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HISTOPATHOLOGICAL AND BIOCHEMICAL CHANGES IN GRASS CARP GILL AND MUSCLE TISSUES DUE TO NICKEL EXPOSURE MAY BE RELATED TO THE INDUCTION OF OXIDATIVE STRESS

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ABSTRACT
The present study was designed to investigate the Nickel (Ni) induced biochemical and histopathological changes in gill and muscle tissues of fresh water fish grass carp. These Fish were exposed to 35mg/L of Nickel sulphate for 7 And 14 days. The activity of lipid peroxidation markers, antioxidant defense system, total protein, lipids, estimation of glucose and glycogen were assayed in Gill. Fish exposed to nickel for 7 and 14 days a significant ($p < 0.05$) increase in the levels of LPO along with significant ($p < 0.05$) decrease in the levels of SOD, GSH and GPx and siginificant ($p < 0.05$) decrease in the level of total protein, lipids, glucose and glycogen. These results indicate that 14 days Ni exposed fish were increase in the level of LPO along with decrease in the level of SOD, GSH, GPx, protein, lipids, glucose and glycogen when compared to 7 days Ni exposed fishes. The histopathological studies in the gill and muscle tissues of fish also supported the view that sub lethal dose of Nickel for 14 days significantly increased the morphological alterations when compared with 7 days Ni exposed fish. Our results clearly suggest that even at a very low sub lethal dose of Nickel exposure to grass carp for 7&14 days increased the level of metabolic stress, oxidative stress, antioxidant depletion and morphological alterations in the selected tissues.

KEYWORDS: Nickel, Oxidative stress, Antioxidants, Lipid peroxidation, Gill.

INTRODUCTION
The fluctuating environmental conditions and human interference has greatly affected the flow of environmentally deleterious changes by loading with toxicants to the aquatic system. The toxicants especially heavy metals and pesticides contamination of aquatic system has gained the attention of environmentalists throughout the globe. Heavy metals have been recognized as serious pollutants of the aquatic environment that are causing earnest deterioration in metabolic, physiological and structural systems of organisms. The accumulation of metals in an aquatic environment has direct impacts on the sustainability of the ecosystem. Involvement of metals, which are required for metabolic activities in organisms, lies in the narrow range between their importance and toxicity. Household waste water and industrial effluents used to irrigate agricultural lands comprise large quantities of different heavy metals which usually penetrate and concentrated in different tissues of fish body viz. skin, gills and intestine through water and food.[1]

Nickel (Ni) is a silvery white metal that takes on a high polish. It is a transition metal, hard and ductile. The properties of nickel and its environmental distribution have been summarized by the US Agency for Toxic Substances and Disease Registry (ATSDR). Ni accumulates and proves to be a multi target toxicant causing damage to many organs such as gill, kidney, lung, brain and testis.[2] The main pathological lesions and specific biochemical changes related to Ni toxicity is the reflective of Ni concentration and oxidative stress in tissues. Recent studies suggest that reactive oxygen species (ROS) generation associated with Ni exposure leads to oxidative stress, which is an important factor involved in Ni induced apoptosis.[3] It is postulated that the genotoxic effects of Ni can result from the generation of oxygen radicals in the reaction between metal and proteins. These radicals can subsequently interact with DNA, inducing damage to its bases, DNA strand breaks and DNA-protein cross-links.[4]

Fishes are considered as one of the most significant indicators in freshwater systems for the estimation of metal pollution.[5] The commercial and edible fish

species have been widely investigated in order to check heavy metal contamination particularly those that are hazardous to human health. With the growth of fishes, the heavy metals accumulate in the internal organs like muscles, liver and intestine in a considerably higher concentration, make the fish unsuitable for human consumption.[6] While the essentiality of Ni remains in question, the deleterious effects of excess Ni on aquatic animal health are not well recognized. In fresh water biota the effects of Ni include the impairment of gas exchange, inhibition of ion regulation and the promotion of metabolic stress etc., But the works are limited in the area of Ni induced oxidative stress and anti oxidant imbalance in grass carp upon sub chronic exposure. With this in mind, the present study has been designed to investigate the Ni induced oxidative stress in some selected organs of grass carp.

MATERIALS AND METHODS

Toxicant

Reagent grade nickel sulphate ($NiSO_4 \cdot 6H2O$) (Sigma-Aldrich Company, USA) was used as the toxicant. Stock solution was prepared by dissolving a necessary amount of nickel sulphate in distilled water, the final concentration being recalculated according to the amount of heavy metal ion.

Animals

The fresh water fish, Grass carp were collected from fish farm located in thokkanampakkam village, cuddalore district, Tamilnadu. Healthy fishes of comparable body weight (28±3.45g) and length (12±3.55cm) were selected for the study. This fishes were brought to the laboratory and transferred to the rectangular plastic tank (100 × 175cm) liters capacity containing chlorine free aerated well water.

Fishes were acclimated to laboratory conditions prior to start of experiment. Fishes were fed twice a day with crumbled feed (30 % Digestible Protein and 2.70 Kcal g-1 Digestible Energy) during adaptation, but they were not fed during last 24 hours of adaptations and throughout the test duration. Leftover feed and feacal wastes were removed from all the aquaria through vacuum pumps. After acclimation to laboratory conditions the fish were transferred to the glass aquaria for toxicity tests. Chemically pure compounds of nickel ($NiSO_4 \cdot 6H_2O$) were dissolved as per desired weight in deionized water and stock solutions were prepared. Ten (10) fishes were kept in each glass aquarium with three replicates for each test dose. In order to eliminate stress to fishes, the concentration of metal mixture in each aquarium was increased gradually and 50 percent test concentration was maintained within 3.5 hours and full toxicant concentration in 7 hours. Constant air was supplied to all aquaria.

Experimental Design

Group I: Control group, fish were reared in tap water and fed with fish commercial diet.

Group II: Fish exposed to 35mg/L of Nickel Sulphate (Ni) (7days).

Group III: Fish exposed to 35mg/L of Nickel Sulphate (Ni) (14days).

Tissue samples were obtained from fish after the expiry of 7 & 14 days for the investigation of the biochemical parameters and oxidative stress. In addition, tissue specimens were collected for detection of the pathological changes in gill and muscle of exposed fish.

Biochemical Analysis

Estimation of Total Proteins

The total protein contents in the tissues was estimated by the method of Lowry et al. [7] To 500 mg of the sample 0. 9 ml of 10% TCA was added to precipitate the proteins and centrifuged. To the residue 1. 0 ml of 1N NaOH was added. 0. 5 ml of the above sample and 40 ml of alkaline copper reagent was mixed and kept at room temperature for 10 minutes. After 10 minutes 0. 5 ml of diluted Folin-phenol reagent was added to the mixture and was kept at room temperature for 20 minutes and read at 640 nm. Standards (Bovine albumin) and blanks were also treated in the same manner.

Estimation of Total Lipids

Lipid content was estimated by the semimicro determination method of Pande et al.[8] The tissues from the experimental animals were isolated on ice and were quickly weighed. 25 mg of the tissue was homogenised in cold chloroform-ethanol mixture (2:1). Filter paper soaked in chloroform-methanol mixture was used to filter the homogenate. One ml of filterate was taken into a glass stoppered graduated tube (6″× 3/4″) and the solvent was removed by rapid evaporation under reduced pressure in vacuum desiccator. After complete removal of the solvent the vacuum was released carefully. The tubes were removed and 3.0 ml of 2% potassium dichromate in 98% sulphuric acid was added. The tubes were placed in a boiling water bath for 15 minutes and then cooled in ice water bath. When the contents were cooled 4. 5 ml of distilled water was added and the tubes were cooled again in running tapwater. The colour intensity was measured at 590 nm in a spectrophotometer against a reagent blank and the values are expressed a mg/g wet weight of the tissue.

Estimation of Glucose and Glycogen

The estimation of glucose and glycogen was carried out following the methods of Kemp and Kits Van Heijhigen.[9] The tissues were isolated and homogenized in 5.0ml of 80% methanol and centrifuged at 3000rpm for 15min. The supernatant containing free glucose was decanted into a calibrated test tube. The residue was set apart for the quantitative estimation of glycogen.

Estimation of Glucose

To the decanted supernatant, 10ml of activated animal charcoal powder was added. The methanol was allowed to evaporate by warming the solution in a water bath.

Deproteinising solution (5g of TCA in 100 ml distilled water) was added to the residue to bring the total volume to 5. 0 ml. the suspension was centrifuged at 2000rpm for 10min and the clear supernatant was used for the estimation of glucose.

1. 0 ml of supernatant was taken in a separate test tube and 3. 0 ml of concentrated sulphuric acid was added to it. The mixture was heated in a boiling water bath and subsequently cooled in running tap water. The intensity of colour developed was measured in a spectrophotometer against the reagent blank (3. 0ml of concentration sulphuric acid) at 520 nm. The quantity of glucose present in the sample was read from the standard graph drawn from known quantities of glucose. The quantity of glucose present in the sample is expressed in mg/g wet weight of the tissue.

Estimation of Glycogen

For the estimation of glycogen, the residue left after methanol extraction was resuspended and homogenized in 5. 0 ml of deproteinising silver nitrate solution (5g TCA and 10mg Ag_2SO_4 in 100ml of distilled water and heated at 100°C in a water bath for 15 minutes. The mixture was cooled and made upto 5. 0 ml with deproteinsing silver sulphate solution once again and centrifuged at 2000rpm for 10 minutes. The clear supernatant was collected for the estimation of glycogen.

1. 0 ml of the supernatant was taken in a separate test tube and 3. 0ml of concentrated sulphuric acid was added to it. The mixture was heated in a boiling water bath for 6. 5 minutes and subsequently cooled in running tap water. The intensity of colour developed was measured in a spectrophotometer against the reagent blank (3ml of concentrated sulphuric acid) at 520nm. The quantity of glycogen present in the sample was read from known quantities of glycogen. The quantity of glycogen present in the sample is expressed in mg/g wet weight of the tissue.

SOD Assay

SOD activity was analysed as described earlier by Parihar et al.[10] The tissue homogenate (1:8 w/v) for SOD activity assay was treated with 1 ml of triton X-100 (1%) for 30 min to ensure that full activity was released and then centrifuged at a speed of 1600 x g at 4°C. The pellet was discarded and the supernatant was assayed for SOD activity, which involves the ability of enzyme to inhibit the autooxidation of pyrogallol. The assay system contained 1 mM DTPA, 50 mMtris-HCl buffer (pH 8. 2) and tissue homogenate (0. 5 ml). The assay mixture was transferred to a 3 ml cuvette and the reaction was initiated by the addition of freshly prepared 2. 6 mM pyrogallol solution in10 mM HCl. The enzyme kinetics was carried out at 420 nm for 10 min at room temperature (27°C) on a Perkin-Elmer UV spectrophotometer. One unit of SOD is described as the amount of enzyme required to cause 50% inhibition of pyrogallol autooxidation per 3 ml of assay mixture. Calculations were made per gram wet weight of tissue.

GPX Assay

GPx activity was measured in spectrophotometer by a coupled enzyme procedure at 27°C, monitoring loss of NADPH at 340 nm as described by Lawrence and Burk.[11] The homogenate was centrifuged at 4°C at 5000 rpm for 15 min and resulting supernatant at 10, 000 rpm for 20 min. The post mitochondrial supernatant (PMS) (the resulting supernatant at 10, 000 rpm) was used for enzyme assay. The enzymatic reaction was conducted in 3 ml quartz cuvettes of 1 cm path length in a Perkin-Elmer spectrophotometer. The reaction mixture contained 50 mM phosphate buffer (pH 7. 4), 4 mM sodium azide, 1 mM EDTA, 4 mM reduced glutathione (GSH), 0. 2 mM NADPH, 1 unit glutathione reductase, 0.1 ml of supernatant sample and 0.71 mM H_2O_2 as substrate. Reactions were initiated by the addition of H_2O_2. Direct proportionality was seen with GSH-Px enzyme activity overtime and with sample concentration. Activities were estimated from the decrease of optical density at 340 nm and to NADPH oxidation between 2 and 4 min after the start of the reaction. The results are expressed as unit/g wet weight of tissue. One unit of GPx defined as the amount of the enzyme necessary to catalyse 1 nmol NADPH min/g wet weight at 27°C.

GSH Assay

The GSH was quantitated by the method of Jollow et al.[12] The homogenate for GSH assay was centrifuged at1600 g for 15 min at 4°C. The supernatant (0. 5 ml) was added to 4 ml of ice cold 0. 5 M solution of 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB) in 0. 1 M phosphate buffer (pH 8.0). The optical density was read at 412 nm in a spectrophotometer (Perkin-Elmer, USA). A calibration curve was prepared using GSH as a standard.

Estimation of Lipid Peroxidation

TBARS in tissues were analysed by the method of Ohkawa et al.[13] To 0.2 ml of tissue homogenate, 0.2 ml of 8.1% sodium dodecyl sulfate and 1. 5 ml of 20% acetic acid were added. The pH of mixture was adjusted to 3. 5 with sodium hydroxide, then 1.5 ml of 0. 8% aqueous solution of (TBA) was added to the mixture and the volume was made upto 4 ml with distilled water. The reaction mixture was heated in water bath at 95ºC for 60 minutes. After cooling in tap water, 1.0 ml of distilled water and 5. 0 ml of n-butanol pyridine mixture were added and shaken vigorously. After centrifugation at 4000 rpm for 10 minutes, the organic layer was removed and absorbance was read at 535 nm. The level of TBARS on tissues was expressed in nmol/ mg protein.

Histological Analysis

Tissue samples from control and treated fishes were fixed in 10% neutral buffered formalin and then processed for routine wax histological evaluation (dehydrated and embedded in paraffin). Sections of 5 µ

were taken and stained with hematoxylin and eosin stains.

Statistical Analysis

Results were expressed as Mean ± SEM for six animals in each group. Statistical analysis was completed by one-way analysis of variance (ANOVA) test and followed by Tukey's post hoc test using the Prism 5.0 (San Diego, CA, USA) statistical package program. A value of $p< 0.05$ was considered to be statistically significant.

RESULTS

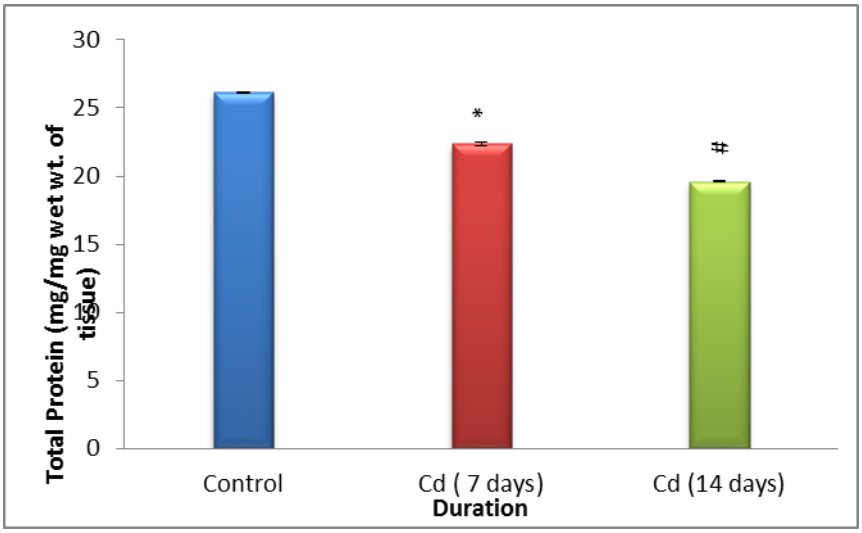

Fig 1: The quantity of total protein in gill of Ni in different duration (7&14 days).

Fig. 1 demonstrates the levels of total protein in gill of control and experimental Carp fish. The levels of Protein were significantly ($p<0.05$) decreased in gill of Ni intoxicated fish. The values were highly decreased in 14 days when compared to 7 days.

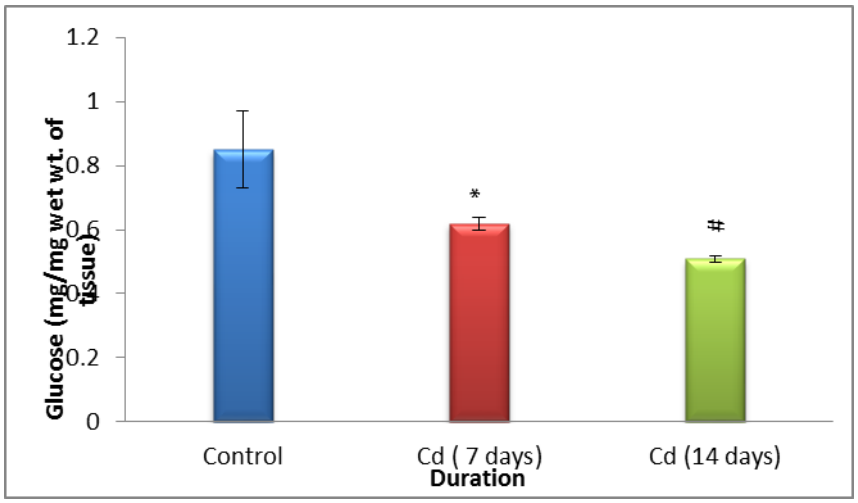

Fig 2: The quantity of glucose in gill of Ni in different duration (7&14 days).

Fig. 2 demonstrates the levels of glucose in gill of control and experimental Carp fish. The levels of glucose were significantly ($p<0.05$) decreased in gill of Ni intoxicated fish. The values were highly decreased in 14 days when compared to 7 days.

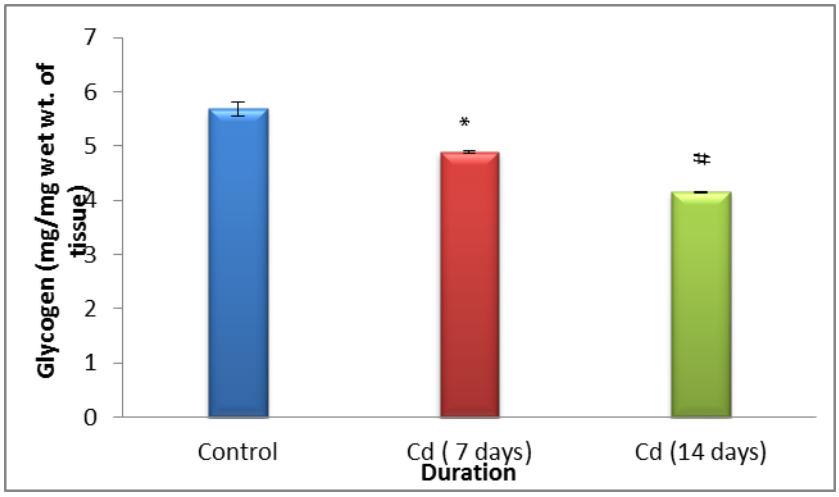

Fig 3: The quantity of glycogen in gill of Ni in different duration (7&14 days).

Fig. 3 demonstrates the levels of glycogen in gill of control and experimental Carp fish. The levels of glycogen were significantly ($p<0.05$) decreased in gill of Ni intoxicated fish. The values were highly decreased in 14 days when compared to 7 days.

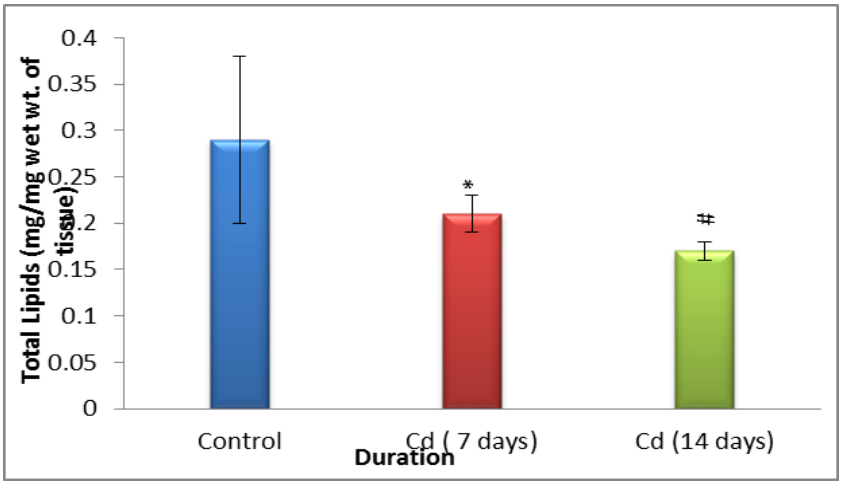

Fig 4: The quantity of total lipids in gill of Ni in different duration (7&14 days).

Fig. 4 demonstrates the levels of lipids in gill of control and experimental Carp fish. The levels of lipids were significantly ($p<0.05$) decreased in gill of Ni intoxicated fish. The values were highly decreased in 14 days when compared to 7 days.

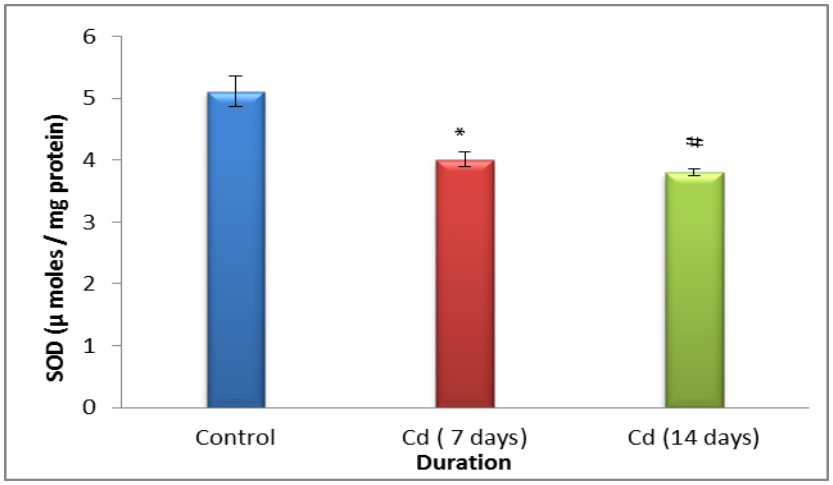

Fig 5-8: The quantity of Oxidative biomarkers & Antioxidant enzymes in gill of Ni in different duration (7&14 days)

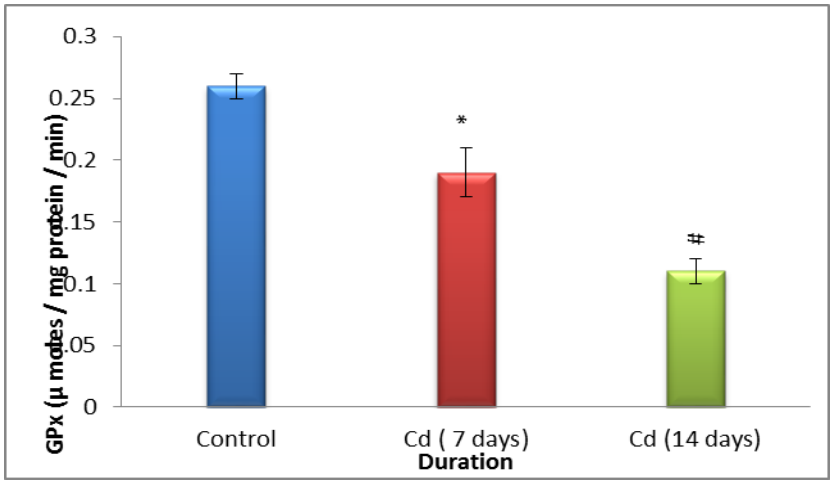

Fig 5&6: demonstrates the activities of enzymic antioxidants SOD & GPx were significantly (p<0.05) decreased in the gill of Ni treated Carp fish. The values of antioxidant enzymes were highly decreased in 14 days when compared to 7 days.

The values are expressed as mean ± sd. n=6, values that do not share a common superscript symbol in the same coloum differ significantly at p<0.05 DMRT. *con vs 7th day, #con vs 14th day.

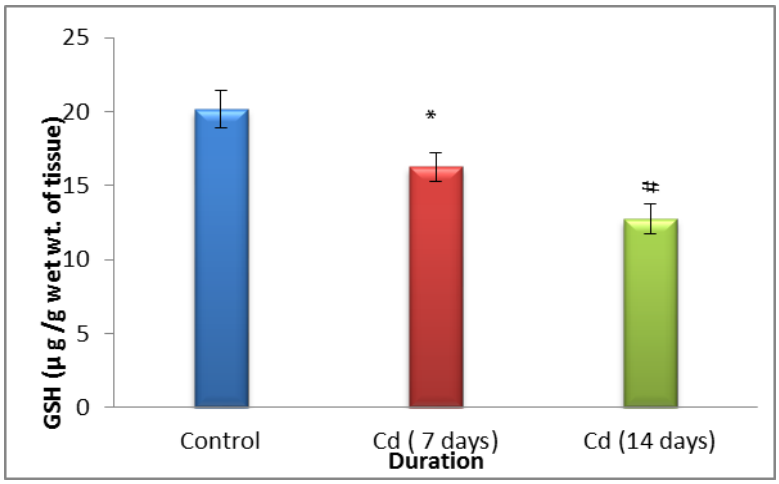

Fig. 7: demonstrates the activities of Non-enzymic antioxidant GSH were significantly (p<0.05) decreased in the gill of Ni treated Carp fish. The values of Non-enzymic antioxidant enzyme were highly decreased in 14 days when compared to 7 days.

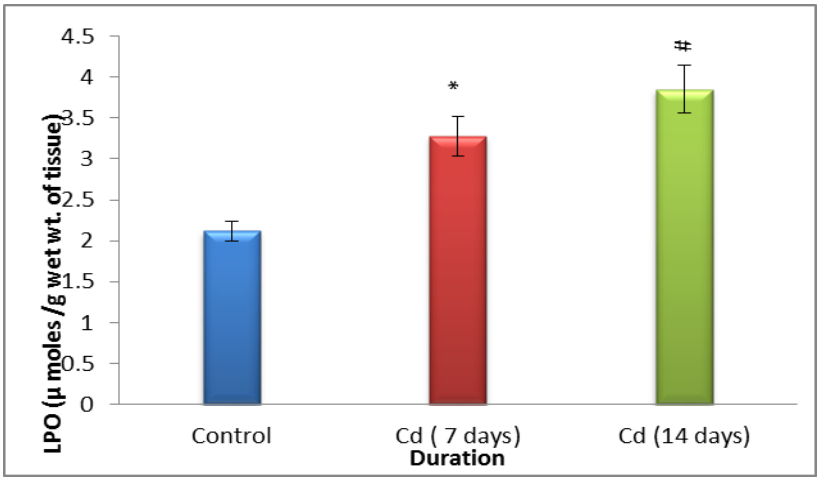

Fig 8: Demonstrates the levels of lipid peroxidation were significantly (p<0.05) increased in the gill of Ni treated Carp fish. The values of Non-enzymic antioxidant enzyme were highly increased in 14 days when compared to 7 days.

The values are expressed as mean ± sd. n=6, values that do not share a common superscript symbol in the same coloum differ significantly at p<0.05 DMRT. *con vs 7th day, #con vs 14th day.

Fig. 9 HISTOPATHOLOGY OF GILLS

Fig. 9 a. Control fish Gill H &E 40X

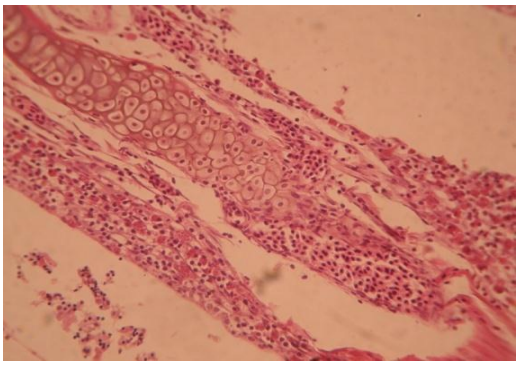

Fig. 9 b. Ni Treated Fish Gill (7 day) H &E 40X

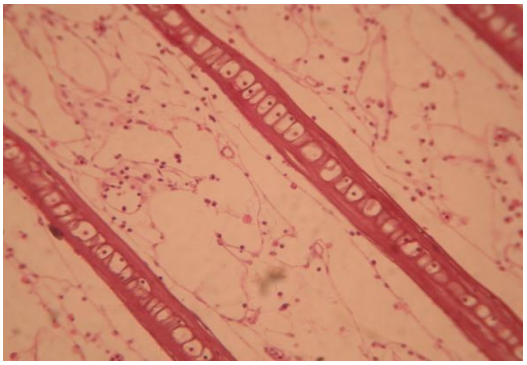

Fig. 9 c. Ni Treated Fish Gill (14 day) H &E 40X

Groups	Observations
Control	Blood capillary, mucous cells, primary lamellar epithelium and secondary lamellae have been observed.
Ni Toxicity (7 days)	Blood congestion, sub epithelial oedema, epithelial hyperplasia, hypertrophy of pavement cell and Vacuolar degeneration of cytoplasm is occurred.
Ni Toxicity (14 days)	Severe congestion shortening in length and tearing of subepithelial oedema and Lamellar fusion has been observed.

Histopathology of Gill

Fig. 10 HISTOPATHOLOGY OF MUSCLE

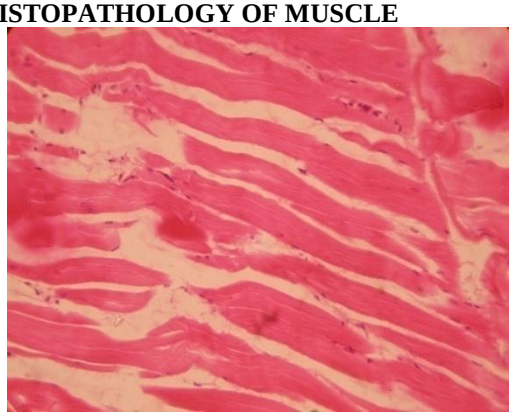

Fig. 10 a. Control fish Muscle H &E 40X

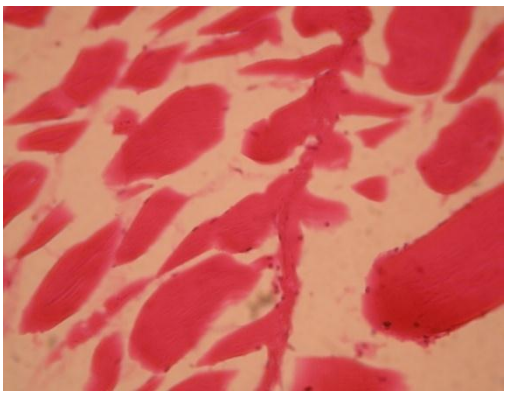

Fig. 10 b. Ni Treated Fish Muscle (7 day) H &E 40X

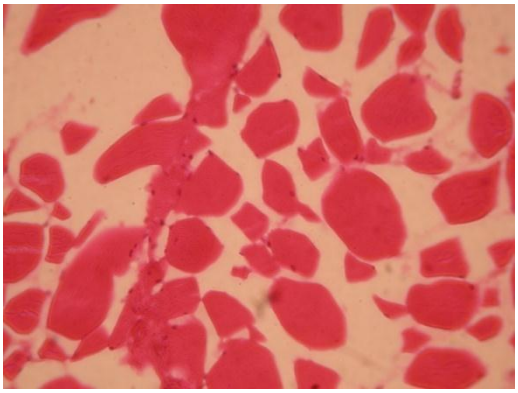

Fig. 10 c. Ni Treated Fish Muscle (14 day) H &E 40X

Histopathology of Muscle

Groups	Observations
Control	Normal muscular bundles were seen
Ni Toxicity (7 days)	Necrosis and aggregations of inflammatory cell are observed.
Ni Toxicity (14 days)	Disorganisation of muscle fiber, necrosis, and Chronic inflammation is occurred.

DISCUSSION

Nickel is used in a wide variety of metallurgical processes such as electroplating and alloy production as well as in nickel-cadmium batteries. Ni accumulates and proves to be a multi target toxicant causing damage to many organs such as gill, kidney, lung, brain and testis. Ni produced a significant oxidative stress to the treated fishes, which was evident from the increased levels of lipid peroxidation and thereby increased in the levels of serum hepatic markers, oxidative stress markers in liver.

Proteins are involved in major physiological events therefore the assessment of the protein content can be considered as a diagnostic tool to determine the physiological phases of organism. Proteins are highly sensitive to heavy metal poisoning.[14] The depletion of total protein content may be due to breakdown of protein into free amino acid under the effect of mercury chloride of lower exposure period due to their utilization in the formation of mucoproteins which are eliminated in the form of mucus.[15]

Epidemiological and experimental studies suggest that exposure to Ni leads to alteration of lipid metabolism and occurrence of cardiovascular diseases.[16] Since the gills plays a pivotal role in lipid homeostasis, the accumulation of Ni in gill could be responsible for the dysfunction of gill metabolites has been observed in our study.

Antioxidant enzyme are considered to be the body's primary defense, which prevents biological macromolecules from oxidative injury and removes peroxides, free radicals and superoxide anion generated inside the cell. GSH, GPx and GST constitute mutually a supportive team of defense against reactive oxygen species (ROS). The decrease in the activities of antioxidant enzymes might be due to binding of Ni with sulphydryl groups of enzymes and oxidative modifications of amino acid chains, which alters the enzyme structure and leads to inactivation or decreased activity of enzyme. Thus, the decreased activities of tissue antioxidant systems clearly intimates the accumulation of free radicals and increased level of LPO, which increase the oxidative stress by depleting the antioxidants in tissues.

The biochemical analysis showed the depletion of total protein, glucose, glycogen and lipid contents in gill. The disturbance in the protein, carbohydrate and lipid metabolism was considered as one of the most outstanding biological lesions due to the action of Ni and utilization to meet excess energy demand as well as the condition and response of the test organism to Ni, the degree of retention and the rate of excretion. The Lipid peroxidation marker Viz., MDA was also increased with increasing days of exposure.

In the present study, the gills showed extensive fusion of gill epithelium, hyperplasia and hypertrophy of various cell types, extensive vacuolization, and disintegration of various cell types and invasion of epithelia by phagocytic cells. All these alterations may be the attempts made by the fish to prevent the entry of Ni.

CONCLUSION

The toxic responses elicited by grass carp upon Ni exposure in a sub lethal dose showed a statistically significant differences in all the biochemical and morphological endpoints in both the exposure periods. It may be concluded that the introduction of sublethal levels of nickel in freshwater fish grass carp results in oxidative stress, antioxidant imbalance and depletion of energy depots in the exposed tissues of grass carp. This oxidative stress-mediated metabolic changes are also contributed by stress-induced biochemical action of

nickel on fish gill function leading to hypoxia. The indiscriminate disposal of nickel effluents by industry may have profound adverse effects on the survival potential of freshwater fishes.

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