Short Communication

Studies of micronuclei and other nuclear abnormalities in red blood cells of *Colossoma macropomum* exposed to methylmercury

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Abstract

The frequencies of micronuclei (MN) and morphological nuclear abnormalities (NA) in erythrocytes in the peripheral blood of tambaqui (*Colossoma macropomum*), treated with 2 mg.L^{-1} methylmercury (MeHg), were analyzed. Two groups (nine specimens in each) were exposed to MeHg for different periods (group A - 24 h; group B - 120 h). A third group served as negative control (group C, untreated; n = 9). Although, when compared to the control group there were no significant differences in MN frequency in the treated groups, for NA, the differences between the frequencies of group B (treated for 120 h) and the control group were extremely significant (p < 0.02), thus demonstrating the potentially adverse effects of MeHg on *C. macropomum* erythrocytes after prolonged exposure.

Key words: cytotoxicity; genotoxicity; methylmercury; micronuclei assay.

Heavy metals represent a significant ecological and public health threat, through their toxicity and ability to accumulate in living organisms (Çavas, 2008). The amply described adverse effects therefrom, in both the natural environment and under laboratory conditions give testimony to their toxicity in certain concentrations (Russo *et al.*, 2004). Furthermore, environmental contamination through the bioaccumulation of compounds containing heavy metals is a potential cause of damage to genetic material (Prá *et al.*, 2006).

Mercury is considered one of the most dangerous of the heavy metals, through its high toxicity, bioaccumulative properties, and other deleterious effects on biota, this including genetic alteration or mutagenesis (WHO, 1990). Among other mutagenic properties, mercury and certain organomercurial compounds exert an adverse effect on tubulin, the structural subunit of microtubules involved in cytoplasm organization, and also a component of spindle fibers. Mercury impairs tubulin polymerization, thereby causing the contraction of metaphasic chromosomes, a delay in centromere division, and slower anaphasic move-ment (Thier *et al.*, 2003). Methylmercury (MeHg) is classified by the International Agency for Research on Cancer (IARC) as a group 2B substance, thereby indicating a tendency to being carcinogenic for humans (Hallenbeck, 1993).

Biomonitoring, a promising tool for identifying pollutants (bioindicators) that affect human and environmental health, is especially useful with organisms thus exposed in biological systems (biomarkers) (Silva *et al.*, 2003). The effects of genotoxic substances on fish genomes have been the theme of many studies, especially when seeking to establish the response of genes to environmental stimuli (Bücker *et al.*, 2006). Since fish response to toxicants is often similar to that in the higher vertebrates, they can be useful in screening for chemicals potentially capable of inducing teratogenic and carcinogenic effects in humans (Al-Sabti and Metcalfe, 1995).

The micronucleus test, one of the most popular and promising tests of environmental genotoxicity, has served as an index of cytogenetic damage for over 30 years (Fenech *et al.*, 2003). In recent years, considerable attention has been paid to the simultaneous expression of morphological nuclear abnormalities (NAs) and micronuclei (MN) in the piscine micronucleus test (Çavas and Ergene-Gozukara, 2003). Among current cytogenetic techniques, NAs and MNs are considered as indicators of cytotoxicity and

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genetic toxicology, respectively (Çavas *et al.*, 2005; Grisolia *et al.*, 2009).

Abundant data indicating the increase in genotoxic and cytotoxic damage induced by mercury have been divulged in scientific literature (Ayllon and Garcia-Vazquez, 2000; Çavas, 2008, Guilherme *et al.*, 2008; Rocha *et al.*, 2009). As the genotoxic and cytotoxic potential of mercury compounds is a well-known phenomenon, the main aim of the current study was to assess nuclear responses in fish treated with methylmercury chloride.

Colossoma macropomum (Cuvier, 1818), commonly known as tambaqui was chosen as a model. This species of the Characidae is the biggest Characiforme in the Solimões/Amazonas River system, reaching a size of 1 m in native environment and weighing approximately 30 kg (Araújo-Lima and Goulding, 1998).

Juvenile specimens of *C. macropomum*, with an average length of 14.3 ± 1.04 cm and an average weight of 42.5 ± 8.20 g, were obtained from the Pisciculture Station of the Federal Rural-Amazon University in Castanhal, Pará State, Brazil. The fishes were transported to the laboratory, where they remained for one-month acclimatization at a density of three specimens per 30-L aquarium under constant aeration, with a 12-h light/dark photoperiod and chlorine-free water, at pH 6.5 ± 0.29 and a temperature of 26 ± 1.3 °C.

Methylmercury chloride (Pestanal®, analytical standard, approximately 100% pure), was purchased from Sigma-Aldrich®. Exposure to the contaminant was facilitated via water, with a sub-lethal MeHg concentration of 2 mg.L^{-1}. This concentration equals the greater found during the Minamata disaster (Japan). Two groups (nine specimens in each) were exposed for different lengths of time (group A: 24 h; group B: 120 h), with a third group as negative control (group C, untreated; n = 9).

Peripheral blood, obtained with heparinized syringes, was immediately smeared. After fixation in ethanol (100%) for 20 min, the slides were initially left to air-dry, and after stained with 10% Giemsa. Four thousand erythrocytes per fish were examined at 1000 x magnification. MNs were defined as round or oval intracytoplasmatic bodies neither linked nor connected in any way to the main nucleus, with a diameter of 1/30-1/10 of that of the major nucleus and on the same optical plane (Al-Sabti and Metcalfe, 1995; Ayllon and Garcia-Vazquez, 2000). The NAs were used as cytotoxicity biomarkers. Three NAs were considered, viz., buds, lobes and invaginations (Ayllon and Garcia-Vazquez, 2000; Bolognesi *et al.*, 2006). The frequencies of MN and morphological NAs were calculated from the same microscopic slides.

The Kolmogorov-Smirnov test of MN and NA data for goodness of fit (p-value > 0.05) revealed no significant departure from normality. After assessing the normality of data distribution, parametric tests were applied for detecting differences at the 0.05 significance level. Differences

between mean values were compared by means of one way ANOVA and the Least Significant Difference (LSD) test. All the data were expressed as means ± standard deviation (SD). All the analyses were undertaken with the BioEstat 5.0 statistical package (Ayres *et al.*, 2007).

MN and NA frequencies in red blood cells of *C. macropomum* treated with MeHg are shown in Figure 1. Although initially no significant differences were observed in MN frequencies between unexposed control fishes and those exposed, a statistically significant increase in erythrocytes with altered nuclear morphology was indeed observed after exposure for 120 h.

Whilst spontaneous (or basal) MN frequency in fish is normally very low (Ferraro *et al.*, 2004), appreciable interspecies differences have been reported. Thus, when considering frequency per 1,000 cells, MNs were 0.08 ± 0.13 in *Hoplias malabaricus* (Ferraro *et al.*, 2004), 0.1 ± 0.316 in *Eigenmannia virescens* (Bücker *et al.*, 2006), 3.17 ± 0.48 in *Carassius auratus* (Çavas and Könen, 2007), and 2.4 ± 1.19 in *Colossoma macropomum* (present paper). Furthermore, in MN assaying undertaken by Ramsdorf *et al.* (2009), using *H. malabaricus*, there were no MNs, only nuclear morphological alterations.

The high interindividual variability associated to the low baseline frequency for this biomarker, confirms the need for scoring a consistent number of cells (at least 4000) per specimen, in an appropriate number of animals (Bolognesi *et al.*, 2006), which was the case in the present study. Nevertheless, in the literature, divergence is wide as to the ideal quantity.

Although not statistically significant, in the present experiment, group A (exposed to MeHg for 24 h) presented fewer micronucleated erythrocytes than the control group.
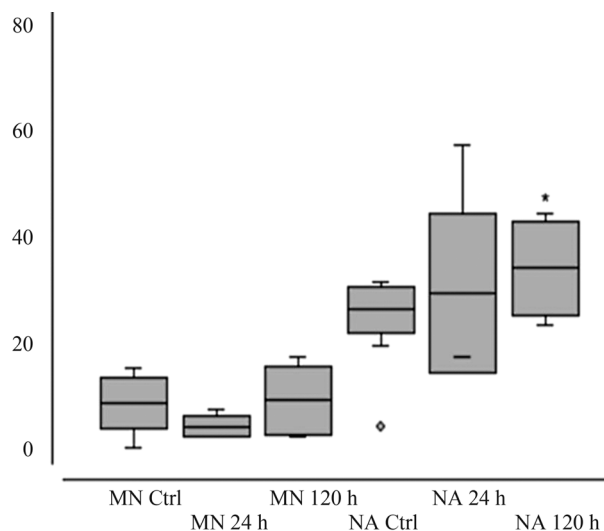
Figure 1 - Micronuclei (MN) and nuclear abnormality (NA) frequencies (per 4000 red blood cells) in *Colossoma macropomum* treated with MeHg (2 mg.L^{-1}). *Indicates significant differences compared to the control group (ANOVA; $p < 0.02$).

This may be for several reasons, such as either the ecto-thermic nature of the fish, the effects of erythropoesis and seasonal variability erythrocyte nuclear morphology, the lifespan of the circulating erythrocytes, or the elimination of old erythrocytes and those containing micro- (Udroiu, 2006) and irregular shaped nuclei.

Erythrocytic NA frequency and its affinity with total mercury concentration (Hg_t) in the blood were seasonally assessed in the mullet *Liza aurata* from the Laranjo basin, Aveiro, Portugal. Surprisingly, no NA induction was found during the winter, notwithstanding the high blood Hg_t, possibly explainable by dynamic haematological alterations, viz., reduced erythropoesis and/or increased erythrocyte elimination, capable of masking genotoxicity expression (Guilherme *et al.*, 2008).

Reports are contradictory as to the genotoxic potential of mercury compounds. For example, *Phoxinus phoxinus* treated with mercury nitrate presented no significant increase in nuclear damage (Ayllon and Garcia-Vazquez, 2000). Similar findings were also reported for *Hoplias malabaricus* exposed to trophic doses of methylmercury (Lopes-Poleza S, 2004, Master Dissertation, Universidade Federal do Paraná, Belém). Notwithstanding, mercury compounds seem to be genotoxic for other fish species, as *Poecilia latipinna* exposed to mercury nitrate (Ayllon and Garcia-Vazquez, 2000), and *Carassius auratus* exposed to mercury chloride (Çavas, 2008). In other fish-species, such as killifish (*Fundulus heterociclitus*), MN induction was obtained by exposure to methylmercury derivatives, more active over a short period of time than inorganic mercury salts (Perry *et al.*, 1988). According to Grisolia *et al.* (2009), one should be aware of the differential sensitivity and responses of aquatic organisms to genotoxic agents, and their relationships within the aquatic ecosystem.

Among current cytogenetic techniques, MN and certain other NAs are considered to be sensitive indicators of genotoxicity and cytotoxicity, numerous reports indicating that mercury induces a greater number of NAs than MNs (Ayllon and Garcia-Vazquez, 2000; Çavas, 2008; Rocha *et al.*, 2009; and Lopes-Poleza S, 2004, Master Dissertation, Universidade Federal do Paraná, Belém). In the present case, *C. macropomum* only seems to be sensitive to mercury cytotoxicity due to the induction of morphological NAs.

Acknowledgments

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