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Basic Violet-1 Dye Induced Variation in Antioxidative Enzymes Leads to Neurotoxicity in *Labeo rohita*

Rajbir Kaur[1], Arvinder Kaur[2] and Satinder Kaur[1]*

[1]PG Department of Zoology, Khalsa College Amritsar, Amritsar – 143002, Punjab, India; kaursatinder570@gmail.com

[2]PG Department of Zoology, Guru Nanak Dev University, Amritsar – 143005, Punjab, India

Abstract

The present study explored the potential use of brain anti-oxidative enzymes as pollution biomarkers in fish *Labeo rohita*. Experiments were conducted to see the neurotoxic effects of dye Basic violet-1 on fish brain enzymes such as lactate dehydrogenase, alkaline phosphatase and acid phosphatase. Acute toxicity tests were performed for 96 hrs with dye concentrations of 0.2 (LC_{20}), 0.4 (LC_{40}), 0.6 (LC_{60}), 0.8 (LC_{80}) and 1 (LC_{100}) mg/l to study biochemical changes. Behavioural observations were also recorded. Recovery experiments were run for 15 and 30 days. Exposure to the dye adversely affects the brain of the fish as evidenced by altered swimming behavior. Significant dose and duration-dependent increase in enzyme activity was recorded for alkaline phosphatase and acid phosphatase while lactate dehydrogenase activity showed a decrease when compared to the control. Lactate dehydrogenase was found to be a maximally affected enzyme as the percentage change over control is 301.26%. The results indicate prolonged neurotoxic effects of the dye as indicated by the altered swimming behaviour and enzymatic profile of fish. The present study recommends the use of *L. rohita* as sentinel species in water quality studies and supports the incorporation of fish biochemical profiles as a biomarker in water/wastewater monitoring programs.

Keywords: Antioxidative Enzymes, Azo Dyes, Biomarkers, *Labeo rohita,* Neurotoxicity

1. Introduction

A diversity of organic and synthetic substances is being introduced into the environment in the form of industrial wastes, domestic sewage, agricultural runoff and chemical spills. Most of these substances are washed away and carried by rains and floods to nearby aquatic ecosystems that serve as a sink for different pollutants[7]. Finally, these pollutants get dissolved in water where they tend to accumulate in the body tissues of inhabiting aquatic organisms. They accumulate these pollutants in their bodies, where the magnitudes of these pollutants are several times more than the amount present in water. The impregnated pollutants badly affect the organisms at physiological, behavioural, morphological and genetic levels. Hence, the accumulated mutations and other changes decrease the survival of individuals[16].

Azo dyes are an important group of xenobiotic compounds that are being used in a wide variety of daily-use products such as food and medicines. Different studies

**Author for correspondence*

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confirmed their toxic and persistent nature which makes them potent carcinogens and mutagens[2,18]. Along with industrial effluents, large quantities of unbound dyes (approximately 10-15% dye) reach different water bodies[27] and causes acute/chronic effects on fish and other aquatic fauna. Fish holds the highest position in the aquatic chain, so are greatly affected by such pollutants entering the water. These dyes cause alterations in biochemical and haematological parameters of fishes and other aquatic organisms due to their close contact with the contaminated water[8].

These tissue alterations are triggered by redox reactions generating free radicals, especially free oxygen radicals and Reactive Oxygen Species (ROS) due to dye contamination of water bodies[26]. To counteract the toxic effects of ROS, aerobic organisms use both enzymatic and non-enzymatic antioxidants. When ROS generation exceeds the capacity of the cellular antioxidants, it causes oxidative stress and oxidative damage in the target organism. The antioxidant system protects cells against the deleterious effects of oxy-radical generation by maintaining endogenous reactive oxygen species at relatively low levels and attenuating the damages related to their high reactivity. But some toxic chemicals affect the physiology and reproductive biology of the fish and thereby lead to disturbed integrity of ecosystems. At the same time contaminated fish poses a direct threat to human health because it is an important link in the food chain of humans[11].

In the present study, the acute toxic potential of Basic Violet-1 (BV-1) was evaluated in *Labeo rohita,* an Indian major carp. Fish are found abundantly in Indian rivers and are cultivated at a large scale in Punjab. *L. rohita* is considered a good experimental model because they are very sensitive to a vast variety of pollutants, entering the fish body from the water and affecting the biochemical and physiology of fish[10]. Levels of antioxidant enzyme, Lactate Dehydrogenase (LDH), Alkaline Phosphatase (AKP) and Acid Phosphatase (ACP) were estimated in the brain after 96h of exposure as an indicator of the stress of the selected lethal doses of the dye. To date, very little work has been reported on the effect of azo dyes on antioxidative enzymes in the brain of fish. Therefore, it is proposed to explore these biomarkers in fish as early indicators of toxicity of Basic Violet-1 (BV-1). Fish is a vertebrates and can accumulate or metabolize toxicants like human beings therefore the study will also throw light on the probable toxicity of this dye on the enzymes of human beings.

2. Materials and Methods

2.1 Chemicals

All Analytical Reagents (AR) grade chemicals used for the present study were purchased from SRL, Sigma-Aldrich and Himedia. Azo dye, BV-1 (CI: 42535) was purchased from the local market, in Amritsar, Punjab, India.

2.2 Animal Care

Fingerlings of *L. rohita* (9.6±0.8 cm in length and 19.01±1.02 g in weight) were procured from the ponds of Government Fish Farm, Rajasansi, Amritsar. Fish was subjected to an acclimation period of three weeks in plastic pools of 200 L capacity in the laboratory. Fish were fed on Toya floating pellets during acclimation as well as the experimental period except for 24 h preceding exposure and during the bioassay. Tap water was dechlorinated to remove chlorine from water so that chlorine will not interfere with the results obtained and also to make it safe for fish to consume. Tap water after dechlorination was used as diluent and control, and the test water was changed every day.

2.3 Experimental Design

Static renewal toxicity and recovery bioassays were performed with fish *L. rohita* (n = 15 fish/conc; N = 270). Fifteen fish were exposed to each concentration of 0.2 (LC_{20}), 0.4 (LC_{40}), 0.6 (LC_{60}), 0.8 (LC_{80}) and 1 (LC_{100}) mg/l of BV-1 for a duration of 96 h and each experiment was conducted in triplicates. Simultaneously, a control group was run using tap water having an equal number of fish. After completion of the test duration, five fish were sacrificed and ten fish were transferred to tap water for recovery periods of 15 and 30 days. Fish were fed during recovery periods and water was changed every day. The level of the enzymes was estimated in the fish brain after exposure durations of 96 h and recovery periods of 15 and 30 days with Systronic dual beam spectrophotometer-Genesis 10 UV. The brain was dissected out and kept in respective buffers, dried, weighed and homogenized in the cold buffer. Ice was kept around the tissue to avoid heating and denaturing the enzymes. The homogenate was centrifuged at 10,000×g for 45 min at +4°C and the

supernatant was collected for estimation of the levels of anti-oxidative enzymes.

2.3.1 Lactate Dehydrogenase

LDH activity was determined according to[25]. LDH is an oxidoreductase enzyme that catalyzes the interconversion of lactate and pyruvate. When disease or injury affects tissues containing LDH, the cells release LDH into the bloodstream, where it is identified at higher than normal levels. Therefore, LDH is most often measured to evaluate the presence of tissue or cell damage. Homogenate (10%) was prepared in Tris/NaCl solution (pH 7.2), the reaction mixture comprised 2.5 ml of 0.244 mM NADH, 0.5 ml of 9.76 mM pyruvate and 50 µl enzyme samples. The decrease in absorbance due to the oxidation of NADH was recorded at 340 nm for 5 min; the specific activity was calculated as U/min/mg protein.

2.3.2 Acid Phosphatase (ACP) and Alkaline Phosphatase (AKP)

Method[13] was used for measuring the activity of ACP and AKP. It is based on the principle that ACP catalyzes the hydrolysis of sodium-α-naphthyl phosphate to α-naphthol. Fast red salt reacts with α-naphthol to produce an end product which is an azo dye and was measured in a spectrophotometer at 540 nm at 30^{0}C. Homogenate (3.5%) was prepared in extraction buffer. 2 ml substrate solution was pre-incubated at 30^{0}C in a water bath for 10 min and 0.2 ml supernatant was added to it. The reaction mixture was incubated for another 30 min at 30^{0}C. Post-coupling solution (2 ml) was added to stop the reaction and the mixture was incubated for 1 hour at 30^{0}C. In the case of blank, the enzyme sample was replaced with an equivalent amount of distilled water. The formation of the end product was measured at 540 nm after one hour of the addition of the post-coupling solution. The procedure for estimating alkaline phosphatase was the same as that for acid phosphatase with the exception that acetate buffer was replaced with 0.05 M Tris buffer (pH 8.6) and 5% homogenate was prepared in extraction buffer. The specific activity was calculated as U/min/mg protein.

2.3.3 Protein Measurement

The Concentration of protein in the extract was measured by the method of of MacIntyre using Bovine serum albumin as standard[12].

2.4 Statistical Analysis

Data were subjected to ANOVA (SPSS and MINITAB) for finding out the differences in the activity of enzymes before and after exposure to the toxicant and within the groups. Tukey test was used for finding differences among the specific activity of anti-oxidative enzymes of fish. The biochemical results are reported as Mean±S.E. The differences were regarded as statistically significant when P<0.001 to P<0.05.

3. Results

A wide range of altered fish behaviours such as increased aggressiveness, decreased feeding ability, irregular movement and loss of coordination were recorded after 96 hr exposure duration. The fish showed avoidance behaviour to dye toxicant as fish moved to the corner of the aquarium and finally localized to the bottom of the aquarium. Behaviours such as loss of coordination, irregular and erratic swimming movements and loss of equilibrium followed by hanging vertically in water were also recorded.

Recorded enzyme activity values after 96 h test exposure and 15th and 30th day recovery periods are depicted in Tables 1-3.

Fish mortality was recorded at the highest dye concentrations of 0.8 and 1 mg/l within 96 hrs and in recovery experiments at a dye concentration of 0.6 mg/l. So, for these concentrations, the observations could not be recorded even after 96h exposure and for recovery experiments at a toxicant concentration of 0.6 mg/l.

3.1 Lactate Dehydrogenase (LDH)

Data for the change in LDH activity have been presented in Table 1.

Table 1. Effect of BV-1 on the activity of LDH (U/min/mg protein, Mean±S.E) after exposure and recovery durations

Conc./Specific activity	96 h	15th Day	30th Day
Control	6.38±0.1^a	4.46±0.02^a	6.69±0.08^a
0.2	5.37±0.1^a	2.18±0.02^b	4.37±0.08^b
0.4	3.67±0.04^b	1.18±0.02^b	3.59±0.09^b
0.6	1.59±0.07^c	----------	----------

Values with different superscripts (a, b, c and d) are significantly different at P<0.05.

Table 2. Effect of BV-1 on the activity of ACP (U/min/mg protein, Mean±S.E) after) after exposure and recovery durations

Conc./Specific activity	96 h	15th Day	30th Day
Control	0.249±0.004[a]	0.06±0.001[a]	0.22±0.01[a]
0.2	0.44±0.009[b]	0.11±0.002[b]	0.4±0.01[b]
0.4	0.562±0.006[b]	0.16±0.002[c]	0.59±0.02[c]
0.6	0.641±0.006[b]	----------	----------

Values with different superscripts (a, b, c and d) are significantly different at P<0.05.

Table 3. Effect of BV-1 on the activity of AKP (U/min/mg protein, Mean±S.E) after exposure and recovery durations

Conc./Specific activity	96h	15th Day	30th Day
Control	0.06±0.001[a]	0.23±0.01[a]	0.17±0.03[a]
0.2	0.22±0.006[b]	0.45±0.02[b]	0.36±0.03[b]
0.4	0.28±0.003[b]	0.54±0.002[b]	0.57±0.01[c]
0.6	0.56±0.016[c]	----------	----------

Values with different superscripts (a, b, c and d) are significantly different at P<0.05

The activity of LDH decreased dose-dependently over control (P<0.05) in the brain after 96 h exposure as well as till the end of the recovery period. The recorded values of LDH in test fish at the highest concentration were 1.59±0.07 as compared to control values of 6.38±0.1. A maximum decrease over control was observed in the brain after 96h exposure (301.26%) as well as on the 15th day (277.97%) of the recovery period

3.2 Acid Phosphatase (ACP)

A significant increase over control (0.249±0.004) (P<0.001) was observed in the activity of ACP in brain after 96 h (0.641±0.006) exposure as well as till the end of the recovery period (Table 2). The maximum increase in ACP activity was noticed in the brain on the 30th day (62.71%) of the recovery period.

3.3 Alkaline Phosphatase (AKP)

96 h exposure to the dye induced a dose-dependent increase over control (P<0.001) in AKP activity of the brain with a maximum increase (89.29%) after 96 h exposure (0.56±0.016) (Table 3). A similar trend of AKP activity was observed in the brain till the end of the recovery period. The maximum increase was observed in the brain on the 15th (57.41%) and 30th (70.18%) days of the recovery period.

4. Discussion

In the current study, exposure to BV-1 induced a variable amount of oxidative damage in the brain of *L. rohita*. It was observed that ACP and AKP increased while LDH decreased in the brain of the fish after 96h as well as till the end of the recovery period. This dye was observed to have a long-lasting effect on the defence system of the fish as hardly any reversal in the activity of selected enzymes was observed in the brain of the exposed fish even after 30 days of the recovery period. The altered physiological reaction of organisms by stressors is due to the biomarker changes, for example, decreased behavioural activity of organisms might be due to a reduction in respiration and Electron transport system which may result in oxidative damage to the tissues[4].

Decline over control in LDH activity after 96 h exposure as well as till the end of the recovery period could be due to the formation of an enzyme-inhibitor complex that led to impairment of carbohydrate metabolism[3] or reduction in de novo synthesis of proteins (enzymes) in the brain of the dye exposed fish. LDH converts lactate to pyruvate and has a very important role in carbohydrate metabolism, decrease in LDH activity in the current study can be directly correlated to the lethargy shown by the dye-exposed fish throughout the exposure and the recovery period. It was observed that on exposure to the present dye, the test fish swam vigorously for some time but hardly swam thereafter and ate very less during exposure as well as the recovery period[11]. LDH is the terminal enzyme of anaerobic glycolysis, and therefore is of crucial importance to physiology, particularly in conditions of chemical stress when high levels of energy may be required for a short period[14]. Sekar *et al.*, reported that a decrease in LDH activity reflected a possible decrease in the glycolytic capacity of the tissues[22]. Reduction in LDH activity in the liver, gill, muscle and brain of chlorpyrifos-exposed *Heteropneustes fossilis* has been reported by Vassault[24]. They suggested that the binding of the pesticide or its metabolites with the enzyme molecule was responsible

for the decline in LDH activity. A decrease in LDH activity has also been observed in *Clarias gariepinus and L. rohita* on exposure to lead nitrate and AB-1 dye, respectively[11,17].

A significant increase over control in the activity of ACP and AKP in the brain throughout the present study could be due to dye-induced cell damage as the increase was dose-dependent. When a cytotoxic agent like an azo dye induces necrosis/apoptosis in the exposed animals it leads to an increase in the mobilization of lysosomes, which in turn leads to an increase in the ACP and AKP in the tissues[21]. An increase in ACP activity was observed in the brain of *Spiralothelphusa hydrobromic* on exposure to textile dye industry effluent by by Tripathi and Shasma[23]. Amin *et al.*, reported a dose-dependent increase in AKP activity in Tartrazine-treated rats[1]. Increased ACP and AKP activity in stressed fishes has been reported by other workers[6,11]. A significant increase of acid phosphatase activity was reported in *Cirrhinus mrigala* exposed to lead acetate[19]. Kaur and Dua reported increased acid phosphatase activity in the gill, and liver tissues of *Oreochromis mossambicus* exposed to heavy metals[9]. Rao reported the level of acid phosphatase increased in the gill and liver tissue of *O.mossambicus* exposed to nickel electroplating effluent[20]. Tripathi and Shasmal reported a significant increase in the level of acid phosphatase activity in the *Channa punctatus* exposed to mercuric effluent[15].

5. Conclusion

The enzymatic defence system of the test fish was strongly affected by BV-1 because it caused significant changes in the activities of antioxidant enzymes in the brain of *L. rohita.* The present dye had a long-lasting effect as the alterations were prolonged till the end of the recovery period of 30 days. The activity of antioxidant enzymes changed in the brain of the fish under the influence of dye but maximum alteration was observed in the activity of LDH. Therefore LDH can act as an effective stress indicator of the present dye. This study will help in determining the consequences of direct as well as indirect exposure of man to BV-1 as the fish respond to toxins like higher vertebrates and form an important link in the food chain of man. The present study recommends the use of fish *L. rohita* as sentinel species in various water pollution studies. The study further supports the incorporation of a biochemical profile as a bio-marker in water/wastewater monitoring programmes.

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