets in *Hoplias malabaricus*. Representative individual blood cells from group C, stained with ethidium bromide and photographed using an epifluorescence microscope. (a) Class 0 cells - no damage, (b) Class 1 cells - minor damage, (c) Class 2 cells - moderate damage, and (d) Class 3 cells - major damage.

based on the DNA migration (Figure 1). Some interindividual differences were detected, in agreement with other studies (Mitchelmore and Chipman, 1998). Our results also agreed with those of Abd-Allah *et al.* (1999) showing that the comet assay is an effective, simple and rapid method for assessing genetic damage caused by heavy metals in fish.

The few reports that have examined fish chromosomal aberrations have been limited to studying the effects of radiation on some cichlid species (Manna and Som, 1982; Som and Manna, 1984). This lack of information may reflect the difficulty in using conventional methods to study species with small chromosomes. A solution to this problem could be the use of certain neotropical cyprinodontiform fish species that have a small number of relatively large chromosomes (Alink *et al.*, 1980). The viability of this approach was confirmed here for *H. malabaricus,* which has a low diploid number and relatively large, biarmed chromosomes, but no heteromorphic sex chromosomes.

In conclusion, this report is one of the first to use the comet assay to evaluate DNA damage following chronic exposure to Pb^{2+} in the diet. The results obtained show that the neotropical fish *H. malabaricus* may be a useful natural model for screening the clastogenic effects of lead on fish chromosomes.

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