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Research Article

GENOTOXIC TESTING OF LEAD NITRATE IN AIR-BREATHING TELEOST *CHANNA PUNCTATUS* (BLOCH)

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ABSTRACT : The *in vivo* genotoxic potential of lead nitrate was evaluated in a freshwater fish teleost *Channa punctatus* by investigating mitotic index (MI%) in the kidney cells and micronucleated erythrocytes in the peripheral blood. Fishes were divided into seven groups. Fishes of group I constituted untreated controls. Fishes of group II consisted positive controls treated with cyclophosphamide @ 5 mg/l. Fishes of group III-VII were exposed to sub-lethal concentrations (1.16, 1.55, 2.32, 4.64 and 5.8 mg/l) of Pb $(NO_3)_2$ dissolved in the distilled water. For genotoxicity assay, hematopoietic cells from the kidneys and caudal vein of fishes were examined after 24, 48, 72 and 96 h of exposure. In the cells of kidney mitotic index decreased with increase in the concentration of lead nitrate. The frequency of micronucleated erythrocytes in the peripheral blood increased concomitantly with the increase in concentration and period of exposure. Two-way analysis of variance (ANOVA) test revealed statistically significant effect of concentration of lead nitrate as well as duration of treatment on mitotic index and frequency of micronucleated erythrocytes.
Keywords: lead nitrate, mitotic index, micronuclei, *Channa punctatus*

INTRODUCTION

Pollution of aquatic ecosystem has become a global problem because the aquatic environment is the ultimate destination for almost all kinds of waste materials. The quality of both surface and groundwater has been severely affected by these waste products. Among all types of aquatic pollutants, heavy metals are of greatest concern because after reaching in the aquatic bodies they not only deteriorate the life sustaining quality of water but also cause damage to both flora and fauna by affecting various physiological, biochemical and cellular processes [1-4]. Due to their long half-life period and non-biodegradability, bioaccumulation and biomagnifications the problem increases many folds. Among the heavy metals, lead is a major aquatic pollutant in many parts of the world. It is one of the most toxic and non-biodegradable elements. Lead occurs naturally in the earth's crust, rock, soil, and water. It enters into aquatic ecosystems through diverse natural sources as well as anthropogenic activities. Most of the waterborne lead derives from human activities such as mining and smelting, coal burning, cement manufacturing, and use in gasoline, batteries, and paint. The toxic effect of lead is primarily the inactivation of enzymes and proteins by the binding wth sulfhydryl group.

The fish are excellent material for the study of the genotoxic potential of contaminants present in water, because they respond with high sensitivity to waterborne pollutants and their response to toxicants is very similar to other higher vertebrates. The Toxico-pathological impact of lead on fishes have been well documented [5-6], however, only scanty information is available regarding the genotoxicological manifestations among fishes especially in freshwater air breathing fishes. In the present study genotoxic, potential of lead nitrate in aquatic environment has been studied by assessing the mitotic depression in the rapidly dividing hematopoietic cells in the kidney and frequency of micronucleated erythrocytes in the peripheral blood of a freshwater, air-breathing teleost *Channa. punctatus.*

MATERIALS AND METHODS

Healthy specimens (12-16cm length and 50-55g weight) of *Channa punctatus* (Bloch) 2n = 32, (Class - Actinopterygii, Order – Perciformes, Family – Channidae) were collected from a single population from local fish market and were treated with 0.05% $KMnO_4$ solution for 2 minutes to avoid any dermal infection. Fishes were left for acclimatization for 15 days in the laboratory in glass aquaria containing 30 l non-chlorinated tap water. The fishes were fed *ad libitum* with minced boiled chicken eggs. Feeding was stopped 24 h prior to the commencement of the experiment and the fishes were kept starved throughout the period of the experiment. Water of the aquaria was changed after every 24 h, leaving no fecal matter, unconsumed food or dead fish, if any.

Prior to the commencement of the experiment median lethal concentration (96 h LC50) was determined by employing short-term static toxicity assay. For toxicity test six aquaria of 50 liter capacity were taken having 30 l of dechlorinated tap water (Physico-chemical properties, pH = 7.3±0.2; Temp. = 26±2^{0}C; Alkalinity = 65±4.5 mg/L; Total Hardness = 265±2.5 mg/L; D. O. = 7.0±0.2 mg/L). Eight concentrations of Lead nitrate viz. 2, 4, 6, 8, 10, 12, 14, 16 mg/l was prepared for toxicity test. 30 adult fishes of similar size and weight were exposed to each test concentration. Proper aeration was maintained in test as well as control aquaria by air pumps and stone diffusers throughout the experiment. Mortality was recorded after 24 h interval each day at the same time up to 96 hrs. Experiment was carried out in three replicates. The LC50 values of various intervals were calculated according to Spearman Karber's Method [7].

Five equally spaced concentrations of lead nitrate $(PbNO_3)_2$ which were below the determined LC 50 value for 96 h were selected for treatment of fishes. Acclimated fishes were divided into seven groups and treated for 24, 48, 72 and 96 h, as follows:

Group I: control fishes kept in aquarium containing distilled water,

Group II: positive control fishes kept in aquarium containing 5 mg/l cyclophosphamide (positive control).

Group III-VII: were kept in separate aquaria containing one of the five concentrations of $Pb(NO_3)_2$ which are 1.16 (LC50/10), 1.55 (LC50/7.5), 2.32 (LC50/5), 4.64 (LC50/2.5) and 5.8 (LC50/2) mg/l.

After the exposure periods of 24 h, 48 h, 72 h and 96 h, five fishes from the respective experimental as well as control aquaria were sacrificed. The fishes were injected 0.05 % colchicine, dissolved in glass distilled water, intramuscularly (@ 1 ml/100 gm of body weight) one and half hour prior to sacrifice. The kidneys were taken out, cut into small pieces and homogenized in 10 ml hypotonic solution (0.56% KCl) in glass tissue homogenizer. The cell suspension was poured in a 15 ml centrifuge tube and incubated for about 25-35 minutes at room temperature for swelling of the cells. The hypotonic treatment was stopped by adding 1 ml of freshly prepared, chilled Carnoy's fixative (methanol: glacial acetic acid, 3 : 1). The cell suspension thus obtained was centrifuged at 1200 rpm for 10 min, supernatant discarded and the cell pellet was re-suspended in 7-8 ml of chilled fixative and again centrifuged for 10 min at 1200 rpm. The process of washing of the cell pellet with fixative was repeated thrice to get clear whitish pellet. Slides were prepared by air drying technique and stained with 10% Giemsa in phosphate buffer (pH 6.8). 5000 cells were counted at a magnification of 100 X for number of mitotically dividing cells. For micronuclei (MN) assay blood was drawn from the heart regions by cardiac puncture using the cold hypodermic micro syringes pre-rinsed with heparin (anticoagulant). The collected blood from the control and experimental groups was expelled on clean glass slides and thin smears were prepared. The slides were air-dried for 24 h, fixed in methanol for 10 minutes and stained in 10% Giemsa (v/v). Five slides were prepared for each fish. Slides were coded and scored blind under 100 X oil-immersion lens. 1000 cells were scored from each slide for the presence or absence of micronuclei in their cytoplasm. Micronuclei were identified as small (diameter less than one-third of the main nucleus) non-refractive, circular or ovoid chromatin bodies separated from the main nucleus and have similar staining as the main nucleus

All data are expressed as mean and SEM. Dunnet multiple comparisons test was conducted to test the significance of difference between control and treated animals. Two-way analysis of variance (ANOVA) test was done to determine the significance of the effect of concentration and period of treatment on mitotic index and micronuclei formation. All statistical analyses were performed by using Graphpad prism 3 software.

RESULTS

Table 1 displays data obtained for the mitotic index (% MI) in the kidney cells of control, positive control and lead nitrate treated fishes. The mitotic index evaluated as percentage of dividing cells in control fishes (group I) was 7.10± 0.010. The cytotoxicity of cyclophosphamide (group II) to the fish was evident as highly significant ($p<0.01$) decrease in mitotic index when compared to control fish of group I. Treatment of fish with lead nitrate resulted in concentration related decrease in the mitotic indices, as compared to control. The two lower concentrations of lead nitrate (1.16 and 1.55 mg/l) did not produce any significant effect on the mitotic index. Statistically significant reduction in mitotic index was recorded at 2.32, 4.64 and 5.80 mg/l for 24 and 48 h. After 72 h of exposure only the two higher concentrations i.e. 4.64 mg/l ($p<0.05$) and 5.80 mg/l ($p<0.01$) and after 96 h of exposure only the highest concentration i.e 5.80 mg/l caused significant reduction in mitotic index as compared to control. Fig 1 compares inhibition of mitotic activity (percent that of control) in the treated animals. It is evident from the figure that the highest concentration (5.8 mg/l) of lead nitrate and shortest duration (24 h) of exposure caused maximum inhibition in the mitotic activity (more than 54%) while the lowest concentration and the longest period of treatment caused minimum inhibition (7.52%) in mitotic activity in the kidney cells.

Table 1 Effect of lead nitrate treatments on the cell division in the kidney cells of *Channa punctatus*

Concentration	24 h	48 h	72 h	96 h
Control	7.18 ± 0.44	7.18 ± 0.44	7.04 ± 0.26	7.00 ± 0.12
CP	3.88 ± 0.23**	4.34 ± 0.25**	4.84 ± 0.48**	5.46 ± 0.55**
1.16 mg/l	5.54 ± 0.34	5.84 ± 0.26	6.24 ± 0.68	6.64 ± 0.26
1.55 mg/l	5.36 ± 0.33	5.58 ± 0.26	6.04 ± 0.44	6.30 ± 0.18
2.32 mg/l	4.26 ± 0.59**	4.84 ± 0.39**	5.42 ± 0.36	5.84 ± 0.49
4.64 mg/l	3.82 ± 0.72***	4.28 ± 0.57**	4.98 ± 0.52**	5.32 ± 0.63
5.80 mg/l	3.24 ± 0.48***	3.78 ± 0.48***	4.06 ± 0.68**	4.54 ± 0.64**

CP, Cyclophosphamide used as positive control
1000 cells per fish and total 5000 cells have been scored in each case
Mitotic index (MI %) = Number of dividing cells / total No. of cells scored X 100
* ($p<0.05$) and ** ($p<0.01$) differ significantly from the control in Dunnet multiple Comparisions test.

Table 2: Two-way analysis of variance (ANOVA) of mitotic index showing significant variation between treatments as well as periods of treatment

Sources of Variation	df	Mean Square	F-value
Between periods	3	1.25	16.22***
Between treatment	5	5.14	66.67***
Residual	15	0.07	

*** significant at $p<0.001$

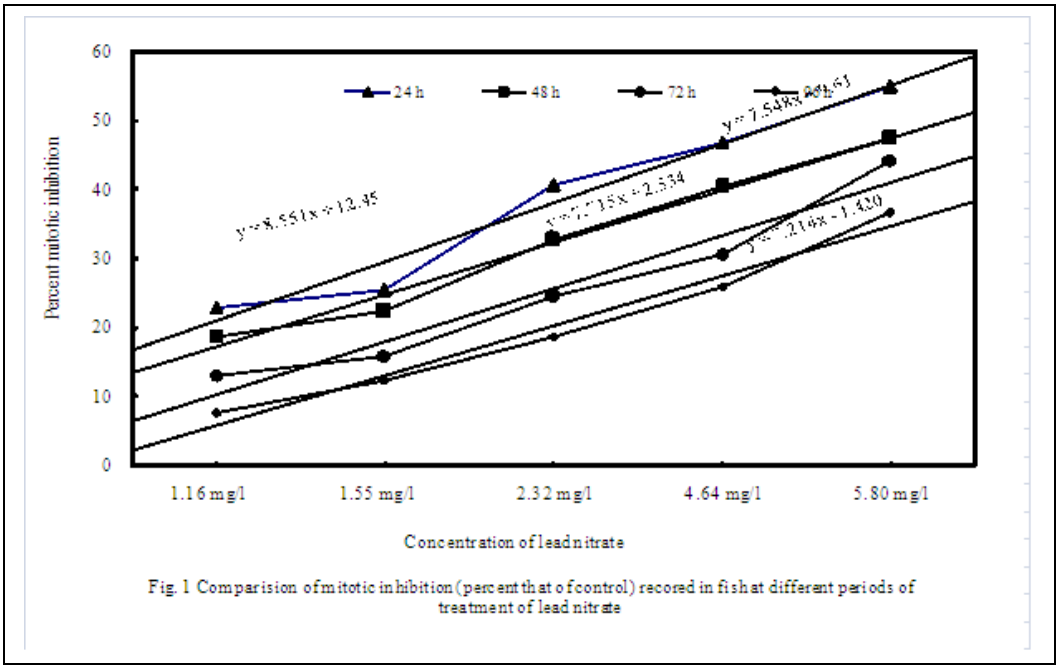

Fig. 1 Comparision of mitotic inhibition (percent that of control) recored in fish at different periods of treatment of lead nitrate

Two-way analysis of variance test (ANOVA test) revealed highly significant ($p<0.01$) difference between the dosage of lead nitrate as well as periods of treatment (Table 2). and period of exposure related decrease in mitotic index.

Frequencies of micronucleated erythrocytes in fishes exposed to 1.16, 1.55, 2.32, 4.64 and 5.8 mg/l of lead nitrate as well as parallel control and positive control are summarized in Table 3. It is evident from the table that treatment of resulted in concentration and period of treatment related increase in the frequency of micronucleated erythrocytes in the peripheral blood of fishes when compared to untreated control. The differences between mean number of micronucleated erythrocytes of lead nitrate treated and control fishes were statistically significant at 24, 48 and 72 h (2.32, 4.64 and 5.8 mg/l), and at 96 h exposure. Two-way analysis of variance test (ANOVA test) revealed highly significant ($p<0.001$) difference between the concentrations of lead nitrate as well as periods of treatment (Table 4).

Table 3 Frequency of micronucleated erythrocytes in blood of freshwater fish *Channa punctatus* treated with different concentrations of lead nitrate.

Chemical /	Frequency (%) of micronucleated erythrocytes [a]			
Dose	24 h	**48 h**	**72 h**	**96 h**
Control	0.024 ± 0.016	0.021 ± 0.013	0.030 ± 0.020	0.019 ± 0.011
CP	0.36 ± 0.092**	0.048 ± 0.135**	0.59 ± 0.135**	0.68 ± 0.082**
1.16 mg/l	0.069 ± 0.030	0.090 ± 0.025	0.11 ± 0.032	0.16 ± 0.021*
1.55 mg/l	0.079 ± 0.038	0.108 ± 0.028	0.12 ± 0.010	0.18 ± 0.029**
2.32 mg/l	0.13 ± 0.051*	0.16 ± 0.027*	0.21 ± 0.030**	0.24 ± 0.025**
4.64 mg/l	0.18 ± 0.033*	0.22 ± 0.019**	0.25 ± 0.066**	0.37 ± 0.039**
5.80 mg/l	0.23 ± 0.022**	0.31 ± 0.058**	0.38 ± 0.028**	0.47 ± 0.048**

[a] 1000 cells per fish and total 5000 cells have been scored in each case
* ($p<0.05$) and ** ($p<0.01$) differ significantly from the control in Dunnet multiple comparisions test.

Table 4: Two-way analysis of variance (ANOVA) of mitotic index showing significant variation between treatments as well as periods of treatment			
Sources of	df	Mean Square	F-value
Variation			
Between periods	3	0.0169	12.27***
Between treatment	5	0.0518	37.64***
Residual	15	0.05	
*** significant at <0.001 respectively			

DISCUSSION

In recent years, awareness of the potential hazards of pollutants discharged in the aquatic environment has stimulated much interest in the use of fishes for the biomonitoring of water pollution. Fishes are among the most sensitive aquatic organisms for evaluating genotoxic effect of chemical pollutants present in polluted water bodies. Genotoxic studies using cytogenetic markers in fishes have been undertaken by a number of workers in recent past. These studies have clearly demonstrated that chemical pollutants produce genotoxic effects in fish [8-17].

There are very few published reports on the *in vivo* cytogenetic effects of lead nitrate in freshwater teleost. All the tested concentrations of lead nitrate produced inhibitory effects on the mitotic activity of kidney cells as is evident by the reduction in the mitotic index. Maximum inhibition in the mitotic activity was recorded after 24 h of exposure and thereafter the inhibitory effect gradually decreased with the lapse of exposure period. This is probably due to sudden exposure to lead nitrate might have caused severe stress on the exposed animals, but gradually with the lapse of time the animals became adapted to such situation. It has been suggested that a family of highly conserved cellular proteins referred to as the heat shock proteins (HSPs) characterizes the stress response in fish at the cellular level [18]. Extensive studies have revealed that HSPs assist the folding of nascent polypeptide chains, act as a molecular chaperone, and mediate the repair and degradation of altered or denatured proteins and are also active in supporting various components of cell signaling, including the cytoskeleton, enzymes, and steroid hormone receptors [19-20]. HSPs are also involved in the most basic cellular processes, such as cell division and growth. Inhibition of mitotic activity as observed in the present study may be due to suppressive effects of lead nitrate on the activity of HSPs. In addition, arrest of mitosis due to failure of repair of genetic damage may have contributed to decreased values of MI.

The micronucleus assay in the hematopoietic cells is one of the most sensitive tools to evaluate the genotoxic property of water contaminants. The test has been applied for both *ex situ* and *in situ* monitoring of genotoxic effects due to exposure to environmental pollutants. Micronuclei are formed due to condensation of acentric chromosome fragments or lagging chromosomes that fail to incorporate into daughter cell nuclei during cell division. Therefore, cytogenetic damage that results in chromosome breaks or spindle abnormalities leads to micronucleus formation and thus the incidence of micronuclei serves as an index of genotoxic effect. The results of the present study indicate that exposure of fishes to lead nitrate resulted in increased frequency of erythrocytes with micronuclei in the peripheral blood.

CONCLUSION

The overall results of the present study show that lead nitrate is genotoxic because this compound induced cytotoxicity and micronuclei formation. Since, mitotic index is decreased; lead nitrate has highly cytotoxic effect. This compound has also clastogenic and aneugenic effects as evident by induction of micronuclei in peripheral blood cells. Therefore, it should be always kept in mind that excess discharge of heavy metals in aquatic bodies may be very dangerous for the fish and other aquatic fauna.

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