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Genotoxicity Assessment of Mercuric Chloride in the Marine Fish *Therapon jarbua*

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Abstract

The aim of the present study was to standardize and to assess the predictive value of the cytogenetic analysis by Micronucleus (MN) test in fish erythrocytes as a biomarker for marine environmental contamination. Micronucleus frequency baseline in erythrocytes was evaluated in and genotoxic potential of a common chemical was determined in fish experimentally exposed in aquarium under controlled conditions. Fish (*Therapon jaruba*) were exposed for 96 hrs to a single heavy metal (mercuric chloride). Chromosomal damage was determined as micronuclei frequency in fish erythrocytes. Significant increase in MN frequency was observed in erythrocytes of fish exposed to mercuric chloride. Concentration of 0.25 ppm induced the highest MN frequency (2.95 micronucleated cells/1000 cells compared to 1 MNcell/1000 cells in control animals). The study revealed that micronucleus test, as an index of cumulative exposure, appears to be a sensitive model to evaluate genotoxic compounds in fish under controlled conditions.

Keywords: genotoxicity; mercuric chloride; micronucleus

1. Introduction

In India, about 200 tons of mercury and its compounds are introduced into the environment annually as effluents from industries (Saffi, 1981). Mercuric chloride has been used in agriculture as a fungicide, in medicine as a topical antiseptic and disinfectant, and in chemistry as an intermediate in the production of other mercury compounds. The contamination of aquatic ecosystems by heavy metals and pesticides has gained increasing attention in recent decades. Chronic exposure to and accumulation of these chemicals in aquatic biota can result in tissue burdens that produce adverse effects not only in the directly exposed organisms, but also in human beings.

Fish provides a suitable model for monitoring aquatic genotoxicity and wastewater quality because of its ability to metabolize xenobiotics and accumulated pollutants. A micronucleus assay has been used successfully in several species (De Flora, *et al.*, 1993, Al-Sabti and Metcalfe, 1995). The micronucleus (MN) test has been developed together with DNA-unwinding assays as perspective methods for mass monitoring of clastogenicity and genotoxicity in fish and mussels (Dailianis *et al.*, 2003).

The MN tests have been successfully used as a measure of genotoxic stress in fish, under both laboratory and field conditions. In 2006 Soumendra *et al.*, made an attempt to detect genetic biomarkers in two fish species, *Labeo bata* and *Oreochromis mossambica,* by MN and binucleate (BN) erythrocytes in the gill and kidney erythrocytes exposed to thermal power plant discharge at Titagarh Thermal Power Plant, Kolkata, India.

The present study was conducted to determine the acute genotoxicity of the heavy metal compound $HgCl_2$ in static systems. Mercuric chloride is toxic, solvable in water hence it can penetrate the aquatic animals. Mutagenic studies with native fish species represent an important effort in determining the potential effects of toxic agents. This study was carried out to evaluate the use of the micronucleus test (MN) for the estimation of aquatic pollution using marine edible fish under lab conditions.

2. Materials and methods

2.1. Sample Collection

The fish species selected for the present study was collected from Pudhumadam coast of Gulf of Mannar, Southeast Coast of India. *Therapon jarbua* belongs to the order Perciformes of the family Theraponidae. The fish species, *Therapon jarbua* (6-6.3 cm in length and 4-4.25 g in weight) was selected for the detection of genotoxic effect

Table 1. Physicochemical parameters of the test water

Parameter	Exposure Time			
	24 hrs	48 hrs	72 hrs	96 hrs
pH	7.5	7.5	7.5	7.5
Temp (°C)	29	29	29	29
Salinity (ppm)	38	38	38	38
DO (mg/L)	4.2	6.4	6.4	6.4

among other major edible marine fish species, which are regularly trapped by the local fish farmers for human consumption.

Healthy specimens of 200 fish were procured from local sources and were treated with 0.05% $KMnO_4$ solution for 2 min to clear any dermal infection. Fishes were then transferred 15 days prior to exposure to flow-through systems of 80 L capacity for acclimation to laboratory conditions. Fish were maintained under normal day- night light duration and were fed on boiled chicken eggs and small pieces of goat liver on alternate days during the acclimation period. Every effort was made to provide healthy condition for fish and no mortality occurred during this period. Exploratory range of concentrations of the test chemical was used in a series of range finding experiments (APHA/ AWWA/WPCF, 1998). The experiments were repeated three times. The stock solution of $HgCl_2$ (99.5%) was prepared by dissolving it in water. Initially, a preliminary range finding bioassay was performed. Based on these results a concentration gradient series was prepared from the stock solution. Finally, five final test concentrations (0.125, 0.25, 0.5, 1, 2 ppm) were selected and the negative control (unexposed) was also set in all experiments. Mortality was recorded at 24, 48, 72 and 96 hrs. Dead specimens were recorded and removed immediately from the tanks. 10 fish were tested for each test concentration and no specimen was used more than once. The test chemical used in the present study was $HgCl_2$ and the lethal concentration was specified in table 2.

2.2. Physical and chemical analysis

Salinity, temperature, dissolved oxygen and pH was measured at the beginning and end of the test in the control and test containers by following the method of Grasshoff *et al.,* (1999)

2.3. Determination of sublethal concentration

Stock solution (1 mg/ml) was prepared by dissolving analytical grade mercuric chloride ($HgCl_2$) in double distilled water. Test concentrations were given in parts per million of $HgCl_2$. Test concentrations were prepared by diluting appropriate aliquots of the stock solution. The fish (20 numbers in each tank) were exposed to $HgCl_2$ in static systems (80 L capacity as recommended by Parrish (1985). Fish were not fed during the experimental period as recommended by Ward and Parrish (1982) and Reish and Oshida (1987). LC_{50} values (20 numbers for each concentration for control, 0.125, 0.25, 0.5, 1, 2 ppm) were calculated from the data obtained in acute toxicity bioassays, by Finney's (1971) method of probit analysis.

2.4. Determination of heavy metal in seawater and fish tissue

For dissolve metal analysis, the sea water was filtered by 0.45 μm Millipore filter paper and stored in a pre cleaned, acid washed polyethylene bottle. It was acidified with nitric acid (65%, Suprapur,

Table 2. Mortality of *Therapon jarbua* exposed (No: 10) to Mercury chloride (mg/L) in Definitive test

Concentration in mg/L	Exposure Time				% of mortality
	24 hrs	48 hrs	72 hrs	96 hrs	at 96 hrs
Control	0	0	0	0	0
0.125	0	0	0	0	0
0.25	0	0	2	4	40
0.5	1	5	7	8	80
1	2	6	9	10	100
2	8	10	10	10	100
Calculated LC_{50}	1.339	0.649	0.411	0.310	-
95% Confidence limits	0.959 – 2.227	0.452 – 0.921	0.296 – 0.570	0.228 – 0.421	-

Table 3. Concentration of mercury chloride after 96 hrs exposure in *Therapon jarbua* (mg/g.dw) in whole body tissue

Concentration	Water		Tissue
	0 hr	96 hrs	96 hrs
Control	0	0	0
0.125 ppm	0.125 ppm	0.018 ppm	0.018 ppm
0.250 ppm	0.250 ppm	0.018 ppm	0.029 ppm

Merck) at a pH between 2 and 3. From this seawater, heavy metal was analysed by the APDC-MIBK Extraction method (Grasshoff *et al*., 1999).

The whole body tissue (3-5 fishes sacrificed, anesthesia using ethylene glycol) was washed with Milli-Q water and dried at 95°C and ground to a fine powder and transferred to a glass beaker. To the tissue 8-10 ml of concentrated acid in the ratio 1:1 (60% HNO_3: 70% $HClO_4$) was added, such that the tissue was totally wet with a slight excess of acid. This was covered and left at room temperature for 12 hrs. The digested sample was heated to 180°C, till the sample volume reduced to 2-3 ml.

2.5. Micronucleus test

After exposure for 96 hrs, blood samples were obtained by caudal vein puncture using a heparinized syringe from five fishes. Two microscopic slides were prepared for each fish. Micronucleus test was proceded based on Bahari *et al*. (1994). Slides were scored by a single observer using blind review, in light microscope, lens using 100X oil immersion magnification. For MN scoring purpose, only non-refractive small nuclei (≥1/3 of the diameter of the main nucleus) located close to the main oval nucleus

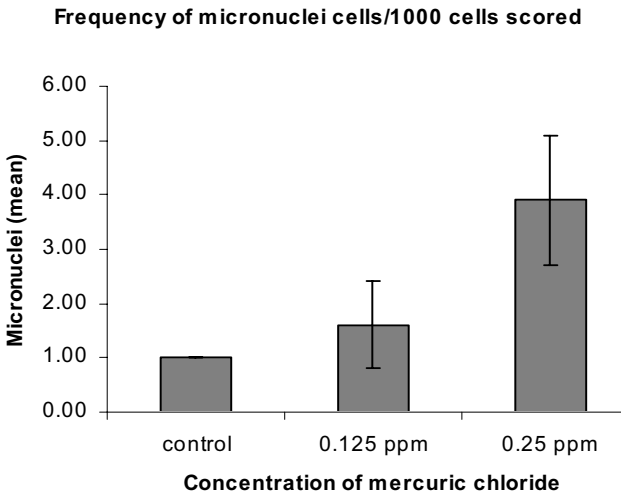

Figure 1. Micronuclei frequency induced by Mercuric chloride (Means ± SD, *n=3*)

of round erythrocytes with intact cytoplasm were considered. Mean MN frequencies and standard deviation were expressed as the number of MN per 5000 erythrocytes (500 cells/slide), for each concentration for five fish (mean of 10 slides).

2.6. Statistical Analysis

To determine statistically significant differences between experimental and control groups, all the mean values of data were analyzed using one-way ANOVA test.

3. Results and Discussion

Environment plays a vital role in marine fish hence the physicochemical parameters were maintained throughout the experiments. The physicochemical characteristics of test water are listed in Table 1.

Fish showed abnormal behavior during the experimental period. At the start of the exposure, fish were alert, and stopped swimming and remained static in position. After some time they tried to avoid the toxic water by fast swimming and jumping. In tanks with concentrations 0.25, 0.5 and 1 ppm the fish swam unsteadily with jerky movements and hyper excitability. Their fins became hard and stretched possibly due to stretching of body muscles. Ultimately, fish lost its balance, became exhausted, or lost consciousness and became lethargic. Finally, they remained in a vertical position for a few minutes with the anterior side or terminal mouth up near the surface of the water, trying to swallow the air and their tail in the downward direction. Soon they settled at the bottom of the tank and after some time their bellies turned upward and died.

The LC_{50} values of $HgCl_2$ were 1.339, 0.649, 0.411 and 0.310 (µg/L) at 24, 48, 72 and 96 hrs, respectively (table 2). The accumulation of Hg was analyzed by mercury analyzer-AAS (Atomic Absorption Spectrophotometer) in both water and tissue samples as listed in Table 3.The frequency of micronuclei was 1.00/1000 erythrocytes in the controls. After exposure to mercuric chloride, the highest induction of micronuclei, 3.86/1000 erythrocytes, was found in the highest concentration (0.25 ppm) of mercuric chloride e-4) (Fig. 1). The results of this study indicated that the MN test in fish can be a suitable biomarker for *in- situ* monitoring of genotoxic pollution in the marine environment.

The micronuclei formation between different exposure concentrations of mercuric chloride was significantly vary ($p>0.001$; $F_{2,8} = 5.14$).

A number of studies deal with the evaluation of genotoxic effects of mercury and mercury compounds on mammalian cells in culture and experimental animals, as well as in exposed human populations. Soumendra *et al.,* 2006 studied the frequencies of micronucleated (MN) and binucleated (BN) erythrocytes of two fishes, *Labeo bata* and *Oreochromis mossambica* inhabiting ponds with highly significant differences noticed for MN frequencies in gill and kidney erythrocytes of experimental fishes, where kidney erythrocytes showed highly significant difference ($p<0.001$) values than gill erythrocytes.

Fish serve as useful genetic models for the evaluation of pollution in aquatic ecosystems (Park *et al*., 1993). The erythrocyte micronucleus test has been used with different fish species to monitor aquatic pollutants displaying mutagenic features (De Flora *et al*., 1993; Pantaleao *et al.,* 2006).

Studies of the micronuclei rates of various fish species showed that they generally peaked between the first and fifth days after treatment (Al-Sabti and Metcalfe, 1995) depending on the drug used for the induction of micronuclei. It is concluded from this study that gill erythrocytes can be used for estimating the genotoxic effects of waterborne pollutants

The frequencies of micronuclei and binuclei in the erythrocytes of fish from pond located at industrial vicinity were significantly higher than that of the control groups. Several authors (Bahari *et al.,* 1994; Al-Sabti, 2000; Atteq *et al.,* 2002; Abul Farah *et al*., 2003; Porto *et al*., 2005) have suggested that some physical and chemical agents (PCP, 2,4- dichlorophenoxyacetic acid, temperature) are responsible for causing genotoxic effects in fish.

The present study showed behavior abnormalities of fish and subsequent death. It implifies that the toxic effect is mediated through the disturbed nervous and enzyme systems affecting the respiratory function nervous system, which involves control of almost all vital activities similar to many earlier studies which have demonstrated that pesticides inhibit Ca^{2++} - ATPase in the gills of the fish. Heavy metals are known to have neurotoxic action on the calcium pump in the fish brain. The LC_{50} values reported in the present study are similar to those reported by Khangarot (1981). Al-Sabti and Metcalfe (1995) who demonstrated that micronucleus induction normally occurred one to five days after exposure,

The results of this study indicate that the MN test in fish is a suitable biomarker for *in-situ* monitoring of genotoxic pollution in the marine environment.

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