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Copper Toxicity: haematological and histopathological changes and prophylactic role of vitamin C in the fish, *Anabas testudineus* (Bloch, 1792)

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Abstract

The sub-lethal effects of copper on the humoral, enzymological and histopathological parameters in the teleost fish, *Anabas testudineus* and the curative capacity of vitamin C were investigated. The 96 hour LC₅₀ value of copper was determined using Probit method and was found to be 1.74 mg/L. Fish blood from two sub-lethal concentrations, vitamin supplemented media and toxicant and vitamin free control were analyzed on 7th, 14th, 21st and 28th day of exposure. The haemoglobin (Hb), haematocrit (Hct), erythrocyte count (RBC) and oxygen carrying capacity were reduced compared to the control values up to the day 21 followed by a significant increase ($p < 0.0001$) in all test concentrations on the day 28. The microscopic observation of RBCs in higher concentrations and long exposure showed crenation, immature RBCs with enlarged nuclei, hypochromia and cytoplasmic vacuolation. The plasma lactate dehydrogenase (LDH) activity increased ($p < 0.0001$) progressively up to 21st day of exposure and then decreased significantly towards the control value on the 28th day. Copper exposure caused a dose and duration based increase in hepatotoxic biomarkers like glutamate oxalo transaminase (GOT) and glutamate pyruvate transaminase (GPT) in all the samples studied. The liver of exposed fish showed drastic architectural disruption such as hepatocyte degeneration, cell necrosis, inflammation with sinusoid dilation and thrombus formation. The present investigation clearly reveals toxicity of copper even at sub-lethal concentrations on the physiology and histology of the fish. The betterment of humoral, enzymological and histological aspects in vitamin treated fish illustrates the curative and prophylactic role of the vitamin against copper intoxication.

Keywords: Copper, Vitamin C, hematological and enzymatic parameters, histopathology, *Anabas testudineus*.

1. Introduction

Among all types of pollution, aquatic pollution is of greater concern as each and every kind of the life depends on water. Among all types of aquatic pollutants, heavy metals are of greatest concern. Heavy metals when reach the aquatic bodies deteriorate the life sustaining quality of water and cause damages to both flora and fauna (Nriagu^[1]).

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Heavy metals are considered as the main cause of aquatic pollution and the magnitude of environmental degradation they caused is far beyond the recommended threshold limit values (Abdel-Meguid et al.^[2]).

Eventhough most of the heavy metals are micronutrients, they exert a prominent role in environmental deterioration. The heavy metal and pesticide contamination of aquatic ecosystems has increased manifold in the last few decades due to their extensive use in agricultural, chemical and industrial processes and is a real threat to the aquatic fauna. Fishes are rather more vulnerable since they are frequently exposed to aquatic pollutants. Since fishes occupy the top of aquatic food chain, they are suitable bioindicators of metal contamination. Metals are well known inducers of oxidative stress, and assessment of oxidative damage in fishes can reflect metal contamination of the aquatic environment.

Among metals, copper is used in industries manufacturing organic chemicals, fertilizers, iron and steel works, electrical works, antifouling paints, pulp and paper industries, pesticides, fungicides and automobile accessories. Even though copper is an essential trace element required in low concentrations, it is discharged into freshwater environments in large concentrations as an industrial effluent and severely affect the freshwater fauna, especially fishes. Copper sulphate is widely used as an algacide for controlling phytoplankton in fish ponds and lakes as well as a herbicide used in aquatic weed control since 1882 (Carbonell and Tarazona^[3]). Copper intoxication increases mortality of fish offspring and weakens their condition, resistance and viability (Jeziarska and Witesca^[4]). Intensive industrial developments in the last few decades have increased the concentration of copper in aquatic ecosystems and that affected fish and deteriorate the natural resources. The major water bodies and rivers in Kerala are not free from the pollution stress of varying degrees due to the influx of effluents from nearby factories, pesticides and fertilizers from cultivating areas, besides urban and rural sewages. Periyar, a typical freshwater ecosystem and the longest river in Kerala is gradually undergoing eco- degradation due to various anthropogenic reasons and the concentration of copper in water and sediments was recorded as 0.075-2.59 microgram/litre and 0.055-4.32 microgram/gram respectively. Fishes acquire copper by the gills from the surrounding water, as well as from the diet by the digestive tract (Kamunde et al.^[5]). It is necessary for the synthesis of Hb and act as a component of many enzymes such as cytochrome oxidase.

Elevated aquatic copper levels cause a range of negative effects on fish such as reduced growth, interference with whole body iono-regulation and endocrine disruption. Many of the toxic responses in fish are in part due to the high reactivity of copper with H₂O₂ and potential to undergo redox reactions to form reactive oxygen species (ROS), that may cause irreversible cellular damage and death. High concentration of copper can alter hematological constitution and cause retarded growth and inhibition of spawning (James et al.^[6]). Specific information on the toxic effects of heavy metals on the fishery and the prophylactic and curative efficacy of vitamin C, a non enzymatic antioxidant is greatly lacking. The quantum of work on freshwater teleost fishes especially *Anabas testudineus* in relation to copper is relatively low. It was in this context that the present study was undertaken.

2. Materials and Methods

A static renewal bioassay method was adopted to determine the 96 hr. LC₅₀ (Sprague^[7]). *Anabas testudineus*, a common fresh water teleost was used for the study. The fish is grey green on the dorsal and lateral sides and yellowish green on the ventral side. Body is covered with large overlapping cycloid scales. In front of the gills are the accessory respiratory organs called labyrinthiform organs for aerial breathing.

Fishes were collected and acclimatized in the laboratory for a period of one month in 200 L. tanks disinfected with potassium permanganate solution before the start of the experiment. The physico-chemical parameters of the water were, temperature - 27.1 °C ± 2.4 °C, P^H - 7.2 ± 0.07 and dissolved oxygen - 7.74 ± 0.34mg/L. The oxygen saturation was maintained by aerating the holding tank with aquarium pump. Fishes irrespective of sex with a weight of 40 – 45 gm and length 8 – 10 cm. were selected for the experiment. They were fed once daily with a commercial feed and the water was changed one hour after feeding. Copper stock solution was made from hydrated copper sulphate (CuSO₄.5H₂O) manufactured by Merck Specialities Private Limited, Annie Besant Road, Worli, Mumbai, India and added subsequently to the water in experimental tanks to obtain desired test concentrations. Prior to the toxicity experiment, a range finding test was carried out. The acute toxic levels of copper were determined by static renewal test as suggested in (APHA^[8]).

Three replicates each containing twelve fish of equal size were exposed to different concentrations of the toxicant for a period of four days and mortality was noted after every 24 hours. Based on the result, concentrations for the definitive toxicity experiments were selected. Twelve healthy and active fishes were randomly selected and were transferred to each experimental tank containing 20L of dechlorinated tap water. The fish were observed regularly and the number of death in all media were recorded daily for a period of 96 hours. The number of dead organisms in each of the test series was recorded. Probit values were plotted on probit paper and the concentrations of copper that killed 50% of the test organisms (LC_{50}) for a period of 96-hour exposure with a 95% confidence limit were calculated (Finney^[9]) and was derived as 1.74 mg/L by following the computerized statistical package, SPSS 16.0. Based on the 96-hour LC_{50} value, two sub-lethal concentrations of copper such as $1/5^{th}$ and $1/15^{th}$ of 96 hour LC_{50} (0.34 mg/L and 0.113mg/L) and another set of sub-lethal concentrations supplemented with the antioxidant, vitamin C (Ascorbic acid, 2.5 mg/L) manufactured by Merck Specialities Private Limited, Annie Besant Road, Worli, Mumbai, India were maintained to evaluate the prophylactic and curative effect of vitamin C against heavy metal intoxication. A control medium was also maintained. All the media were renewed every 24 hours.

Fishes were caught on the 7th, 14th, 21st and 28th and anaesthetized for collection of blood samples for hematological studies. Blood was treated with EDTA to prevent coagulation. All the hematological analyses were performed using standard techniques. RBC count was performed haemocytometrically with Neubauer chamber (Davidson and Henry^[10]). Hb was determined by cyanmethaemoglobin method (Drabkins^[11]). The serum LDH was determined kinetically (Thomas^[12]). Serum GOT and GPT were determined kinetically (Rej and Vanterlind, Wolf and Williams^[13,14]).

Histopathological techniques and staining procedures were done by standard methods (Bucke, Bullock^[15,16]). Liver samples were collected on the 14th and 28th day of exposure after immobilizing the fishes and liver tissues were dissected out, cleaned in saline and fixed in 10% neutral buffered formalin for 24 h. After fixation, the tissues were graded in an ascending alcohol series and cleared in xylene. The tissues were embedded in paraffin wax. After paraffin infiltration, the sections were cut to a 5-micron thickness using a rotary microtome and sections were examined under the microscope and photographs were taken. Mayer's hematoxylin staining method was used.

3. Result

The hematological response of *A. testudineus* on exposure to sub-lethal concentrations of copper showed significant decrease ($p < 0.0001$) in RBC count, Hb and oxygen carrying capacity in the blood of fish exposed to sub-lethal concentrations of copper in comparison with the control group. The microscopic examination of the RBCs in higher concentrations depicted obvious morphological alterations such as crenation and enlargement of nuclei, hypochromia and immature RBCs (Fig.1.1) and cytoplasmic vacuolation and membrane degeneration. The results of the ANOVA indicated a significant reduction ($p < 0.0001$) of RBC count in all sub-lethal concentrations and the decrease was also significant with increase in exposure time. The supplementation of vitamin C in both sub-lethal concentrations enhanced the RBC count significantly ($p < 0.0001$) in all exposures. As a response of fish to copper, the Hb, RBC count and oxygen carrying capacity were improved significantly ($p < 0.0001$) and attained values near to the control in all test concentrations on the 28th day of exposure. The administration of vitamin C in all the sub-lethal concentrations brought these indices near to the control values.

As a toxic response, the serum LDH level increased with increasing copper concentrations in all test concentrations during the first three exposure periods in the present study. Up to the 21st day of exposure, serum LDH level showed prominent and progressive increase ($p < 0.0001$) compared to the control values. However on the 28th day, in both experimental media serum LDH level decreased markedly.

The enzyme activity was found to be affected significantly as a consequence of copper intoxication. Vitamin C administration brought the serum LDH level towards the control values. The serum GOT and GPT activity were high in the sub-lethal concentrations in comparison with the control values and their increase was significant ($p < 0.0001$) as per the results of the ANOVA. The increase in serum GOT and GPT activity to combat the copper induced stress in test concentrations were found to be proportionate to the increase in concentration of copper and the period of exposure. In vitamin C supplemented fish, serum GOT and GPT activity decreased significantly towards the control values. The changes in the humoral and enzymological parameters in experimental and control fishes are presented in Table 1.

Table 1: Variation in haematological and Enzymological parameters of *Anabas testudineus* on exposure to copper with and without supplementation of vitamin C.

Sub-Lethal Concentrations						
Hematological Parameter	Duration of exposure	Control	0.34 mg Cu./L	0.34 mg Cu./L+2.5 mg Vitamin C	0.113 mg Cu./L	0.113 mg Cu./L+2.5 mg Vitamin C
Haemoglobin (Hb)- (g%)	7 days	14.818 ±0.025	8.748±0.011*	9.31± 0.003*	10.791 ±0.005*	13.175 ±0.019*
	14 days	14.622 ±0.011	7.844 ±0.004*	8.26 ±0.008*	9.41 ±0.004*	10.57 ±0.003*
	21 days	13.94 ±0.024	6.317 ±0.003*	7.12±0.004*	8.117 ±0.004*	8.89 ±0.003*
	28 days	13.762 ±0.013	8.32 ±0.004*	9.24 ±0.006*	10.182 ±0.004*	10.98 ±0.012*
Erythrocyte (RBC)- (10 ⁶ /mm ³)	7 days	3.121 ± 0.004	2.42 ± 0.004*	2.53 ± 0.004*	2.752 ± 0.004*	2.92 ± 0.023*
	14 days	3.101 ± 0.001	2.195 ± 0.004*	2.301 ± 0.002*	2.588 ± 0.006*	2.69 ± 0.007*
	21 days	2.994 ± 0.002	1.87 ± 0.006*	2.092 ± 0.004*	2.282 ± 0.004*	2.425 ± 0.005*
	28 days	2.965 ± 0.002	2.322 ± 0.007*	2.31 ± 0.004*	2.682 ± 0.005*	2.838 ± 0.010*
Oxygen carrying capacity (ml O ₂ /g Hb)	7 days	18.52±0.032	12.221 ± 1.288*	11.637 ± 0.004*	13.489 ± 0.006*	16.446 ± 0.031*
	14 days	18.278 ± 0.013	9.805 ± 0.005*	10.324 ± 0.005*	11.762 ± 0.006*	13.212 ± 0.004*
	21 days	17.419 ± 0.033	7.896 ± 0.004*	8.901 ± 0.006*	10.146 ± 0.005*	11.112 ± 0.004*
	28 days	17.203 ± 0.017	10.399 ± 0.006*	11.549 ± 0.008*	12.728 ± 0.005*	13.724 ± 0.015*
Glutamate Pyruvate transaminase (GPT) -- (IU/L)	7Days	51.95 ± 0.12	90.57 ± 0.38*	76.2 ± 0.06*	70.84 ± 0.25*	58.12 ± 0.30*
	14Days	52.78 ± 0.27	108 ± 0.11*	90.21 ± 0.19*	81.61 ± 0.12*	69.07 ± 0.26*
	21Days	54.14 ± 0.23	121.22 ± 0.24*	112.24 ± 0.19*	93.87 ± 0.14*	78.82 ± 0.14*
	28Days	54.88 ± 0.07	139.75 ± 0.11*	124.64 ± 0.31*	103.20 ± 0.10*	93.67 ± 0.13*
Glumate Oxalate transaminase (GOT) -- (IU/L)	7Days	118.37 ± 0.26	245.22 ± 0.57*	201.84 ± 0.26*	124.62 ± 0.08*	121.2 ± 0.22*
	14Days	118.78 ± 0.32	291.88 ± 0.16*	168.85 ± 0.35*	139.04 ± 0.23*	128.4 ± 0.08*
	21Days	116.98 ± 0.16	308.72 ± 0.34*	178.45 ± 0.46*	147.35 ± 0.51*	137.54 ± 0.14*
	28Days	123.04 ± 0.21	321.25 ± 0.16*	198 ± 0.36*	158 ± 1.52*	145.91 ± 0.26*
Lactate dehydrogenase (LDH) (IU/L)	7Days	269.72 ± 2.09	408.31±1.46*	371.97 ± 0.56*	302.68 ± 0.52*	263.08 ± 0.35*
	14Days	268.37±0.27	433.2 ± 1.43*	387 ± 0.86*	323.88 ± 0.55*	283.57 ± 0.29*
	21Days	269.65 ± 0.25	456.85 ± 0.64*	416.51 ± 1.00*	346.27 ± 0.51*	303.52 ± 0.68*
	28Days	274.15 ± 4.03	422.07 ± 0.28*	382.55 ± 0.56*	321.54 ± 0.43*	277.77 ± 0.94*

Each value is the average of seven observations ± SE. * All values are significant at (p<0.0001)

Light microscopic study of the fish liver exposed to copper for 28 days showed several pathological changes. The liver cells were degenerated and the normal architecture of liver was markedly disorganized. Congestion of central vein, clumped erythrocytes and haemorrhages were observed in the liver of exposed fishes (Fig.3.1, 3.2). Hypertrophy of hepatocytes with pycnotic nuclei was quite evident in liver of fish exposed to both metal concentrations (Fig.4.1). Obvious degenerative changes observed against control fishes were marked increase in numbers of Kupffer cells, dilation and congestion of blood vessels and enlargement of sinusoids (Fig.5.1, 5.2) Furthermore, the hepatocytes exhibited focal necrosis resulting in complete disintegration of cellular components as evidenced by the presence of darkly

stained eosinophilic debris in liver of fish exposed to 0.34 mg/L for 28 days (Fig.4.1,3.1) There were typical changes in the size of hepatocytes and their nuclei were displaced to the periphery as a sign of vacuolization (Fig.3.2, 4.1). The cell membrane of some cells were ruptured resulting into fusion of two or more cells giving binucleate or even multinucleate appearance of hepatocytes. In some places hypertrophy and hyperplasia of bile duct cells and sign of blood vessel fibrosis were noted at higher concentration (Fig.3.1). Vitamin C treatment reduced degenerative changes such as vacuolar degeneration, fatty degeneration, nuclear pyknosis and necrotic changes to a great extent when compared to those fishes exposed to heavy metal intoxication.

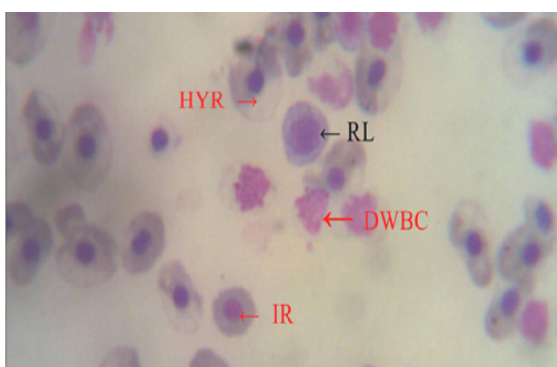


Fig 1.1: Blood of *A. testudineus* exposed to 0.34 mg/L copper for 21 days. hypochromic RBC's (HYR),immature RBC's (IR),reactive lymphocytes (RL) and degenerated WBC's (DWBC). Giemsa stained. (100X)

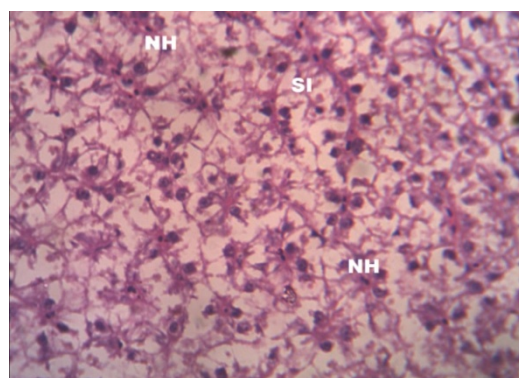


Fig 2.1: Normal histology of Liver of *A. testudineus*. normal hepatocyte (NH), sinusoid (SI). (H&E 400)

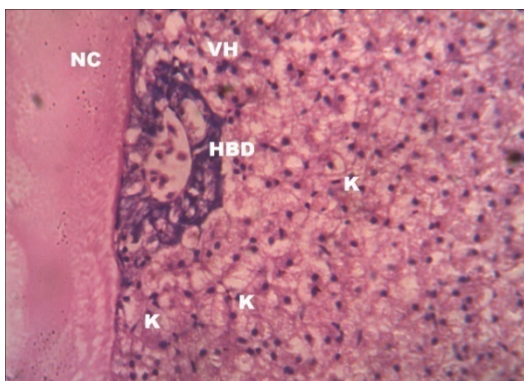


Fig 3.1: Histopathological alterations of Liver of *A. testudineus* exposed to 0.34 mg/L Copper of for 28 days. Necrosis (NC), vascular haemorrhage (VH), hypertrophied bile duct (HSB). kupffer cell (K). (H&E 400)

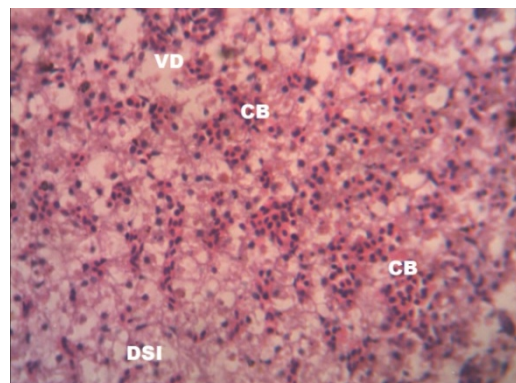


Fig 3.2: Histopathological alterations of the Liver of *A. testudineus* exposed to 0.34 mg/L Copper of for 14 days dilated sinusoids (DSI). Vacuolar degeneration (VD), congested blood vessel (CB). (H&E 400)

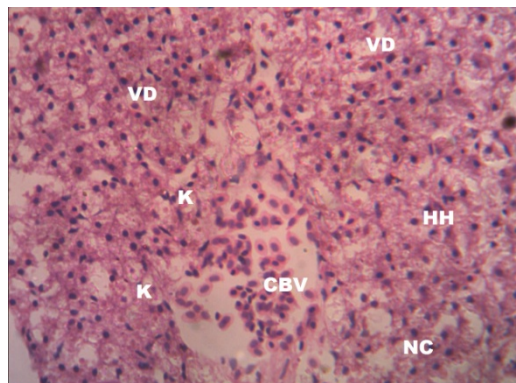


Fig 4.1: Histopathological alterations of the Liver of *A. testudineus* exposed to 0.11 mg/L Copper of for 28 days. Vacuolar degeneration (VD), necrosis (NC), hypertrophied hepatocyte (HH), congested blood vessel (CB). (H&E 400)

4. Discussion

The determination of acute toxicity is regarded as an initial screening step in the assessment and evaluation of the toxic characteristics of all compounds. The heavy metal contamination in aquatic bodies cause potent harm on the ecological equilibrium of the environment in general and organisms in particular not only due to their capacity to diminish the productivity and fecundity of organisms but the health of man also, being the final consumer in food chain, the contamination of fresh water ecosystems became a serious matter of concern. Environmental stressors such as metals may change the biochemical parameters in exposed fish. Therefore the measurement of serum biochemical parameters can be useful as a diagnostic tool in toxicology to find out their general health status and target organs affected by the toxicants (Zikic and Stajn^[17]).

The RBC count and Hb content decreased in *A.testudineus* in both sub-lethal concentrations of copper in the present study. It might be due to the destruction of mature RBCs or inhibition of erythropoiesis due to degeneration of erythropoietic tissues in kidney and spleen (Hota^[18]). Significant decrease in these parameters in a study in *Etrophus maculatus* on exposure to lindane was attributed to haemolytic anaemia or inhibition of aerobic oxidation that curtails the de novo synthesis of Hb (Bijoy Nandan and Nimila^[19]). Under the stress condition fish exhibits asphyxiation due to respiratory failure and anaerobic glycolysis is enhanced. The decrease in humoral parameters such as RBC count and Hb content could also be due to the impairment in iron synthesizing machinery and defective uptake and absorption of iron causing microcytic hypochromic anaemia in *Channa striatus* exposed to metasytox (Natarajan^[20]). The manifestation of anaemia due to fall in Hb could be attributed to impairment of erythropoietic tissues such

as kidney and spleen or accelerated erythroclasia as a result of change in membrane permeability or increased mechanical fragility or defective Fe metabolism or impairment of intestinal absorption of Fe due to mucosal defects (Gill and Eppele^[21]).

Morphological changes in RBCs may be taken as a serious indicator of heavy metal intoxication in freshwater fish (Witesca^[22]). The enlargement of RBC nuclei in exposed fishes in the present study could be the initial sign of disintegration of the nuclei followed by gradual shrinkage. Membrane degeneration was also detected in RBC's in the blood of fishes exposed to highest nominal concentration. Peroxidation of membrane lipid is a possible reason for the damage of erythrocyte membrane in fishes exposed to heavy metals (Ribarov and Benov^[23]). The increase in population of immature RBCs on a time and dose dependent manner might be a compensatory homeostatic mechanism against increased degeneration of cells in fishes under metallic stress. Significant decrease in hematological indices in *Carassius auratus* on intraperitoneal injection of microcystins and suggested that the decrease may be associated with impaired oxygen uptake due to gill damage (Zhang et al.^[24]). The reduced oxygen carrying capacity of blood in all tested concentrations and exposures up to the third week might be due to fall in RBC count, intense haemolysis and haemodilution by copper stress. As a long term toxic response to copper stress, a shift from anaerobiosis to aerobiosis induced by excitation of erythrocytosis or compensatory erythropoiesis could be the reason for increase in RBC count and Hb along with oxygen carrying capacity on the day 28 in the present study is well in agreement with the observation of (Kori-Siakpere and Ubogu^[25]). As a curative agent, vitamin C supplementation enhanced all of the hematological indices in copper exposed fishes when compared to control group.

Exposure of *A. testudineus* to copper showed a time and dose dependent elevation in the activities of serum GOT, GPT and LDH. Among transaminases, GOT is the principal enzyme that interferes the TCA cycle in a major way (Al-Attar^[26]), and is concerned with the molecular rearrangement of amino acids in citric acid cycle at two points such as oxaloacetic acid and alpha ketoglutaric acid, to provide sufficient reduction equivalents like NADH and NADP to mitochondria for phosphorylation for meeting the high energy crisis caused by heavy metal stress (Parthiban and Muniyan^[27]). A rise in GOT activity indicate the occurrence of greater energy demand normally associated with synthetic activities of the cell (Meister^[28]). GPT is more predominant in organs such as liver concerned with intense glycogenesis. The increased activity of these enzymes in blood is correlated either with leakage of these tissue specific enzymes from the damaged hepatic cells in to the blood circulation or increased synthesis of these enzymes or enzyme induction as a result of heavy metal toxicosis (Shakoori and Alam^[29]). The liberation of these enzymes in to the blood stream is regarded as the detrimental effect of heavy metals on the hepatic parenchyma cells (Harvey et al.^[30]). The rise in GOT and GPT as biomarkers of liver damage as evidenced by histopathological evaluation of liver of exposed fishes in the present study coincides with the works on *Cyprinus carpio* exposed to curacron (Baby Joseph and Justin Raj^[31]). The increase in GOT, GPT and LDH due to necrotic changes and hypofunction of liver as toxic response of copper could be regarded as a strong indication of hepatotoxicity (Ozgun Firat^[32]). Pathological and degenerative changes observed in the liver of exposed fishes in the present study are in agreement with these reports.

LDH enzyme is present in most of the animal tissues and is involved in the inter conversion of pyruvic acid to lactic acid and act as a vital enzyme between glycolytic pathway and tricarboxylic acid cycle. The synthesis of more lactic acid in copper induced hypoxia might be the reason for the increase in LDH activity in the present study corroborates the findings in *Anabas scandens* (Mary Chandravathy and Reddy^[33]). The lactic acid synthesized at hypoxic condition in muscle and other tissues may be transported to the liver through blood. The hyperglycemia, reduced Hb, fall in RBC count, reduced oxygen carrying capacity of blood and increased LDH activity in all sub-lethal concentrations up to the day 21 in the present study might be an indication of the metabolic shift from aerobiosis to anaerobiosis as a result of metallic stress (Sathyaparameshwar^[34]). However on the 28th day of exposure, The increase in Hb content, RBC count and oxygen carrying capacity along with parallel decline in

serum LDH in the present study reveals that the fish requires a considerable span of time to recover from stress and to reinstate the normal aerobic mode even at sub-lethal concentrations.

Liver is the principal organ concerned with detoxification and biotransformation processes. Because of the diversity of function and it's crucial role in the circulatory system, it is one of the organs most affected by contaminants in the water. The liver of control fish exhibited normal architecture with hepatocytes having homogenous cytoplasm with centrally placed nucleus (Fig.2.1). In the present study, liver tissues completely lost their architecture on exposure to copper. Similar observations of copper induced histopathological changes in liver, kidney and gills on Nile tilapia (*Oreochromis niloticus*) have been reported (Abdel Tawwab et al^[35]). In the present study, the cumulative effect of two sub-lethal concentrations of copper on the histology of the liver was investigated. Histological changes in specimens exposed for four weeks showed toxic responses such as hypertrophy of hepatocytes, dilation of sinusoids and congestion of blood vessels, suggesting that exposure of fish to different copper concentrations exert great stress on the fish and elicits severe changes in histology. These histological changes are not considered metal specific but are generally associated with the response of hepatocytes to toxicants. Severe necrosis, haemorrhage nuclear pyknosis and degeneration of hepatocytes were witnessed in the liver tissue of *Labeo rohita* exposed to zinc (Loganathan et al.^[36]). Alterations in liver hepatocytes associated with stress have been well studied and reported the formation of vacuoles in the hepatocytes (Metelev et al.^[37]). Vacuolar degeneration and disrupted hepatocytes detected in exposed fishes substantiates the potency of copper in causing liver damage (Fig.4.1, 5.1). Hypoxia due to gill degeneration to be the most common cause of cellular degeneration in the liver (Eder and Gedigk^[38]). In the present study, the gills showed degeneration as the epithelial lining proliferated with a reduction in the total respiratory surface reducing diffusion of oxygen across the gill epithelium. According to the results obtained, it is evident that the magnitude of pathological changes in liver of copper intoxicated fishes is correlated with the dose and time of exposure (Hawkes^[39]). In this context it is imperative to study the histological deviations of liver in exposed fishes as a reliable biomarker of metal toxicity. Studies on *Oreochromis niloticus* exposed to reported discrete pathological changes and suggested that such changes are common in fish exposed over short-term exposure periods while regenerative responses are observed in fishes exposed to long-term periods (Abdel Warith et al.^[40]).

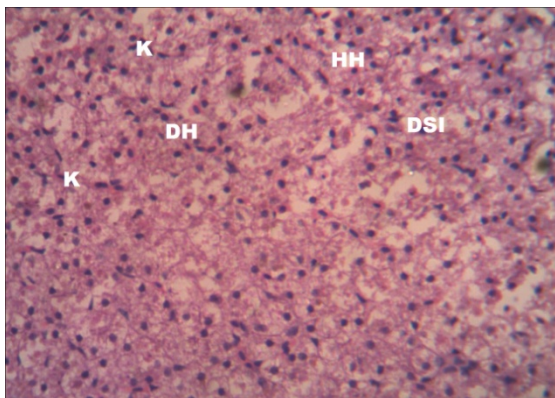


Fig 5.1: Histopathological alterations of the Liver of *A. testudineus* exposed to 0.34 mg/L Copper supplemented with vitamin C for 28 days. Degenerated hepatocyte (DH), dilated sinusoid (DSI), hypertrophied hepatocyte (HH), vacuolar degeneration (VD). (H&E 400)

The results obtained in the present study revealed that the metal intoxicated fish recovered at a faster rate on supplementation of vitamin C. Supplementation of vitamin C in copper exposed fishes significantly reduced the serum GOT, GPT and LDH activity compared to fishes in vitamin free medium clearly reveals the prophylactic role of vitamin C as a typical antioxidant protecting fishes from oxidative stress and tissue injury to a great extent. These results agree with earlier findings^[36]. Vitamin C is one among the most important biological antioxidants. Majority of animals synthesize vitamin C from D-glucose. However most of the fishes are incapable of self-synthesis of vitamin C. Therefore it could be concluded that vitamin C administration is efficient for reducing copper toxicity in fish. Vitamin C is closely related to the immunological system performance and has antioxidant properties favouring the integrity and fluidity of membranes and capable of controlling the oxidizing reactions of fatty acids, thus keeping cellular respiration and avoiding cell death (Brake^[41]).

It is also suggested that the antioxidant activity of vitamin c makes it a hunter of free radicals, thus preventing the auto-intoxication of immunological cells such as macrophages which are the first processors of the information about the alien bodies and maximizing the defensive capacity of fish (Brake^[41]). Study of antioxidant property of vitamin C on *Clarias gariepinus* found that it reacts directly with superoxide radicals and hydroxyl radicals (Adham et al.^[42]). The use of vitamin C is efficient in toxicity reduction, prevention of diseases and enhancement of fish tolerance to environmental stress^[36]. It is concluded that the results with reference to tissue injury in copper intoxicated fishes showed demonstrable recovery when supplemented with vitamin C justifying it's role as a powerful antioxidant and cytoprotective agent.

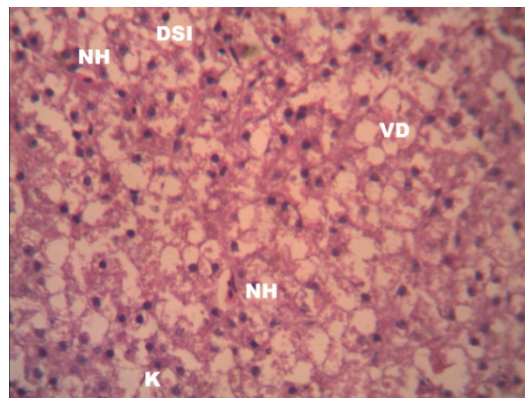


Fig 5.2: Histopathological alterations of the Liver of *A. testudineus* exposed to 0.11 mg/L Copper supplemented with vitamin C for 28 days .normal hepatocyte (NH). (H&E 400)

Increasing the P^H of the water body and formation of copper precipitate in alkaline medium followed by its removal are reliable methods to reduce copper ion concentration and the burden of copper in water (Sreenivasa Reddi et al.^[43]). In this context it is advisable that the industries should take care of treating the effluents more alkaline rather than discharging them as such into the water bodies.

5. Conclusion

It is obvious from the present study that copper is toxic even at sub-lethal concentration and it is enough to elicit serious physiological and histological alterations in fish. The supplementation of vitamin in copper exposed fish showed symptoms of restorative responses in haematological, enzymological and histological parameters elucidating the curative and prophylactic role of vitamin C against copper intoxication.

6. Acknowledgements

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