



Structural Analysis of the Erythrocytes of *Channa punctatus* (Bloch) exposed to Mercuric Chloride using Scanning Electron Microscopy

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Abstract

Fish are susceptible to harmful effects of heavy metals and other effluents which influence the hematological profile. Hematological parameters are considered as pathophysiological indicators and are important in diagnosing the structural and functional status of fish exposed to toxicants. Ultrastructural analyses can be used to study the alterations in erythrocytes due to environmental stress. The aim of the present study was to evaluate the toxicity of mercuric chloride on erythrocytes of fish, *Channa punctatus*. The experiments were conducted in accordance with the standard methods given in APHA (2005) and EPA (1996). Erythrocytes of the fish are nucleated and elliptical in shape. Scanning electron micrographs revealed the presence of spherocytes, crenation of membrane, membrane internalization and development of lobopodial projections in the erythrocytes of exposed fish. The study substantiates the hypothesis that assessment of morphological damage to erythrocytes through scanning electron microscopy can be effectively used as a toxicological tool.

Keywords: *Channa punctatus*, erythrocytes, mercuric chloride, scanning electron microscopy.

Introduction

Water pollution is of major concern to environmentalists as well as toxicologists as it is a potential threat to aquatic organisms. Aquatic ecosystem gets contaminated with toxic chemicals released from industrial wastes and agricultural run-off. Due to bioaccumulation, heavy metal pollution has always been a global concern. Heavy metals in water bodies induce structural and functional alterations in the organisms and pose serious threat to non-target species (Mwachiro and Druve, 1997; Fazio *et al.*, 2014). Mercury is discharged into the environment by effluents of various industries like paper and pulp, electroplating, thermal power stations, metal industries, electronic wastes etc. It is also used as fungicide in agriculture, in chemistry for production of various mercury compounds, in manufacturing of paints and dental amalgams and is used in medicines as tropical antiseptic.

Various reports have documented the toxicity of mercuric chloride and its impact on fish species. Hedayati and Ghaffar (2013) reported significant changes in hematological, biochemical and immunological parameters in silver carp, *Hypophthalmichthys molitrix* exposed to mercuric chloride. Behavioral manifestation and alterations in

biochemical content in mercury treated fish *Danio rerio* was reported by Vutukuru and Basani (2013). Further, light microscopic studies were previously performed on liver, gills and testis in fish, *Gymnotus carapo* (Vergilio *et al.*, 2012) and electron microscopic studies on gills in *Channa punctatus* (Gupta and Dua, 2002). Saloch *et al.* (2014) observed hematological changes in fish *Clarias gariepinus* exposed to mercuric chloride for a period of 60 days.

Fish lives in direct contact with water so they are vulnerable to physico-chemical change occurring in them (Faggio *et al.*, 2014; Fazio *et al.*, 2013a; Fazio *et al.*, 2013b). They are highly sensitive to environmental changes and occupy different trophic levels in the food chains. Therefore, fish are considered as reliable indicators of heavy metal pollution. Fish serve as a good protein source for human consumption. Hence, unhealthy fishes become health hazard for human population.

Hematology is a valuable tool to evaluate pathological condition of the fish. Hesser (1960) recommended the use of hematological parameters as indicators of fish health. Erythrocytes are important subject for toxicological studies, as it has no internal compartments. Thus morphological alteration in erythrocytes in response to water pollution can be studied through ultrastructural analysis. According to

Juneja and Mahajan (1983) pollutants as well as other environmental factors can induce morphological changes in the blood parameters.

Scanning electron microscopy (SEM) is considered as a powerful tool for analyzing the effects of toxicants and pollutants on the organism. SEM provides information about structural variations in the cells of organism exposed to toxicants. In view of such considerations, in this study the morphological alterations in the erythrocytes of *Channa punctatus* exposed to sublethal concentrations of mercuric chloride were evaluated by means of scanning electron microscopy.

Materials and Methods

Test Organism

Healthy specimens of fish, *Channa punctatus* (Bloch) were procured from local market, Amritsar and carefully brought to the laboratory. Fish were acclimated in plastic tanks (60x35x60cm) containing tap water under normal laboratory conditions (Photoperiod- natural 12-h light:dark cycle, Temp- $17.4 \pm 0.1^\circ\text{C}$, pH- 7.19 ± 0.01 , dissolved oxygen- 6.65 ± 0.02 mg/L, Salinity- 0.29 ± 0.01 mg/L and Electrical Conductivity- $601.67 \pm 0.6 \mu\text{S/cm}$) for 15 days. Each tank was stocked with fish with stocking density 0.8g/L as recommended by EPA (1996) and APHA (2005). These were static bioassay studies. The species was selected for experimental study because it is available throughout the year, easy to maintain in laboratory and sensitive to pollutants.

Chemicals

Mercuric chloride (AR) manufactured by SD fine chemicals limited, Mumbai, India having a molecular weight of 271.50 was used during the present study. Mercuric chloride solutions having concentration i.e. 0.020, 0.027 and 0.040 mg/L were prepared using double distilled water.

Chronic Toxicity Bioassay

Specimens of freshwater fish, *Channa punctatus* having weight = 16.74 ± 0.6 mg, total length = 11.56 ± 0.03 cm and fork length = 9.53 ± 0.14 cm were used for bioassay. 96h LC50 value of mercuric chloride for *Channa punctatus* was calculated using Probit analysis. Static non-renewal chronic toxicity bioassays were conducted according to standard methods of the U.S. Environmental Protection Agency (U.S. EPA, 2002) and APHA (2005).

Fish were transferred to experimental tanks ($n = 7$ per treatment and duration; total fish = 84) containing sub lethal concentrations of mercuric chloride (1/40, 1/30, 1/20 of LC50) i.e. 0.020, 0.027, 0.040 mg/L. Simultaneously control tank containing tap water was run for the same exposure durations containing fish ($n = 7$ per treatment and duration; total = 28). All the tests were carried out in triplicates. Fish were exposed to mercuric chloride for a period of 15, 30, 60 days and 30 days of recovery. During

recovery, fish from three exposed groups were transferred from experimental tanks to tanks containing normal tap water. Water in all the four tanks was changed after every two days and individuals were fed with boiled egg protein daily during recovery period of 30 days.

Sample Collection and Processing

Blood samples were collected from the heart region by cardiac puncture using syringes having needle size 0.55x25mm/24Gx1" (Hindustan syringes & medical devices ltd, Faridabad, India) from both control and exposed groups on completion of 15, 30, 60 days of exposure as well as 30 days of recovery. Few drops of blood were immediately fixed in 2.5% glutaraldehyde prepared in 0.1M phosphate buffer (pH 7.4) for 2-3h in ciklok micro centrifuge tubes (Tarsons product pvt ltd, Kolkata, India) and the sample was centrifuged at 1500 rpm for 5 min. Supernatant was discarded and the erythrocyte pellet was washed 2-3 times with phosphate buffer. The pellet was then suspended in small volume of distilled water and dehydrated with increasing concentrations of ethanol. A small drop of erythrocyte suspension was applied to a coverslip (circular, 10mm). Air-dried samples were sputter coated with gold and examined under scanning electron microscope (Zeiss EVO LS10, Carl Zeiss, Germany) at an accelerating voltage of 15-20 kV.

Results

Scanning electron micrograph of erythrocyte in the control group showed elliptical shape (Figure 1A). On exposure to mercuric chloride, alterations in the morphology of erythrocytes were observed.

Change in the shape of erythrocytes was observed from elliptical to spherical (spherocytes), when the fish was exposed to 0.020 mg/L for 15 days (Figure 1B). An increase in the frequency of spherocytes was noticed with increasing exposure duration i.e., 30 days (Figure 1F) and 60 days (Figure 2B). Increasing concentration of the toxicant for different exposure period also enumerated an increase in the number of such cells. Number of elongated cells also increased with exposure concentration (Figure 1C-D).

Some RBCs developed lobopodial protrusions of the membrane at an exposure period of 30 and 60 days (Figure 2E). Irregularly shaped RBCs were also observed at different concentrations and time durations. Crenated cells having serrated margins were also recorded at higher concentrations (Figure 2A, 2C). Higher exposure duration leads to the irregular shape of RBC at 0.020 mg/L for 30 days (Figure 1E). Membrane internalization was also observed at 0.04 mg/L at 60 days (Figure 2D). Echinocytes, elongated and oval cells having lobopodial projections were noticed during the study. The extent of morphological alterations was found to be directly proportional to the exposure duration and

concentration. The recovery experiments after 30 days showed a little improvement but still alterations were observed (Figure 2F, 3A-B).

Discussion

The results of the present study explicitly exhibit substantial changes in the morphology of erythrocytes of fish *Channa punctatus* exposed to sublethal concentrations of mercuric chloride. Marked increase in the alterations was observed in a duration and dose dependent manner. Stress condition such as presence of heavy metals in water lead to physiopathological

changes, which can be studied through hematological profile of the organism (Nussey *et al.*, 1995). Changes in the morphology of the erythrocytes of fish exposed to mercuric chloride indicate poor health status of the fish. Round and swollen RBCs are named as spherocytes (Sawhney and Johal, 2000). According to Dey *et al.*, 1999; Sawhney and Johal, 2000; Massar *et al.*, 2012, alterations in the morphology of erythrocyte in the blood of fish are associated with pesticide toxicity as well as heavy metal pollution.

Mercury has high affinity for sulfhydryl groups (SH). According to Ribarov *et al.* (1983), binding of mercury to sulfhydryl groups leads to their

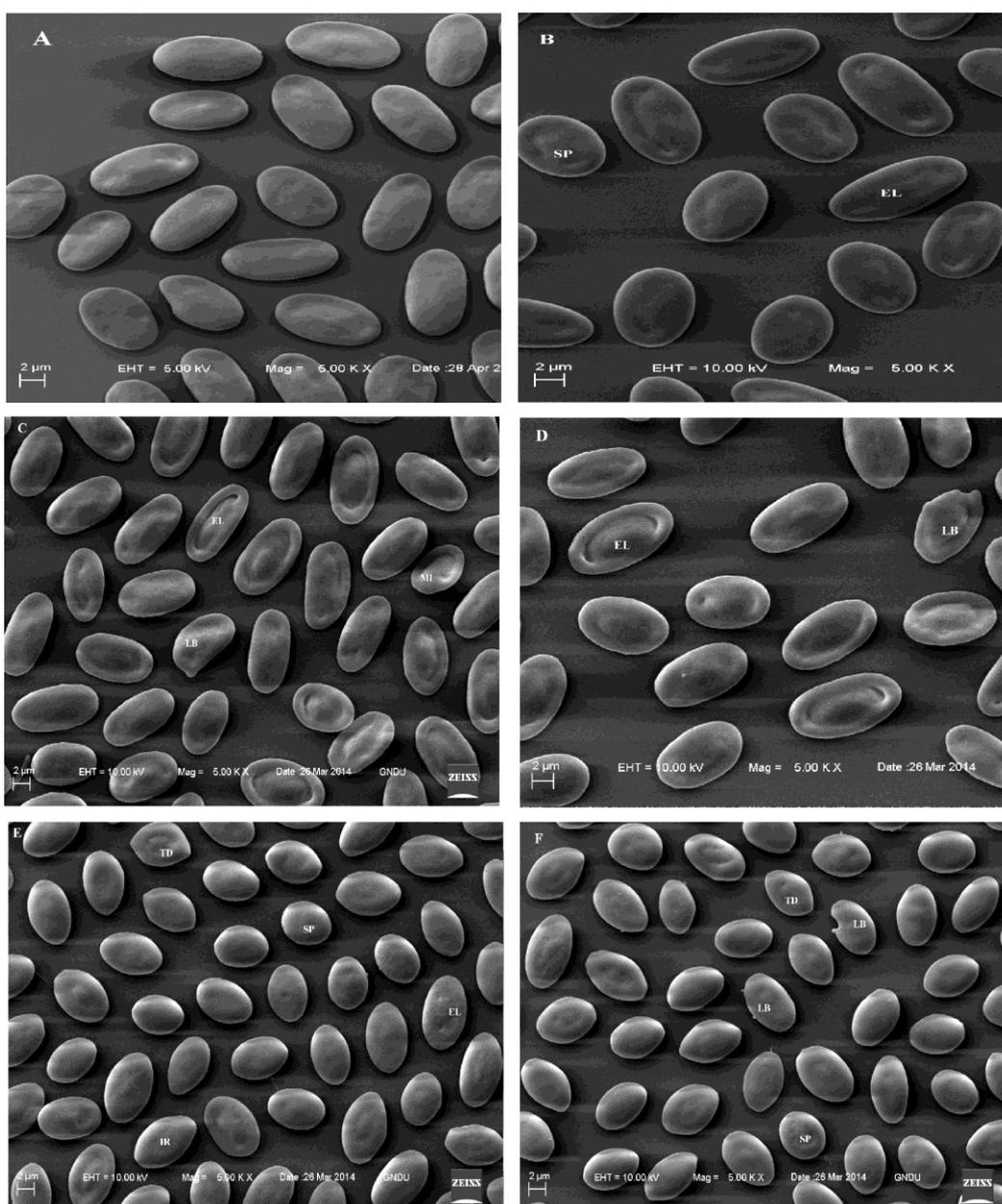


Figure 1. Scanning electron micrograph of erythrocytes of *Channa punctatus*: (A) control group – elliptical shape; (B) spherocytes (SP) and elongated cell (EL) at 0.020 mg/L, 15d; (C) lobopodial projection (LB), elongated cell (0.027 mg/L, 15d); (D) increased number of elongated cells (0.040 mg/L, 15d); (E) tear drop (TD) and irregular (IR) shape cell (0.020 mg/L, 30d); (F) lobopodial projection at two points at 0.027 mg/L, 30d.

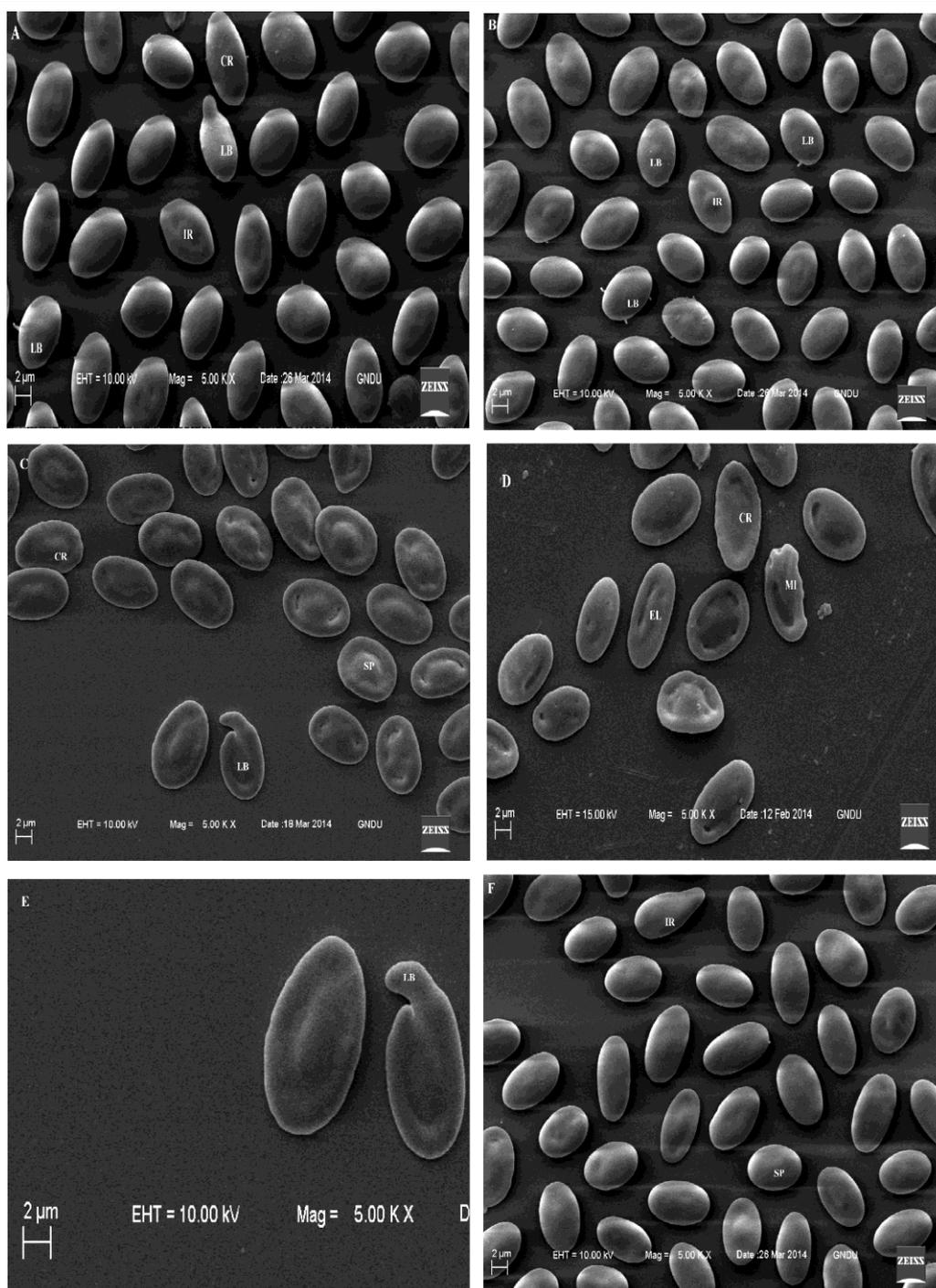


Figure 2. Scanning electron micrographs of erythrocytes from treated fish showing: (A) crenated cell (CR), irregular shaped cell (IR) at 0.040 mg/L, 30d; (B) spherocytes (SP) increased in number, lobopodial projection (LB) at two or more points (0.020 mg/L, 60d); (C) crenated cell and lobopodial projection (0.027 mg/L, 60d); (D) membrane internalization (MI) at 0.040 mg/L, 60d; (E) lobopodial projection (0.027 mg/L, 60d); (F) cells from fish under recovery for 30d (0.020 mg/L).

inactivation, which further decreases antioxidant activity of the membrane. Calcium homeostasis is also affected by inorganic mercury. There is a rapid increase in the intracellular calcium in the cells exposed to mercury due to influx from the extracellular medium. ATP depleted cells as well as cells having increased Ca^{2+} produce echinocytes but

restoration of appropriate ATP and calcium levels result in the return of normal shape of RBC (Nelson *et al.*, 1983). Due to interlacing of the spicules of the crenated erythrocytes, blood flow as well as viscosity is affected (Reinhart and Chien, 1986).

The transformation of normal red blood cells of human to crenated cells can be attributed to extrinsic

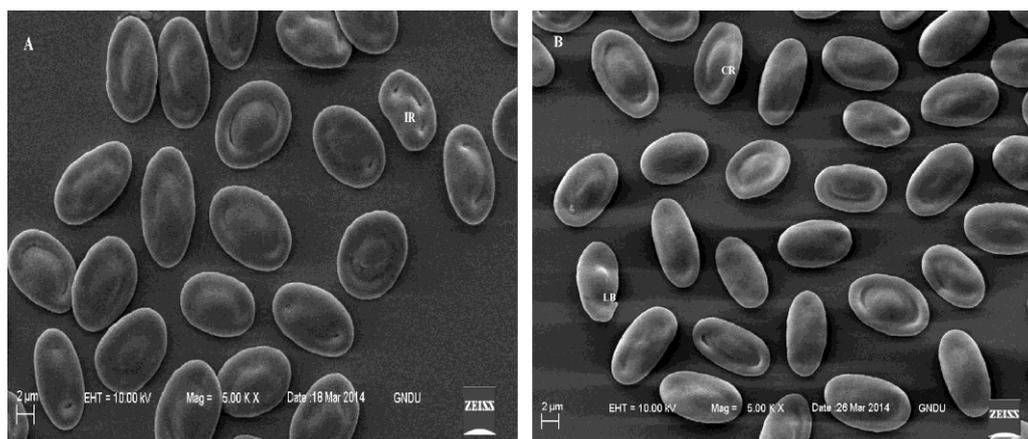


Figure 3. Scanning electron micrographs of erythrocytes exposed to recovery: (A) 0.027 mg/L for 30d; (B) 0.040 mg/L, 30d.

factors or intrinsic factors, such as ATP depletion, which is associated with the ageing of red cells as testified by Brecher and Bessis (1972). The amount of ATP acts as determinant factor for the genesis of echinocytes (Birchmeier and Singer, 1977; Feo and Mohandas, 1977; Reinhart and Chien, 1987). According to Nikinmaa and Huestis (1984), adequate amount of ATP is required for the maintenance of structural integrity of nucleated RBC.

The present observations suggest that mercuric chloride have echinocytogenic properties, as crenation is the early stage of echinocyte formation. Crenation of RBC membrane has been reported in fish, *Cyprinus carpio*, inhabiting a polluted lake in North East India by Massar *et al.* (2012). Echinocyte formation results in the expansion of plasma membrane, which leads to swelling of RBC before lysing was also observed by Isomaa *et al.* (1986). Therefore, various alterations induced by mercuric chloride in the current study can be correlated with altered surface membrane area to volume ratio of the cell as reported by Naskar *et al.* (2006) in *Clarias batrachus* due to aluminium toxicity.

Hemolysis of erythrocytes may be witnessed at the higher concentrations of mercuric chloride or other echinocytogenic agents. Spherocytes observed in the current study were also reported by Agarwal *et al.* (1990) on giving single as well as repeated exposures of methyl isocyanate to rat. According to Tanaka and Nakai (1977), the normal biconcave shape of rat erythrocyte changes to spherocyte on exposure to mercuric chloride.

The formation of lobopodia and contraction of erythrocyte from one side as observed in the present study has also been observed in the fish, *Channa punctatus* exposed to malathion (Sawhney and Johal, 2000). Similar results have also been reported by Massar *et al.* (2012). Abnormally shaped red blood cells, codocytes observed in the current study has been reported by Naskar *et al.*, 2006.

Zeni *et al.* (2002) in a study on fish *Ictalurus melas* observed occurrence of echinocytosis when exposed to sublethal concentrations of an anionic

detergent. The ultrastructural changes on exposure to azo dye, basic violet- 1(CI: 42535) in erythrocyte of *Labeo rohita* were irregular margins, echinocytosis, lobopodial projections, spherocytosis and tear- drop shaped cells as reviewed by Kaur and Kaur (2015). The frequency of such alterations increased with increasing concentration and time duration.

According to Barnhart *et al.* (1983), alterations in the morphology of RBC symbolize the pathologic state. On exposure to the toxicant, fish counteract reduced oxygen level by increasing the blood components that are responsible for uptake of oxygen (Gill *et al.*, 1991). Therefore alteration in the red cell morphology can be correlated to the fulfillment of oxygen requirement of the animal. Escudero *et al.* (1998) suggested that the change in the morphology of surface membrane could be attributed to the deviation in the normal membrane lipid composition.

In conclusion, the scanning electron microscopic observations revealed morphological damage to the erythrocytes of *Channa punctatus*. The degree of damage is directly proportional to the exposure duration and concentration of toxicant and is developed to help fish to encounter hypoxic and toxic ambient conditions. The monitoring of morphological alterations in fish blood cells is a highly sensitive way to assess the effects of toxicants.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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