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MUTAGENIC EFFECTS OF TOXIC RESIDUES ON THE MICRONUCLEI OF FRESH WATER TELEOSTEI

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RESUMO

Neste estudo foi aplicado o teste de micronúcleo em teleósteos que habitam o Reservatório de Americana, rio Piracicaba, São Paulo, para verificar o grau com que os resíduos agrotóxicos presentes naquele sistema aquático estão afetando o material genético dos peixes que ali vivem e se reproduzem. O teste foi também aplicado num grupo controle constituído por teleósteos coletados em ambientes não poluídos localizados nos rios Atibaia e Atibainha, tributários do rio Piracicaba. Os resultados mostraram que a freqüência de micronúcleos nos eritrócitos, quando comparado com o controle, foi significantemente maior nos peixes do reservatório, indicando que as suas águas estão contaminadas com resíduos tóxicos num grau capaz de causar mutagênese nos peixes.

Palavras-chave : teleósteos, poluição aquática, micronúcleos, mutagênese.

ABSTRACT

In this study, the micronucleus test was applied to teleostei inhabiting the Americana reservoir on the Piracicaba river in São Paulo, in order to evaluate the effects of the toxic agricultural residues present in the reservoir on the genetic make-up of the fish that live and reproduce there. The test was also applied to a control group of teleostei collected in non-polluted environments in the Atibaia and Atibainha rivers, tributaries of the Piracicaba. The results showed that the frequency of micronuclei in the erythrocytes was significantly greater in fish from the reservoir in comparison with those from the control group. This indicates that the waters of the reservoir are contaminated with toxic residues in amounts capable of causing mutagenesis in its fish.

Keywords : teleostei, aquatic pollution, micronucleus, mutagenesis.

INTRODUCTION

When organisms are exposed to mutagenic agents, they may suffer DNA lesions which increase the risk of the development of tumors, if not repaired by its defense systems. Genotoxic agents include the phenol compounds (Stich, 1991), N-nitrosamine (Ashby *et al*., 1991), sulphur-35 (Monakhov, 1991), and the ionizing radiation procedure (Schimid & Bauchinger, 1980; Natarajan *et al*.; 1986; Jong *et al*., 1988; Eisele *et al*., 1991; Doloy *et al*., 1991). The indiscriminate use of agro-toxins is of special concern, because toxic compounds, such as heavy metals, accumulate in the soil and from there, are washed into rivers and water tables, with serious consequences for the species at the top of the food-chain.

The development of techniques which permit the precocious identification of the presence of mutagenic agents is of great importance for the identification of environments that have been polluted. Cytogenetic analysis is one of the most common of these techniques, which consists of preparing cell cultures of exposed individuals, or analyzing the cells of the hematopoietic system directly. Clastogenic

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agents rupture the chromosomes and micronuclei, while aneugenic agents interfere with the mitotic spindle apparatus through numeric alterations to the chromosomes. These alterations are due to the interruption of chromosome migration to the poles, which originates micronuclei.

Micronuclei are formed during mitotic cell division, in the transition from metaphase to anaphase, when chromosomes and acentric fragments not attached to the spindle apparatus, or delayed during the mitosis to the poles, remain in the cytoplasm in the form of small nuclei. These micronuclei are useful for biomonitoring, because they indicate exposure to mutagenic agents. The toxic effects of substances, such as glyphosate, dichlowos, surfactants, mixtures of chemical compounds, and heavy metals like cadmium and copper, have been verified in aquatic organisms through the presence micronuclei (McKlim *et al*. 1970; Folmar *et al*. 1979; Ariyoshi et al. 1990; Horsberg & Hoy, 1990; Ensenbach & Nagel, 1995; Gardner & Yevich, 1970; Tort & Torres, 1988).

In recent decades, the increasing use of chemical products has caused an accumulation of residues in the environment, which have serious consequences in aquatic habitats (Blaxhall, 1972; Pritchard, 1993). The aim of the present study is to evaluate the effects of the pollution of the waters of the Americana reservoir in the municipality of Americana, São Paulo, through analysis of the frequency of micronuclei in the erythrocytes of the peripheral circulation in local fish.

MATERIALS AND METHODS

The focus of this pilot study is to verify the number of micronuclei in the erythrocytes of fish, based on the assumption that all individuals inhabiting an environment polluted with toxic residues will have more micronuclei in comparison with those from unpolluted habitat. For this, two groups of fish (control and experimental) were collected within the same region of the State of São Paulo. Five species were collected in both groups: *Astyanax faciatus* and *Serrasalmus spilopleura* (Characidae), *Hoplias malabaricus* (Erythrinidae), *Pimelodella gracilis* (Pimelodidae) and *Geophagus brasiliensis* (Cichlidae)

Control group specimens were collected at two sites on the Atibainha river, near Piracaia, and one on the Atibaia river, upriver from Bom Jesus dos Perdões. These sites were chosen because of the general lack of pollution in the area. The experimental group was collected at three sites in the Americana reservoir, on the Piracicaba river, in the municipality of Americana (22º44'S, 47º14'). The reservoir is formed by tributaries of the Piracicaba river, which pass through a number of agricultural areas, characterized by high levels of pollution. The reservoir suffers considerable local impact, from the dumping of urban and industrial sewage by the city of Campinas, to increasing agricultural activity, which produces toxic residues that drain directly into the aquatic environment. All specimens were collected with fishnets and hooks, in order to obtain living individuals in good condition. Samples were collected during the dry season (March to August) and the rainy season (September to February).

Blood samples were collected from the tail vein or directly from the heart, using a hypodermic syringe and needles previously heparinized. Slides were prepared with the blood smears and left to dry outdoors. They were then fixed with methanol PA during 3 minutes, and stored in boxes to be taken to the Cellular Biology Laboratory of the Pontifical Catholic University of Campinas, where they were dyed with Schiff reagent for analysis. Dyeing, by the Feulgen technique, consisted of hydrolyzing slides in HCl 1N immersed in water at 60ºC during 8 min. Slides were then immediately immersed in iced H_2O at approximately 4ºC, to halt hydrolysis. Slides were then washed in running tap water for 10 minutes, and quickly rinsed in distilled water. After being dried well, the slides were dipped in Schiff reagent in the dark for one hour at room temperature, and then mounted with Permount and cover slips, and left to dry until the following day. The cells were analyzed under binocular microscopes.

From each species of both groups (control and experimental) we took 10 samples in order to verify the amount of micronuclei, 5 samples in each dry and rainy season. In each sample we analyzed 3,000 erythrocyte cells, establishing in this way the number of micronuclei per species in the two seasons for both groups (Table 1). The frequency of micronuclei was determined according to erythrocytes of the peripheral circulation, following the technique proposed by Schimid (1975), adapted to fish, observing the criteria proposed by Titenko-Holand *et al*. (1997). In order to evaluate the genotixic effect, we only considered positive (mutagenic) the following associated conditions: occurrence of at least 3 micronuclei for every 1000 cells, micronuclei coloration identical to that of the nuclei and smaller structures, or equal to 1/3 of the main nucleus and clearly separated in the cytoplasm (Grassi et al., 2002). The number of micronuclei was grouped for all species, because the question is focused primarily on the variation of these micronuclei between seasons and polluted-unpolluted areas. The results were analyzed through a two-way Anova (pollution and precipitation as the two factors) followed by the Tukey test, with a 5% significance level

RESULTS

Table 2 shows the results of the analysis of variance. As expected, the mean number of micronuclei was significantly greater in the experimental group (Americana reservoir) in comparison with the control group ($F_{0.05(1)1,56} = 889.24$, $p<0.01$). The mean number of micronuclei was also significantly larger in the dry season in comparison with the wet ($F_{0.05(1)1,56} = 20.01$, $p<0.001$), without interaction between factors ($F_{0.05(1)1,56} = 3.11$, $p>0.05$), which reflects that the difference among levels of one factor is constant at all levels of the other. These results indicate clearly that the reservoir is very polluted with genotoxic material, either clastogenic or aneugenic or both, and that the dry season was the period of highest toxicity in both environments. Table 3 shows the significance of the multiple comparisons between the means of the micronuclei frequencies from both environments and between seasons.

DISCUSSION

Mutagenetic studies based on the observation of micronuclei are both practical and reliable, due to the fact that all clastogenic and aneugenic agents cause alterations in chromosome numbers or morphology. Such alterations may be detected with greater precision through karyotype analysis, which pinpoints the smallest alterations, such as gaps, which cannot be identified using the micronucleus technique. The latter method is nevertheless favored by its simplicity, dependability, and speed, not to mention the fact that it does not cause suffering to the animals. As similar

Table 1. Frequency distribution of micronuclei between the factors pollution and precipitation for statistics: Dry Control x Dry Experimental, I, II, III grouped and Wet Control x Wet Experimental, I, II, III grouped.

Season	Species	Control Group (Unpolluted)			Experimental Group (Polluted)		
		I	II	III	I	II	III
Dry	*A.faciatus*	6	7	7	18	23	26
	H.malabaricus	5	5	6	17	21	23
	P.gracilis	6	8	7	21	23	23
	G.brasiliensis	6	6	8	20	24	26
	S.spilopleura	5	7	7	19	21	24
Wet	*A.faciatus*	4	4	6	16	17	21
	H.ma labaricus	3	5	5	16	17	20
	P.gracilis	3	6	6	18	20	21
	G.brasiliensis	5	5	6	17	19	23
	S.spilopleura	5	6	7	17	19	22

I, II, III – collection sites

conclusions can be drawn from micronucleus testing, it is clearly preferable over karyotyping.

Our analysis shows clearly that the Americana reservoir is heavily polluted and that its fish are contaminated, endangering their populations and the health of the humans that eat them. Despite the greater leaching of toxins by rainwater during the wet season, fewer micronuclei were recorded during this season, as reported by Grassi (2002). A possible explanation for this is that the reduced volume of standing water in the dry season reduces flow and increases the precipitation of toxins in the bottom sediments, resulting in greater exposure for the fish. In the subsequent rainy period, the renewed flow of water dilutes and removes the accumulated toxins.

It is pertinent to ask why some cells have more than one micronucleus. Do these cells receive micronuclei from the previous cell division, or does each new nucleus form its own micronuclei, which migrate to the same cell? Another possibility is that several acentric fragments get together randomly to form a macronucleus. Both clastogenic and aneugenic agents may provoke the formation of a single micronucleus, but they may sometimes form two or more. This is normally rare, however, under low concentrations of mutagenic agents. Soma (2000) showed that cells receiving higher doses of X radiation tended to have more micronuclei.

The mutagenic agents may be natural or artificial. The former originate from natural sources, such as radiation from the chemical elements that form the soil. Artificial agents are synthetic chemicals, or those derived from synthetic reactions. Each year, the number of synthetic compounds increases, due to industrialization and the technological development of manufacturing, transportation, and agriculture, part of which ends up in the environment. In fish, as well as other vertebrates, hematopoietic tissues respond rapidly to genotoxic agents, within four to five days of exposure. In fish, the hematopoietic organ is the cephalic kidney, in contrast with mammals, in which it is the bone marrow. These organs respond quickly because they are subject to a constant process of intensive cell division, making their cells vulnerable to mutation and the formation of micronuclei.

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Table 2. Analysis of Variance summary between the distribution of micronuclei of the treatments (polluted and unpolluted groups) and blocks (dry and wet seasons).

Source of Variation	Degrees of freedom	Sum of square	Mean square	F
Between Treatments A	1	3226.67	3226.67	889.24***
Between Blocks B	1	72.6	72.6	20.01***
Interaction A x B	1	11.27	11.27	3.11 ns
Error	56	203.2	3.63	

*** $p<0.001$; ns, no significant at 5% level

Table 3. Tukey multiple comparisons of the means of micronuclei according to environment and seasons.

Samples	means		q
Control (Co) x Reservoir (Re)	5.74 (Co)	20.4 (Re)	41.89***
Wet (We) x Dry (Dr)	11.97 (We)	14.17 (Dr)	6.28***

*** $p<0.001$

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