Genetics and Molecular Biology, 23, 2, 489-492 (2000)

Micronucleus frequencies in *Astyanax bimaculatus* (Characidae) treated with cyclophosphamide or vinblastine sulfate

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Abstract

Two known mutagenic drugs, cyclophosphamide and vinblastine sulfate, were tested using the micronucleus test in the native fish species, *Astyanax bimaculatus*, in order to determine which of these drugs and the doses which would be the most adequate for use as positive controls in this species. This Brazilian fish species was chosen because few toxicity studies have used native fish species and this particular species is widely consumed in various regions of Brazil. Three thousand erythrocytes per specimen were scored. Doses of 16 and 8 mg/kg body weight of cyclophosphamide and vinblastine sulfate, respectively, were the most effective in causing micronuclei. Cyclophosphamide was the most mutagenic of the two drugs and is recommended for use as a positive control in *A. bimaculatus*.

INTRODUCTION

Fish are excellent subjects for the study of the mutagenic and/or carcinogenic potential of contaminants present in water samples since they can metabolize, concentrate and store waterborne pollutants (Al-Sabti, 1991). Since fish often respond to toxicants in a similar way to higher vertebrates, they can be used to screen for chemicals that are potentially teratogenic and carcinogenic in humans. The main application for model systems using fish is to determine the distribution and effects of chemical contaminants in the aquatic environment (Al-Sabti and Metcalfe, 1995). In Brazil, a few native species have been used in acute or chronic toxicity assays. Mutagenic studies with native fish species represent an important effort in determing the potencial effects of toxic agents on the ichthyofauna.

The use of negative and positive control groups is recommended in all mutagenicity tests. According to Preston *et al*. (1987), positive controls are included to establish the ability of the analyzers to correctly determine aberrations and to ascertain the expected test-to-test and animal-to-animal variations, and to establish the sensitivity of a particular test.

Cyclophosphamide is a clastogenic agent for various animal species. Chorvatovicová and Sandula (1995) recommended the use of this drug in chromosome aberration tests, sister chromatid exchanges and micronucleus (MN) formation *in vitro* and *in vivo*. This drug, and vinblastine sulfate, an aneugenic agent, are mutagenic drugs usually used as positive controls in *in vivo* tests of short duration. The dose of cyclophosphamide and vinblastine sulfate used in routine mammalian test systems has been established, but this has not been done for native Brazilian fish. Preliminary studies on this species treated with cyclophosphamide or vinblastine sulfate showed that the maximum response for micronucleus induction by these agents was 24 h after injection. As a preliminary step in monitoring the levels of genotoxic pollutants in Brazilian rivers, we tested for the optimum dose of cyclophosphamide and vinblastine sulfate to use in native fish species. Such monitoring is particularly important since many species of Brazilian freshwater fish are widely consumed by humans.

In this study *Astyanax bimaculatus* was choosen because it is a common, small, detritivorous fish of considerable economic importance.

MATERIAL AND METHODS

Fish

Adult specimens of *Astyanax bimaculatus* (Characidae), popularly known as lambari, weighing 5 to 25 g were treated with vinblastine sulfate (N = 36) or cyclophosphamide (N = 48). The specimens were obtained from the fish hatchery station of the Universidade Estadual de Londrina.

Acute treatment with cyclophosphamide or vinblastine sulfate

The fish were acclimatized for a week in a 600-liter tank with well-aerated water at 22 ± 1°C. They were fed every two days with appropriate pelleted food which was withdrawn 24 h before the experiments. The fish were divided into four and three groups of 12 for treatments with cyclophosphamide and vinblastine, respectively. Each group of fish received an intraperitoneal injection of cyclophosphamide (4, 8, 16 or 32 mg/kg body weight) or vinblastine sulfate (8, 16, or 32 mg/kg body weight) diluted in distilled

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water. Subsequently, the fish were transferred to 140-liter tanks to give an ideal density of one gram of fish to one liter of water (Apha and WPC, 1981).

Blood samples were collected from a caudal vessel with a syringe (previously washed with liquemine (Roche), 24 h after beginning treatment with cyclophosphamide or vinblastine sulfate. The negative control group was housed in 140-liter tanks of water and blood samples were collected 24 h later.

Slides

Three fine blood smears were prepared for each fish. Two of these were fixed with absolute methanol after 24 h and then stained for 10 min with Giemsa diluted in phosphate buffer (1:20). The third slide was stained with the Feulgen reaction (1 N HCl for 11 min at 60°C followed by washing with distilled water and incubation in Schiff reagent for 2 h). Since fish erythrocytes are nucleated, these two staining procedures were used to compare the frequency of MN obtained with the Giemsa and Schiff stains, and to be sure that DNA was being analyzed. The slides were mounted using Entelan (Merck).

Cytological analysis

Randomized, coded slides were scored using a Nikon microscope and a 100X objective. Three thousand erythrocytes were analyzed from each fish (2000 stained with Giemsa and 1000 stained with Feulgen). The following scoring criteria were used (Huber *et al*., 1983; Titenko-Holland *et al*., 1997): 1) the cell had an oval appearance and intact cytoplasm, 2) oval nuclei with an intact nuclear membrane, 3) micronuclei less than or equal to one third the size of the main nuclei, 4) micronuclei clearly separated from the main nuclei and 5) micronuclei were never refringent.

The frequency of micronucleated cells per 3000 erythrocytes was determined for each fish. The number of MN was expressed per thousand erythrocytes.

Statistical analysis

The frequencies of MN in fish treated with different doses of cyclophosphamide or vinblastine sulfate and in the control group were compared by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. All tests were done as described by Zar (1974) with the level of significance set at 5%.

RESULTS

Since cytological analysis did not show differences in the frequencies of MN in slides stained with Giemsa or Schiff reagent, the data were pooled for statistical analysis. The number of cells analyzed was different for each group of the animals treated with mutagenic drugs because some fish died within 24 h after injection (Table I).

There were significant within-treatment differences (ANOVA) for both cyclophosphamide ($F = 8.38$; $P \leq 0.05$) and vinblastine sulfate ($F = 8.54$; $P \leq 0.05$), and also when compared with their respective control group. The Student-Newman-Keuls test indicated that the cyclophosphamide doses of 4 and 32 mg/kg body weight did not alter the frequency of MN compared to the control, whereas the other doses (8 and 16 mg/kg body weight) significantly increased the frequency of MN. The frequencies of MN for cyclophosphamide concentrations of 8 and 32 mg/kg body weight were similar and did not differ significantly to the values observed with the dose of 4 mg/kg body weight. Likewise, the responses to 8 and 16 mg/kg body weight were also similar.

There were no significant differences in the frequency of MN among the different doses of vinblastine sulfate used. However, the MN frequencies with all of the doses were significantly higher than in the control group.

DISCUSSION

Cytogenetic methods are probably the most sensitive and efficient means of detecting the effects of genotoxins.

Table I - Frequency of micronucleated erythrocytes (MN) in *Astyanax bimaculatus* exposed to different concentrations of cyclophosphamide and vinblastine sulfate for 24 h.

	Frequencies of MN (per thousand)					
	Cyclophosphamide			Vinblastine sulfate		
Treatment (mg/kg body weight)	N	No. of cells analyzed	$\bar{X} \pm$ SD*	N	No. of cells analyzed	$\bar{X} \pm$ SD*
Control	12	36,000	0.31 ± 0.27^{a}	12	36,000	0.31 ± 0.27^{e}
4	7	21,000	$0.57 \pm 0.60^{a,b}$	-	ND	-
8	6	12,000	$1.22 \pm 0.66^{b,c,d}$	10	30,000	1.13 ± 0.53^{f}
16	10	30,000	1.53 ± 0.71^{c}	8	24,000	0.96 ± 0.45^{f}
32	5	15,000	$0.60 \pm 0.37^{a,d}$	8	24,000	1.21 ± 0.59^{f}

*Mean MN frequency ± standard deviation (SD). ND, Not determined. Results with same superscripts do not differ significantly from each other ($\alpha = 0.05$).

However, fish are not normally very useful for certain cytogenetic techniques, such as chromosome aberration tests and sister chromatid exchanges, because they have a large number of small chromosomes (Belpaeme *et al.*, 1996).

The MN test has been used successfully as a mutagenic assay since it is simple, reliable, sensitive, and is not strongly dependent on any karyotypic characteristics (Heddle *et al.*, 1983). The methods using fish erythrocytes are not time consuming and can be done without causing suffering to the animals (Minissi *et al.*, 1996). For these reasons, the micronucleus test using fish erythrocytes is a promising assay for investigations in environmental mutagenesis (Al-Sabti and Metcalfe, 1995). Indeed, several studies have used the MN test to evaluate the exposure of fish to different pollutants under laboratory conditions (Manna *et al.*, 1985; Das and Nanda, 1986; Al-Sabti *et al.*, 1994; Odeigah and Osanyipeju, 1995; Belpaeme *et al.*, 1996; Nepomuceno *et al.*, 1997).

In the present study, the drugs tested were cyclophosphamide and vinblastine sulfate. Cyclophosphamide, an alkylating agent used in chemotherapy, requires metabolic activation before it can act as a mutagenic agent to promote chromosomal aberrations (Anderson *et al.*, 1995). Vinblastine sulfate, an aneugenic agent derived from *Vinca* alkaloids and used here as a positive control, inhibits the polymerization of the tubulin dimer *in vitro*, thereby preventing cell proliferation at low concentrations (Kallio *et al.*, 1995). Treatment with cyclophosphamide or vinblastine sulfate led to the death of some fish within 24 h after injection, making it impossible to analyze the same number of cells for each group (Table I). This mortality rate was not dose dependent for the treatment with cyclophosphamide or with vinblastine sulfate (Table I) and therefore, there was no relation to the concentration of the mutagenic drugs. One explanation for the mortality rate could be the injection trauma in the treated groups as there were no deaths in the control group which did not receive any type of injection. This hypothesis was not confirmed in a recent experiment, where we injected control fish with saline solution (data not shown).

The maximum response for micronucleus induction by cyclophosphamide or vinblastine sulfate was 24 h after injection. This interval is the same as that usually recommended in rodent tests (Preston *et al.*, 1987). The frequencies of spontaneous and induced MN were low (Table I). According to Rizzoni *et al.* (1987), a dose- and time-dependent response has been observed in many studies using fish, although the MN frequencies are lower than in mammals.

Vinblastine sulfate induced micronuclei but there was no dose dependence in this response (Table I). For cyclophosphamide, there was a gradual increase in the frequency of MN at doses of 4-16 mg/kg body weight. In contrast, with 32 mg/kg, there was a significant reduction in the MN frequency (Table I). As pointed out by Nepomuceno *et al.* (1997) in their work with fish exposed to mercury, this decrease suggests that the toxic and inhibitory effects of the higher dose of cyclophosphamide affected cell division, with a subsequent hindrance in the passage of the affected cells into the peripheral circulation since they tend to be removed from the organism faster than undamaged cells.

Despite the large standard deviation of the data (Table I) statistical analyses indicated that the cyclophosphamide dose of 16 mg/kg body weight and a vinblastine sulfate dose of 8 mg/kg body weight were the most adequate for use as positive controls in this species because these doses showed a high frequency of MN and a low mortality rate.

Kallio and Lahdetie (1993) observed that mice spermatids treated with vinblastine sulfate have a very low frequency of MN when compared with spermatids exposed to mitomycin C (a clastogenic drug). Although vinblastine sulfate produced a significant increase in the frequency of MN when compared with its negative control in the present study, the frequencies were still very low. This may explain why vinblastine sulfate is not frequently used as a positive control. Our results indicate that cyclophosphamide is more suitable than vinblastine sulfate as a mutagenic agent for *A. bimaculatus* (Table I).

Previous studies on three introduced species of fish treated with cyclophosphamide, mitomycin C, 5-fluorouracil, and bleomycin also proved that cyclophosphamide is one of the most effective drugs in inducing MN (Grisolia *et al.*, 1996).

Chorvatovicová and Sandula (1995) recommended the use of cyclophosphamide in chromosome aberration tests, sister chromatid exchanges and MN formation *in vitro* and *in vivo*. The present study confirmed this recommendation in *Astyanax bimaculatus*.

ACKNOWLEDGMENTS

The authors thank Dra. Claudia B.R. Martinez for her help with some of the statistical analyses. We thank Mr. Carlos Lourenço and Mr. João Francisco dos Santos for technical assistance. The authors also thank the fish hatchery station of the State University of Londrina for providing the fish. This research was supported by the State University of Londrina and financed by Klabin Fabricadora de Papel e Celulose Ltda.

RESUMO

Duas drogas reconhecidas como mutagênicas, ciclofosfamida e vimblastina sulfato, foram avaliadas usando o teste do micronúcleo em uma espécie de peixe nativa, *Astyanax bimaculatus*, para detectar que droga e quais doses são as mais adequadas para serem usadas como controles positivos para esta espécie. Esta espécie de peixe brasileira foi escolhida devido à escassez de estudos toxicológicos com espécies de peixes nativos e também porque ela é amplamente consumida em algumas regiões do Brasil. Um total de 3000 eritrócitos por espécimen foram contados. As doses de 16 e 8 mg/kg de peso corporal de ciclofosfamida e de vimblastina sulfato, respectivamente, foram as mais

efetivas na indução de micronúcleos. A ciclofosfamida mostrou ser o melhor agente mutagênico para ser usado como um controle positivo para *Astyanax bimaculatus*.

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(Received May 24, 1999)