

Effect of sublethal levels of nitrite on some blood parameters of juvenile *Labeo rohita* (Hamilton-Buchanan)

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Juveniles of *L. rohita* were exposed to sublethal levels of nitrite (0.02, 0.1 and 0.4 mg/l) for 2, 24, 48 and 96 hr. The time of exposure at individual concentrations of nitrite did not show any significant difference in haemoglobin, cortisol, chloride and lactic acid. Haematocrit showed significant reduction with increasing concentration of nitrite irrespective of duration of exposure. Fishes exposed to 0.4 mg/l nitrite showed significantly high levels of glucose beyond 2 hr. The mean erythrocytic fragility of fishes exposed to the 3 concentrations of nitrite for 3 exposure periods showed significant higher sensitivity to osmotic stress. The results suggest decrease in haematocrit and cell wall strength of erythrocytes creating stress to fish.

Keywords: Blood, Erythrocytes, *L. rohita*, Nitrite, Stress

The accumulation of nitrite in the environment is one of the principal problems in freshwater fish culture. Nitrite is highly toxic to freshwater fishes, but its toxic action is poorly understood¹.

Formation of methemoglobin, a substance that interferes with the ability of blood cells to carry oxygen when concentration reach 30-40% of total haemoglobin concentration is the most recognizable sign of nitrite toxicity in humans². Fish exposed to nitrite in the water are able to accumulate the anion in the blood plasma and tissues, with nitrite freely crossing the red cell membrane and oxidizing haemoglobin to methemoglobin. This gives a reddish brown colouration to blood, thereby reducing the functional haemoglobin^{3,4}.

Sublethal effects of nitrite has been studied in salmonids^{5,6}, *Ictalurus punctatus*^{7,8}, *Salmo gairdneri*⁹, fat head minnows¹⁰, *Catla catla*¹¹, *Ctenopharyngodon idella*¹ and in *Mugil platanus*¹².

The present study has been undertaken to evaluate the changes in various blood parameters of the fish *Labeo rohita* (Hamilton-Buchanan) exposed to sublethal levels of nitrite (0.02, 0.1 and 0.4 mg/l) for 96 hr.

Materials and Methods

Hatchery bred fingerlings of *L. rohita* of average length and weight (115 mm and 15.0 g) were

acclimatized in the laboratory in fibre glass tank containing chlorine free tap water at a stocking density of 0.4 g/l with continuous aeration at 12:12 L:D cycle. The water quality conditions were pH (7.7-7.8), water temperature (27°-28°C), alkalinity (136-160 mg/l) and hardness (170-190 mg/l). All the fishes were fed tubifex worms *ad libitum*. Water was exchanged daily and excretory products were siphoned out. Water quality parameters were measured as per APHA¹³.

After acclimatization for 1 month, 240 healthy fishes without any parasitic infestation were exposed to three sublethal levels of nitrite. The 96 hr LC₅₀ of nitrite for the fishes was found to be 2.5 mg/l. The fishes were divided into four groups. The fish in group 1 were not exposed to any treatments and served as control. Fishes in groups 2-4 were exposed to 0.02, 0.1 and 0.4 mg/l nitrite respectively for 96 hr.

All the experimental fishes were kept at the rate of 30 fish in 450 l of tap water in duplicate tanks with continuous aeration. The pH of the all tanks was maintained at 8±0.02 units with the temperature being at 28°C. Nitrite was introduced as reagent grade sodium nitrite and concentrations were confirmed by an azo-dye method¹³.

To measure red cell fragility, 50 µl of freshly drawn heparinised blood was added to each 10 tubes containing 3 ml unbuffered saline solution of 0.9, 0.7, 0.6, 0.5, 0.45, 0.4, 0.35, 0.3, 0.2, 0.1 g/dl NaCl. After 5 min the tubes were vortexed and centrifuged to

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sediment the whole red cells, cell fragments and blood clots. The absorbance of the decanted supernatant was determined at 460 nm using a 0.9% saline blank. Maximum haemolysis occurred at 0.1 g/dl NaCl. A plot of haemolysis versus saline concentration produced a sigmoid curve and the concentration at which 50% haemolysis occurred was the median corpuscular fragility in g/dl NaCl¹⁴.

Blood sampling and analysis—Ten fishes from each group were sacrificed after 2, 24, 48 and 96 hr from the start of the experiment. To obtain blood, *L. rohita* fingerlings were netted gently and rapidly anaesthetized using MS 222 (ethyl m-amino-benzoate methane sulphonate) at the dose of 60 mg/l and the fishes were immobilized within 1 min of application¹⁵. Blood was collected by severing the caudal peduncle. Heparin was used as an anticoagulant. Immediately after collection blood was centrifuged at 3000 rpm for 5 min in cold and plasma separated out was either used for analysis immediately or stored at -20°C for analysis later. Sampling procedure of netting, anaesthesia and plasma storing was completed within 10 min to avoid influence of netting, combined with anaesthesia on the basal cortisol levels^{16,17}. The methods employed for estimation of various blood parameters are glucose¹⁸, plasma chloride¹⁹, lactic acid²⁰, haematocrit²¹, haemoglobin (Sahli's method). Plasma cortisol was measured by a direct immunoenzymatic determination kit manufactured by Equipar SRL via G. Ferrari, 21/N 21047, Saronno, Italy using a 96 well microtitre plate read by ELISA microplate reader (Model EL311SX, Biotech Instruments Inc.). All the values of various parameters are expressed as mean $\pm$ SE. Analysis of variance (ANOVA) was performed to compare the means.

Results

Exposure of nitrite @ 0.02 mg/l to fishes revealed reduction in haemoglobin (Fig. 1a) and haematocrit (Fig. 1b) after 24 hr of exposure. However, haemoglobin value returned to control level after 48 hr. No significant change in the levels of cortisol, glucose, chloride, cholesterol, lactic acid was noticed up to 96 hr of exposure.

In 0.1 mg/l exposed fishes, lowering of haemoglobin and haematocrit level was observed at 24 hr (Fig. 1a, b). Haemoglobin and haematocrit values recovered within 96 hr of exposure. No substantial change in other parameters was found upto 96 hr of exposure.

In 0.4 mg/l nitrite exposed fishes, reduction in haemoglobin and haematocrit value was observed after 2, 24 and 48 hr of exposure (Figs. 1a, b). After 96 hr, recovery in their levels were recorded. Enhancement in the levels of cortisol (Fig. 1c), plasma glucose (Fig. 1d) and lactic acid (Fig. 1f) was found after 24 hr. Hypochloremia was recorded after 24 hr and 48 hr of nitrite exposure (Fig. 1e). Stabilization of plasma chloride level and also of other parameters was noticed after 96 hr in the exposed fishes. Analysis of variance revealed that the different levels of nitrite did not show any significant difference over time of exposure in haemoglobin, haematocrit, cortisol, chloride and lactic acid. In haematocrit nitrite application showed significant reduction in the value irrespective of duration of exposure. Exposure of nitrite @ 0.4 mg/l to fishes showed significantly high levels of glucose over control beyond 2 hr.

Osmotic fragility—Statistically significant higher sensitivity to osmotic stress, compared to control (0.375 mg/l) was observed (Table 1). However, after 2 hr of exposure, the 0.02 mg/l nitrite exposed fishes were less sensitive to osmotic stress than control with mean erythrocytic fragility (MEF) being 0.365 g/dL of NaCl.

Discussion

It is evident that exposure to nitrite in water reduced the levels of haemoglobin in the fishes. The nitrite ions in water enter fish through the chloride cells^{22,23}. From the blood plasma, nitrite diffuses into red blood cells, where it oxidizes the iron in haemoglobin to the +3 oxidation state, which lacks the capacity to bind oxygen reversibly^{24,25}. Degeneration of red cell structural and functional properties may cause an increased removal of red cells from the circulation and contribute to the

Table 1—Mean erythrocytic fragility (MEF) in *L. rohita* exposed to different nitrite levels
[Values expressed as g/dl are mean $\pm$ SE]

Nitrite concentration (mg/l)	2 hr	24 hr	96 hr
Control	0.375 $\pm$ 0.0058	0.375 $\pm$ 0.0058	0.375 $\pm$ 0.0058
0.02	0.365 $\pm$ 0.0033	0.425 $\pm$ 0.0029	0.435 $\pm$ 0.0076
0.1	0.420 $\pm$ 0.0029	0.435 $\pm$ 0.0058	0.452 $\pm$ 0.0049
0.4	0.435 $\pm$ 0.0025	0.430 $\pm$ 0.0087	0.450 $\pm$ 0.0029

decrease in total haemoglobin seen in nitrite-exposed fish²⁶. Shrinkage in red blood cells was evident in blood films of nitrite-exposed fishes. This may have reduced the haematocrit levels in various exposed fishes. This is in agreement to the observation Jensen *et al.*²⁶.

The elevations in cortisol and glucose at 0.4 mg/l nitrite exposed fishes were a result of general stress related physiological changes. Elevation of

hypothalamus pituitary interrenal (HPI) axis causes the rise in blood cortisol²⁷, which in turn enhances the blood glucose level²⁸. Elevation of plasma cortisol and glucose have been well documented as primary and secondary stress responses respectively, in fish²⁹. Carmichel *et al.*³⁰ reported an increase in plasma cortisol and glucose in stressed large mouth bass.

Reduction of plasma chloride observed is a characteristic effect in nitrite-exposed fish²⁶ where

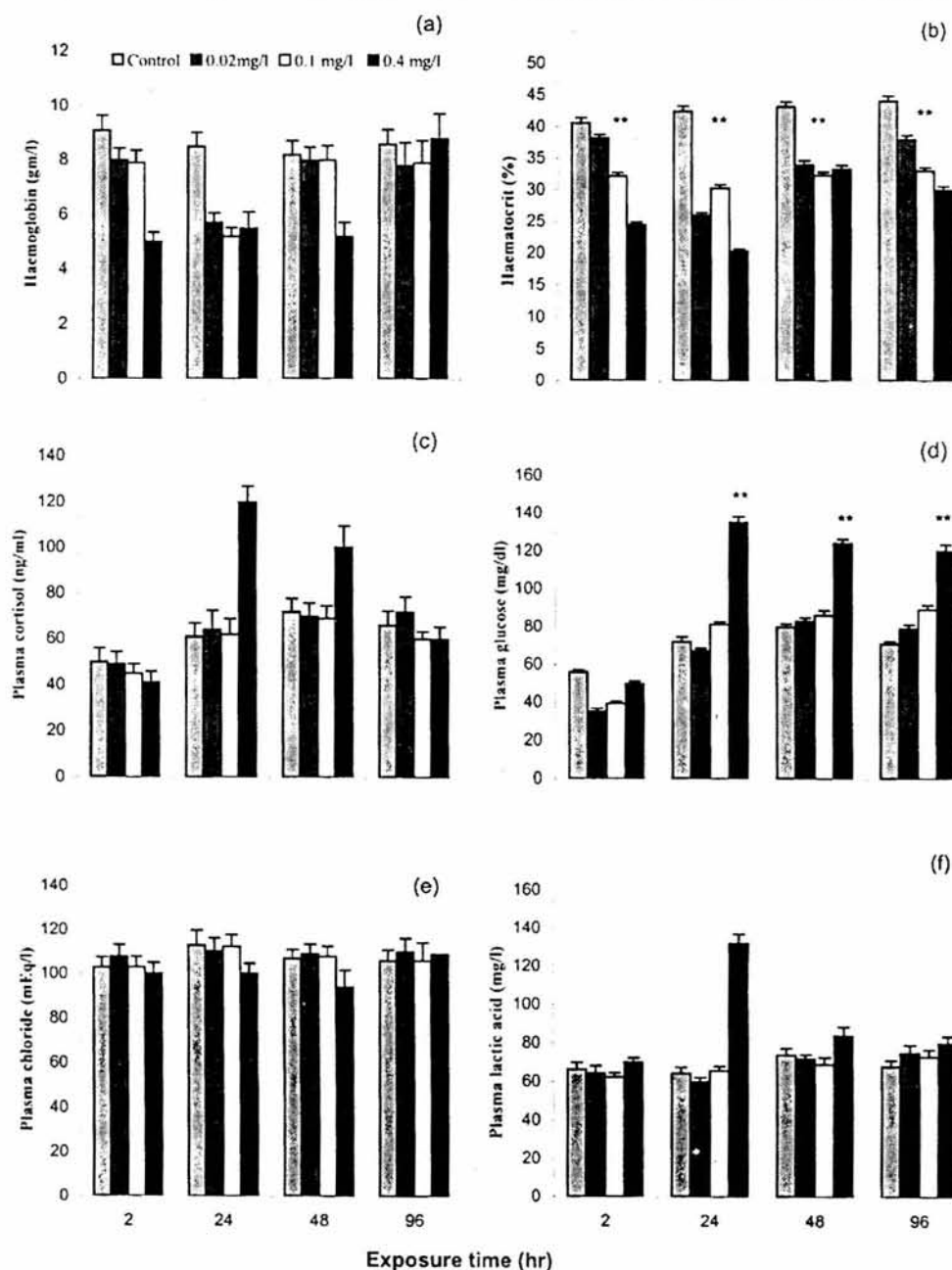


Fig.1—Changes in the physiological parameters of *L. rohita* fingerlings of control and subjected to sublethal nitrite (0, 0.02, 0.01 and 0.4 mg/l) for 2, 24, 48 and 96 hr (a: haemoglobin, b: haematocrit, c: plasma cortisol, d: plasma glucose, e: plasma chloride, f: plasma lactic acid). **Significant at 1% level.

passive efflux of Cl^- ions takes place and active branchial Cl^- uptake is partially converted to NO_2^- uptake. Perrone and Meade³¹ proposed that nitrite present in the environment is actively pumped across the gills at the chloride uptake sites (chloride cells) of fishes. Nitrite enters the fish via the same route as chloride by being a competitive inhibitor of the active chloride uptake mechanism of gills³². This uptake mechanism rationalizes why an elevation of environmental chloride levels protects against nitrite accumulation and toxicity³. Nitrite increases chloride cell activity to maintain ion homeostasis³³. Cortisol is known to cause proliferation of chloride cells on the gills³⁴. The increased cortisol levels as observed in 0.4 mg/l exposed fishes possibly trigger the elevation in number of chloride cells. An increased chloride cells density may enhance the chloride uptake capacity in response to the chloride depletion inside the system, which at the same time may be maladaptive by increasing nitrite uptake from the environment.

Except in fishes exposed for 2 hr in 0.02 mg/l concentration of nitrite, all fishes at different exposure period and at different concentrations exhibited decrease in cell wall strength. But the haemoglobin levels recovered possibly by recruitment of new RBC as a result of stress induced adrenaline stimulation of spleen¹⁴.

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