MICRONUCLEI AND OTHER NUCLEAR ABNORMALITIES IN ACARÁ 
Aequidens tetramerus (PERCIFORMS: CICHLIDAE) EXPOSED 
TO COPPER SULFATE

ABSTRACT

We have investigated micronuclei and other nuclear abnormalities in peripheral blood erythrocytes of saddle cichlid, Aequidens tetramerus, in order to evaluate the genotoxic and mutagenic effects of copper sulfate. Specimens (n = 4) were subjected to copper sulfate 7,975 mg.L⁻¹ with an equal control group (n = 4). The Mann-Whitney test was used to compare nuclear abnormality frequencies between control and treatment groups. Results showed statistically significant difference of altered cells amongst the exposed acará and the control group. Our results emphasize the importance of the micronucleus test in the assessment of genotoxic effects in fish, which can be used as bioindicators of the exposure of human populations to chemicals with a genotoxic potential found in drinking water.

PALAVRAS-CHAVES:
Teste do Micronúcleo; Genotoxicidade; Aequidens.

RESUMO

Neste trabalho, investigamos a frequência de micronúcleos e outras anormalidades nucleares em eritrócitos periféricos do acará-sela Aequidens tetramerus, com o objetivo de avaliar os efeitos genotóxicos e mutagênicos do sulfato de cobre. Quatro espécimes foram submetidos ao sulfato de cobre 7,975 mg.L⁻¹ e outros quatro constituíram o grupo controle. As freqüências de anormalidades nucleares foram comparadas pelo teste de Mann-Whitney. Os resultados apresentaram diferença estatisticamente significativa de células alteradas entre os acará expostos e o grupo controle. Nossos resultados reforçam a importância do teste do micronúcleo na avaliação de efeitos genotóxicos em peixes, os quais podem ser utilizados como bioindicadores da exposição de populações humanas a produtos químicos com potencial genotóxico presentes na água de consumo.
INTRODUCTION

Many studies have described the effects of transition metals, both the natural environment and in laboratory conditions, concluding that these are toxic in certain concentrations (RUSSO et al., 2004). Environmental contamination with compounds containing transition metals is of concern because in addition to high toxicity, such metals undergo bioaccumulation and have a potential to cause damage to genetic material (PRA et al., 2006). Contact with copper may occur in agriculture due to the use of fungicides and algaecides and in copper production industries, in manufacturing fungicides and in metal smelting (BANU et al., 2004). Copper sulfate is widely used in aquaculture to control the blooming of algae and macrophyte infestation, with application rates ranging from 0.5 to 2.0 mg.L\(^{-1}\) (BOYD; MASSAUT, 1999). In fact, it is the most widely used algicide in aquaculture (VALENTI, 1998).

Pavanelli et al. (2002) recommend the use of copper sulfate to control saprolegniose in fish, a disease caused by fungi of the genus Saprolegnia, with one-hour baths in concentrations of 15 or 25 mg.L\(^{-1}\). The same authors recommend a concentration of 0.75 mg.L\(^{-1}\) of copper sulfate solution for 14 days to fight protozoan Amyloodinium ocellatum.

Copper can also strike the lentic and lotic environments through the runoff and entrainment with rainwater, being adsorbed or dissolved in the organic matter, as well as through groundwater contamination. The waters of the Amazon have unique physical and chemical properties that directly interfere with concentration, competition and complexation of metals by altering the toxicity thereof (BEVILACQUA, 2009). According to the same author, the Biotic Ligand Model (BLM) is an efficient computational model for predicting the toxicity of copper in waters with pH above 5, as observed, for example, in the Solimões and Amazon rivers. However, in the waters of the Negro river, with pH values below 4.5, the model does not show the same reliability and it should be adjusted to acidic water.

Many researches with \textit{in vitro} studies and cell cultures assert the capability of copper to initiate oxidative damage in addition to peroxidation of lipid membranes, thus interfering with important cell functions and properties (KRUMSCHNABEL et al., 2005). Biomonitoring is a promising tool for the identification of pollutants that affect human and environmental health, especially with organisms exposed to such pollutants (bioindicators) using tests in biological systems (biomarkers) (SILVA et al., 2003).

The effects of genotoxic substances on fish genome have been the subject of many studies, especially those seeking to establish the response of genes to environmental stimuli (BÜCKER et al., 2006).

Bioassays allow for the study of toxic effects of various contaminants in isolation or in combination, so as to significantly reduce the influence of environmental variables. The use of aquatic organisms, particularly those of endemic species, is of fundamental importance in biomonitoring studies. However, using such organisms requires the standardization of protocols for the detection of DNA damage (FERRARO et al., 2004). The growing interest in environmental
genotoxicity studies has led to the development of various tests to detect genotoxic agents in the aquatic environment. The micronucleus test is one of the most popular and promising environmental genotoxicity tests and has been considered an index of cytogenetic damage for nearly 30 years (FENECH et al., 2003). The micronucleus test, developed by Schmid (1975) using bone marrow cells of mammals, has been applied extensively to test the genotoxicity of chemical compounds. Excellent results have been obtained with this test in invertebrates, fish and amphibians in the monitoring of contaminated areas (CAMPANA et al., 2003).

Genotoxicity tests using fish species can be performed in vitro or in vivo. As for the latter, the tested agents are injected into fish or added to water or food (COTELLE; FERARD, 1999). In the past decade, some studies have been published with the micronucleus test used to assess genotoxic effects of copper salts on exposed fish (CAVAS et al., 2005; ANDREIKÈNAITË et al., 2007; OBIAKOR et al., 2010).

This paper presents the results of a study of the neotropical fish Aequidens tetramerus, which was exposed to copper sulfate (CuSO₄) in water. The effects of such exposure were assessed by the frequency of typical micronuclei (MN) and other nuclear abnormalities, more specifically morfonuclear changes (aMN) in peripheral erythrocytes.

**MATERIAL AND METHODS**

The fish species selected as a model was Aequidens tetramerus (Heckel, 1840), known as saddle cichlid, a neotropical fish native to several basins in South America, which consumes mainly foodstuffs of animal origin (MOREIRA; ZUANON, 2002). Cytogenetic studies show that this species has a diploid number 2n = 48, with karyotype made up of submetacentric and acrocentric only (MARESCALCHI, 2004).

In our study, we used eight adult specimens of A. tetramerus, with average weight and length of 64.64 ± 28 g and 15 ± 1.76 cm, respectively. Initially, the saddle-cichlid specimens were acclimated for 30 days in individual 15-liter aquariums. Water temperature and pH were maintained around 28°C and 5.5, respectively; constant aeration was provided and a photoperiod of 12/12 h. After acclimation, subjects were divided into two groups of four, one group being exposed to copper sulfate and the other, not exposed, was used as a control.

The specimens were exposed to copper sulfate dissolved in the aquarium water at a concentration of 7.975 mg.L⁻¹; the water was not renewed during the exposure period. The selection of concentration
was based on the work of Prá et al. (2006) and corresponds to an intermediate value amongst those used in aquaculture. Blood samples for the MN test were collected after 24 hours of exposure and the protocol used was based on Benincá (2006).

The collected blood was immediately dripped on slides to make the smears (technique that involves scattering whole blood on a glass slide), which dried at ambient temperature. The slides were subjected to a 20-minute bath in ethanol (100%) for fixation. Next, the slides were washed with distilled water and stained for 10 minutes in 10% Giemsa diluted in phosphate buffer (pH 6.8); the slides were then washed with distilled water and air dried at room temperature. After drying, the slides were observed under optical microscope with magnification of 1,000x.

Analysis of 1,500 erythrocytes per specimen was conducted, including those erythrocytes with typical micronucleus (MN), such that as shown in Figure 1, and changes in nucleus shape (morfonuclear changes - aMN). Only cells with intact plasma membrane were considered. The aMN considered hereby, according to Carrasco et al. (1990); Ayllon e Garcia-Vazquez (2000) and Bolognesi et al. (2006) were three, namely: blebbed nuclei (nuclei with one or more small nuclear membrane evaginations ranging in size from the strip of small bumps to completely circumscribed structures, similar to micronuclei, but still connected to the main nucleus); lobed nuclei (nuclei with wider evaginations than those described for blebbed); notched nuclei (nuclei that have a well-defined cut in their shape, usually with an appreciable depth and that appears to be delimited by the nuclear envelope).

Figure 1 - Fish erythrocytes stained with Giemsa at 1,000x magnification. The arrow indicates a micronucleus.
The results were expressed as a mean ± standard deviation. Then, all data were tested for normality using the Kolmogorov-Smirnov test. Because the data did not show a normal distribution, the statistical significance of differences between exposed fish and control group fish was assessed using the nonparametric Mann-Whitney test. For the statistical analysis, we used the BioEstat 5.0 software (AYRES et al., 2007), with a significance level of 0.05.

RESULTS AND DISCUSSION

The use of different tests and biological systems ensures a more accurate screening of the effects caused by contaminants in the aquatic environment. Accordingly, several biomarkers are being developed and among these genetic biomarkers have gained wide acceptance (RAMSDORF, 2007).

The micronuclei frequencies and nuclear morphological changes after treatment with copper sulfate 7.975 mg.L⁻¹ are presented in Table 1. The spontaneous frequency of micronuclei in fish is usually very low (FERRARO et al., 2004). In our experiment, the typical micronuclei (Figure 1) showed low frequencies both in the control group and in the exposed fish group; however, it may be noted that exposure to CuSO₄ significantly increased the occurrence of micronuclei (p <0.05).

<table>
<thead>
<tr>
<th>TABLE 1 - Mean ± Standard Deviation (SD) of Micronuclei (MN) and nuclear morphological changes (aMN) in A. tetramerus exposed to CuSO₄ at a concentration of 7.975 mg.L⁻¹.</th>
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<tr>
<td><strong>MEAN ± SD for MN AND aMN/1500 CELLS</strong></td>
</tr>
<tr>
<td>MN</td>
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<td>-------------------------------</td>
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<tr>
<td><strong>Control</strong></td>
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<td><strong>Exposed</strong></td>
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* P <0.05, ** p <0.02 compared against control (Mann-Whitney test).
According to our proposal, the morfonuclear changes were also analyzed and appeared a little more frequently. The difference between the exposed fish and the control group as to the morfonuclear changes (p < 0.02) was even more significant than that observed for typical micronuclei.

The occurrence of other nuclear abnormalities in addition to micronuclei has also been considered as a truly useful indicator in the assessment of the genotoxic and cytotoxic effects of contaminants on aquatic organisms (CAVAS; ERGENE-GOZUKARA, 2003; DAILIANIS et al., 2003; FERRARO et al., 2004; BARŠIENĖ et al., 2006; MATSUMOTO et al., 2006).

Considering all the changes found in our experiment, i.e. typical micronuclei + morfonuclear changes, the Mann-Whitney test showed a very significant difference between the exposed and control groups (p < 0.02). Such difference can be seen in Table 1 and in the chart in Figure 2.

Several studies have shown that the peripheral erythrocytes of fish have a high incidence of micronuclei after exposure to different pollutants.
under laboratory conditions (BARŠIENĖ et al., 2005; ANDREIKĖNAITĖ et al., 2007; NAGPUR et al., 2008). In some studies, however, it was not possible to detect significant mutagenic effects on the erythrocytes analyzed (FERRARO et al., 2004; BUCK et al., 2006; MATSUMOTO et al., 2006; ROCHA et al., 2009), unless the frequency of micronuclei was analyzed along with the morfonuclear changes (FERRARO et al., 2004; MATSUMOTO et al., 2006; ROCHA et al., 2009).

On the other hand, although there are few publications reporting copper sulfate genotoxicity tests in fish, Cavas et al. (2005) found that the dose of 0.25 mg.L\(^{-1}\) induced an increase in the frequency of micronuclei in peripheral blood of Cyprinus carpio and Carassius gibelio, in the cells of the gills and liver, showing higher sensitivity in these tissues in relation to peripheral blood. In a more recent experiment, Obiakor et al. (2010) evaluated the effect of copper sulfate and zinc sulfate (isolately and in their binary mixture) in erythrocytes of species Synodontis Clarias and Tilapia nilotica. All treatments resulted in a significant increase in the frequency of micronucleated erythrocytes, but the highest level was observed in the fish exposed to the mixture of said metals.

Even though some studies of MN show greater sensitivity of the epithelial cell of gills in relation to erythrocytes, as in Hayashi et al. (1998), Cavas et al. (2005) and Cavas (2008), the use of peripheral blood cells has prevailed due to the technical ease in obtaining the tissue, because this is possible without sacrificing the animals, and especially because it is possible to make actual comparisons of results with a larger number publications in the area.

Therefore, we conclude that the results presented hereby regarding the assessment of genotoxic effects in fish have shown that the micronucleus test in fish in captivity can be used as a biomarker and the organisms as sentinels for the occurrence of pollution in aquatic ecosystems, in addition to being an indirect indication of a possible exposure of human populations to metal pollutants with a genotoxic potential contained in drinking water.

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