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Phenotypic Alterations in Erythrocytes of *Ctenopharyngodon idellus* (Cuvier & Valenciennes) Induced by Chlorpyrifos: SEM Study

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Abstract

Erythrocyte morphology is one of the most sensitive indicators of toxic impact of various environmental factors on fish. Present investigation deals with the assessment of deleterious effects of an organophosphate, chlorpyrifos on the structural alterations of RBC's of a freshwater fish, *Ctenopharyngodon idellus* (Cuvier & Valenciennes). On exposure of the fish to the toxicant at different sublethal concentrations (1.44 µg/l and 2.41 µg/l) for 15, 30 and 60 days, the erythrocytes exhibited marked alterations in structure such as lobopodial projections, shrinkage of cell membrane and clumping of cells. Different alterations observed were echinocytes, spherocytes, discocytes, acanthocytes, fusiform cells and stomatocytes. The frequency of occurrence of these alterations was found to be directly proportional to the toxicant concentration. From the study of blood which plays vital metabolic functions, it is apparent that the pesticide leads to physiological malfunctioning due to cellular damage, ultimately hampering the normal growth of the fish.

Keywords: *Ctenopharyngodon idellus*, chlorpyrifos, toxicity, erythrocytes, organophosphate, SEM.

1. Introduction

The contamination of fresh waters with a wide range of pollutants has become a matter of great concern over the last few decades, not only because of the threat to public water supplies, but also to the damage caused to the aquatic life. Pesticides enter aquatic environments through industrial effluents and surface runoff of rain water.

Depending upon the pesticide exposure, the concentration of these toxicants may be lethal or sublethal to aquatic organisms (Jindal and Kaur, 2014).

Fish erythrocyte morphology is one of the most sensitive indicators of toxic impact of various environmental factors. Many fish species are susceptible to the deleterious effects of xenobiotics, as reflected in the blood changes, including anaemia, eosinophilia, lymphocytosis, alterations in erythrocyte morphology and branchial and renal lesions (Gill and Pant, 1987; Jindal and Rani, 2005). In fish, toxic substances taken up from the water enter the blood and therefore, blood cells are among the first target of toxicity, immediately after the gill epithelium. Blood may be considered as target and carrier of chemicals since the lipid moiety of erythrocyte membrane is likely a site of interaction. Toxicants usually enter the blood stream of the fish after absorption from the skin, gills and gastrointestinal tract. Increased haemolysis as a result of cellular injury by many xenobiotics has been associated with lipid peroxidation of red cells. So, blood analysis is of vital diagnostic value. No other tissue gives so true picture of the varying metabolism of the body, as does the blood. Thus, haematology offers a good parameter for determining many pathological states. However, relatively little attention has been paid on the toxicant induced morphological alterations of erythrocytes of the fish. In recent years, fish haematology has become a common and easy tool to assess the physiological conditions, disease of the animal and toxicological screening of fish as test species.

2. Materials & Methods

The live specimens of *Ctenopharyngodon idellus* (Cuvier & Valenciennes) were brought from Nanoke Fish Seed Farm, Distt. Patiala, Punjab. They were acclimatized to the laboratory conditions for 15 days. For chronic toxicity tests, three groups (Grp I-control, Grp II with lower concentration and Grp III with higher concentration of the toxicant) were set up and to each group 10 healthy fish were introduced. One-third (2.41 µg/l) and one-fifth (1.44 µg/l) of the 96 h LC_{50} value of the pesticide as reported in our previous work (Jindal and Kaur, 2014) were taken as sub-lethal concentrations. The physico-chemical characteristics of water used in the experiment were determined in accordance with the standard methods (APHA, 2012) (pH 7.2±0.1, dissolved oxygen 8.0±0.3 mg/L, temperature 25±2 °C, total alkalinity 175±10 mg/L and total hardness 18±0.5 mg/L). Experiment was conducted for a period of 60 days and the observations were taken at an interval of 15, 30 and 60 days. For this, at the end of each exposure period, fish from control and toxicant treated tanks were randomly collected and the blood samples were taken from the caudal vein of the fish using a heparinised syringe. 2-3 drops of blood were put in the fixative (2.5% glutaraldehyde + 2% paraformaldehyde) for 2 h, centrifuged at 1000 rpm for 10 min and the supernatant was discarded, phosphate buffer was added and shaked well. It was repeated for 3 times and a drop of erythrocytes was put over double adhesive tape attached to aluminium stub and allowed to settle. The extra liquid from the sides was soaked by filter paper, allowed to air dry, sputter coated with gold and viewed under JSM JEOL 6100 Scanning Electron Microscope (at RSIC, CIL, Panjab University, Chandigarh).

3. Results & Discussion

The studies on the morphological features of erythrocytes have great importance for assessing their functional state, vitality and kinetics. Erythrocytes are fundamentally capable of few stereotypic responses to a variety of environmental perturbations, which have sometimes vital physiological significance and it is implicated that morphological abnormalities of erythrocytes represent the pathological conditions (Barnhart et al., 1983). These changes in various cell types may be due to abnormal erythropoiesis, inadequate haemoglobin formation and damage to red cells after they leave bone marrow (Bessis, 1972) or alterations in biochemical and enzymological parameters in the blood (Samuel and Sastry, 1989).

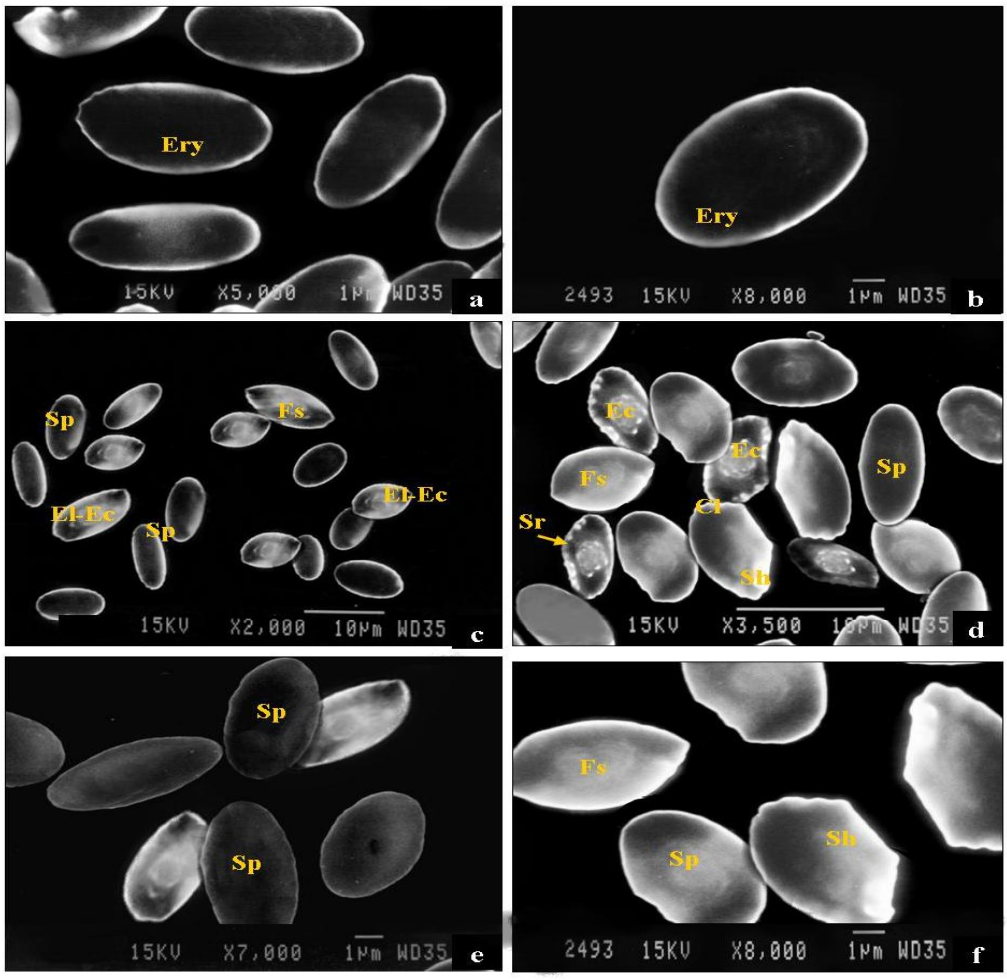

Fig 1 Scanning electron micrographs of erythrocytes of *Ctenopharyngodon idellus* on exposure to chlorpyrifos at 1.44 µg/l and 2.41 µg/l concentration on 15th day.

a, b : Control
c-e: at 1.44 µg/l
d -f: at 2.41 µg/l

Abbreviations:
Ery-Erythrocyte, Sp-Spherocyte, Fs-Fusiform cell, Ec-Echinocyte, Cl-Clubbing of Ery, Sh-Shrinkage, El-Ec-Ellipto-Echinocyte, Sr-Serrations.

In the present work, SEM was done to study the toxic effects of chlorpyrifos on the phenotypic alterations in erythrocytes of the fish. The studies revealed erythrocytes of control fish appeared elliptical with an oblong nucleus (Fig 1 a, b). On exposure of the fish to 1.44 µg/l of chlorpyrifos for 15 days, some erythrocytes were found to be swollen and became spherical in shape and are referred as spherocytes. However, some erythrocytes got shrinked and assumed different irregular semi-oval shape (Fig 1 c-e). At higher dose of the toxicant, numerous spherocytes with irregular outline and shrinkage were observed.

The projections of the membranes of cells were sharp or blunt and there was presence of evenly spaced spicules around their circumference. These cells are referred as echinocytes, also known as Burr cells. Conversion of erythrocytes into echinocytes may be attributed to induced cholesterol level caused by liver dysfunction (Remia et al., 2008). Lipids speculated the surface receptors on the red blood cells bind with cholesterol which induced the shape change. Also, cells with widen middle part and tapering ends were found, called fusiform cells (Fig 1 d-f).

Sherman (1979) illustrated that change in membrane lipid composition could be the key reason for such deformities in shape of blood cells in response to various chemical treatments. The plausible explanation of all these altered forms of blood cells could be attributed to the severity of "toxic stress" induced by chlorpyrifos on the basis of reports available by earlier workers (Naskar et al, 2006; Witeska et al, 2011)

Alterations in the erythrocyte size after exposure to certain toxicants in fish have been reported. An increase in erythrocyte diameter was observed by Lone and Javaid (1976), whereas these were found to shrink initially and then expanded in the blood of *Channa punctatus* (Chakarbarty and Banerjee, 1988; Johal and Grewal, 2004). The most complex change was that erythrocytes formed chain pattern (Sawhney and Johal, 2000). During present investigation, slight clumping of cells in the erythrocytes has been seen which increased with increase in the exposure period. The cytoplasmic content was also found to start oozing out resulting in the formation of crenate cells with projections known as echinocytes. This could be attributed to the effect of the toxicant leading to the bursting of cells. As observed during the present investigation, shrinkage of cell membrane of RBCs of the fish exposed to the pesticide has also been reported in *Anabas testudineus* exposed to rogor (Singh et al., 1979).

On exposure of the fish to the toxicant for 30 days, the contraction from both sides of erythrocytes resulted in flattening. Fusiform cells having thin ends and round discocytes with biconcave shape have been observed which may be attributed to cholesterol enrichment. More clumping and shrinkage has also been noticed. Erythrocytes developed lobopodial projections due to protrusions of cytoplasm in the form of protuberances from the surface of cell. Fusiform cells, acanthocytes (cells with irregularly-spaced abnormal thorny projections, resembling burr cells), rhomboidal shaped cells, and cell with small mouth formation called stomatocyte were observed (Fig. 2 c, e). On exposure of the fish to the higher concentration, the cells were found to be swollen, ruptured with irregular outline. Serrations on the surface of echinocytes and acanthocytes were found commonly (Fig 2 f). Jindal and Rani (2005) also reported cytoplasmic disturbances and protuberances while studying the impact of methyl parathion on RBC's of *Cyprinus carpio.*

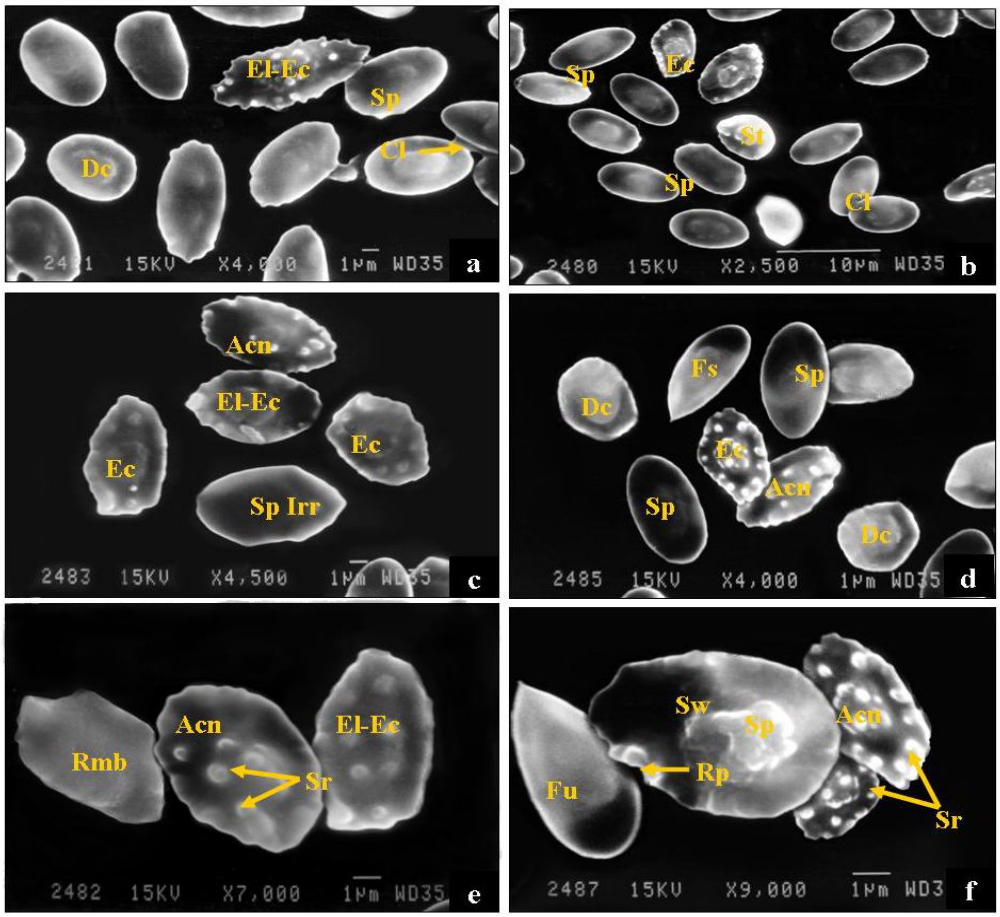

Fig 2 Scanning electron micrographs of erythrocytes of *Ctenopharyngodon idellus* on exposure to chlorpyrifos at 1.44 µg/l and 2.41 µg/l concentration on 30th day.

a, c, e: at 1.44 µg/l
b, d,-f: at 2.41 µg/l
Abbreviations:
Ery-Erythrocyte, Sp-Spherocyte, Fs-Fusiform cell, Ec-Echinocyte, Cl-Clubbing of Ery, Sh-Shrinkage, El-Ec-Ellipto-Echinocyte, Sr-Serrations, Acn-Acanthocyte, Rp-Ruptured, Sw– Swollen, Rmb– Rhomboid shaped cell, Sp Irr– Iregular Sperocyte, Dc-Discocyte, St-Stomatocyte.

On 60th day exposure of the fish to the toxicant, more pronounced effects were observed in the shape of erythrocytes. Fusiform cells and bud formation from one side of erythrocyte was noticed. The other changes were noticed were shrinkage, increased number of spherocytes and discocytes. Echinocytes with severe serrations were present. Rhomboidal shaped cells and cells with lobopodial projections were also found (Fig 3 a,c,e). At higher concentration of the toxicant, the cells with folded membrane and acanthocytes with severe serrations were observed. Extreme shrinkage of spherocytes turned them into cuboidal shaped cells. Also some ovalocytes (oval shaped cells) were observed (Fig 3 b, f). The modifications observed in the shape and size of erythrocytes due to effect of the pesticide could be explained as a common morphological abnormality that occurred in pathological conditions (Barnhart et al.,

1983). Similar findings have been made on the impact of organophosphate pesticides on *Channa punctatus* (Chakarabarty and Banerjee, 1988). Esteban et al. (2000), Strunjak-Perovis et al. (2009), Serezli et al. (2011) and Gupta & Poddar (2014) reported alterations in morphology of red blood cells of fishes. From the above altered shapes of red blood cells, it could be inferred that these effects might be due to disturbed lipid microenvironment of the membrane and due to increased lipid peroxidation as explained by Akahori et al. (1999). Lipid peroxidation is known to produce malondialdehyde which can interact with free amino groups of proteins and phospholipids causing structural and functional alterations of biomembranes (Teresa et al., 2002). Further, Sawhney and Johal (2000) documented that malathion induced abnormalities in red blood cells in *Channa punctatus* occurred due to depressed ATP under hypoxic condition.

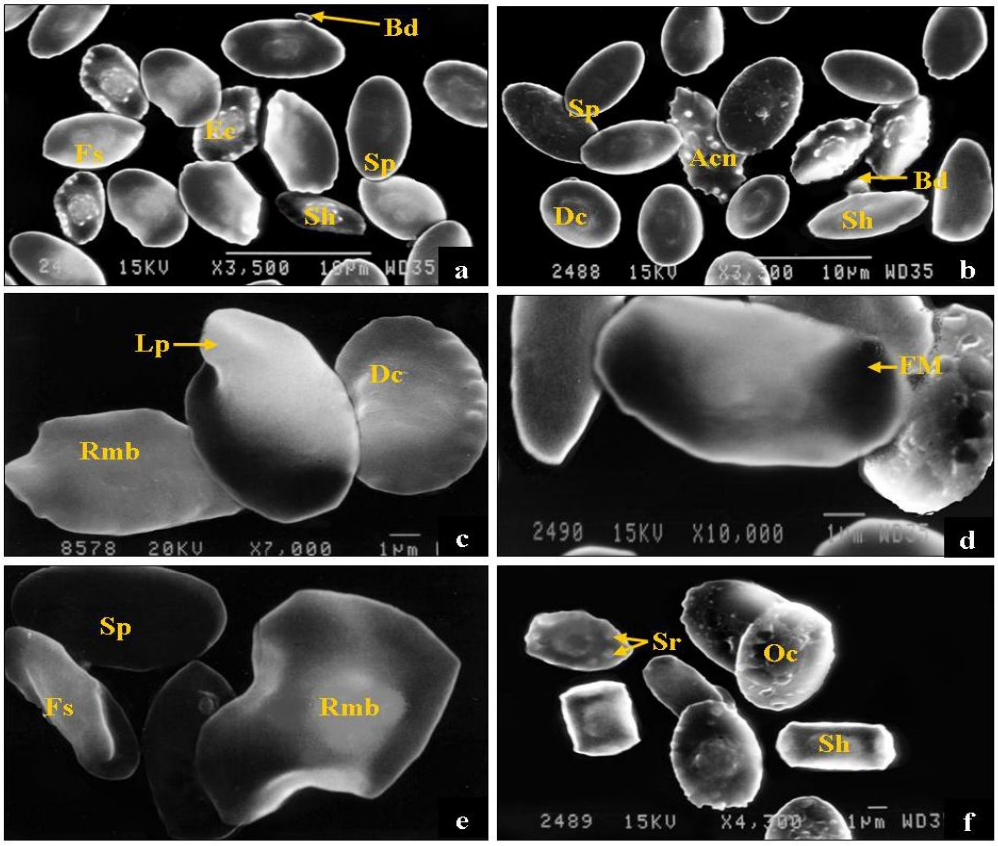

Fig 3 Scanning electron micrographs of erythrocytes of *Ctenopharyngodon idellus* on exposure to chlorpyrifos at 1.44 µg/l and 2.41 µg/l concentration on 60th day.

a, c, e: at 1.44 µg/l
b, d, f: at 2.41 µg/l

Abbreviations:
Ery-Erythrocyte, Sp-Spherocyte, Fs-Fusiform cell, Ec-Echinocyte, Cl-Clubbing of Ery, Sh-Shrinkage, Sr-Serrations, Acn-Acanthocyte, Rp-Ruptured, Sw–Swollen, Rmb– Rhomboid shaped cell, Sp Irr– Iregular Sperocyte, Dc-Discocyte, St-Stomatocyte, Oc-Ovalocyte, LP-Lobopodial Projection, FM-Folded Membrane, Bd-Bud formation.

So, from the present findings it could be concluded that chlorpyrifos caused a huge effect on the morphology of RBCs and affected the fish adversely.

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