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Micronucleus Test in Fish from a Pampasic Pond (Argentina): An Estimation of the Presence of Genotoxic Compounds

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The Los Padres pond is one of the commonly shallow, polymictic lakes from the so-called depressed Pampa (Argentina). Its watershed includes one affluent, named Los Padres creek, which flows through horticultural lands wherein great amounts of pesticides are applied. Opposite to this stream, the pond drains into La Tapera creek that is the effluent running toward the sea. Many studies have confirmed the capacity of various pesticides to induce genetic damage. The use of micronucleus (MN) tests in fish has enabled us to detect the presence of contaminants in the lake water and to evaluate their genotoxic effects. For this purpose, water samples were collected during April, August, and December 1999 from both creeks characterized by different environmental conditions. In the laboratory, specimens of tetras *Cheirodon interruptus* (Pisces, Characidae) were reared in water samples from the two creeks. Control fish were kept in drinking water. Fifteen individuals from each experimental group were sacrificed after 24-, 48-, and 72-hour exposure intervals. Micronucleus frequency in fish erythrocytes was determined, and the Kruskal–Wallis test for statistic analysis was used. We made the following observations: (1) Highly significant differences occurred in MN frequency between the control group and the samples from both creeks. (2) An increase in MN frequency was evident in specimens sampled from the affluent input during the month of December. These results allowed us to conclude that the increase in MN frequency observed in fish belonging to both sampling sites would indicate the existence of genotoxic compounds in the Los Padres pond. The high MN frequency in fish collected near Los Padres creek inlet might be related to the polluted load transported by the affluent and discharged into the lake's surface waters. Future work would allow us to develop efficient methods for predicting the presence of genotoxic contaminants. It would be possible then to propose strategies for regulating and decreasing the sources of pollution that affect human health.

KEYWORDS: genotoxicity, micronucleus test, fish erythrocytes, *Cheirodon interruptus*, water pollution

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Introduction

As a result of the continuous release of genotoxic compounds into inland and coastal waters, the need for sensitive assays to monitor their accumulation and impact has increased.[1] In recent years, there has been a growing interest in the use of biomarkers for assessing and monitoring natural ecosystem quality. Fish are suitable organisms for this purpose because they play different roles in trophic webs.[2] Water pollution control is thus vital to protect aquatic ecosystems.

Among the many mutagenesis assays, the micronucleus (MN) test has been successfully used in a wide range of organisms such as invertebrates, fish, and amphibians, as a biological dosimeter of polluted areas (in situ assays), and in the genotoxicity evaluation of test compounds after direct or indirect exposure (in vivo, such as used in fish studies[3–7]).

Micronuclei essentially consist of chromosomal fragments or whole chromosomes, which have not migrated to poles during mitosis. They result either from chromosome breaks (clastogenic effects), or from dysfunction of the spindle apparatus, or centromere kinetochore complexes leading to the elimination of whole chromosomes (aneugenic effects).

In the present paper, MN frequency in fish erythrocytes has been evaluated in a characid, *Cheirodon interruptus* from Los Padres lake, and compared with the values observed in erythrocytes of fish reared under controlled conditions. Small water bodies (e.g., ponds and creeks) are often the first to receive runoff containing pollutants from a number of sources including pesticide use, sewage contaminants, and industrial effluents.

Within the frame of a wider investigation on the trophic status of Los Padres lake, the present work was designed to estimate the presence of genotoxic compounds in this pampasic pond by means of the MN test. Its purpose was to evaluate the sensitivity of this rapid and non-expensive test system and its suitability as a biological monitor.

Material and Methods

Study Area

Los Padres pond is a shallow, polymictic lake, situated at 37° 56′ 30″ S and 57° 44′ 30″ W (in the Buenos Aires province, Argentina), with an area of 2.16 km^2 and 1.24 m of mean depth.[8] It is surrounded by agricultural and natural soils. Surface water contribution to Los Padres watershed is provided by one affluent named Los Padres creek, which flows through an important agricultural–livestock area carrying a significant load of suspended particulate matter. Opposite to the affluent, the pond drains into La Tapera creek, the effluent running toward the sea. Most of the littoral zone is covered with the bulrush, *Schoenoplectus californicus*, particularly developed in Los Padres creek delta.[9,10]

Fish and Experimental Design

Cheirodon interruptus, widely distributed both in lotic and lentic environments of Argentina,[11] was chosen because it is relatively easy to handle and acclimate to laboratory conditions.

For the experiments, water was collected in April, August, and December 1999 from two sites of the pond (Fig. 1), which have different trophic status: Station 1 at La Tapera creek outlet and Station 2 at Los Padres creek inlet (delta).[10]

Approximately 300 specimens (1.19 g ± 0.947 and 4.3 cm ± 0.988) were acclimated in the laboratory. All the studies were carried out in compliance with our Institutional Regulation for Animal Care and Use. The fish were randomly divided into two aquaria containing water from each sampling site. Fifteen

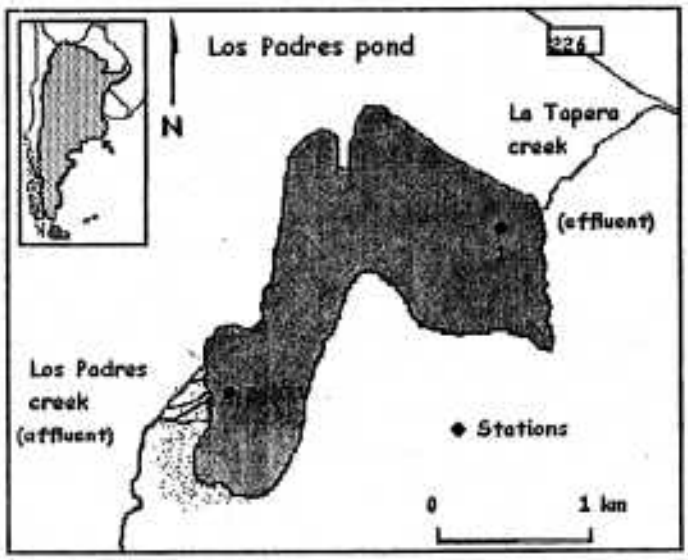

FIGURE 1. Map of Los Padres pond showing sampling locations.

individuals were used for each experimental condition. A total of 40 fish kept in tap water served as controls. To avoid pain, the fish were submerged in a 5% benzocaine solution before being punctured. Fish were sacrificed after 24-, 48-, and 72-hour exposure intervals because, in a previous work on the same fish species, the greatest induction of micronuclei was observed between 24 and 72 hours.[6]

Blood Smear Preparation

Although modified by us, the MN test in fish is similar to that of the mammalian.[12] Blood was obtained through heart puncture with heparinized tips and peripheral blood smears, two per fish, were immediately made by applying a drop of blood on clean slides, fixed in absolute methanol for 15 minutes, and air dried. Twenty-four hours later, the material was stained with 15% Giemsa solution for 10 minutes.

Two thousand erythrocytes were analyzed from each animal, under 1000 × magnification to determine the number of micronuclei. Only cells with one micronucleus clearly detached from the nucleus were scored. Coded and randomized slides were scored using a blind review by a single observer.

Statistical Analysis

All data from the assays were tested for normality using the Wilks test. Because data did not show normal distribution, the nonparametric Kruskal–Wallis test was used to detect statistically significant differences in the frequency of micronucleated erythrocytes between exposed specimens and the controls. A value of $p < 0.05$ was considered to indicate statistical significance.

Results

Erythrocytes were clearly distinguishable in the slides analyzed. The mature erythrocytes of *Cheirodon interruptus* have large and oval nucleated cells. Micronuclei observed showed the features described by Schmid[12] (Fig. 2).

Tables 1 and 2 summarize the results. No statistically significant differences were found in the results of assays carried out at 24-, 48-, and 72-hour intervals for each sampling site and month (Table 1). However, when data of the three intervals were commingled, statistically significant differences were found in the December sampling.

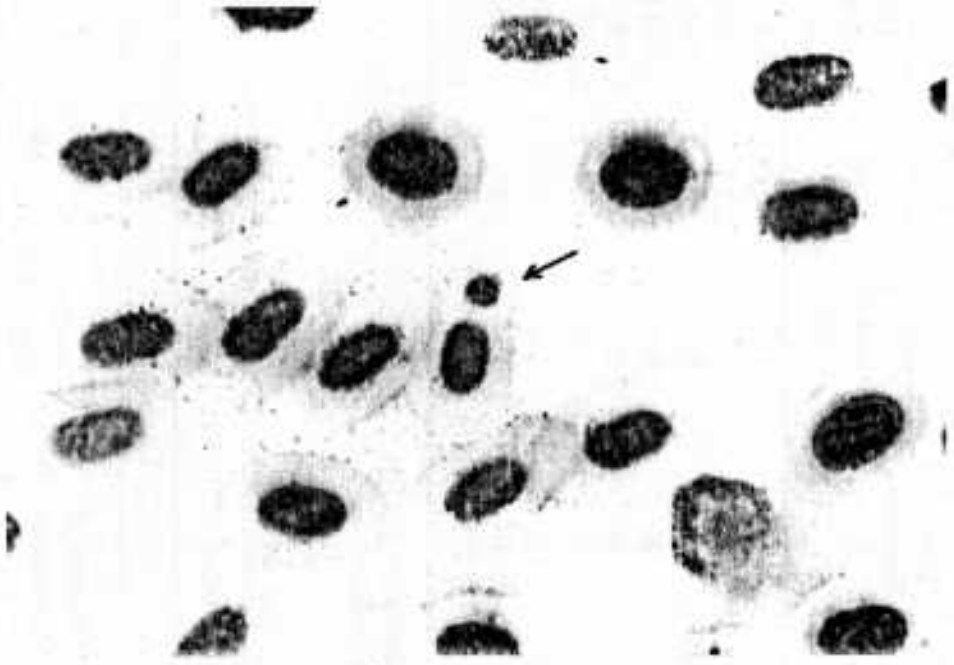

FIGURE 2. Micronucleated erythrocyte (arrow) in *Cheirodon interruptus*. Giemsa-stained blood smear, 1000×.

TABLE 1. Micronucleus Frequency at 24-, 48-, and 72-Hour Exposure Intervals in *Cheirodon interruptus* Erythrocytes

Month	Time of Exposure (h)	Station 1			Station 2			K–W* 24 vs 48 vs 72 h	
		Number of specimens	Total MN	MN/2000 erythrocytes (mean ± SE)	Number of specimens	Total MN	MN/2,000 Erythrocytes (mean ± SE)	Sta 1	Sta 2
April	24	14	19	1.3571 ± 0.3249	15	17	1.133 ± 0.3217	n/s	n/s
	48	15	14	0.9333 ± 0.2063	13	19	1.4615 ± 0.3125		
	72	15	15	1.000 ± 0.239	14	19	1.3571 ± 0.2891		
August	24	14	21	1.500 ± 0.3101	14	24	1.7143 ± 0.3219	n/s	n/s
	48	13	20	1.5385 ± 0.2433	15	28	1.8667 ± 0.2364		
	72	13	17	1.3077 ± 0.3077	13	28	2.1538 ± 0.3897		
December	24	15	24	1.6000 ± 0.3352	19	39	2.0526 ± 0.2902	n/s	n/s
	48	15	16	1.0667 ± 0.2063	15	28	1.8667 ± 0.3217		
	72	15	20	1.3333 ± 0.2520	12	34	2.8333 ± 0.4578		

* K–W: Kruskal–Wallis test.
n/s = not significant.

TABLE 2. Comparison of the MN Frequency between *Cheirodon interruptus* Specimens Reared under Lake-Water Samples and Those Used as Controls

Month	Control			Station 1				Station 2			
	Number of specimens	Total MN	MN/2000 erythrocytes (mean ± SE)	Number of specimens	Total MN	MN/2000 erythrocytes (mean ± SE)	Control vs Station 1 p	Number of specimens	Total MN	MN/2000 erythrocytes (mean ± SE)	Control vs Station 2 p
April	40	21	0.5250 ± 0.1339	44	48	1.0909 ± 0.1483	0.0106	44	55	1.3095 ± 0.1754	0.0005
August				40	58	1.4500 ± 0.1639	9.9×10^{-5}	42	80	1.9048 ± 0.1797	2.4×10^{-7}
December				45	60	1.3333 ± 0.1557	0.0012	46	101	2.1957 ± 0.2029	0.0000

TABLE 3. Comparison of the Mean MN Frequency between Specimens Belonging to Los Padres Creek Inlet and La Tapera Creek Outlet[a]

Month	Station 1			Station 2			K–W[b] P
	Number of specimens	Total MN	MN/2,000 erythrocytes (mean ± SE)	Number of specimens	Total MN	MN/2000 erythrocytes (mean ± SE)	
April	44	48	1.0909 ± 0.1483	42	55	1.3095 ± 0.1754	0.3424
August	40	58	1.4500 ± 0.1639	42	80	1.9048 ± 0.1797	0.0659
December	45	60	1.3333 ± 0.1557	46	101	2.1957 ± 0.2029	0.0011[c]

[a] Because no differences were observed between specimens from both sampling locations at 24-, 48-, and 72-hour exposure intervals, the data were commingled.

[b] K–W: Kruskal–Wallis test.

[c] Comparison between Los Padres creek and La Tapera creek specimens was highly significant statistically only in the December sampling.

Fish exposed to samples of water from the two sampling locations (Station 1 and Station 2) showed a statistically significant increase in the number of micronucleated erythrocytes compared with the control group ($p < 0.01$ in all cases; control versus Station 1 and control vs. Station 2; Table 2).

Although in specimens exposed to the water of Station 2 a progressive increase in the MN frequency was observed from April to December, these differences were statistically significant only in December in relation to Station 1 (Table 3).

Discussion and Conclusions

Within the past 10 years, the MN test in fish has played an important role in assessing the effect of the exposure to water pollutants and has provided an early warning of genotoxic threat to fish, their ecosystems, and then to humans.[13,14]

Micronuclei measurements in fish were shown to be a better parameter than chromosomal aberrations in environmental studies under laboratory and field conditions.[3,15–17] Compared with the chromosomal aberration assay, the MN assay has several advantages including low cost, relatively fast slide analysis, and a clear visualization of micronuclei in the cells. In a previous work, we have demonstrated that the frequency of MN in *Cheirodon interruptus* can serve as a valuable indicator in assessing genotoxic effects after exposure to the pyrethroid insecticide lambda-cyhalothrin.[6]

In the present study, when compared with the control group, the increase in the MN frequency observed in fish from both sampling sites showed the existence of genotoxic compounds in the Los Padres pond. The high MN frequency in fish collected near the Los Padres creek inlet might be related to the polluted load transported by the affluent and discharged into the lake's surface waters.

Studies made on this ecosystem have shown the presence of the organochlorine pesticides (OCPs) [dichlorodiphenyl-trichloroethane (DDT), hexachlorocyclohexane (HCH), chlordane, heptachlor, aldrin] and polychlorinated biphenyls (PCBs) in organisms from different trophic levels,[18] which support the observation that the lake receives these pollutants through local or regional atmospheric dispersion and run-off. PCBs in bulrush, grass-shrimp, and fish inhabiting this water body have also been detected and quantified.[19] Likewise, the presence of most organochlorine pesticides in the soil surrounding the horticultural watershed of Los Padres pond has been reported[20] and it was suggested that the agricultural soil could be an important source of OCPs in the nearby pond.[21] These studies demonstrate that the Los Padres creek delta is a heavily contaminated area.

On the other hand, it has been demonstrated that PCBs and DDTs can increase the frequency of MN in fish.[22,23] Moreover, several PCB congeners are capable of forming DNA adducts after metabolic activation, which can be detected by the ^{32}P-postlabeling technique.[24] The significant differences found in the

micronucleated cells between control and exposed organisms can be correlated with the load of pollutants.

Future advances in this area of ecologic studies would allow development of efficient methods for predicting the presence of genotoxic contaminants. It would then be possible to propose strategies for regulating and decreasing the sources of pollution that affect human health.

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