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Sodium Fluoride Toxicity in the Fresh Water Cat Fish *Clarias batrachus* (Linn.): Effects on the Erythrocyte Morphology and Antioxidant Enzymes

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ABSTRACT

Fluoride is a naturally occurring compound present in the earth's crust which enters the ground and surface waters through natural and anthropogenic sources. Aquatic life is continuously exposed to high concentrations of fluoride in surface waters and harmful effects ensue when fluoride enters the food chain. Fluoride tends to be accumulated in the exoskeletons of invertebrates and in the bone tissues of fishes. The fluoride ions act as enzymatic poisons, inhibiting enzyme activity and, ultimately, interrupting metabolic processes, such as, glycolysis and synthesis of proteins. The present study consists of toxic effects of sub lethal concentration of NaF on the morphology of blood cells and antioxidant enzymes of the edible catfish *Clarias batrachus* under laboratory condition. Results indicate concentration and duration of exposure dependent induction of oxidative stress and subsequent alternations in the activities of antioxidant enzymes, superoxide dismutase and catalase. Changes in the morphology of blood cells like appearance of agglutination of blood cells, anisocytosis, schizocytosis, echinocytosis, hypochromasia, crenation, pyknocytes and cell fragmentation suggest activation of apoptotic machinery in the blood cells and these results appears to be related to a persistent condition of oxidative stress.

Key words: *Clarias batrachus*, sodium fluoride, morphological changes, blood cells, antioxidant enzymes, super oxide dismutase, catalase

INTRODUCTION

Fluoride is a naturally occurring compound present in the earth's crust in various forms including-Fluorspar (CaF_2), Cryolite ($NaAlF_6$) and Fluoroapatite [$Ca_{10}F_2(PO_4)_6$] and enters the ground and surface waters through natural and anthropogenic sources. Natural sources are runoff from weathering of fluoride containing rocks, whereas, anthropogenic sources include atmospheric deposition of emissions from coal-fired power plants and other industries. Although, fluoride is typically found in freshwater at concentrations less than 1.0 mg L^{-1}, however, natural concentrations may exceed even 50.0 mg L^{-1} (McNeely *et al*., 1979). The World Health Organization (WHO) has recommended the upper limit of Fluoride to be 1.5 mg L^{-1}. In humans, low concentration of fluoride contributes beneficially in the hardening of teeth and protection from caries while excess of fluoride is responsible for fluorosis due to its retention within the body and deleterious integration into biochemical pathways, often as a substitute for calcium (Barbier *et al*., 2010).

Aquatic life is continuously exposed to high concentrations of fluoride in surface waters and harmful effects ensue when fluoride enters the food chain and accumulates in the exoskeleton of invertebrates and bone tissue of fishes. The fluoride ions act as enzymatic poisons, inhibiting enzyme activity and interrupting metabolic processes, such as, glycolysis and protein synthesis (Camargo, 2003). Fishes make excellent bioassay animal models in toxicological studies (Hollis *et al*., 1999; Kumar *et al*., 1999). Presence of pollutants in low concentrations although may not result in fish mortality but may still be toxic to them. The toxic effects of elevated levels of fluoride (F) on various aquatic species are well documented by Sigler and Neuhold (1972), Pillai and Mane (1984), Gikunju (1992) and Azmat *et al*. (2007). Other works include Narwaria and Saksena (2012) on behavioral and Srivastava *et al*. (2012) on biochemical (glucose, total protein, triglyceride, cholesterol, SGOT, SGPT and ALP) response to sodium fluoride. Comparative toxicological studies of textile dye wastewater on *Gambusia affinis*, revealed cytotoxic effects on RBCs as changes in their shape and variation in their size (Soni *et al*., 2006). However, no work has been done on the effect of fluoride on blood cell deformation in fishes.

The present study describes the toxicity of sodium fluoride on the fresh water catfish *Clarias batrachus* (Linn.) in terms of changes on the morphology of erythrocytes and activities of antioxidant enzymes, using standard techniques.

MATERIALS AND METHODS

Exposure to toxicant: Healthy catfishes (*Clarias batrachus*) within a range of 100-150 g b.wt. were procured live from the local fish markets and acclimatized for seven days in glass aquaria under laboratory conditions with continuous oxygen supply and daily feed. After acclimatization, the fishes were subsequently divided into four groups comprising of one control and three experimental groups (E1, E2 and E3) of 10 fishes each. Fishes of the control group were exposed to tap water while those from the experimental groups were exposed to sodium fluoride solutions for a short term duration of 96 h and concentrations 100 (E1),300 (E2) and 600 (E3) ppm.

Collection of blood samples: Blood was collected in two separate tubes-(A) 1 mL in heparin lithium coated tubes and (B) 4-5 mL in plastic centrifuge tubes at 24, 48, 72 and 96 h by cutting the caudal peduncle and processed further for studies on the following aspects:

- **Studies on morphology of erythrocytes:** Smears were prepared from the blood collected in the heparin lithium coated tubes (A), dried in air and fixed in absolute methanol. Staining of dried blood films was done in 0.2% Leishman stain prepared in absolute methanol (Dacie and Lewis, 1975). Microscopical examination of the slides was done at 100x magnification with immersion oil and photographs taken
- **Estimation antioxidant enzymes:** For the determination of antioxidant enzymes, blood collected in tube (B) was further centrifuged at 3000 rpm for 15 min to extract serum. Superoxide dismutase activity in serum was measured by the Pyrogallol auto-oxidation method employed by Marklund and Marklund (1974) and the activity of SOD expressed as units mg^{-1} protein min^{-1}. Activity of catalase was assayed by measuring the rate of breakdown of H_2O_2 at 570 nm according to the method of Sinha (1972) and measured as the amount of H_2O_2 consumed min^{-1} mg^{-1} of protein

Statistical analysis: A maximum of ten replicates were taken in CAT assay and six replicates in SOD assay for both control and experimental. The statistical significance of difference was measured by one way analysis of variance (ANOVA) using SPSS software for windows.

RESULTS

Short term exposure of *Clarias batrachus* to various concentrations of sodium fluoride (100, 300 and 600 ppm) at 24, 48, 72 and 96 h showed the following features.

Morphology of blood cells: Erythrocytes of control fishes showed the presence of normocytic and normochromic cells at 24, 48, 72 and 96 h (Fig. 1a-d). On the other hand, time and concentration dependent changes in the morphology of erythrocytes could be observed in response to fluoride toxicity and a large number of abnormal erythrocytes were visible depending upon the extent of toxicity. At 100 ppm NaF intoxication, hyperchromasia was observed to develop along with the presence of unusual deeply stained cells with increased thickness and pyknotic nuclei (Fig. 2a-d). At 300 ppm the cells showed agglutination, anisocytosis, schistocytosis, fragmentation and degeneration (Fig. 3a-d). Intoxication by 600 ppm of NaF showed hypochromasia and polychromasia (signs of immaturity), echinocytes in blood cells and numerous fragmentations at 96 h of exposure (Fig. 4d).

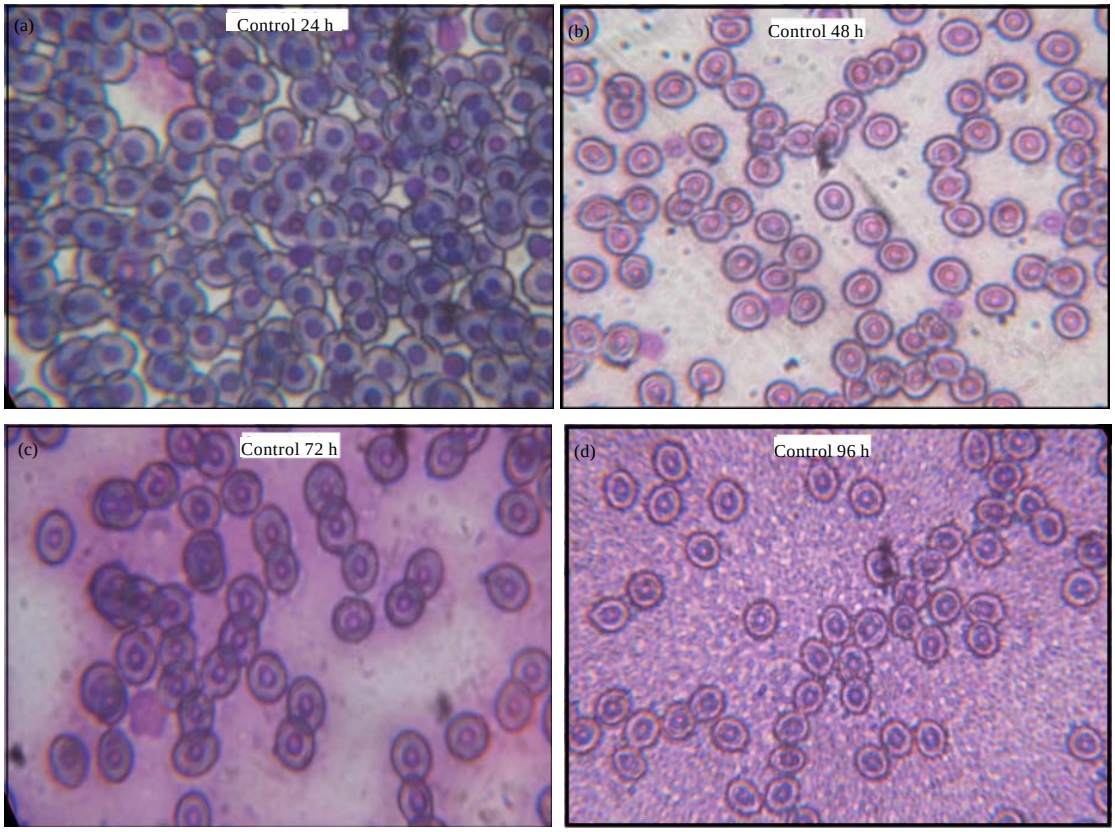

Fig. 1(a-d): Photomicrographs of morphology of blood cells. Control: Healthy erythrocytes showing normocytic and normochromic cells at (a) 24, (b) 48, (c) 72 and (d) 96 h, respectively

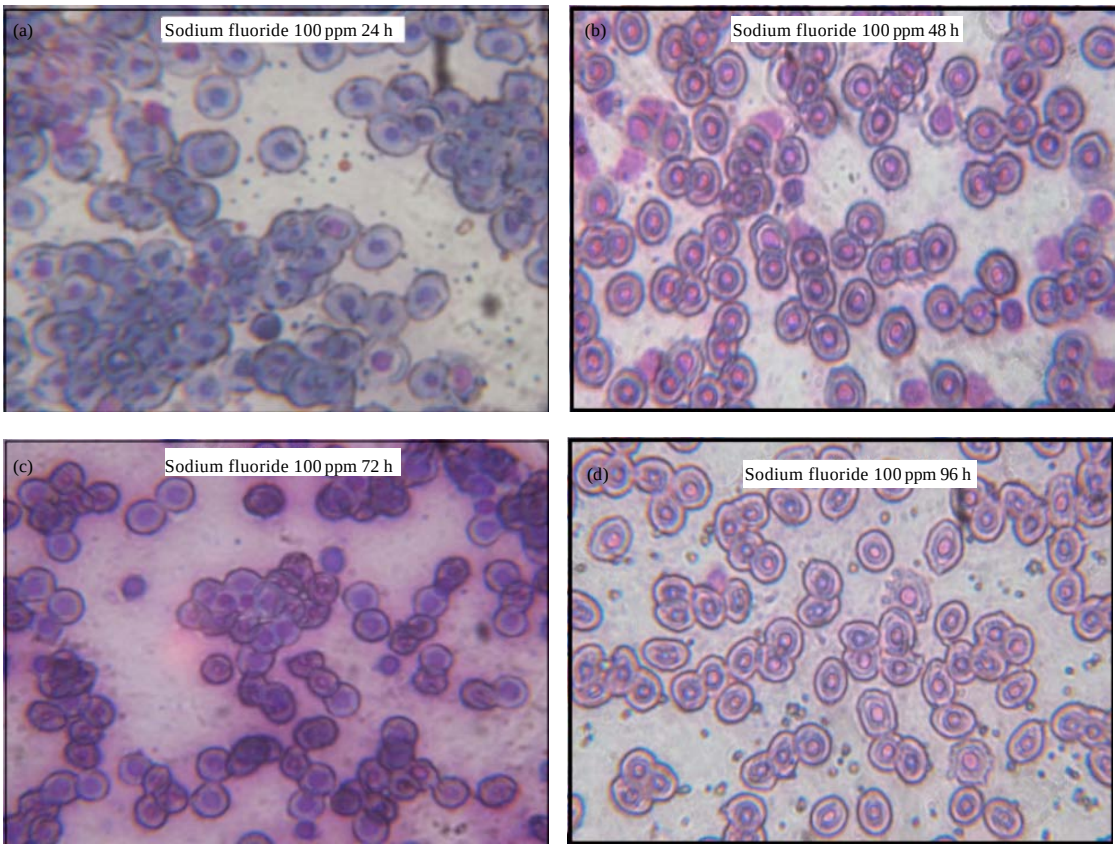

Fig. 2(a-d): Changes in morphology of blood cells after 100 ppm NaF intoxication showing abnormal changes in erythrocytes, anisocytosis, cells agglutination, hyperchromasia and pyknocytes at (a) 24, (b) 48, (c) 72 and (d) 96 h duration of exposure, resectively

Antioxidant enzymes: A significant decrease in the catalase activity with increasing the concentration of NaF and duration of exposure compared with control ($p<0.001$) was observed in case of catalase (Fig. 5), However, SOD activity showed a significant augmentation with increasing the concentration of NaF for a period of 24 h ($p<0.001$) and afterwards significant increase in SOD activity with increased concentration of NaF but with no significance with the duration of exposure (Fig. 6).

DISCUSSION

The present study shows concentration and duration of exposure dependent morphological changes in the blood cells in the form of hyperchromasia, agglutination, fragmentation and degeneration, besides presence of poikilocytosis, ranging between anisocytosis, schistocytosis and formation of echinocytes. Similar poikilocytosis and anisocytosis has been observed by Soni *et al.* (2006) in *Gambussia affinis* exposed to textile dye effluent.

In mammals, permeability of erythrocyte membrane to pollutants not only destroys them but also alters their shape and size by affecting the structure and function of cell membrane (Moss and Hatway, 1964; Udden, 2000; Suwalsky *et al.*, 2004). Qualitative and quantitative abnormalities

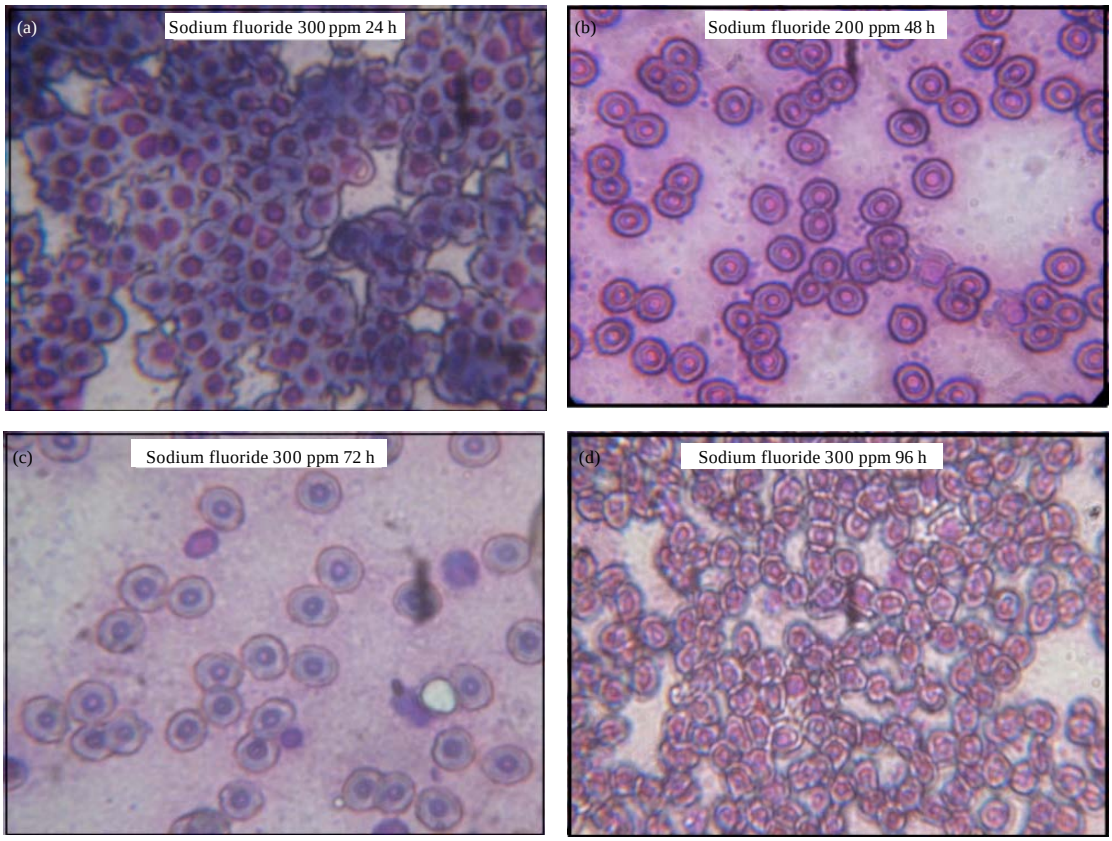

Fig. 3(a-d): Changes in morphology of blood cells after 300 ppm NaF intoxication showing higher number of cell agglutination in (a) and development of crenation in membranes of blood cells (Echinocytosis), schistocytosis with pyknotic nuclei, cell fragmentation and nuclear degeneration at (a) 24, (b) 48, (c) 72 and (d) 96 h duration of exposure, respectively

of spectrin and membrane protein band 4.1 have been reported to be associated with elliptocytosis in humans (Salsbury *et al*., 1968). Presence of echinocytes in blood cells has been reported by Susheela and Jain (1986) in rabbits ingesting excess fluoride. Shape changes and disorganization of spectrin network have been observed after addition of 1 mM sodium fluoride and 10 μM aluminium chloride in human red blood cells, where cells lost their membrane material and became smaller (Strunecka *et al*., 1991). Scanning electron microscopy of human RBC in response to fluoride toxicity by Shashi and Meenakshi (2012) also shows similar morphological alterations in their shape and presence of significant positive correlation between blood fluoride level and erythrocyte morphology. Hence, it is beyond doubt that the morphological abnormalities in erythrocytes observed in the present work are a consequence of fluoride toxicity.

Excessive fluoride ingestion has been identified as a risk factor for fluorosis and oxidative stress. It has been found to cause chronic or acute Reactive Oxygen Species (ROS) overproduction, exceeding the capacity of cellular antioxidant defense systems, cause oxidative damage to macromolecules such as DNA and proteins, peroxidation of membrane phospholipids and

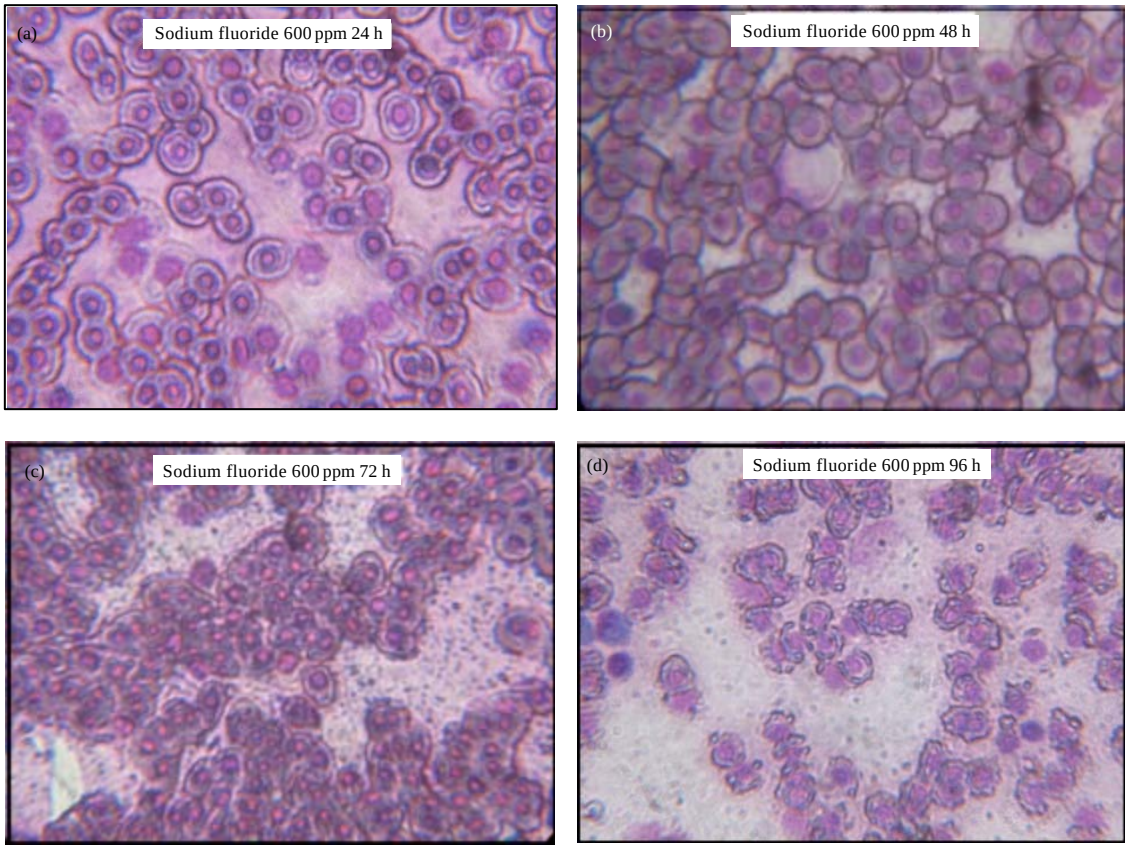

Fig. 4(a-d): Changes in morphology of blood cells after 600 ppm NaF intoxication showing highest number of abnomal blood cells, polychromasia echinocytes cells, hypochromic cells, highest cell fragmentation (Schistocytosis) and nuclear degeneration at (a) 24, (b) 48, (c) 72 and (d) 96 h duration of exposure, respectively

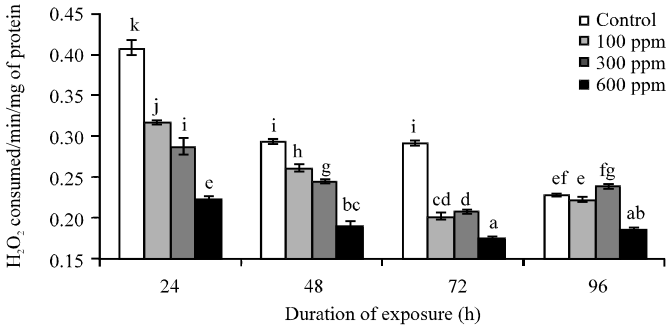

Fig. 5: Activity of catalase (H_2O_2 consumed/min/mg of protein) as function of duration of exposure (h) and concentration of NaF (ppm), factor, duration of exposure: df = 3.144, F = 371.18, $p<0.001$. Factor concentration of NaF: df = 3.144, F = 436.478, $p<0.001$: Factor duration of exposure×concentration of NaF: df = 9.144, F = 42.811, $p<0.001$ from ANOVA. Means bearing similar letters are not statistically significant from each other within variables (based on Duncan's multiple-range test)

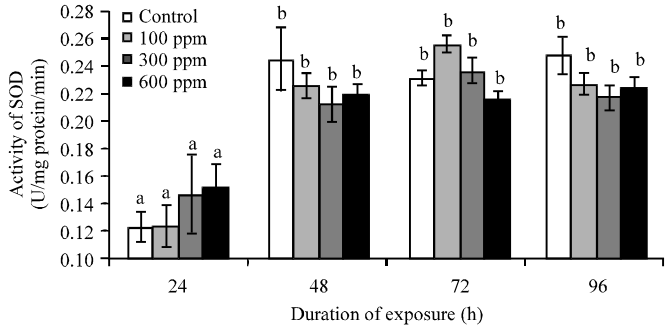

Fig. 6: Activity of SOD (U/mg protein/min) as function of duration of exposure (h) and concentration of NaF (ppm), factor, duration of exposure: df = 3.80, F = 50.19, p<0.001. Factor concentration of NaF: df = 3.80, F = 0.363, p = 0.78. Factor duration of exposure×concentration of NaF: df = 9.80, F = 1.484, p = 0.168 from ANOVA

mitochondrial depolarization, thus initiating cell disintegration and cell death as well as organ lesions (Agalakova and Gussev, 2012). Studies on the antioxidant enzymes in the present case also show a significant decline in the serum CAT activity of exposed fishes with increasing NaF concentration and duration of exposure and are suggestive of inhibition of catalase activity in response to Fluoride intoxication. Similarly, significant augmentation in SOD activity, with increasing concentrations in first 24 h followed by inability to cope up with its need in the subsequent exposure periods is indicative beyond doubt that fluoride affects the antioxidant enzymes. Generation of free radicals, lipid peroxidation and altered antioxidant defence systems are thought to play an important role in the toxic effects of fluoride (Guan *et al.*, 2000; Shivarajashankara *et al.*, 2001; Guven and Kaya, 2005). We agree with Chlubeka (2003) and Shanthakumari *et al.* (2004) that fluoride induces excessive production of oxygen free radicals and causes the depletion in biological activities of Super Oxide Dismutase (SOD) and catalase. Morphological deformations and degenerative changes in the blood cells suggest activation of apoptotic machinery as a consequence of persistent oxidative stress.

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