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Impact of Nitrite Toxicity on Histopathological Profile To Freshwater Fish, Cirrhinus Mrigala.

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----------ABSTRACT----------

The present study was to evaluate nitrite toxicity in fish gills during long-term exposure of sublethal concentration of sodium nitrite (NaNo2). The effect of nitrite on histopathological parmeters were evaluated exposing Indian freshwater fish, Cirrhinus mrigala to a sublethal concentration of nitrite (28.31ppm) for different period from 7 to 35 days (7, 14, 21, 28, 35) days. During 7th day exposure, the primary gill lamellae swollen due to hyperplasia of epithelial cells. At the end of 14th day, mucous cells were located in the secondary lamellae and on 21st and 28th day hypertrophied epithelial, lamellar hyperplasia in the middle part of secondary lamella, epithelial lifting, aneurysms lifting, lamellar fusion, and bulging was detected. The observed changes were severe as the exposure period of gill at the end of 35th days.

KEYWORDS: *Sodium nitrite, Cirrhinus mrigala, fish gills.*

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I. INTRODUCTION

Aquatic environment from natural process, waste water from industrial process releases nitrite continuously. Nitrogen constitute a core group like (Nitrite, Nitrate and Ammonia) of aquatic pollutants. The crux of the problems lies infact that these elements not only accumulate in water and sediment but also concentrate in the tissues causing alteration at various functional levels of the organisms (Vutukuru et al., 2005). In fish nitrite are absorbed by the gill and digestive tract (Verbost et al., 1989) and can accumulate in different organs such as kidney, liver and internal tissues (Levesque et al., 2003). Fish are useful experimental models that have been widely used to evaluate the health of aquatic ecosystems and toxicologic pathology (Korkmaz et al., 2009). Histopathology is an important component of several measures of fish health and histopathological markers have been recommended for field application, more often as a generalized, nonspecific response to severe stressfull stimuli (Teh et al., 1997). Gills are the most delicate structure of the teleost body that they are liable to damage by any irritant material in water whether dissolved or suspended. Gills therefore are potentially useful to monitor the health of fish (Palaniappan et al., 2010).

Fish gills comprise a large part of fish body that contacts the external environment and play an important role in the gas and ion exchange between the organism and environment (Witeska et al., 2006; Oliva et al., 2009). The apparatus and the skin, which are continuously exposed to aquatic medium, are the principal sites activity of numerous toxic substances (Brunelli et al., 2008). The gill surface is more than half of the entire body surface area. In fish the internal environment is separated from the external environment by only a few microns of delicate gill epithelium and thus the branchial function is very sensitive to environmental contamination (Cengiz, 2006). Since gills are considered the most vulnerable organ to external pollutants owing to their direct contact with water, facilitating the lamellar epithelial penetration and ingress into blood circulation (Gernhofer et al., 2001). Several histological studies have been carried out inorder to determine the physiological and morphological changes in gills (Mallat, 1995). Gill lesions as indicators of exposure to contaminants have previously been used in numerous laboratory and field studies around the world (Tophon et al., 2003). Gill therefore, are potentially useful to monitor the health of fish (Lacroix et al., 1993). The gills in fish can be a valuable model for assessing the effects of toxicants on cells and tissues are stated by Mallat (1985) and Mallat et al. (1995). Structural alterations in gills of fish inhibiting polluted water has been studied by many workers (Bucher and Hofer, 1993; Cengiz, 2006; Kellya Janz, 2009).

Krishnani et al. (2003) observed gill epithelial necrosis in Lates calcarifer exposed to copper. Das et al. (2004) in Labeo rohita observed gill epithelial necrosis due to nitrite exposure. The changes in gills were observed in P. goniontus in the thickening of primary lamellar epithelium and clubbing of secondary lamella exposed to cadmium (Wangsonsak et al., 2007). Mishra and Mohanty (2008) observed the changes in gills of Channa punctatus like epithelial necrosis, desquamation and aneurism exposed to chromium. Nile tilapia exposed to cypermethrin treatments snows lifting of epithelia, edema and hypertrophy of epithelial cells (Korkmaz et al., 2009). Cengiz, (2006) reported desquamation and necrosis in the gill of Cyprinus carpio to

deltamethrin. Although toxicant impairs the metabolic and physiological studies alone do not satisfy the complete understanding of pathological conditions of tissues under stress. Hence it is useful to have an insight into histological analysis. The extent of severity of tissue damage is a consequence of the concentration of toxicant and is time dependent (Tilak et al., 2001). Also the severity of damage depends on the toxic potential of particular compounds on salt accumulated in the tissues (Jayanth Roa, 1985). Microscopic examination of target tissues is an important end point in the evaluation of toxic potential and risk assessment of chemicals in the environment. Hence the present study was aimed to investigate the nitrite toxicity at acute and sublethal concentration on histopathological lesions in gill tissues of Cirrhinus mrigala.

II. MATERIALS AND METHODS

Histopathological profiles of gills were studied by the methods Pearse (1968), Roberts (1978) and Humason (1979). The changes in physico-chemical characteristics, such as temperature, PH, Dissolved oxygen, alkalinity, hardness, salinity, calcium and magnesium of experimental water were recorded throughout the experimental period. Freshwater fish Cirrhinus mrigala, weighing 5.0-6.0 gm and measuring 7-8 cm were collected from Tamilnadu fisheries development corporation limited, Aliyar fish farm, Aliyar, Tamilnadu, India. Fish of same age and size which hatched from the same lot of eggs were collected, the age of fish being 2 to 3 months old. They were safely brought to the laboratory in well packed polythene bags containing aerated water and stocked in a large cement tanks (36' x18'x19'). Fish were acclimatized for about 20 days before the commencement of the experiment. During acclimatization period, fish were fed with ad libitum, with rice bran and ground nut oil cake in the form of dough once in daily. Water replaced every 24h after feeding in order to maintain a healthy environment for the fish. This ensures sufficient oxygen supply for the fish and the environment is devoid of any accumulated metabolic waste. The feeding was withheld for 24h before the commencement of the experiment and to keep the specimens in the same metabolic state. The feeding was withheld for 24h before the commencement of the experiment and to keep the specimens in the same metabolic state. The fish were introduced into glass aquarium (26'x18'x18.5') cm which was washed thoroughly and maintained in the laboratory. Separate circular plastic tubs of 50 litres of water capacity were taken and different concentrations of nitrite were added. 10 healthy fishes were introduced into each tub. A control tub (no toxicant) with 50 litres of water and 10 fishes were also maintained. Three replicates were maintained for each concentration groups. The mortality/ survival of fish in control and nitrite treated tubs was recorded after 24h and the concentration at which 50% mortality of fish occurred was taken as the median lethal concentration (Lc50) for 24h. Sublethal values were found to be 28.31 ppm. For histopathological studies fish were treated with nitrite and various steps were followed for this study. Fixation, washing, dehydration, clearing, infiltration, embedding, sectioning, staining.

FIXATION

The gills were dissected out from the control and nitrite treated fish were cut into bits of 1 to 2 cm in diameter. These tissues were immediately put in Bouin's fluid for 24h to avoid post-mortem changes and shrinkage during further process like dehydration, embedding and sectioning.

WASHING

After fixation the tissues were taken and the excessive fixative was removed by transferring the tissues to 50% alcohol (to prevent interference with subsequent process).

DEHYDRATION

In dehydration process the tissues were put into alcohol series like 30%, 50%, 70%, 90% and absolute alcohol and duration of 30 min. was given in each alcohol series. To ensure complete removal of water from the tissues a minimum of two or three changes were given in absolute alcohol at an interval of 30 minutes.

CLEARING

During clearing alcohol was replaced from the tissues by using a clearing agent xylene. The tissues were kept in xylene for about 30 min to 1h until they become transparent.

INFILTRATION

During infiltration the tissues were kept in a paraffin embedding bath (metal cups filled with paraffin at 58-60^{0}C). Then the tissues were transferred directly from xylene to molten paraffin. A minimum of three changes were given in paraffin wax with 30 minutes duration in each.

EMBEDDING

For embedding, L blocks were filled with molten paraffin wax. Then the tissues were placed with proper orientation. After embedding process, the blocks were kept in water overnight to ensure complete solidification. Finally, blocks were removed for sectioning.

SECTIONING

Tissues were cut with 7µ thickness using a rotator microtome. Then the sections were spread on a glass slide using egg albumin as an adhesive. After complete spreading, the sections were placed in an oven overnight at 37^0C and the sections were taken for staining.

STAINING

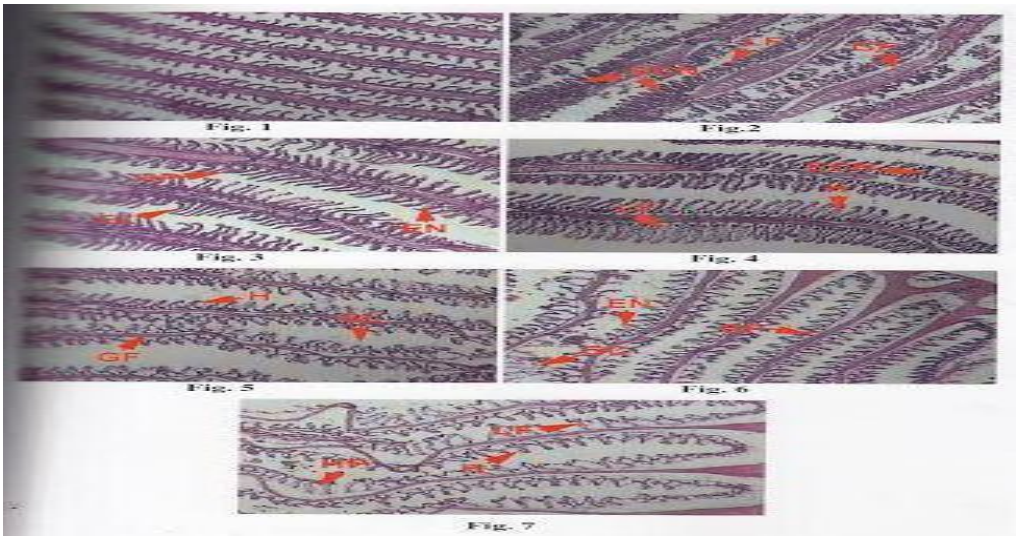
During staining paraffin wax from the sections was removed by dewaxing using xylene. Then the sections were hydrated by immersing in descending grades of alcohol (absolute alcohol, 90%, 70%, 50%, 30%) for about 1-2 min. in each. Then, the sections were stained in Heidenhain's iron haematoxylin stain for 2 to 5 min, washed in tap water until the sections become bluish black in colour and then stained in 1% Eosin.

For destaining, 1% iron alum was used. The sections were dehydrated through ascending grades of alcohol (30%, 50%, 70%, 90%, absolute alcohol) for about 5 sections in each. Subsequently, the sections were cleared in xylene and mounted permanently with cover glass using DPX mountant. The histological results of gills of fish from control and nitrite treated were given as photomicrographs in appropriate places in the text.

III. RESULTS AND DISCUSSION

In control fish, gill filament were seen and secondary lamellae were lined along both sides of the gill filament. The surface of the control gill lamellae was covered with epithelial cells running parallel along the surface. This epithelium consisted of different cells types including pavement (epithelial), mucous (goblet), and chloride cells (Ph.m.1). Histopathological structure of Cirrhinus mrigala exposed to acute concentration of nitrite was shown in Ph.m.2. During acute treatment, fish showed increased mucous secretion, hyperplasia of the epithelial cells, hyperplasia and fusion of secondary lamella.

Ph.m.3 shows the gill morphological structure of Cirrhinus mrigala when exposed to sublethal concentration of nitrite for 7 days. During the exposure period, the primary gill lamellae swollen due to hyperplasia of epithelial cells and fusion of the marginal areas of adjacent were noticed. At the end of 14^{th} day, mucous cells were located in the secondary lamellae and distally in the primary lamellae, while chloride cells were only observed on the basement of the secondary gill lamellae (Ph.m.4). Ph.m.5 and 6 shows the gill morphological structure of Cirrhinus mrigala when exposed to sublethal concentration (21^{st} and 28^{th} days) of nitrite. During above treatment, hypertrophied epithelial, lamellar hyperplasia in the middle part of the secondary lamella, epithelial lifting, aneurysms lifting, lamellar fusion, and bulging was detected. The observed changes were severe as the exposure period extended showing a complete rupture of gill at the end of 35^{th} day (Ph. m. 7).



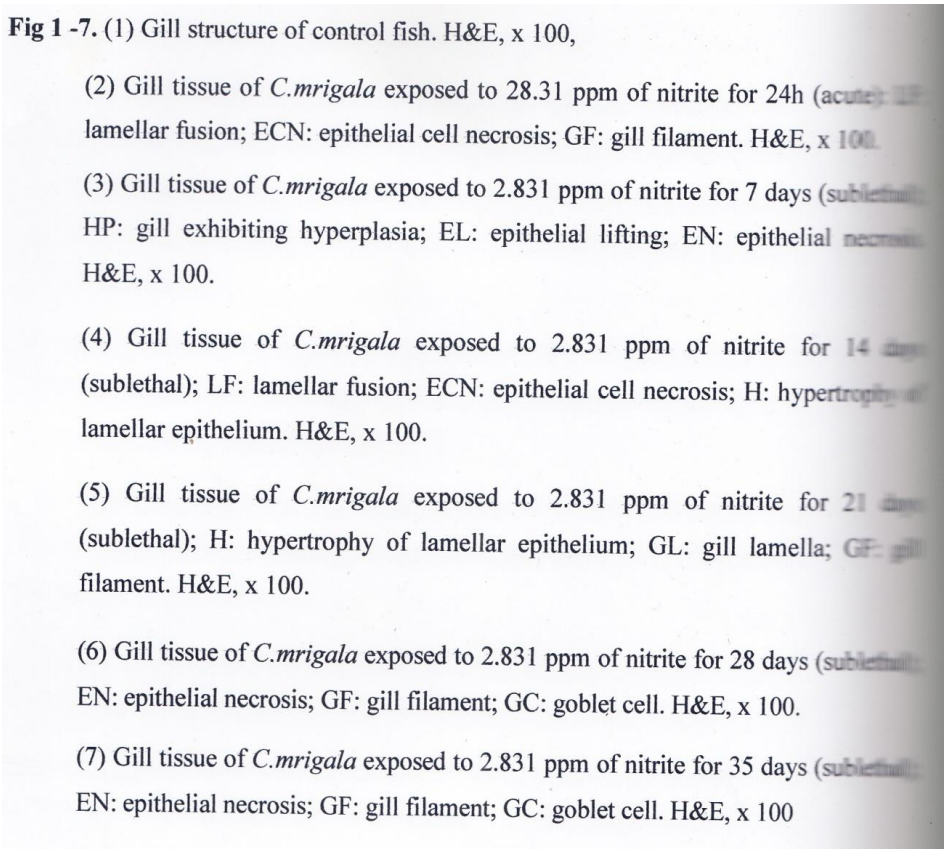


Fig 1 -7. (1) Gill structure of control fish. H&E, x 100,

(2) Gill tissue of *C.mrigala* exposed to 28.31 ppm of nitrite for 24h (acute): LF: lamellar fusion; ECN: epithelial cell necrosis; GF: gill filament. H&E, x 100.

(3) Gill tissue of *C.mrigala* exposed to 2.831 ppm of nitrite for 7 days (sublethal): HP: gill exhibiting hyperplasia; EL: epithelial lifting; EN: epithelial necrosis. H&E, x 100.

(4) Gill tissue of *C.mrigala* exposed to 2.831 ppm of nitrite for 14 days (sublethal); LF: lamellar fusion; ECN: epithelial cell necrosis; H: hypertrophy of lamellar epithelium. H&E, x 100.

(5) Gill tissue of *C.mrigala* exposed to 2.831 ppm of nitrite for 21 days (sublethal); H: hypertrophy of lamellar epithelium; GL: gill lamella; GF: gill filament. H&E, x 100.

(6) Gill tissue of *C.mrigala* exposed to 2.831 ppm of nitrite for 28 days (sublethal): EN: epithelial necrosis; GF: gill filament; GC: goblet cell. H&E, x 100.

(7) Gill tissue of *C.mrigala* exposed to 2.831 ppm of nitrite for 35 days (sublethal): EN: epithelial necrosis; GF: gill filament; GC: goblet cell. H&E, x 100

Gills represent a thin and extensive surface (up to 90% of the total body surface) in intimate contact with water. Due to the constant contact with the external environment, gills are the first target of waterborne pollutants (Poleksic and Mitrovic-Tutundzic, 1994; Fernandes and Mazon, 2003). Gills are well known target organs in fish, being the first to react to unfavourable environmental conditions (Benli et al., 2008). Fish gills have a large surface area covered by a thin layer of epithelial cells, and are well supplied with blood to facilitate gas exchange. Hence, gills are often most affected by exposure to toxicant and histological examination of gill tissue may reveal sublethal effects, such as toxicant – induced structural changes (Mallat,1985). In the present study control individuals did not show any histopathological changes in the tissues examined by the light microscope. However during acute and sublethal treatment of nitrite the fish Cirrhinus mrigala shows histological alterations such as hyperplasia of the epithelial cells, hemorrhages with rupture of the lamellar epithelium and lifting of the lamellar epithelium. During acute treatment the hyperplasia was more severe, resulting in the fusion of some secondary lamellae. Further, alterations such as hypertrophy of epithelial cells, lamellar aneurysms and lamellar disorganization were also observed. Similar observation was made by many authors (Benli et al., 2008). During prolonged exposure edema and hyperplasia of the respiratory epithelium of secondary lamellae was observed. The present investigation during sublethal treatment lifting and swelling of the lamellar epithelium and lesions, separation of respiratory epithelium, and alterations of pavement cells were observed. The above structural alterations were severe when the exposure period extended. Most differences between control and sublethal nitrite exposed fish were observed in the gills which include lamellar deformations; fusion secondary lamellae and telangiactasis compare to control group.

Histological damage to gill surfaces by nitrite is attributed to high accumulations in gills, irritation due to elevated mucous secretion, increased ventilation volume and decreased gill oxygen uptake efficiency. Engelhardt et al., (1981) observed epithelial lifting and lamellar fusion in rainbow trout Oncorhynchus mykiss exposed to petroleum residues. Winkaler et al. (2001) found anomalies such as hyperplasia, hypertrophy, dilation of the marginal channel and aneurysms in Neotropical fish Astyanax altiparanae collected from contaminated water. Miron et al. (2008) reported edema and fusion of the secondary lamellae in gills of silver catfish exposed to ammonia levels above 96 h-LC50. They also suggested that all these lesions may reduce gill functional surface for gaseous exchange, impairing respiratory function.

According to Mallat (1985) such alterations are non-specific and may be induced by different types of contaminant. He concluded that the most common gill lesions induced by toxic substances and other chemicals are necrosis, hyperplasia, hypertrophy and rupture of gill tissues, lamellar fusion, hyper secretion and proliferation of mucous cells, alterations in chloride cells and vascularisation. Alterations in epithelial lifting, hyperplasia and hypertrophy of the epithelial cells, besides partial fusion of some secondary lamellae are examples of defence mechanisms (Velasco-Santamaria and Cruz-Casallas, 2008). Since, in general, these results in the increase of the distance between the external environment and the blood and thus serve as a barrier to the entrance of contaminants (Mallat, 1985; Poleksic and Mitrovic Tutundzic, 1994; Fernandes and Mazon, 2003). In ordered to increase the epithelial area for diffusion and thus reduce the absorption of pollutants in to the blood, epithelial hyperplasia is considered as a protective response to toxics, irritants and environmental stressors (Mallat, 1985; Albassam et al., 1987). Lamellar aneurism, on the other hand, represents a lesion that can result from the rupture of pillar cells, and this corresponds to the deleterious effect of xenobiotics on branchial tissue (Martinez et al., 2004).

Hinton et al. (1992) reported that most part of the gill lesions cost by sub lethal exposures affects lamella epithelium however, some alterations in blood vessels may also occur, when fishes suffer a more severe type of stress. In this case, damaged pillar cell can result in an increased blood inside the lamellae, causing dilation of the marginal channel, blood congestion or even an aneurysm (Takashima and Hibiya, 1995). The formation of an aneurysm is related to the rupture of pillar cells (Martinez et al., 2004) due to a bigger flow of blood or even because of the direct effect of contaminants on these cells. This is a severe type of lesion, recovery from which is possible, but more difficult than the epithelial changes (Poleksic and Mitrovic – Tutundzic, 1994). Earlier authors divided the commonly reported gill lesions into two groups; (1) the direct deleterious effects of the irritants and (2) the defence responses of the fish. In the present investigation the observed epithelial necrosis and desquamation of the gill epithelium are direct responses induced by the action of nitrite. The defense responses noticed are lifting up of the epithelium and lamellar fusion. The lifting of the epithelium increases the distance through which the toxicant has to travel to reach the blood stream. Lamellar fusion could be protective in that it diminishes the amount of vulnerable gill surface area. Gill hyperplasia might serve as a defensive mechanism leading to a decrease in the respiratory surface and an increase in the toxicant blood diffusion distance.

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