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RESEARCH ARTICLE

PROTECTIVE EFFECT OF VITAMIN C ON THE VARIATIONS IN BIOCHEMICAL AND HISTOPATHOLOGICAL PARAMETERS INDUCED BY COPPER IN THE TELEOST FISH, *ANABAS TESTUDINEUS* (BLOCH, 1792)

[1]Bhaskaran Nair Girish Kumar and [2]*Sivasankaran Bijoy Nandan

[1]Post graduate and Research Department of Zoology, NSS Hindu College, Changanacherry, Kottayam, Kerala, India

[2]Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, Fine Arts Avenue, Cochin. 682016, Kerala, India

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ABSTRACT

The toxic effects of copper and ameliorating capacity of vitamin C on biochemical and histopathological parameters in the teleost fish, *Anabas testudineus* were investigated. The 96 hour LC_{50} value of copper was determined by Probit method and was found to be 1.74 mg/L. Fishes from two sub-lethal concentrations of copper, two vitamin supplemented and control media were analysed. The decreased ($p > 0.0001$) haemoglobin (Hb), red blood cells(RBC) and oxygen carrying capacity(OC) up to 21st day depicted hypoxia with hypochromic, microcytic anaemia followed by the gradual increase on 28th day of exposure could be the symptom of restoration of aerobic respiration from copper induced anaerobia. The increase of plasma glucose, cortisol and LDH (lactate dehydrogenase) ($p < 0.0001$) up to 21st day parallel to decrease in liver and muscle glycogen in both test concentrations and exposures justified hyperglycaemia due to glycogenolysis to combat high energy demand. Copper exposure induced obvious histopathological changes in gill architecture. Since gills are main route of metal uptake with extensive surface area interacting with toxic metal ions, the intensity of degenerative changes were more profound in copper intoxicated fishes. The symptoms of improvement of humoral and histological parameters in vitamin supplemented fishes reiterates the curative and prophylactic capacity of vitamin C.

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 (Samanta *et al*., 2005). Heavy metals are considered as one of the main causes of pollution in aquatic ecosystems at present having the highest environmental stress index, often in excess of the recommended threshold limit values (Abdel – meguid *et al.,* 2002). Trace metal and pesticide contamination of aquatic ecosystems has increased in the last decades due to their extensive use in agricultural, chemical and industrial processes which are becoming threats to living organisms. Fishes are more frequently exposed to pollutants because regardless of where pollution occurs, it's ultimate destination is aquatic environment. Intensive industrial developments in the last few decades have increased the concentration of copper at an alarming rate in aquatic ecosystems and that affected fish and deteriorate the natural resources. Copper is relatively common in all aquatic and terrestrial ecosystems (Nriagu, 1998). However copper becomes toxic to cells when its concentration surpasses natural levels (Theophanides and Anastassopoulou 2002).Copper intoxication increases mortality of fish offspring and weakens their condition, resistance and viability (Jezierska and Witeska, 2001). The major rivers in Kerala are subjected to intense 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. As an instance, a typical freshwater ecosystem, Periyar, the longest river in Kerala is undergoing eco- degradation with a burden of copper in water and sediments as 0. 075-2.59 mg/litre and 0.055 - 4.32 µg/ gram respectively which is far beyond the permissible limit suggested by US Environmental Protection Agency and World Health Organization (Ciji 2012). In freshwater aquatic environments, standard non-toxic values were set to 5-112 µg/L (Chapman 1996); Among metals, copper is used in industries manufacturing organic chemicals, fertilizers, iron and steel works, electrical works, antifouling paints, pulp and

Corresponding author: Sivasankaran Bijoy Nandan
Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, Fine Arts Avenue, Cochin. 682016, Kerala, India.

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.

High concentration of copper cause haematological and physiological changes leading to retarded growth and inhibition of spawning (Sorenson 1991). Copper is an essential micronutrient for all organisms which is acquired by the gills from the surrounding water as well as from the diet by the digestive tract in fishes. The enormous surface area of gills in fishes, and the minimum distance between water and blood in the branchial epithelium are ideal for the intake of toxicants (Hughes 1984). Gills are morphologically and physiologically modified for performing an array of functions, such as gas exchange, ion and water exchange, acid-base balance, nitrogenous waste excretion, toxicant uptake, detoxification, excretion, and several other metabolic transformations (Evans *et al.,* 2005, Tang and Lee 2011). In copper-polluted water, gills serve as an important route of metal uptake and subsequent accumulation may change the morphology of gill (Campbell *et al.,* 1999). Histopathological changes could be the result of the integration of a large number of interactive physiological processes (Van de Oost *et al.,* 2003).The ultrastructure of tissues and organs is severally affected even in sub-lethal concentrations of toxicants. Due to its diverse functions, delicate structure and constant exposure to the external environment, gills are the most sensitive target organ of waterborne pollutants (Perry and Laurent 1993). Copper is well known for high reactivity with H_2O_2 and it's potential to generate reactive oxygen species (ROS) causing irreversible cellular damage and death. Being the most reliable bioindicators of oxidative stress, haemoglobin, oxygen carrying capacity, serum glucose and LDH, liver and muscle glycogen and LDH were determined in the present study. There is not much scientific information available on the toxic effects of trace metals on the fishery. The quantum of work in freshwater teleost fish, *Anabas testudineus* in relation to copper is relatively low. It was in this context that the present study was undertaken.

MATERIALS AND METHODS

Fish Sampling

Fishes irrespective of sex with a weight of 45 – 50 gm and length 8 – 10 cm. were selected for the experiment. They were collected from a pond at Alappuzha, Kerala. They were maintained in 200 L. tanks disinfected with potassium permanganate solution. Before the start of the experiment, fishes were acclimatized for a period of one month. The physico-chemical parameters of the water were, temperature- 27.1^0 C ± 2.4^0C 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 were fed once daily with a commercial feed and water was changed one hour after feeding.

Experimental Design

A static renewal bioassay was followed for the determination of 96 hr. LC_{50} (Sprague 1973). Copper stock solution was made from hydrated copper sulphate ($CuSO_4$. $5H_2O$) AR Grade, manufactured by Merck Specialities Private Limited, Mumbai, India by dissolving weighed amount of salt in double distilled water 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. Acute toxic levels of copper were determined by static renewal test as suggested in APHA (2005). 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 of more or less similar size were randomly selected from the holding tank and were transferred to each experimental tank which contained 20L of dechlorinated tap water. Fishes were observed regularly and the number of death in each media were recorded daily for a period of 96 hours. Observations were made separately for each replicate. 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 according to Finney (1971). The 96-hour LC_{50} value was derived from the mortality data obtained after exposure to 96- hours by following the computerized statistical package, SPSS 16. 0 and was found to be 1.74mg/L. 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} were made for the experiment (0. 34 mg/L and 0.113mg/L respectively) and each was run in triplicate. Another set of same sub-lethal concentrations supplementing vitamin C (Ascorbic acid, 2.5 mg/L) were also maintained to evaluate the prophylactic and curative effect of vitamin C against heavy metal intoxication. The media were renewed every 24 hours.

From each of the copper exposed, copper along with vitamin C exposed and control groups, seven fishes were caught and anaesthetized by stroking on their head and blood samples were collected in small vials pre-rinsed with heparin through puncturing the caudal peduncle for hematological studies. Blood was treated with EDTA to prevent coagulation. All the hematological analyses were performed using standard techniques. Hb was determined by cyanmethaemoglobin method proposed by Cooks (1994). The oxygen-carrying capacity of the fish blood was calculated by multiplying the Hb content by 1.25 oxygen combining power of Hb/g (Johansen, 1970).The serum LDH was determined as per the method proposed by Thomas (1998). Liver and muscle glycogen were determined as per the method of Montgomery (1957). The serum cortisol was determined by enzyme linked immuno fluorescent assay (ELFA) of Levesque *et al.* (2003). The Plasma glucose was determined as per the method of Trinder, (1969).

Histopathological Procedure

Histopathological techniques and staining procedures were adopted from the methods described by Bucke (1972) and Bullock *et al.* (1980). Samples of gill were collected from immobilized fishes on the 14^{th} and 28^{th} day of exposure. Gill tissues were dissected out, cleaned in saline and fixed in 10% neutral buffered formalin for 24 h. After fixation, the tissues

were graded in ascending alcohol series and cleared in xylene. The tissues were decalcified in 30% nitric acid before alcohol grading. The tissues were embedded in paraffin wax. After paraffin infiltration, the sections were cut to a 5-µm thickness using a rotary microtome and sections were examined under the microscope. Delafield's hematoxylin staining method was used.

Statistical Analysis

All data were expressed as mean ± standard error of means (SEM). Data were analysed by one way analysis of variance (ANOVA).

RESULTS

Copper induced humoral response of *Anabas testudineus* in the sub-lethal concentrations depicted significant decrease ($p < 0.0001$) in Hb and oxygen carrying capacity of blood. The prominent decrease in Hb and oxygen carrying capacity of blood are indicative of microcytic, hypochromic, hypoxic anaemia induced by copper intoxication. On the 28th day of exposure, Hb content and oxygen carrying capacity improved significantly ($p > 0.0001$) towards the control in all test concentrations indicative of restorative efforts of fishes against copper intoxication. The serum glucose level registered a significant and parallel increase ($p > 0.0001$) in both test concentrations up to the day 21showing a hyperglycemic response followed by a fall towards the control value on the 28th day as a homeostatic mechanism to overwhelm metal toxicity. Vitamin C administration brought the serum glucose level towards control in both sub-lethal concentrations.

As a toxic response, the serum LDH level increased parallel with increasing copper concentrations up to three weeks followed by a decrease at the end of 4th week in the present study. The serum cortisol also followed the same pattern of dose dependent steady increase from the lowest to the highest nominal concentrations for a period of 21 days as LDH and it was statistically significant ($p > 0.0001$). However cortisol level decreased on the 28th day of exposure in both test concentrations. Supplementation of Vitamin C brought the serum cortisol and LDH level towards control. The liver and muscle of copper treated fishes showed significantly high ($p > 0.0001$) LDH activity over the control throughout the study period. The increase in LDH activity in the liver and muscle in all phases of exposures and test concentrations was found to be proportional to dose and duration. The increase in LDH activity was well pronounced in muscle than liver. Glycogen registered a sharp decline in the muscle of the exposed fishes than the liver. The decrease in glycogen was significant ($p < 0.0001$) in all doses and durations of exposure in the present study. The changes in biochemical parameters of fishes exposed to copper, vitamin supplemented and control are given in Table 1. A. The gill of control fishes is characterised by thin, slender finger like secondary lamellae attached on either side of the primary gill lamellae. The secondary gill lamellae are highly vascularised and surrounded by a thin layer of epithelial cells (Fig.1.1). Copper caused discrete pathological changes in the gills of the exposed fishes. Edematous changes charecterised by epithelial detachment, degeneration of lamellae, hyperplasia and necrosis of epithelial lining, dilation and congestion in blood vessels, epithelial lifting, lamellar aneurism and lamellar fusion were observed in the gills of fish exposed to 0.17mg/L for 14 and 28 days (Fig. 2.1 and 2.2).

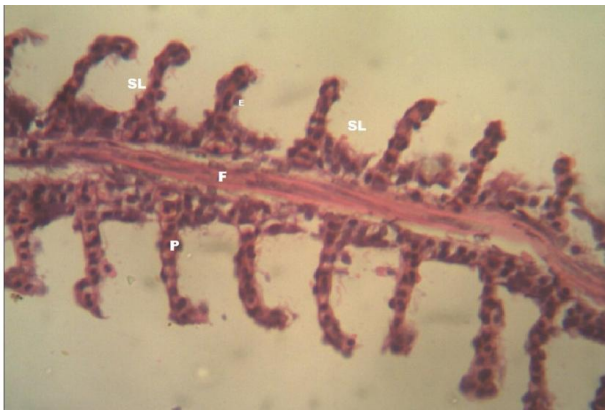

Fig.1.1. Histology of normal Gill of *A. testudineus*. Secondarylamella (SL), Pillar cell(P), Primary gill filament (F). (H&E 400)

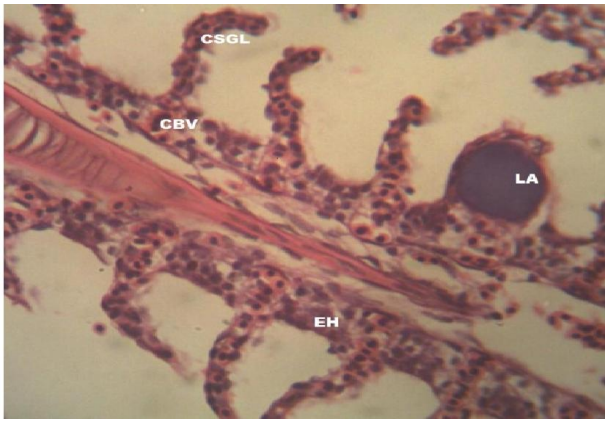

Fig.2.1. Histopathological alterations of Gill of *A. testudineus* exposed to 0.17 mg/L Copper of for 14 days. Clubbed secondary gill lamella (CSGL), Congested blood vessel (CBV), Lamellar aneurism(LA), Epithelial hyperplasia(EH). (H&E 400) FGH

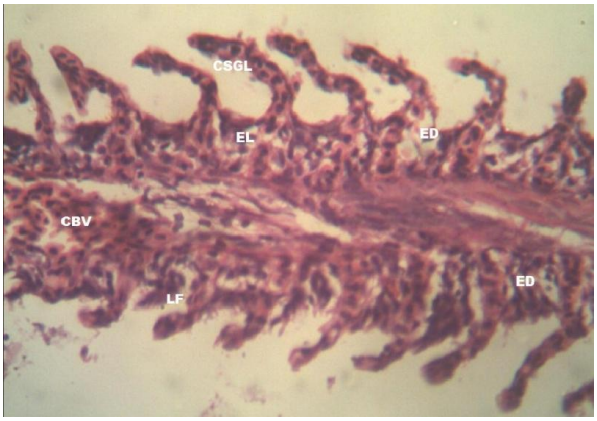

Fig.2.2. Histopathological alterations of Gill of *A. testudineus* exposed to 0.17 mg/L Copper of for 28 days. Clubbed secondary gill lamella(CBV), Congested blood vessel(CBV), Epithelial lifting (E L), Epithelial detachment (ED). (H&E 400) FGH

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

			Sub-lethal Concentrations			
Parameter	Duration of exposure	Control	0.34 mg Cu./L	0.34 mg Cu./L+2.5 mg Vitamin C	0.17 mg Cu./L	0.17 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*	10.09±0.008*	11.24 ±0.007*
	14 days	14.622 ±0.011	7.844 ±0.004*	8.26 ±0.008*	8.68 ±0.008*	9.2 ±0.003*
	21 days	13.94 ±0.024	6.317 ±0.003*	7.12±0.004*	9.54 ±0.005*	8.15 ±0.006*
	28 days	13.762 ±0.013	8.32 ±0.004*	9.24 ±0.006*	9.164 ±0.004*	10.02 ±0.006*
Erythrocyte (RBC)- (10^6/mm^3)	7 days	3.121 ± 0.004	2.42 ± 0.004*	2.53 ± 0.004*	2.61 ± 0.007*	2.734 ± 0.004*
	14 days	3.101 ± 0.001	2.195 ± 0.004*	2.301 ± 0.002*	2.418 ± 0.005*	2.527 ± 0.006*
	21 days	2.994 ± 0.002	1.87 ± 0.006*	2.092 ± 0.004*	2.181 ± 0.005*	2.31 ± 0.006*
	28 days	2.965 ± 0.002	2.322 ± 0.007*	2.31 ± 0.004*	2.534 ± 0.004*	2.682 ± 0.005*
Oxygen carrying capacity (ml O_2/g Hb)	7 days	18.52±0.032	12.221 ± 1.288*	11.637 ± 0.004*	12.612 ± 0.010*	14.04 ± 0.009*
	14 days	18.278 ± 0.013	9.805 ± 0.005*	10.324 ± 0.005*	10.849 ± 0.011*	11.499 ± 0.003*
	21 days	17.419 ± 0.033	7.896 ± 0.004*	8.901 ± 0.006*	9.424 ± 0.006*	10.187 ± 0.008*
	28 days	17.203 ± 0.017	10.399 ± 0.006*	11.549 ± 0.008*	11.455 ± 0.005*	12.524 ± 0.008*
Glucose – (mg%)	7 Days	101.27 ± 0.66	125.04 ± 0.71*	118.55 ± 0.17*	117.37 ± 0.35*	110.61 ± 0.35*
	14 Days	109.22 ± 0.39	139.24 ± 0.31*	129.68 ± 0.13*	129.75 ± 0.16*	122.58 ± 0.10*
	21Days	105.16 ± 0.22	147.44 ± 0.36*	132.97 ± 0.19*	138.84 ± 0.19*	124.6 ± 0.18*
	28Days	105.77 ± 0.20	136.9 ± 0.28*	128.74 ± 0.28*	129.48 ± 0.52*	121.38 ± 0.27*
Lactate dehydrogenase (LDH) (IU/L)	7Days	269.72 ± 2.09	408.31±1.46*	371.97 ± 0.56*	323.08 ± 6.80*	310.24 ± 0.43*
	14Days	268.37±0.27	433.2 ± 1.43*	387 ± 0.86*	347.54 ± 0.63*	317.95 ± 0.42*
	21Days	269.65 ± 0.25	456.85 ± 0.64*	416.51 ± 1.00*	372.72 ± 0.92*	334.81 ± 0.75*
	28Days	274.15 ± 4.03	422.07 ± 0.28*	382.55 ± 0.56*	349.1 ± 0.92*	291.22 ± 0.65*
Cortisol (microgram/dl)	7Days	130.31 ± 0.17	190.51 ± 0.29*	174.97 ± 0.43*	165.35 ± 0.97*	158. ± 0.61*
	14Days	136.85 ± 0.31	229.94 ± 0.25*	193.11 ± 0.68*	194.78 ± 0.26*	176.07 ± 1.19*
	21Days	132.41 ± 0.17	252.65 ± 0.37*	213.24 ± 0.37*	233.12 ± 0.28*	201.34 ± 0.20*
	28Days	129.58 ± 0.25	201.12 ± 1.27*	166.27 ± 0.56*	172.47 ± 0.29*	156.11 ± 0.53*
Glycogen-Liver-mg/gm	7Days	46.085 ±0.133	31.442+0.229*	36.758 ± 0.040*	36.228 ± 0.250*	41.171 ± 0.074*
	14Days	46.657+0.104	26.071±0.178*	32.042 ± 0.042*	31.3 ± 0.165*	36.157 ± 0.072*
	21Days	46.142 ±.048	21.367 ±0.077*	26.657 ± 0.479*	26.522 ± 0.053*	31.342 ± 0.084*
	28Days	46.642 ±0.081	25.844 ± 0.202*	23.27 ± 0.074*	22.4 ± 0.127*	26.042 ± 0.081*
Glycogen-Muscle	7Days	14.64 ± 0.021	10.71 ± 0.014*	11.771 ± 0.018*	11.628 ± 0.018*	12.828 ± 0.028*
	14Days	14.101 ± 0.019	8.426 ± 0.009*	9.74 ± 0.014*	9.22 ± 0.004*	11.103 ± 0.019*
	21Days	13.8 ± 0.006	7.139 ± 0.001*	8.858 ± 0.005*	8.738 ± 0.005*	9.922 ± 0.019*
	28Days	12.29 ± 0.066	5.31 ± 0.017*	7.784 ± 0.002*	7.542 ± 0.018*	9.772 ± 0.015*
LDH-Liver-unit/mg protein	7Days	245.51 ± 0.288	468.5 ± 0.287*	381.27 ± 0.179*	389.24 ± 0.242*	328.85 ± 0.479*
	14Days	231.8 ± 0.856	641.68 ± 0.120*	525.85 ± 0.199*	518.44 ± 0.310*	393.74 ± 0.486*
	21Days	224.771 ± 0.140	709.27 ± 0.346*	564.042 ± 0.375*	568.52 ± 0.196*	446.22 ±1.91*
	28Days	218.017 ± 0.221	620.8 ± 0.133*	520.38 ± 0.371*	484.27 ± 0.199*	419.814 ± 0.133*
LDH-Muscle	7Days	148.457 ± 0.178	366.71 ± 0.206*	294.914 ± 0.270*	284.51 ± 0.234*	218.542 ± 0.319*
	14Days	142.285 ± 0.159	528.371 ± 0.217*	458.528± 0.233*	389.057 ± 0.1*	322.7 ± 0.080*
	21Days	139.71 ± 0.231	678.228 ± 0.104*	490.472 ± 0.375*	518.8 ± 0.10*	376.25 ± 0.533*
	28Days	134.842 ± 0.139	752.042 ± 0.184*	543.314 ± 5.648*	583.81 ± 0.221*	442.928 ± 0165*

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

The magnitude of degenerative changes were more prominent in highest concentration. However the gills showed atrophic lamellae with erosions and thickening, signs of haemorrhage, shortening and clubbing of lamellar tips on exposure to 0.17mg/L for 14 days. Pathological lesions were intensified in the gills of fishes exposed to highest concentration. Shortening of secondary lamellae were common in fishes exposed to highest concentration of copper for 28 days.

DISCUSSION

The copper exposure in *A.testudineus* impaired the oxygen carrying capacity of blood in all test concentrations up to 21st day of exposure. The reduced oxygen carrying capacity in the present study may be attributed to the fall in RBC due to copper stress. The reduction in RBC and Hb in *Anabas testudineus* in both sub-lethal concentrations of copper might be due to the destruction of RBCs, or the inhibition of erythropoiesis due to degeneration of erythropoietic tissues in the kidney and spleen resulting haemolytic anaemia (Hota, 1995) or inhibition of aerobic oxidation that curtails the de novo synthesis of Hb (Bijoy Nandan and Nimila, 2011) as in *Etroplus maculatus* on exposure to lindane. The sign of improvement of Hb content and oxygen carrying capacity on the day 28 might be the gradual shift from anaerobiosis to aerobiosis as a sign of compensatory homeostatic adjustment of test organism against copper intoxication. The obtained results revealed that fishes recovered faster with improved hematological indices such as Hb, RBC count and oxygen carrying capacity compared to those exposed to copper, elucidating the curative role of vitamin C in copper intoxicated fishes. Plasma glucose is one of the most sensitive indices of oxidative stress in organisms and the change in serum glucose is taken as a reliable indicator of stress in fishes (Vosyliene,

1999). Hyperglycaemia in exposed fishes in all phases of exposures and test in the present study coincides with the studies on *Cyprinus carpio* exposed to chromium (Kumar Parvathy, 2011). The increase in plasma glucose was directly proportional to increase in concentration of copper and duration of exposure. The increase in plasma glucose concentration is a secondary stress response of fish to acute toxicity as observed by Sepici-Dincel *et al.* (2009). The increase in glucose over the control in the present study could be the result of intense glycogenolysis or glucogenesis elicited by glucocorticoid and catecholamine or extrahepatic tissue protein induced by the heavymetal (Zikic, 2001 and Almeida, 2001) or the mobilization of energy sources to cater the increased demand of energy as suggested by (Vijayan *et al.,* 1997). Hyperglycaemia on copper exposure reveals oxidative stress and intense fishes utilize energy reserves such as liver and muscle glycogen extensively. Hyperglycaemia by inhibition of neuroeffector sites in adrenal medulla leading to hypersecretion of adrenaline accelerating glycogenolysis observed by Adel Shalaby (2004) in *Oreochromis niloticus* exposed to ochratoxin supports the present study. Increase in serum glucose level could also be due to increase in rate of transportation of glucose through the blood probably from the liver to the muscle to cope with the high energy demand experienced in the muscle as suggested by (Ravichandran *et al.,* 1995). Contrary to the present finding, decrease in plasma glucose level has been reported by Obuotor *et al.* (2011) in *Clarius gariepinus* exposed to copper. The decrease in plasma glucose parallel to the increase in oxygen carrying capacity on the day 28 might be the symptom of glycogenolytic reversal to overwhelm the toxic effect of copper.

As glucose, serum cortisol is also taken as one of the most important stress indicators in fish (Jen-Lee-Yang and Chen, 2003). The significant and steady increase in serum cortisol up to the day 21 in all test concentrations followed by a decrease on the day 28 in the present study agrees with similar findings of Hossam *et al.* (2009) in *Oreochromis* spp. exposed to selenium in which he reported initial increase in serum cortisol up to 8 weeks followed by a decrease as a result of acclimatization to the toxicant medium. The increased trend of serum cortisol in the present study suggests that copper is exercising it's stress response even after 21 days and the fish did not acclimate to copper during this period. It could be assumed that the hypothalamo-hypophyseal-inter renal (HHI) coupling would not be impaired and cortisol secretion from the head kidney might not be significantly affected by copper exposure. Activation of HHI axis by heavy metals leading to elevation of serum cortisol has been reported by Handy, (2003). Hyperglycaemia due to reduction in uptake, phosphorylation and consumption of glucose by the extra hepatic tissue and increase in liver glycogenolysis by high cortisol level proposed by Ozgur Firat *et al.* (2011) in *Oreochromis niloticus* corroborates the present study. However in the present study, cortisol and glucose showed a decreasing trend on the 28thday of exposure. This is in conformity with the earlier reports of Afaghi *et al.* (2007) in *Cyprinus carpio,* that the blood glucose showed an initial rise for a period of 14 days followed by a fall after the 21st day. It might be because the fish have evolved a gradual adaptation to copper on long term exposure or the metal might have caused exhaustion of HHI axis and atrophy of inter renal tissue causing a decreased cortisol level as suggested by Flodmark *et al.* (2002). It could be inferred that the high concentration of plasma glucose and serum cortisol in blood might be the result of intense utilization of liver and muscle glycogen and subsequently the worsening of the status of the organism as suggested by Afaghi *et al.* (2007). The decrease in the oxygen carrying capacity of blood at all copper concentrations and durations of exposure compared to the control justifies heavy metal induced hypoxia and the possible shift to anaerobic mode of respiration from the normal aerobic mode. In metal poisoned fish supplemented with vitamin C, oxygen carrying capacity, serum glucose and cortisol levels were brought near control values highlighting it's capacity to reduce copper toxicity and enhancement of fish tolerance to heavy metal contamination.

The decrease in liver and muscle glycogen was more obvious with increase in duration of exposure and concentration in the present study. James *et al.* (1992) observed similar decrease in muscle and liver glycogen with hyperglycaemia in *Oreochromis mossambicus* exposed to cadmium. The fall in liver glycogen in experimental fishes is attributed to the increased rate of glycolysis. Decrease in liver and muscle glycogen simultaneous with increase in plasma glucose reported by Hontela *et al.* (1977) agrees with the present observation. Accumulation of heavy metals in the pancreatic tissue causing impairment in the release of insulin and glucagon leading to poor absorption of glucose through intestine might also be a reason for glycogen depletion and hyperglycaemia as observed by Usha rani *et al.* (1989). The intense breakdown of glycogen in the liver of exposed fishes in the present study could be correlated with the induction of cortisol as suggested by Sancho *et al.* (1998). The observed depletion of liver and muscle glycogen in the present study explains the increased demand of these substrates to provide extra energy for fishes under heavy metal strss.

In toxicology and clinical chemistry, the cytoplasmic enzyme, LDH is widely used as marker of organ or tissue lesions. LDH is also used as an indicator of hypoxic conditions in organisms induced by heavy metal toxicosis. LDH enzyme is crucial in the inter conversion of pyruvic acid to lactic acid and act as a linking enzyme between glycolytic pathway and tricarboxylic acid cycle (Thirumavalavan, 2010). The increased LDH activity in exposed fishes may be attributed to synthesis of more lactic acid due to hypoxia in tissues. Blood transports lactic acid synthesized in hypoxia in muscle and other tissues to liver. Mary Chadravathy *et al.* (1994) observed similar increase in LDH activity in *Anabas scandens*. Fall in Hb, RBC count and reduced oxygen carrying capacity, hyperglycaemia and increased LDH activity at increasing copper *concentrations* and duration of exposures might be an indication of a metabolic shift from aerobiosis to anaerobiosis due to metal stress as in the reports of Ali Muhammed Yousafali *et al.* (2011). LDH activity increased in the liver and muscle of copper exposed fish compared to the fishes in control medium.The elevated LDH level in liver and muscle shows acute liver damage (hepatitis) and muscle dysfunction caused due to low oxygen concentration in these tissues. High LDH activity in the liver and muscle of the test organisms might be due to the detrimental effect of copper on these

tissues which contribute short supply of oxygen forcing the cells to derive energy anaerobically as suggested by Padmavathy *et al.* (2003) in *Labeo rohita* exposed to low oxygen concentrations. Increased LDH activity in blood, liver and muscle in the present study undoubtedly justifies the persistence of anaerobia and synthesis of more lactic acid in experimental fishes.Increased LDH activity and accumulation of lactate in the tissue of fishes exposed to xenobiotics have already been documented by many workers (Valermathi and Azariah (2003). LDH activity increased significantly upto the day 21 in the liver of treated fish followed by a decrease on the day 28. The decrease in liver LDH might be the sign of recovery of the fish from metal toxicosis on prolonged exposure.

In the present study, on 7^{th}, 14^{th} and 21^{st} days of exposures in all test concentrations, the increase in glucose, LDH, cortisol, and the fall in Hb, RBC and oxygen carrying capacity substantiated hypoxia and the increased energy demand of fishes perhaps due to the switching from aerobic respiration to anaerobic mode in agreement with previous studies. Severe degenerative changes detected in the gill of copper exposed fishes was sufficient to prove that the target organ level atrocity could be the probable reason for hypoxia. The improvement of Hb content and oxygen carrying capacity in conjunction with a corresponding fall in serum glucose, LDH, and cortisol levels on the day 28 clearly reveals the requirement of a long span of time for the recovery and restoration of normal aerobic mode even at sub- lethal concentrations. However in vitamin C supplemented fishes, all humoral and biochemical parameters displayed a sign of restoration towards control. Yosef *et al.* (2012) in a study on broilers exposed to green tea (*Camellia sinensis*) reported the role of antioxidants in improving the physiological state of the organism by bringing down the damage induced by heavy metals. Vitamin C, vitamin E, vitamin B_6 and β - carotene have been shown to protect organisms against heavy metal induced oxidative stress (El-Tohamy *et al.,* 2010).

Histopathological studies are undisputable screening methods in the evaluation of toxic responses in organisms before the manifestation of severe damages (Jiraungkoorskul *et al.,* 2007). Histopathology of gill of fishes exposed to various heavy metals have been extensively studied and documented by many authors. In polluted water, gills are the important route of metal uptake, and in the initial stages of exposure, metals may change the morphology of gill tissue (Campbell *et al.,* 1999). The ultrastructure of tissues and organs was seriously affected even the waterborne contaminant is very low. Copper exposure induced prominent and rigorous histological changes in the gill structure. Damage of epithelial cells, lamellar swelling, epithelial ruptures, secondary lamellar fusion and hyperplasia of the branchial epithelium were the general histological changes detected in the present study. In 0.34mg/L copper medium, epithelial lifting, atrophic lamellae, congested blood vessels, lamellar fusion, shortening and clubbing of secondary lamellae were observed in 14 and 28 day exposures (Fig.3.1 and Fig.3.2).

Oedematous changes characterized by epithelial lifting and total degeneration detected in both test concentrations on the 28th day in the present study agrees with similar observations in *Oncorhynchus mykiss* exposed to nickel reported by (Pane *et al.,* 2004). Lamellar fusion and hyperplasia of secondary lamellae in higher test concentrations and long exposures in the present study could be the protective mechanism to decrease the susceptibility of the gills. In highest concentration on the 28th day, edematous changes characterized by epithelial lifting and total degeneration were prominent. (24 high) The damage of the secondary lamellae may be attributed to dilation and vascular congestion as observed in the gills.

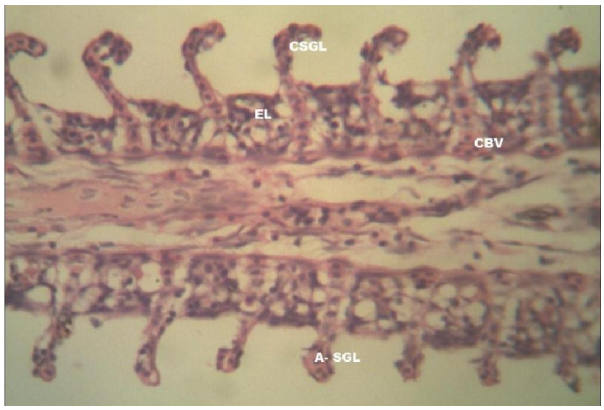

Fig.3.1. Histopathological alterations of Gill of *A. testudineus* exposed to 0.34 mg/L Copper of for 14 days.Clubbed secondary gill lamella(CBV), Congested blood vessel(CBV), Epithelial lifting(EL), Atrophied secondary gill lamella (ASGL). (H&E 400)

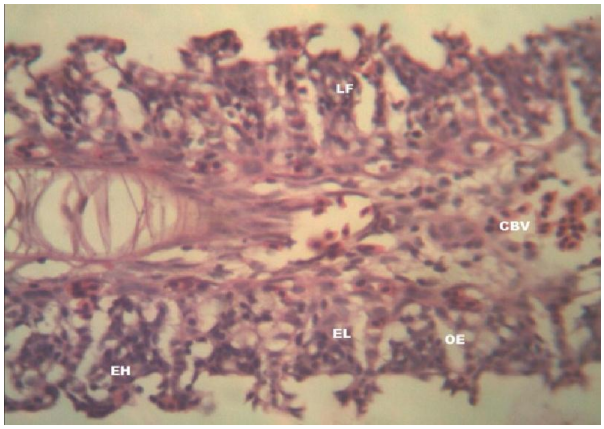

Fig.3.2. Histopathological alterations of Gill of *A. testudineus* exposed to 0.34 mg/L Copper of for 28 days. Congested blood vessel(CBV), Lamellar fusion (LF), Epithelial hyperplasia(EH), Lamellar fusion(LF), Epithelial lifting(EL). (H&E 400)

Lamellar fusion and hyperplasia of secondary lamellae induce suffocation in fish. These structural changes in gills enable fishes to increase the diffusion distance between the blood and the xenobiotics as explained by Hemalatha and Banerjee (1997). Epithelial rupture could lead to a negative ion balance and changes in the hematocrit and hemoglobin and could cause severe disturbances in gill respiration (Peuranen *et al.,* 1994). Observations such as rupture of the branchial epithelium are considered direct, dose-dependent deleterious effects of the pollutant, while hyperplasia, lamellar fusion and mucous

hypersecretion could be the signs of branchial defense responses. Epithelial hyperplasia and curling and fusion of the secondary lamellae were noticed in *C. mrigala* after exposure to monocrotophos (Velmurugan *et al.,* 2007) and in *Gambusia affinis* after 30 days of exposure to deltamethrin (Cengiz and Unlu, 2006). The administration of vitamin C along with copper regained the normal architecture of the gill to a great extent inspite of occasional cases of epithelial lifting and mild oedematous changes in certain parts of the gill lamellae (Fig.4.1 and Fig.4.2). The restorative symptoms of gill lamellae of in vitamin exposed fishes clearly reveals the therapeutic and curative effect of vitamin C as an ideal antioxidant against heavymetal toxicosis.

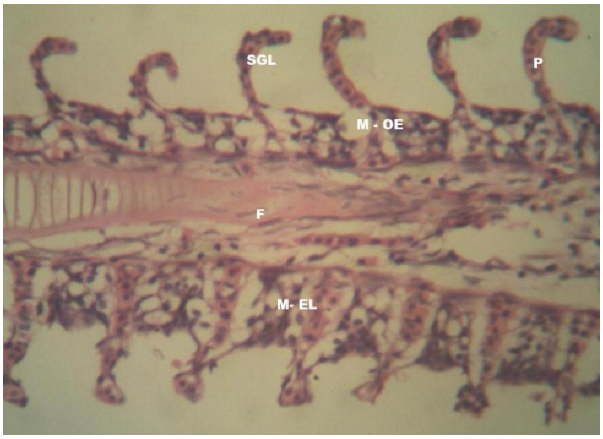

Fig.4.1. Histopathological alterations of Gill of *A. Testudineus* exposed to 0.17mg/L copper supplemented with vitamin C for 28 Days. Secondary gill lamella (SGL), Pillar cell (P), Mild oedema (M OE), Mild epithelial lifting (M EL), Primary gill lamella (F)

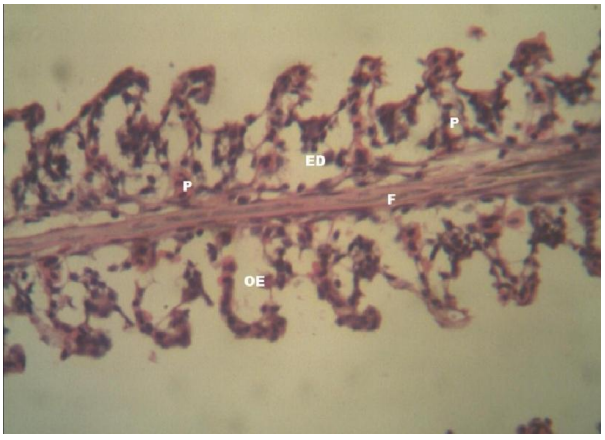

Fig.4.2. Histopathological alterations of Gill of *A. testudineus* exposed to 0.34 mg/L Copper supplemented with vitamin C for 28 days.Epithelialdetachment(ED), Pillar cell (P), Oedema(OE), Primary gill filament (F). (H&E 400)

Conclusion

The 96 hour LC_{50} value of copper for *A. testudineus* was estimated to be 1.74 mg/L. It is concluded from the study that,physiological dysfunctions and the resultant histopathological changes in the tissues of exposed fishes could be a reliable tool in assessing the quality of aquatic environment and helping us to evolve proper strategies in the management and abatement of aquatic pollution. The undesirable changes in humoral, biochemical and histological parameters in fishes even at sub-lethal concentrations reinforces the potency of copper. However supplementation of vitamin C was found effective in reducing the tissue level atrocities caused by the trace metal, copper and the exposed fishes showed signs of restorative responses in biochemical and histological parameters ascertaining the curative and protective role of vitamin C against heavy metal intoxication. Increasing the P^H and formation of alkaline copper precipitate are also strategies recommended for reducing copper ion concentration in water. Hence 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. It is obvious from the present study that, the heavy metals in general and copper in particular are highly detrimental to the fauna and flora due to their long half life period and properties of non-biodegradability, bioaccumulation and biomagnification.

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