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Curative effect of Vitamin C on the Variation in Biochemical and Histopathological Parameters Induced by Copper Exposure in the Teleost Fish, *Anabas testudineus* (Bloch, 1792).

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Abstract: *The biochemical and histopathological alterations in the blood and liver of the teleost fish, Anabas testudineus subjected to two sub-lethal concentrations of copper and the capacity of vitamin C to counteract the toxicity induced perturbations were investigated. The 96 hour LC $_{50}$ value of copper for A. testudineus was determined by Probit method and was found to be 1.74 mg/L. The blood and liver samples were collected from copper exposed and vitamin supplemented media on the 7th,14th,21stand 28thday of exposure. Copper exposure significantly increased (p>0.0001) the activity of biomarkers of hepatotoxicity such as GOT (Glutamate oxalate transaminase) and GPT (Glutamate pyruvate transaminase) in serum and liver compared to vitamin supplemented fishes.The decrease in serum and liver protein of copper exposed fishes justifies increased energy demand due to copper induced oxidative stress. The liver of copper treated fish showed histopathological alterations such as degeneration of hepatocytes,cell necrosis, inflammation with sinusoid dilation,increased incidence of kupffer cells and vacuole formation. Vitamin administration was effective in improving the physiological and histological integrity in copper intoxicated fishes.The present study shows that copper is harmful to Anabas testudineus even at sub-lethal concentrations and it's enrichment in water and sediment is a dangerous threat to aquatic life.*

Keywords: *Copper,GOT,GPT, histopathology, Vitamin C*

I. Introduction

Industrial effluents contributing to aquatic pollution comprise a vast array of toxic substances in which heavy metals are of special concern. Heavy metals are of greatest concern because of their capacity to induce environmental stress and the tendency of accumulation often in excess of the recommended threshold limit values [1]. Indiscriminate discharge of these wastes alter the quality of aquatic ecosystem in general and the fauna and flora in particular. The toxic effects may result from bio-concentration of the metals and their consequent binding with biologically active constituents of the body such as lipids, amino acids, enzymes and proteins [2].The Heavy metal and pesticide contamination of aquatic ecosystems has increased in the last decades due to extensive use of them in agricultural, chemical and industrial processes that are becoming threats to living organisms.Under heavy metal stress situations the fish body elicits immediate responses recognized as primary and secondary responses. The primary response is the perception of an altered state by the central nervous system and the release of the stress hormones like cortisol and catecholamines [3] and secondary responses include the consequence of the released stress hormones [4] causing changes in the blood and tissue chemistry [5].

Copper is an abundant trace metal found at varying concentrations in nearly all aquatic ecosystems [6]. Copper sulphate is widely used as an algaecide for controlling phytoplankton in fish ponds and lakes as well as a herbicide used in aquatic weed control [7]. 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.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.As an instance,Periyar,the longest river in Kerala has a burden of copper in water and sediments as 0.075-2.59 microgram/litre and 0.055-4.32 microgram/gram respectively [8]. Eventhough most of the heavy metals are micronutrients, they exert a prominent role in environmrental deterioration.Industrial effluents and sewage are the major sources of copper in aquatic ecosystems. Copper is an essential micronutrient for all organisms and, in the case of fish, is acquired by the gills from the surrounding water, as well as from the diet by the digestive tract [9]. It is necessary for the synthesis of haemoglobin and act as a component of cytochrome oxidase [10]. Elevated aquatic copper levels cause a range of negative effects on fish. Many of these responses are in part due

to the high reactivity of copper with H_2O_2 and it's potential to undergo redox reactions to form reactive oxygen species (ROS),that may cause irreversible cellular damage and death.Copper has many side effects on metabolism affecting the activity of many enzymes.

The total protein is one of the essential constituent in cells and tissues playing a vital role in normal physiology of organisms. Intense proteolysis to supplement aminoacids to be converted to ketoacids as precursors for the maintenance of carbohydrate metabolism to meet the energy requirement elicited by heavy metal stress has been reported in *Channa punctatus* and *Heteropneustes fossilis* exposed to copper [11,12]. GOT and GPT are cellular metabolic enzymes usually found in small concentrations in plasma derived probably from the regular physiological shedding of cells [13]. Liberation of these enzymes in to the blood stream is a detrimental effect of the heavy metals inducing degenerative necrotic changes and hypofunction of liver[14,15]. GOT and GPT are hence reliable indicators of heavy metal induced hepatotoxicity in organisms and magnitude of aquatic pollution. Increase in GOT and GPT activity has been reported in *Cyprinus carpio* on exposure to cadmium and curacron respectively[16,17]. Histopathology is one of the reliable methods for assessment of short term and long term xenobiotic effects. Histopathological approach are obligatory component of environmental assessment and could be used to formulate monitoring systems [18]. Liver is the principal organ concerned with detoxification and biotransformation processes. Because of the diversity of function it is more likely to be affected by contaminants in water.Copper induced histopathological changes were observed in liver, kidney and gills on Nile tilapia (*Oreochromis niloticus*) [19]. Severe necrosis, haemorrhage, nuclear pyknosis and degeneration of hepatocytes were observed in the liver tissue of Labeo rohita exposed to zinc [20]. The present study was planned to evaluate the efficacy of copper in exercising humoral and target organ level disturbances as well as the ameliorating capacity of vitamin C in the teleost fish,*Anabas testudineus*,The quantum of work in *Anabas testudineus* in relation to copper is relatively low. It was in this context that the present study was undertaken.

II. Materials and Methods.

The proposed study followed a static, renewal bioassay method to determine the 96 hr. LC_{50} [21]. .*Anabas testudineus*, a common fish nicknamed as climbing perch abundantly available in Kerala were used for the study. Fishes were maintained in 200 L. tanks disinfected with potassium permanganate solution. Before the start of the experiment the fish were acclimatized for a period of one month. The physico-chemical parameters of the water were temperature $27.1 \pm 2.4\ ^0C$, PH 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. The fish irrespective of sex with a weight of 45 – 50 gm and length 8 – 10 cm. were selected for the experiment.The fish 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 ($CuSO4.5H_2O$) manufactured by Merck India Limited,Mumbai 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 [22]. Twelve healthy and active fish 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 all media were recorded daily for a period of 96 hours. 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 [23]. The 96-hour LC_{50} value was derived following the computerized statistical package,SPSS 16.0 and was found to be 1.74mg/L. 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 & 0.113mg/L respectively) and each was run in triplicate. Another set of sub-lethal concentrations were also maintained supplementing vitamin C (Ascorbic acid, 2.5 mg/L), to evaluate the prophylactic and curative effect of vitamin C against copper intoxication. A toxicant and vitamin free control medium was also maintained simultaneously.The media were renewed every 24 hours.

Fishes were caught and anaesthetized for collection of serum on 7^{th} ,14^{th},21^{st} and 28^{th} day of exposure.Blood was treated with EDTA to prevent coagulation. Histopathological techniques and staining procedures were done by standard methods [24,25]. Liver samples were collected on the 14^{th} and 28^{th} day of exposure. They were 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.The plasma GOT and GPT were determined as per kinetic method [26,27]. The serum protein was determined as per standard method [28].

III. Result

The humoral response of *Anabas testudineus* on exposure to both sub-lethal concentrations of copper at all duration of exposures depicted significant decrease ($p < 0.0001$) in plasma protein. The protein content decreased tremendously with increase in copper concentrations as well as duration of exposure.The decrease in protein level was more pronounced and significant in higher nominal concentrations and longer exposure periods indicating greater toxic response than in lower concentrations and shorter exposures. Vitamin C administration markedly increased the plasma protein level almost closer to control value.

The serum GOT and GPT activity were high in the sub-lethal concentrations of copper in comparison with the control values and their increase was significant ($p>0.0001$) as per the results of the ANOVA.The increase in 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, GOT and GPT activity were comparatively much lower than those in test concentrations and their activity showed a declining trend in all test concentrations in general and the lowest nominal concentration in particular towards the control value. The alterations in protein content,GOT and GPT of serum and liver in exposed and control fishes are presented in **Table** A.1.and A.2

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

Biochemical Parameter	Duration of Exposure	Control	Sub-lethal Concentrations			
			0.34mg Cu./L	0.34mg Cu./L+2.5mg Vitamin C	0.113mg Cu./L	0.113mg Cu./L+2.5mg Vitamin C
Gluatamate 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*
Protein -(g %)	7Days	7.32 ± 0.05	4.66 ± 0.12*	5.16 ± 0.01*	6.18 ± 0.01*	7.20 ± 0.008*
	14Days	7.33 ± 0.04	4.06 ± 0.02*	5.99 ± 0.04*	5.60 ± 0.01*	7.15 ± 0.01*
	21Days	7.39 ± 0.007	3.48 ± 0.009*	4.40 ± 0.008*	5.14 ± 0.007*	6.49 ± 0.006*
	28Days	7.36 ± 0.01	3.03 ± 0.02*	4.16 ± 0.01*	4.52 ± 0.004*	5.65 ± 0.01*

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

Table A 2. Variations in the protein levels in the liver of *Anabas testudineus* on exposure to copper with and without supplementation of Vitamin C

Tissue	Duration of Exposure	Control	Sublethal Concentrations			
			0.34mg Cu./L	0.34mg Cu./L+2.5mg Vitamin C	0.113mg Cu./L	0.113mg Cu./L+2.5mg Vitamin C
Liver Protein-mg/gm	7D	70.642 ± 0.327	60.7 ± 0.224*	63.014 ± 0.085*	67.442 ± 0.004*	68.228 ± 0.197*
	14D	70.1 ± 0.101	54.471 ± 0.101*	58.842 ± 0.086*	64.685 ± 0.073*	66.485 ± 0.118*
	21D	69.428 ± 0.144	48.442 ± 0.139*	53.628 ± 0.144*	57.7 ± 0.061*	63.028 ± 0.335*
	28D	68.685 ± 0.219	37.585 ± 0.063*	44.928 ± 0.152*	52.528 ± 0.145*	57.357 ± 0.167*
Liver- GOT unit/mg protein	7D	130.171 ± 0.207	225.157 ± 0.154*	182.3 ± 0.361*	163.014 ± 0.179*	138.22 ± 0.091*
	14D	127.171 ± 0.513	267.857 ± 0.186*	230.028 ± 0.144*	197.44 ± 0.230*	166.55 ± 4.227*
	21D	123.514 ± 0.475	312.68 ± 0.208*	273.871 ± 0.106*	246 ± 0.144*	191.057 ± 0.170*
	28D	120.385 ± 0.140	394.18 ± 0.226*	343.057 ± 0.125*	311.92 ± 0.215*	251.78 ± 0.205*
Liver - GPT unit/mg protein	7D	167.55 ± 0.134	216.21 ± 0.145*	191.91 ± 0.194*	182.12 ± 0.094**	171.5 ± 0.160*
	14D	162.51 ± 0.15	231.81 ± 0.204*	216.81 ± 0.35*	194.98 ± 0.162*	182.87 ± 0.128*
	21D	158.31 ± 0.209	287.58 ± 0.225*	234.92 ± 0.086*	227.04 ± 0.078*	205.82 ± 0.074*
	28D	155.042 ± .113	356.67 ± 0.206*	278.34 ± 0.282*	308.457 ± .324*	230.75 ± 0.150*

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

Copper induced discrete pathological changes in the liver of *Anabas testudineus*. The liver of control fish showed normal architecture with homogenous cytoplasm possessing centrally placed nucleus (Fig.1.1). Severe necrotic and inflammatory changes were noticed in the liver of fish in highest concentration and longest exposure. The significant histological changes observed in both test concentrations towards the second half of exposures include the degeneration of hepatocytes, dilation and congestion of sinusoids,hypertrophy and hyperplasia of bile duct,nuclear pyknosis,vascular haemorrhage,vacuolar degeneration etc.(Fig.2.1 and Fig.2.2).Moreover a marked increase in number of kupffer cells was also observed in the highest concentration and longest exposure. However magnitude of degeneration reduced significantly and the liver showed signs of restoration in vitamin treated fishes.

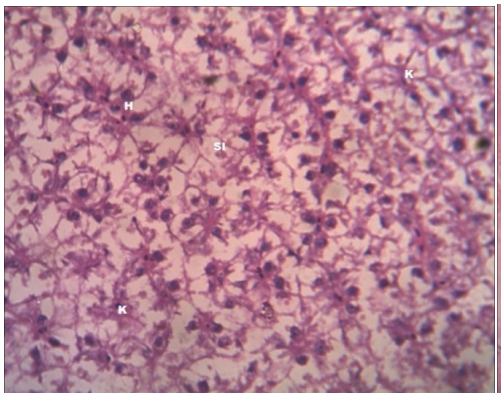

Fig.1.1 Normal histology of Liver of *A. testudineus*. normal hepatocyte (NH), sinusoid (SI). (H&E 400)

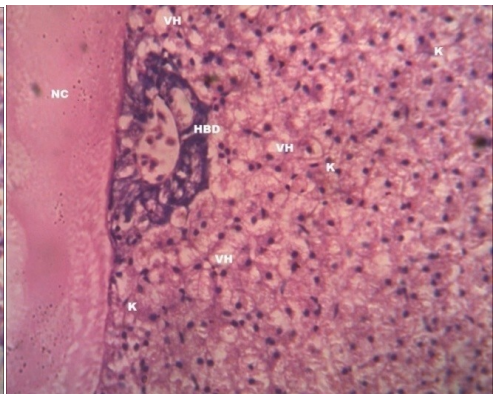

Fig.2.1 Histopathological alterations of Liver of *A. testudineus* exposed to 0.34 mg/L Copper of for 28 days. Necrosis (NC),vascular haemorrhage(VH),hypertrophied bile duct (HBD).kupffer cell (K). (H&E 400)

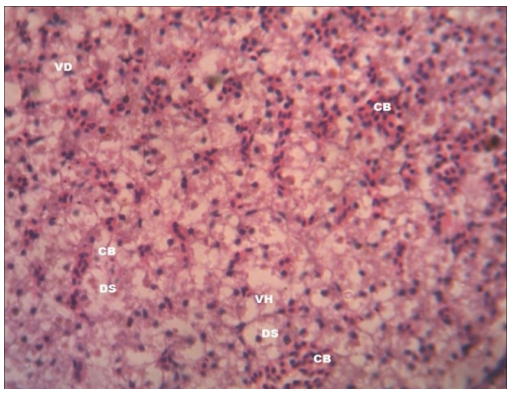

Fig.2.2 Histopathological alterations of the Liver of *A. testudineus* exposed to 0.34 mg/L Copper of for14 days dilatedsinusoid(DS).Vacuolar degeneration(VD),vascular haemorrhage(VH), congested blood vessel(CB). (H&E 400)

IV. Discussion

The aquatic ecosystems are extensively contaminated with heavy metals released from domestic,industrial and other anthropogenic sources causing potent harm on the ecological equilibrium of the environment in general and organisms in particular due to accumulation of toxic metals in their tissues and organs.Since bio-accumulation not only affect the productivity and fecundity of organisms but the health of man also. The various types of stressors in aquatic ecosystems and agriculture practices have been shown to induce changes in the physiological variables of fish [29]. Environmental stressors such as metal exposure may change the biochemical parameters in exposed fish [30]. Therefore the measurement of serum biochemical parameters can be useful as a diagnostic tool in toxicology studies to find out the general health status and target organs affected by the toxicants [31].

The plasma protein decreased significantly at all stages of exposure and concentrations of copper in the present study.The decrease of serum protein was found to be dose and time dependant. The observed decrease of serum proteins could result from the breakdown of protein in to amino acids first to be fed in to the Kreb's cycle to cope up the energy crisis manifested by the metal intoxication [32] and possibly in to nitrogen

and elementary molecules [33]. The depletion in serum protein in test organisms might be due to impaired protein synthesis or the functional deterioration of the liver or excessive loss of protein caused by nephrosis [34,35] corroborates the present study.Similar decrease in serum and liver protein on exposure to copper has been reported in *Labeo rohita* [36]. The decrease in serum protein might also be due to increase in protein breakdown as a consequence of hypersecretion of corticosteroid hormones providing aminoacids for gluconeogenesis to produce more glucose to compensate the increase in energy demand under stress situation. In the present study, vitamin C administration was found to have an ameiliorating effect on copper toxicity enhancing the protein content near to the control.Similar decrease in plasma protein has been documented in *Labeo rohita* exposed to chromium, *Notopterus notopterus* exposed to copper, *Oreochromis niloticus* exposed to copper and in *Rhamdia quelen* exposed to cypermethrin [37,38,39].

The data of the present study showed that copper caused a time and dose dependent elevation in the activities of GOT and GPT in serum and liver in exposed fishes.Among transaminases,GOT plays a crucial role in the TCA cycle [40]. A rise in its activity is indicative of greater energy demand normally associated with synthetic activities of the cell [41]. GOT is concerned with the molecular rearrangement of aminoacids linked to TCA cycle to cater the increased demand of NADH and NADP to mitochondria [42] to synthesize ATP for meeting the high energy crisis [43] induced by copper in the present study. GPT is more predominant in liver as it is concerned with intense glycogenesis. It is also reported that the increase in GOT and GPT in blood may be either due to leakage of these enzymes from the damaged hepatic cells in to the blood or increased rate of synthesis of these enzymes as a toxic response [44,14]. Damage of liver by the accumulation of heavy metals is also a possible reason for increase in GOT and GPT activity in serum and liver [45]. After subchronic dietary copper exposure for forty days increased serum GOT and GPT concentrations with increasing time and dose were observed in the rock fish,*Sebastes schlegeli* [46]. Similar increase in serum GOT and GPT on exposure to various heavy metals were observed and reported by many authors [47,48,49]. The activities of serum GOT and GPT have been commonly used in the diagnosis of fish diseases and tissue degeneration caused by environmental pollution [50]. Increase in activity of GOT and GPT in liver and their subsequent release in to blood stream is indicative of necrotic changes and hypofunction of liver. All these reports strongly supports the conviction that the liver of the heavy metal exposed fish would be severally damaged. The results obtained in the present study revealed that the tissue injury in metal poisoned fish recovered to a great extend on supplementation with vitamin C in bringing serum and liver protein,GOT and GPT activities closer to the control values in the present study coincides with earlier findings [19].

In the present study, the cumulative effect of two sub-lethal concentrations of copper on the histology of the liver was investigated with reference to control. Liver tissues completely lost their architecture on exposure to copper in the present study. Histopathological observation of the liver of exposed fishes in both sub-lethal concentrations after four weeks in the present study showed marked stress responses such as hypertrophy of hepatocytes ,dilation of sinusoids and congestion of blood vessels (Fig.2.1 and Fig,3.1) Severe necrosis, haemorrhage, nuclear pyknosis and degeneration of hepatocytes were witnessed in the liver tissue of *Labeo rohita* exposed to zinc [20]. Alterations in liver hepatocytes associated with stress have been well studied and reported the formation of vacuoles in hepatocytes [51]. Vacuolar degeneration and disrupted hepatocytes detected in exposed fishes substantiates the potency of copper in causing liver damage (Fig.3.1).Vacuolar degeneration and focal necrosis in hepatocytes in the present study coincides with similar observations in *Etroplus maculatus* exposed to lindane [52]. Hypoxia due to gill degeneration is attributed to be the reason for cellular degeneration in the liver [53]. In the present study, the gills showed obvious degenerative changes like proliferation of epithelial lining with reduction in respiratory surface area lowering diffusion of oxygen. In this context it is imperative to study the histological deviations of liver along with biochemical changes in exposed fishes as a reliable biomarker of metal toxicity. All these reports strongly supports the conviction that the liver of the heavy metal exposed fish is severely damaged.The biochemical and histopathological results of the present study revealed that the metal intoxicated fish recovered at a faster rate on supplementation of vitamin C.The liver of fishes from both test concentrations depicted obvious restorative changes after 28 days of exposure (Fig.4.1 and Fig.4.2).

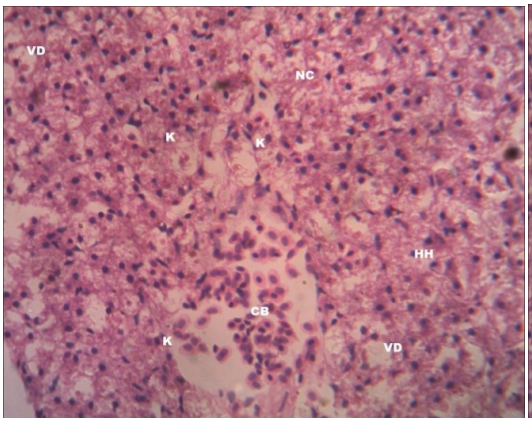

Fig.3.1 Histopathological alterations of the Liver of *A. testudineus* exposed to 0.11 mg/L Copper of for 28 days. Vacuolar degeneration(VD),necrosis(NC),hypertrophied hepatocyte (HH),congested blood vessel(CB). (H&E 400)

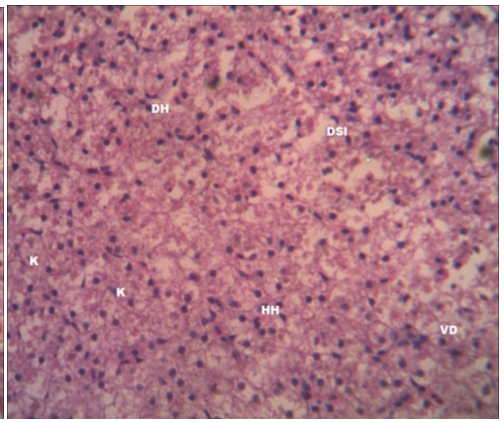

Fig.4.1 Histopathological alterations of the Liver of *A. testudineus* exposed to 0.34 mg/L Copper supplemented with vitamin C for 28 days. degenerated hepatocyte(DH),dilated sinusoid(DSI),hypertrophied hepatocyte(HH),kupffer cell (K),vacuolar degeneration(VD). (H&E 400)

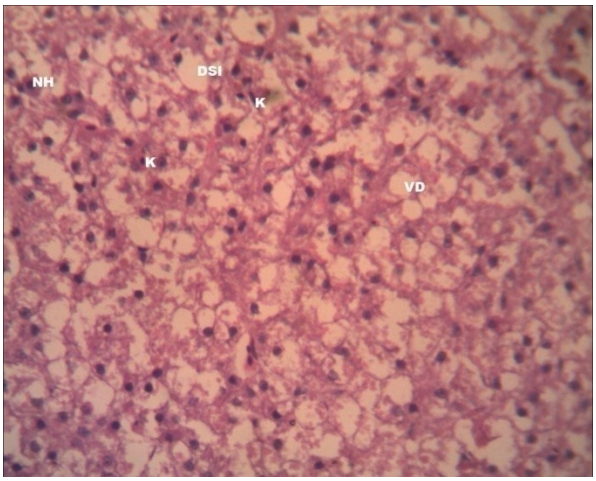

Fig.4.2 Histopathological alterations of the Liver of *A. testudineus* exposed to 0.11 mg/L Copper supplemented with vitamin C for 28 days .normal hepatocyte (NH),dilated sinusoid (DSI),vacuolar degeneration (VD),Kupffer cell (K). (H&E 400)

Vitamin C is a well known non enzymatic biological antioxidant in ameliorating the harm induced by heavy metal intoxication. The efficacy of vitamin C as a scavenger of superoxide radicals and hydroxyl radicals has been demonstrated in *Clarias gariepinus* [54]. Majority of animals synthesize vitamin C from D-glucose but fishes are incapable of self synthesis. Vitamin C is closely related to the immunological system performance and has antioxidant properties by acting as a hunter of free radicals preventing the autointoxication of immunological cells such as macrophages and maximizing the defensive capacity of fish [55] controlling the oxidizing reactions of fatty acids thereby keeping cellular respiration and avoiding cell death [56]. In *Tilapia zillii* on exposure to copper, vitamin C was shown to have protective and therapeutic effects against copper intoxication and prevented the inhibition of GOT and GPT activity [57]. The present study in support of earlier findings justifies the role of vitamin C in toxicity reduction, prevention of diseases and enhancement of fish tolerance to environmental stress. The alterations in serum parameters may be taken as the initial sign of target organ damage and the dysfunction induced by the toxicants and these parameters can thus be used as a rapid and sensitive indicator of monitoring towards the impacts of toxicants on aquatic fauna and ultimately the whole ecosystem.

V. Conclusion

The present study clearly demonstrates the tissue and organ level atrocities induced by the heavy metal,copper,even at sub-lethal concentrations and short term exposures.Copper seems to exert more damage in acidic P^H than alkaline.Hence it is imperative to adopt strategies to treat effluents to more alkaline rather than

discharging them as such in to the water bodies. The toxic response of copper is obviously reflected in the physiology and histology of the exposed fishes in the present study.However the supplementation of vitamin C in copper 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.

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