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Histopathological alterations in spleen of freshwater fish *Cyprinus carpio* exposed to sublethal concentration of sodium cyanide

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Abstract
Aquatic ecosystems in areas with intense mining activity are often subject to cyanide contamination; the present study was aimed to evaluate the harmful effects of sodium cyanide on histoarchitechtural aspect of spleen of freshwater fish *Cyprinus carpio* using an *in vivo* approach. The fishes were exposed to a sublethal concentration of 0.2 mg/L of sodium cyanide for duration of 10 and 20 days and were further allowed to undergo recovery for 14 days in a toxicant free medium. From the present investigation findings like occurrence of haemosiderin pigment, melanomacrophage centers, vacuolation and necrotic eosinophils were evident in all the fishes exposed to sodium cyanide. However, changes were more pronounced in fish subjected to 10 days of exposure, which was followed by 20 days of exposure and 14 days of recovery. The study revealed that there seemed to be the presence of homeostatic mechanism in fish that allows them to stabilize and overcome stress, which in present case is caused by sublethal concentration of sodium cyanide. Since the recovery phenomenon may be adaptive and even strategic, the present investigation also throws a light on adaptive behaviour of fish under stressful environments.
Keywords: Histopathology, Melanomacrophage center, Recovery studies, Sodium cyanide, Spleen.

Introduction

One of the most important and poisonous substances known to man is cyanide. Cyanide is a noxious substance and possesses a property of killing both target and non-target organisms when discharged into the environment (Dube and Hosetti, 2011). The toxicity of cyanide is due to its influence as a respiratory poison in almost all forms of life (Yen *et al*., 1995). Acute doses of cyanide are usually lethal, due to marked susceptibility of the nerve cells of the respiratory centre leading to hypoxia (Greer and Jo, 1995). Chronic cyanide intoxication has been implicated in numerous anomalies such as ataxic neuropathy (Osuntokun, 1981), goitre (Cliff *et al*., 1986) and histopathology (Dixon and Leduc, 1981).

Sodium cyanide being extremely toxic is also very functional in various fields and hence is used in large scale by the international mining community to purify gold and other precious metals through milling of high grade ores and heap leaching of low grade ores. This process adequately needs cycling of millions of gallons of alkaline solutions containing high concentrations of potentially toxic sodium cyanide, free cyanide and metal cyanide complexes, which then openly access the aquatic ecosystems. The discharge of toxic pollutants into water bodies may perhaps result in the chronic toxicity in fish (LeBlanc and Bain 1997).

Cyanide and cyanogenic compounds are crucial toxic components which commonly exist in the environment and cyanide toxicity to the fish can be influenced by a variety of factors including concentration, environmental temperature and dissolved oxygen content (Ballantyne, and Marrs 1987). Earlier studies suggest that the toxicity of cyanide is categorically linked to variations in the enzyme activities of liver (Ma and Pritsos, 1997), and is also associated with the aetiology of goitre (Cliff *et al*., 1986), tropical ataxic neuropathy (Osuntokun, 1981) and epidemic spastic paraparesis (Howlett *et al*., 1990). However, the possible toxic consequence of cyanide on histoarchitechtural aspect of the immunological organ spleen is very limited.

Spleen being a major peripheral lymphoid organ plays an important role in antigen trapping (Hansen, 1997). Spleen serves as one of the primary haemopoitic organs, as teleost fish have no modulatory cavity in their bones (Agius and Roberts, 2003). Lymphocytes and the macrophages are the mainly concentrated areas in earlier histopathoogical studies as they are important for the defence system of the fishes (Fournie *et al*., 2001; Kurtovic *et al*., 2008). Although spleen is known for its vital role in the immune system regulation, comparatively, less attention is attributed to its structure and microanatomy.

A large number of studies have indicated the toxic effect of cyanide on different organs of *Cyprinus carpio* (David and Kartheek, 2014a,b,c). However, little is known about the direct effect of cyanide exposure on spleen. Therefore the present study is

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undertaken to evaluate the toxicological impact of sodium cyanide to immunological organ spleen using histopathological studies.

Materials and Methods

Collection and maintenance of fish

Healthy *Cyprinus carpio* were procured from the State Fisheries Department, Neersagar, Dharwad, Karnataka, India and were acclimatized to laboratory conditions for 15 days at 24 °C. Further they were held in dechlorinated tap water in large cement tanks which were previously washed with potassium permanganate to free the walls from any microbial growth. Fish were fed regularly and 12–16 h of photoperiod was maintained daily during acclimatization. Water was renewed daily, and its physico-chemical characteristics were analyzed following standard methods as suggested by American Public Health Association (APHA, 2005).

Grouping of Experimental Fish

The fishes were set apart into four different groups namely; Group 1 (control), Group 2 (10 day exposure), Group 3 (20 day exposure) and Group 4 (14 day recovery). Each group was maintained in triplicate and consisted of 10 fishes.

Preparation of stock and exposure of fishes

Sodium cyanide of 95% purity was procured from Loba Chemie Pvt. Ltd., Mumbai, India. Stock solution was prepared by dissolving sodium cyanide in double distilled water in a standard volumetric flask. Water was renewed every day over test periods. Henceforth, the replacement of the water medium was followed by the addition of the desired dose of the test compound. The fish were exposed in batches of 10 to a fixed concentration of sodium cyanide with 20 L of water in three replicates for each concentration. One tenth (0.1 mg/L) of the 96 h LC_{50} (1mg/L) was selected as sub lethal concentration for studies and the durations of exposure were 10 and 20 days. Further, the fish were allowed to undergo a recovery period of 14 days. This study was conducted under the guidelines issued by Organization for economic co-operation and development (OECD, 1992) for static-renewal test conditions. At the end of 10 and 20 days of exposure and that of 14 days of recovery, the fish were sacrificed and sampled for histopathological studies.

Histopathological analysis

For the histopathological examination, the method was followed as described by Humason (1972). The animal was dissected and the organ of interest (spleen) was isolated under aseptic conditions. The sample was fixed in Bouin's fluid for 24 to 48 h. Later, the tissue was processed in a series of graded alcohol and embedded in paraffin which was filtered thrice. Organs in paraffin were sectioned into 5 µm thick ribbons by using semi-automated microtome (LeicaRM 2255) and sections were stained primarily with haematoxylin and counter stained with eosin (H & E) for light microscopic examination (Lille, 1969). The sections were observed under 200X magnification. The microscopic view was photographed using an Olympus phase contrast microscope (Olympus BX51, Tokyo, Japan) with attached photography machinery (ProgResC3, Jenoptic-Germany). The photographed images were further observed for differences and the findings were recorded. The studies were carried out at Department of PG studies and research in Zoology after the approval from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Results

The present investigation revealed histopathological alterations in splenic section of fish exposed to sublethal concentration (0.2 mg/L) of sodium cyanide (Group 2 & 3) and relatively less number of lesions was noted in fish that were allowed to recover (Group 4). However, no lesions were observed in sections of spleen of control fish (Group 1).

The water analysis was carried out prior to the exposure studies and the results for the same were found as follows, temperature, 25 ± 2 °C; pH, 7.6± 0.2; dissolved oxygen, 7.7 ± 0.8 mg/L; total hardness, 30.4 ± 3.1 mg as CaCO3/L; salinity, nil; specific gravity, 1.003; conductivity less than 14 µS/cm; calcium, 17.86 ± 0.92 mg/L; phosphate, 0.4 ± 0.004 µg/L and magnesium, 0.8 ± 0.3 mg/L. The findings in the section of spleen of fish exposed to sodium cyanide are illustrated in Figure 1 (200x) and Figure 2 (400x). Various findings like haemosiderosis, melanomacrophage centers (MMC), vacuolation, necrotic eosinophils were observed in the spleen sections of all fish exposed to sodium cyanide. Furthermore, the severity of the findings in Group 2 was the highest followed by Group 3 and Group 4 which were exposed to a period of 10 and 20 days of exposure and 14 days of recovery, respectively.

Discussion

Over the years, spleen structure and function in many vertebrate species, including fish, has been studied due to its importance of involvement in immunity related role. In spleen of fish, white pulp proliferation, lymphocyte depletion, as well as an increase in the size of spleen, haemosiderosis and increase in melanomacrophage centers has often been associated with environmental contamination (Schwaiger *et al*., 1996; Gogal *et al*., 1999; Montero *et al*., 1999; Garcia-Abiado *et al*., 2004).

One of the important physiological features is melanomacrophage centers (MMC) which are seen in the fish spleen (Agius and Roberts, 2003). They are assumed to be the functional substitutes of the germinal centers of spleen (Ellis, 1980).

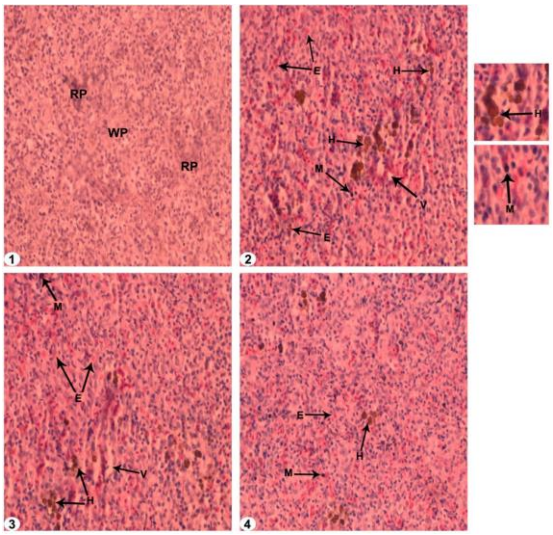

Fig. 1. Section of spleen showing: **1:** Normal architechture, Red pulp (RP), White pulp (WP); **2:** Necrotic eosinophils (E), Melanomacrophage (M), Vacuolation (V), Haemosiderin (H); **3:** Necrotic eosinophil (E), Melanomacrophage (M), Haemosiderin (H), Vacuolation (V); **4:** Necrotic eosinophil (E), Melanomacrophage (M) and Haemosiderin (H). H & E, 200X.

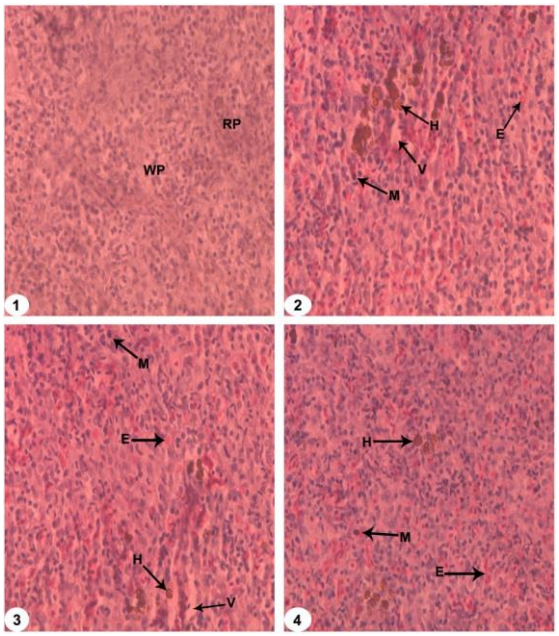

Fig. 2. Section of spleen showing: **1:** Normal architechture, Red pulp (RP), White pulp (WP); **2:** Necrotic eosinophils (E), Melanomacrophage (M), Vacuolation (V), Haemosiderin (H); **3:** Necrotic eosinophil (E), Melanomacrophage (M), Haemosiderin (H), Vacuolation (V); **4:** Necrotic eosinophil (E), Melanomacrophage (M) and Haemosiderin (H). H & E, 400X.

MMC may contain four types of brown pigments: melanin, lipofuscin, ceroid and hemosiderin (Couillard *et al*., 1999). Stressful conditions to the animal often result in increased number of its splenic MMC's (Montero *et al*., 1999), which is in agreement with the present investigation as a large number of MMC's are observed in splenic sections of fish exposed to sublethal concentration of sodium cyanide. Earlier reports by Prashanth and Neelgund (2007), David *et al*. (2008), Dube and Hosetti (2011) and David and Kartheek (2014a,b,c) have suggested the potential biochemical and histopathological toxicity of sodium cyanide towards different freshwater fishes.

Haemosiderin is one of the breakdown products of Hb from senescent and degenerated erythrocytes (Zapata and Cooper, 1990). Haemosiderosis is a pathological condition occurring due to the deposition of haemosiderin. Haemosiderosis is related to an increased rate of erythrocyte destruction in the spleen (Hibiya, 1982) which in present case is perhaps a consequence of sublethal cyanide exposure to the fish *Cyprinus carpio*. This in turn may result in decreased haemoglobin content which is usually attributed to RBC destruction and irregular movement of haemoglobin from the spleen in fishes (Scott and Rogers, 1981). Similar deposition of haemosiderin pigments has been observed in the spleen of fishes exposed to sodium cyanide in the present study. Therefore, it can be said that the findings from previous study and the observations of the present investigation are in a positive accord.

Kaleeswaran *et al*. (2010) suggested the increased severity in the MMC as a homeostatic mechanism of the fish spleen to phagocytose the increasing deposits of haemosiderin and other debris resulting from the destruction of tissues. This matches with the present study wherein the occurrence of MMC could be seen in splenic sections of exposed fish. The present investigation is also in agreement with the findings of Fournie *et al*. (2001) who associated the density of splenic macrophage aggregates in estuarine fishes to exposure to degraded environments. In another study, *Oreochromis niloticus* upon exposure to 1 µg/l of Chlorpyrifos, the sections of pronephros exhibited increased diameter of MMC, the study was reported by Holladay *et al*. (1996) and the reports obtained are in agreement with the present investigation.

The findings obtained in the present investigation are in agreement with reports of Spazier *et al*. (1992) who observed vacuolation in splenic tissue of European eel *Anguilla anguilla* following stress and resulting in impairment of normal physiology of fish. Immune suppression in present case may be a consequence of reduced number of mature lymphocytes. The data is in agreement with Anderson *et al*. (1989) who reported the study on immune-suppression in splenic section of rainbow trout upon exposure to different concentrations of chemical toxicant. Since alterations of the spleen can occur in some pathological

conditions (Gogal *et al*., 1999; Garcia-Abiado *et al*., 2004), it can be inferred that exposure to sodium cyanide in sublethal doses affects the histoarchitechture of spleen of *C. carpio*. But recovery in later periods may be a revitalization phenomenon as every organism strives to overcome the stress to prove its existence. Recovery phenomenon in the present case may be adaptive and even strategic. The findings obtained in the present investigation may also therefore contribute for the future studies in the field of immunotoxicology of fishes.

Conclusion

From the observations made in the present investigation, it may be inferred that sodium cyanide is highly toxic to freshwater fish *C. carpio* when exposed to a sublethal concentration of 0.2mg/L for 10 and 20 days. However, allowing the fishes to recover in toxicant free medium for 14 days may help to overcome stress and restore histoarchitechture of spleen to some extent. We concluded that the histopathological study may prove to be an important biomarker to characterize the toxicological impacts in the environment.

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Conflict of interest

The authors declare no conflict of interest.

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