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Bioconcentration of hexavalent chromium in different organs and induction of micronuclei in peripheral blood cells of *Cirrhinus mrigala* (Ham, 1822)

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ABSTRACT

The fingerlings of *Cirrhinus mrigala* (Ham, 1822) were exposed to different concentrations of chromium in the form of $K_2Cr_2O_7$. LC_{50} for 96 h was calculated as 52.187 mg L^{-1}. For sub-lethal toxicity test fishes were exposed to different concentrations of 10.437, 5.218 and 3.479 mg L^{-1} for 60 days. In comparison to control under the same conditions, the experimental fish showed remarkable change in the accumulation pattern in muscle, gills and liver. The Cr content was measured and the highest concentration was found in liver (61.91±0.73 µg g^{-1}) followed by gills (16.67±0.08 µg g^{-1}) and muscle (8.97±0.06 µg g^{-1}). Frequencies of micronuclei were evaluated in peripheral blood erythrocytes. Cells showed differential sensitivity to the heavy metal treatment. In general, frequencies of MNi cells significantly increased following the exposure for 60 days to chromium. Frequencies of micronuclei formation in erythrocytes varied from 0.83±0.06% to 6.37±0.56%. The results indicated that this heavy metal has genotoxic effects in the fish.

Key words: Bioassay tests, Bioconcentration, Chromium, *Cirrhinus mrigala*, LC_{50}, Micronucleus test

The chromium is recognized as a heavy metal, which is known to be toxic to fish and the human beings. The toxicity of chromium to aquatic life is strongly influenced by the chemical speciation of chromium and water quality (Abbasi and Soni 1985) and considerably varies between and within groups of organisms. Growing awareness about aquatic pollutants generating potential hazards has stimulated much interest in the use of fishes as indicators for monitoring the environmental mutagens, carcinogens and teratogens (Krishnaja and Rege 1982). Fishes are ideal indicators of heavy metal contamination in aquatic ecosystems and these are unique among the vertebrates, because of the potential for metal acquisition from the water. Contamination in the aquatic environment alters their health, size, age structure, composition and number of species found in the aquatic ecosystem. Some heavy metals accumulate in tissues and may pose a health risk to those who frequently consume fish. Chromium (Cr) has a role in glucose, fat and protein metabolism, participating in the insulin action (Anderson 1981). It links directly to macromolecules; fragments the AND (acridinium ditetracyanoquinodimethane) chains and acts in the peroxidation of lipids, generating free radicals and modifying the routes of cell signals. All these processes can contribute to the toxicity and carcinogenicity of Cr compounds (Loyaux Lawniczak *et al*. 2000).

Studies were carried out to evaluate the extent of genetic disorders caused by heavy metals in the fish and developed several techniques to evaluate the genotoxic effect of heavy metals in the organism using a variety of genetic end-points. Micronucleus assays originally developed with mammalian species have been used extensively to test the genotoxic activity of chemicals (Heddle *et al*. 1983). Micronuclei are formed by condensation of chromosomal fragments or whole chromosomes that are not included in the main nucleus following anaphase. Scoring of micronuclei in the interphase is technically much easier and more rapid than the scoring of chromosomal aberrations during metaphase. The micronucleus (MN) test in fish also has potential for detecting clastogenic substances in aqueous media. Since teleost erythrocytes are nucleated, micronuclei have been scored in fish erythrocytes as a measure of clastogenic activity. The study was carried out to determine the LC_{50} value of Cr in *Cirrhinus mrigala* to analyse the impact of chromium on acute static bioassay of *C. mrigala*, which is prerequisite to determine the degree of damage produced by chromium. Hence, the study was carried out to measure the bioconcentration of chromium in *C. mrigala,* and to evaluate the genotoxic effect of chromium in *C. mrigala.*

MATERIALS AND METHODS

The study was conducted in wet lab facility of Central Institute of Fisheries Education (CIFE), Mumbai located at latitude 18° 55'N and longitude 72° 50'E. Analytical

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grade potassium dichromate ($K_2Cr_20_7$) was used as the source of chromium in the experiment. A subchronic exposure of 60 days was carried out for bioconcentration and genotoxic study.

Experimental animals: The fingerlings of *C. mrigala* (6–8 cm, weighing 10–15 g) were brought to the laboratory and were given dip treatment with $KMnO_4$ solution for about 5–10 sec to avoid any possible contamination or transmission of any disease. The fishes were stocked in well-aerated circular tanks of 500 liter capacity for acclimatization for 15 days. The animals were randomly selected and subsequently used for bioassay and sub-lethal exposure.

Acute toxicity tests: A static short term toxicity test was conducted according to standard method (APHA 2005) to determine LC_{50} (median lethal concentration) of chromium for *C. mrigala*, following exposure of 96 h. Initially the range finding test was conducted to ascertain the range, followed by definitive test. Six test concentrations of narrow range i.e. 45, 50, 55, 60, 65 and 70 mg L^{-1} along with a control was maintained to find the LC_{50} value of chromium. Two replicates were maintained for each of these chromium concentrations including the control and 10 fishes were placed in each of the plastic tanks. The concentration of chromium in test solution was maintained during the experimental period. No feeding was done during the experiment. Dead fishes were removed from the tank immediately. Death was assumed when the fishes were immobile and showed no response when touched with a glass rod. The percentage mortality was recorded at 24, 48, 72 and 96 h. Data obtained from the experiment was processed by Probit analysis and the graph was obtained using Microsoft excel spread sheet.

Experimental design: The chromium concentrations in the sub-lethal toxicity study were taken according to the 96 h LC_{50} value of chromium. Three different concentrations of chromium i.e. $1/5^{th}$ (10.437), $1/10^{th}$ (5.218), $1/15^{th}$ (3.479) mg L^{-1} were taken and a control (without chromium) was also maintained. The treatments were as follows:

Treatment 3: 10.437 mg/L chromium
Treatment 2: 5.218 mg/L chromium
Treatment 1: 3.479 mg/L chromium
Treatment T_0: 0.000 mg/L chromium

Three replicates were maintained for each treatment and 30 fish were placed in each of the FRP (fiberglass reinforced plastic) tank of 200 L capacities. Fish were fed with diet having 30% crude protein during the experimental period. The experimental conditions were kept same throughout the study. Experimental tanks were cleaned by siphoning every day to remove the excess feed and the excreta of the fish. The water quality parameters were analyzed during the sampling period. The 50% test solution was replaced every day to maintain the concentration of chromium.

Estimation of chromium: A 10 g of fish muscle, liver and gills were dissected out from the fish. In the specimen where 10 g of sample could not be collected owing to small size more than 1 fish were taken together to collect sample of 10 g. The collected sample was cleaned thoroughly with distilled water and kept in a hot air oven at 60°C for 24 h. The dried samples were ground into fine powder using glass pestle and mortar. About 0.5 g of dried sample was weighed into conical flask for dissolution and to that 10 ml HNO_3 and 2.0 ml HCl was added and kept overnight. The flasks were kept on a hot plate for evaporation until brown fumes were completely given out and white fumes were observed. The completely digested samples were allowed to cool to room temperature following which the samples were transferred to plastic bottles and the volume was made up to 25 ml and stored for further analysis. The digested samples were analyzed in triplicate, using atomic absorption spectrophotometer. The blanks and calibration standard solutions were also analyzed in a similar manner as the samples. The heavy metals were analyzed in the fish samples as per standard procedure. Quantifications were achieved using calibration curves.

Micronucleus test: Peripheral blood samples were drawn by puncturing the caudal vein, using heparinised syringe and smear of blood was made on a pre-cleaned slide. The slides were air-dried in dust and moisture free environment at room temperature and were fixed by dipping it in absolute methanol for 5–10 min. The fixed slides were stained in May-Grunewald's solution for 2–5 min which were washed with double distilled water and dried afterwards. Thereafter the slides were stained with 6–10% Giemsa stain in phosphate buffer (pH- 6.8) for 30 min. It was observed under microscope and the cells, forming micronucleus were counted.

Scoring of micronuclei: The slides were observed under 1,000× magnification and from each slide 1,000 cells were scored. MNi were identified according to the criteria described by Majone *et al.* (1987) and Kirsch-Volders *et al.* (2000). MNi frequency was calculated as follows:

$$\text{MNi (\%)} = \frac{\text{No. of cells containing micronucleus}}{\text{Total no. of cells counted}} \times 100$$

RESULTS AND DISCUSSION

Acute toxicity tests: The results of acute toxicity tests of chromium were expressed in terms of LC_{50} values for 24, 48, 72, and 96 h after Probit analysis using SPSS software and graph method. The results of acute toxicity studies for chromium obtained are shown in Figs 1 and 2. The fingerlings of *C. mrigala,* exposed to various concentration of the chromium, exhibited almost identical and clinical symptoms of varying degree depending on the concentrations of the chromium. The test animals showed restlessness, frequent surfacing, and loss of balance and irregular opercular movement. Gradually they became lethargic and in some cases, excessive mucous secretion was noticed. The LC_{50} values were 227.08, 55.15 and 52.187 mg L^{-1} for 48, 72, and 96 h respectively, showing gradual decrease with increase in time. The acute toxicity values were significant ($P<0.05$) at 24 h and ($P<0.01$) at

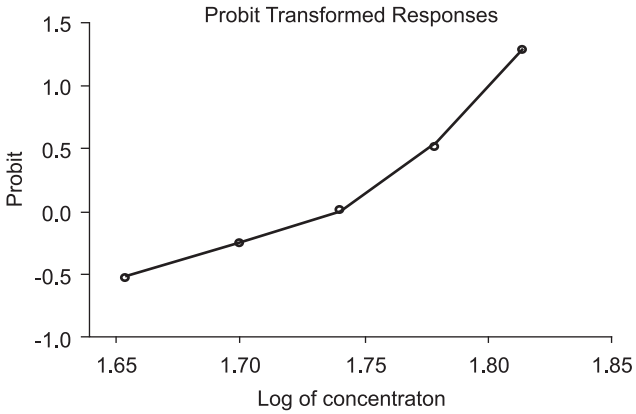

Fig. 1. LC_{50} of chromium for 96 h for *C. mrigala* fingerling.

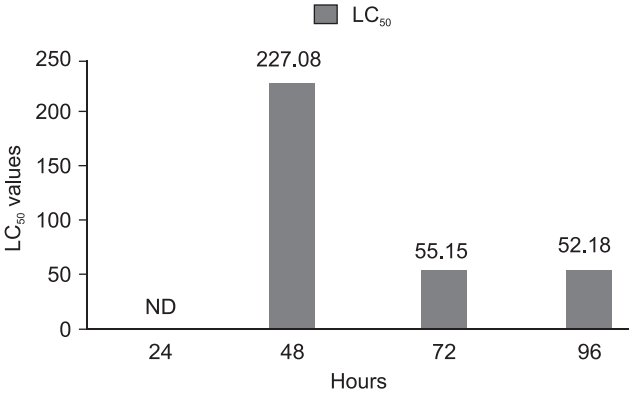

Fig. 2. LC_{50} of chromium for 96 h for *C. mrigala* fingerling.

48, 72, and 96 7 h LC_{50}.

The initial concentration of chromium was negligible in all the 3 analyzed tissue and organs. After 60 days of exposure of fish to different concentrations of chromium, it was found that chromium accumulated in the muscles, gills and liver which depended on duration of exposure and concentration of chromium (Table 1). The highest concentration of chromium was found in liver after 60 days of exposure in treatment T_2 (61.91±0.73 µg/g) and the lowest concentration was found in the muscles in the treatment T_1 (3.05[a]±0.04 µg/g). In gills, the highest concentration was found in T_2 16.67±0.08 µg/gand the lowest concentration was observed in T_1 (4.69±0.02 µg/g) after 60 days of exposure. The accumulation of chromium in liver varied between 10.61±0.20 µg/g to 61.91±0.73 µg/g in T_1 and T_3 treatment, respectively. The fish exposed to the treatment T_3 did not survive beyond 40 days.

There was a significant difference in the formation of micronuclei in the blood cells (Figs 3,4). Number of micronuclei formed in cells varied depending upon the concentration and the exposure duration of chromium. The highest number of micronuclei formation was observed to be 6.37±0.56% while the lowest MNi formation was

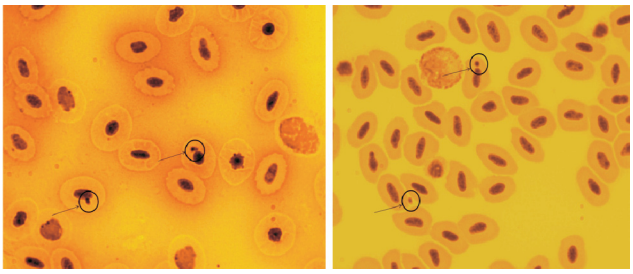

Fig. 3. Effect of chromium on *C. mrigala* showing formation of MNi in cells (arrow marked) of peripheral blood cells.

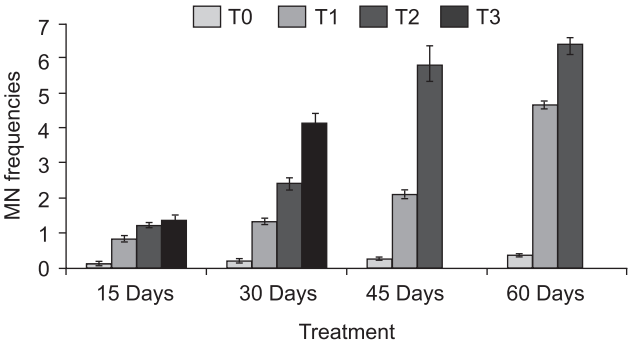

Fig. 4. Micronuclei (%) in blood cells of *C. mrigala* fingerlings exposed to sublethal concentrations of chromium for different durations.

Table 1. Bioconcentration of hexavalent chromiun in different organs of *C. mrigala*

Days / Treatment	T1			T2			T3		
	Gills	Liver	Muscles	Gills	Liver	Muscles	Gills	Liver	Muscles
15	4.69[a]	10.61[a]	3.05[a]	5.64[b]	14.74[b]	3.46[b]	6.17[c]	16.39[c]	4.49[c]
	0.02	0.2	0.04	0.03	0.12	0.04	0.03	0.23	0.09
30	10.52[a]	21.53[a]	5.50[a]	11.59[b]	22.67[b]	6.24[b]	13.53[c]	25.66[c]	7.05[c]
	0.1	0.4	0.16	0.19	0.17	0.03	0.14	0.34	0.04
45	13.29	32.65	7.45	13.83	39.49	8.21	*	*	*
	0.22	0.29	0.21	0.05	0.18	0.25			
60	15.64	41.46	8.56	16.67	61.91	8.97	*	*	*
	0.34	0.78	0.12	0.08	0.73	0.06			

*The fishes could not survive beyond 40 days of experiment in treatment T_3

The values with different superscript differ significantly (P<0.05) (small letters represents variations among the treatment with respect to a particular fortnightly) as Mean ±Standard error (n=3).

0.83±0.06% in the blood cells. On 60th day of exposure, there was a significant difference in the formation of micronuclei between the T_0 and T_2 treatments i.e. micronuclei increased from 0.37±0.03% to 6.37±0.56%.

Our results revealed that hexavalent chromium exerted genotoxic effects on the fish. The 96 h LC_{50} value (52.182 mg/L) was high when compared with cadmium (5.2 mg/L) and lead (32.68 mg/L). Some investigations made on different species of fish showed a differential toxic nature of chromium. The 96 h LC_{50} values, obtained for different species of fish, varied from 4.4 to 97.9 mg/L. The value, observed in the experiment, is in this range. Effect of acute doses (LC_{50}) of chromium on fish was studied in *Salmo giardinerri* (140 mg/Lby Schiffman and Fromm, 1959), *Cyprinus carpio* (250 mg/L; Al-Akel 1996), *Oreochromis mossambicus* (200 mg/L by Jaffri *et al*. 1999), *Labeo rohita* (142 mg /L by Jaffri *et al.* 2003) and *Cyprinus wastoni* (178 mg/ L; Ikhtiar-ud-Din and Hafeez 1996). Variations in values may be due to the several factors such as temperature, pH, dissolved oxygen, water hardness and synergism in addition to different fish species (Al-Akel 1996).

Sanjay *et al*. (2006) exposed air breathing teleost fish *Channa marulius* to Cr (VI) for 96 h. LC_{50} as well as safe level were studied. They observed that toxicity of metal is dose and time dependent i.e., with the increase in metal concentration and time, the mortality also increased. In the present findings Cr (VI) toxicity was noted to increase positively with increase in concentration and duration. Abnormality such as fast jerking movements was noted in fish before death. Abnormal behavioral changes (cough and yawn) at higher concentration might be due to manifestation of the disturbances in the physiological mechanism, which is supposed to initiate, maintain and terminate the behavior. Ahmet *et al.* (2005) stated that increased cough and yawn is due to the increased secretion of mucous, which deposited on the gills to combat the toxicity produced by heavy metals. It also reduced the gaseous diffusion causing less supply of oxygen and causing immediate fish death.

Bio-concentration of chromium in the fish organs (gills and liver) increased depending on the concentration of medium and the exposure time. Giguere *et al*. (2004) reported that heavy metal concentration in the fish increased with age of fish and the exposure time exerted significance impact on the tolerance limit of fish. The highest concentration of chromium, in the present study, was found in liver wheras the lowest concentration was found in muscle. Fish liver exhibited greater tendency to accumulate chromium. Dural *et al*. (2007) and Ploetz *et al*. (2007) reported high levels of cadmium, lead, chromium, copper, zinc and iron in liver and gills of fish species, viz. *Sparus aurata, Dicentrachus labrax*, *Mugil cephalus* and *Scomberomorus cavalla*. Yilmaz *et al*. (2007) reported that in *Leuciscus cephalus* and *Lepornis gibbosus*, cadmium, cobalt and copper accumulations in the liver and gills were high, while these accumulations were least in the fish muscle.

The micronucleus assay in fish is generally performed in enucleated peripheral blood erythrocytes mainly due to its technical feasibility. In most studies it was reported that the short-term bioassay tests for 96 h are sufficient to induce micronuclei in animals, and MNi formation in erythrocytes were reported to be a sensitive biomarker of genotoxicity. However, results of long-term exposure revealed that micronucleus frequencies in erythrocytes decreased after 14 and 21 days. Campana *et al.* (1995) showed that micronucleus frequencies in peripheral erythrocytes of *Cheirodon interruptus* treated with lambda-cyhalothrin and cyclophosphamide, gradually decreased over 15 and 21 days. Similarly, Torres de Lemos *et al.* (2001) reported that micronuclei induction by chromium in erythrocytes of *Pimepheles promelas* exhibited a decrease after the 21 days exposure period. In this study, we observed that the higher doses of chromium increased the micronucleus frequencies in peripheral blood erythrocytes. These differences in the erythrocyte micronucleus frequencies are generally thought to be related to cell kinetics and replacement (Cavas and Ergene-Gozukara 2003).

The study helped to understand the level of bio-concentration in different tissues of fish and demonstrated the induction of genotoxic damage as revealed by the micronucleus assay in fish under long-term exposure to chromium, which provided the early diagnostic tools to detect the toxicity of heavy metal pollution in aquatic environment. This will help in monitoring pollution by suitable management practices such as by setting up emission standards for pollutant, allowable limit of toxicant, and discharge limits in the aquatic environments. However, further studies are needed in this field to develop more efficient and cost effective tools for early diagnosis of toxic effects of environmental pollutants.

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