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Chelating efficacy of CaNa₂ EDTA on nickel-induced toxicity in *Cirrhinus mrigala* (Ham.) through its effects on glutathione peroxidase, reduced glutathione and lipid peroxidation

Rengaswamy Gopal [a,*], S. Narmada [a], Remya Vijayakumar [a], Cheruth Abdul Jaleel [b]

[a] *Division of Toxicology, Department of Zoology, Annamalai University, Annamalainagar 608 002, Tamilnadu, India*
[b] *Stress Physiology Lab, Department of Botany, Annamalai University, Annamalainagar 608 002, Tamilnadu, India*

Abstract

In this age of modern biology, aquatic toxicological research has provided potential tools for ecotoxicologic investigations. Heavy metals primarily affect protein structures and induce a stress in the organisms. The present investigation was carried out to assess the effect of nickel chloride on the selected organs of the freshwater fish *Cirrhinus mrigala* and how CaNa₂ EDTA counters its effects as an antidote. Toxicity experiments were conducted for different exposure periods and also in certain tissues namely gill, liver, kidney and muscle. The total protein content, reduced glutathione, glutathione peroxidase and lipid peroxidation were found to be decreased in the nickel chloride treated tissues and the treatment with CaNa₂ EDTA + nickel chloride returned to near normal levels. Histopathological observations also revealed that after the administration of nickel chloride + CaNa₂ EDTA the chelator induced reduction in nickel toxicity. It has also contributed towards reduction in the pathological damage, thus enabling the organs to attain their near normal histological appearance. The present study shown that CaNa₂ EDTA is an effective chelating agent for the removal of nickel and it has proved efficient in restoring both the biochemical variables and pathological features immediately after a sub lethal exposure of nickel chloride in fish. *To cite this article: R. Gopal et al., C. R. Biologies 332 (2009).*
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1. Introduction

Indiscriminate discharge of raw and partially treated industrial effluents into the aquatic system leads to deterioration of the environment. Among these pollutants the commonest toxins are organic salts of many heavy metals like, copper, zinc, lead, nickel, cadmium, silver and mercury. Some of the heavy metals are biologically important. In trace quantities heavy metals such as iron, copper, manganese, magnesium and zinc are essential for the metabolism of organisms [1]. Essential metals always function in combination with organic molecules, mostly with proteins either bound to metallo-proteins or loosely bound to metal–protein complexes [2]. If the threshold concentration of these metals increases in the environment they may interfere with the metabolic activity in organisms. Hence, the non-essential elements

viz. mercury, cadmium, silver and lead are toxic to aquatic organisms even at very low concentrations.

Polluted water is one of the most significant sub lethal responses of fish [3]. Aquatic population can be affected by aquatic pollutants not only directly but their active retreat out of polluted areas can also result in disturbance of their migrations and distribution patterns [4]. Therefore a reduction of their normal area of habitat as well as their resources can occur [5]. From another point of view, the ability of fish to detect and avoid toxic substances is one of the forms of phenotypic adoption allowing them to survive in altered environment [6].

The rapid pace of industrialization and anthropogenic inputs have contaminated many ecosystems [7] especially the aquatic ecosystem, which receives a wide range of pollutants. Pollution of aquatic habitats seems to be an inevitable problem of universal nature and the intrusion of various pollutants into the aquatic environment affects the survival growth and reproduction of the biological organisms present in the environment. As fish being exclusively aquatic a number of potentially hazardous xenobiotics confront fish life in the sphere of their activities of which, a category of special interest is that of the heavy metals [8].

Heavy metals are extensively used in industries like electroplating, medicine, pesticide and battery manufacturing [9]. Recently, an increase in the concentration of heavy metals (Cd, Cu, Fe, Ni, Mn, Zn, Pb, and Hg) has been reported in water of Vasai Creek, Maharastra [10]. Heavy metal salts constitute a serious type of pollution in freshwater and being stale compounds they are not readily removed by oxidation. Precipitation of other processes can affect the activity in recipient animal [11].

Nickel is one of these heavy metals. The main sources of nickel come from hydrogenation of oil industry and paint factories, motor vehicle, aircraft industry, printing and in some cases the chemical industry. It is also used extensively in electroplating as nickel sulphate and nickel hydroxide is used in nickel–cadmium batteries [12]. In aquatic ecosystem, dissolved Ni concentrations are generally between 0.005 and 0.01 mg l^{-1} [13]. The toxicity of Ni to aquatic life has been shown to vary significantly with organism species, pH and water hardness [14]. Nickel toxicity is generally low [15] but elevated concentration can cause sub lethal effects. In fresh water fish, recorded Ni accumulation values ranged from 10 to 120 µg g^{-1} [16] and distribution of Ni varies significantly between the different tissues [17].

Chelating agents are the most versatile and effective antidotes for metal intoxication. These compounds are usually flexible molecules with two or more electronegative groups that form stable coordinate covalent bonds
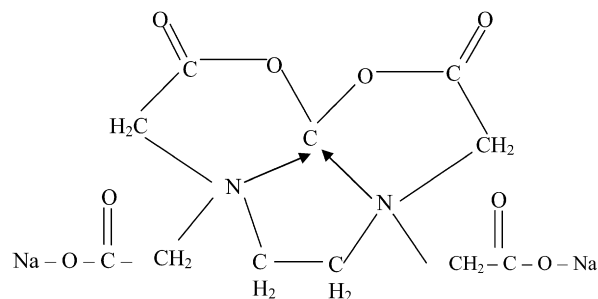

Fig. 1. Structure of calcium di sodium-ethylene diamine tetra acetic acid.

with the cationic metal atom. The body then excretes the complexes thus formed. The greater the numbers of these ligands, the more stable the metal chelator complex. The chelating ligands include functional groups such as –OH, –SH and –NH, which can donate electrons for coordination with the metal [18]. Chelating therapy is widely used in the management of metallic poisoning. Chelating therapy involves applying chelating agents into the blood stream for the purpose of eliminating from the body undesirable substances such as heavy metals, chemical toxins, mineral deposit and fatty plaques.

Calcium disodium complex – Ethylene diamine tetra acetic acid (CaNa$_2$ EDTA) is an efficient chelator of many divalent and trivalent metals, especially for chelation of cadmium [19]. It is a stable water soluble metal chelate, mainly used in food industry, agriculture and horticulture as micronutrient, and calcium is present in a chelated form. It penetrates cell membranes and chelates extra cellular metal ions more effectively than intracellular ions [20]. The structure of CaNa$_2$ EDTA is shown in Fig. 1.

Earlier researchers have concentrated mainly on adult fishes and not much of work has been done on fingerlings, which are more susceptible to environmental contamination. Also, the fingerlings have higher metabolic rates (per gram of body tissue) and therefore are able to take up metals via food and water more rapidly than adult fish. With this in view, in the present study an attempt has been made to investigate the toxic effects of nickel and the chelating influence of CaNa$_2$ EDTA on the selected organs of freshwater, *Cirrhinus mrigala* (Ham.).

2. Materials and methods

2.1. Collection and maintenance of fish

Healthy specimens of *Cirrhinus mrigala* were collected from the farm located in Puther; Nagai, Q. Mil-

leth District, Tamil Nadu, 15 km away from the University campus. They were stocked and maintained in the laboratory conditions ($25 \pm 2\,°C$) for at least 15 days in large cement tanks (capacity 50 liters) previously washed with $KMnO_4$ to free the walls from fungal infections if any. The fishes were fed with cooked rice and groundnut oil cake everyday and water in the tank was renewed every 24 hrs to reduce the quantum of excretory products in the test water. Size of the fish ranging from 10–13 cm in length and 8–12 g in body weight were used in the present study.

2.2. Physical and chemical properties

Nickel is a silvery white ferromagnetic metallic element in Group VIII of periodic table. Nickel has the specific density of 8.90 g/cm^3 and a melting point of 155 °C and boiling point of 28–37 °C. It is insoluble in water, soluble in dilute Nitric acid and Aquaregia and slightly soluble in Hydrochloric acid and Sulphuric acids. Nickel usually has an oxidation state of 2, but also occurs as relatively stable Tri- and Tetravalent ions [21]. Nickel forms complexes (Chelates) that are insoluble in water but soluble in organic solvents. These compounds are often very stable and play an important role in trace analysis.

2.3. Experimental protocol

Following a 24 hr renewal bioassay system for the analysis of sub lethal toxicity did the toxicant exposure. 3 groups of 20 fish each were exposed separately to nickel chloride (35 ppm; 10% of 96 h LC_{50}) and nickel + $CaNa_2$ EDTA (1:3 ratio, APHA, 1999) solution. The experimental medium was prepared by dissolving nickel chloride at 35 ppm in well water having dissolved oxygen 5.8 ppm, pH 7.4, water hardness 30.3 mg/l [22] and water temperature $28 \pm 2\,°C$. Each group was exposed to 50 liter of experimental medium. Parallel group of 20 fish each were kept in separate aquaria containing 50 liter of well water (with out the addition of nickel chloride) and treated as control. Feeding was allowed in the experimental as well as control groups everyday for a period of 3 hrs before the renewal of the medium throughout the tenure of the experiment.

2.4. Calculation of LC_{50}

The LC_{50} value was obtained by the Probit analysis. Method was based on the observed percentage of test animals surviving at concentrations lethal to more than half and less than half of the test subjects [23].
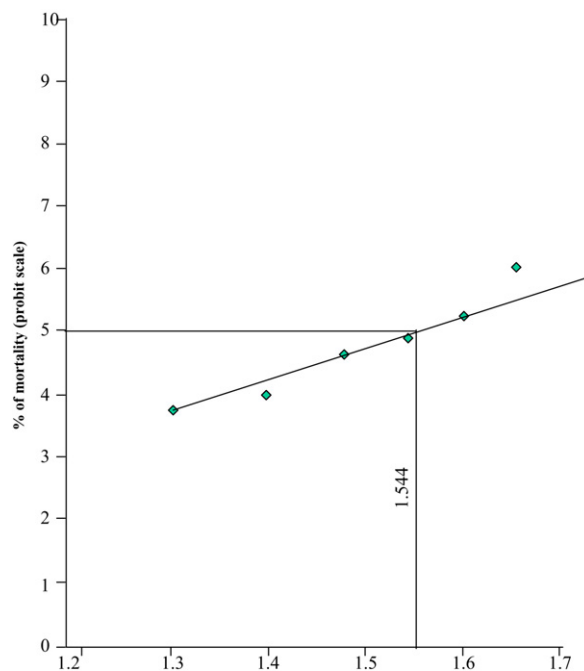

Fig. 2. Probit of mortality of different concentration of nickel chloride.

The plotted dose response data reveal that the relationship of dose to response is non-linear. The Probit model [24] transforms the axis of the percentage of mortality to the Probit scale and the dose axis to the logarithm of the dose. By performing the transformation, the Probit model [24] transforms the dose-response curve to a straight line, which can be used for the calculation of the lethal concentration. The experimental data were analyzed by taking the Probit value of the percentage of mortality to the Y-axis and the logarithm value of the administered dose on the X-axis. A straight line was obtained on plotting the experimental data (Fig. 2). The LC_{50} can be interpolated from the curve by drawing a horizontal line from the 50% mortality point on the ordinate to the concentration-response curve and then drawing a vertical line from its point of intersection with the curve to the abscissa. The vertical line is then read off the graph. The percentages of mortality for different concentration and the obtained 96 hrs LC_{50} for nickel chloride are given in Table 1.

2.5. Biochemical analysis

After the expiry of 28 days of exposure, 3 fishes each from the respective experimental as well as control groups were sacrificed. First pair of Gills (from both sides) liver, Kidneys, and pieces of muscle was excised

Table 1
Percentage of morality of *Cirrhinus mrigala* exposed to different concentrations of Nickel chloride.

Sl. no.	Concentration of NiCl$_2$ (mg/l)	Log concentration	Mortality	% of mortality	Probit scale
1.	20	1.301	2	10	3.7182
2.	25	1.397	3	15	3.9636
3.	30	1.477	7	35	4.6147
4.	35	1.544	9	45	4.8743
5.	40	1.602	12	60	5.2533
6.	45	1.653	17	85	6.0364
7.	50	1.699	20	100	0.0000

Anti log – 1.5522, LC$_{50}$ 96 hrs – 35.66 mg.

from the experimental as well as control fish and were subject to various studies.

2.5.1. Estimation of total protein

Protein content in the selected tissue viz. gill, liver, kidney and muscle was estimated by following the method of Lowry et al. [25]. The tissue was isolated and 2% homogenate of each of the tissue was prepared in 10% TCA. The protein content is expressed in mg/g wet weight of the tissue.

2.5.2. Estimation of reduced glutathione

Reduced glutathione was determined according to the method of Moron et al. [26]. This method was based on the reaction with 5,5′-dithio-(bis)-2-nitrobenzoic acid (DTNB) to give a compound that absorbs at 412 nm. To 1 ml of the homogenate was added 1 ml of distilled water and 1 ml of 5% Trichloroacetic acid. The precipitate was removed by centrifugation. To an aliquot of the supernatant 2 ml of DTNB was added and 0.5 ml of phosphate buffer to a final volume of 3 ml. The absorbances was read at 412 nm in Spectronic – 20 (Bausch & Lomb) against a reagent blank. A series of standards (100–500 μg reduced glutathione) was also run in a similar manner. The amount of reduced glutathione was expressed as μg/mg protein for tissue.

2.5.3. Estimation of glutathione peroxidase (GPx)

The activity of Glutathione peroxidase was assayed by the method of Rotruck et al. [27]. With some modification a known amount of enzyme preparation was allowed to react with H$_2$O$_2$ in the presence of reduced glutathione. After a specified period of enzyme action, the remaining reduced glutathione content was measured by the method of Moron et al. [26].

To the mixture of 0.2 ml of phosphate buffer, 0.2 ml of EDTA, 0.1 ml of sodium azide and 0.2 ml of the enzyme preparation (tissue homogenate) were added and incubated at 37 °C for two minutes. Then 0.2 ml of re-

duced glutathione and 0.1 ml of H$_2$O$_2$ were added to the mixture and at 37 °C exactly for 10 minutes. The reaction was arrested by the addition of 0.5 ml of 10% TCA. Reduced glutathione content was estimated in the supernatant obtained after centrifugation. A blank was prepared similarly to which 0.2 ml of the enzyme was added after the incubation period. The activity of glutathione peroxidase was expressed as μg pf GPx utilized/min/mg protein.

2.5.4. Estimation of lipid peroxidation

The levels of lipid peroxidation in liver, gill, kidney and muscle tissue were estimated by the methods of Hogburg et al. [28]. In this method Malondialdehyde and other Thiobarbituric acid reactive substances (TBARS) were measured by their reactivity with Thiobarbituric acid (TBA) in acidic condition. A pink color was read at 535 nm. Tissue homogenate was prepared in Tris-HCl buffer (pH 7.5). 1.0 ml of the tissue homogenate was combined with 2.0 ml of TBA–TCA–HCl reagent and mixed thoroughly. The mixture was kept in a boiling water bath (60 °C) for 15 minutes. After cooling, the mixture was centrifuged at absorbance of the chromophore at 535 nm against the reagent blank. Values were expressed as mM/100 g tissue.

2.6. Statistical analysis

The data obtained from the biochemical analysis as well as accumulation studies were subjected to standard statistical processing based on random sampling of four different samples of experimental as well as control groups of each tissue at each sampling period. The observations were analyzed using Student 'T' test.

3. Results

3.1. Acute toxicity study

The acute toxicity values for the fish, *Cirrhinus mrigala* exposed to nickel were determined at different time intervals. The LC$_{50}$ values for 24, 48, 72 and 96 hrs were estimated as 20, 25, 30, 35, 40, 45 and 50 ppm respectively (Table 1). The relationship between the median lethal concentrations (LC$_{50}$) of nickel with the exposure periods is given in the toxicity curve (Fig. 2).

3.2. Acute bioassay of Cirrhinus mrigala exposed to nickel chloride

Acute toxicity tests are generally used to assess the potential hazards of chemical contamination in aquatic

organisms. In fishes and other aquatic organisms, the effects of xenobiotics that have occurred within a short period of time are considered to be acute and relatively scarce. Data obtained in acute toxicity tests are generally used to compare the sensitivity of different species to potentialities of chemicals using LC_{50} values and thereby to decide safe environmental concentration. In the present study, the LC_{50} values for different exposure hours were studied. The percentage of mortality in different concentrations was also studied. It was observed that there is no mortality in the control as well as in the lowest concentrations below 96 hrs.

The logarithm of 50% lethal concentration is obtained by finding the value on the abscissa for straight line which assumes the probit value 5. The concentration resulting in 50% mortality and slope of the probit line were calculated for specific period of exposure as described by Finney (1971). The percent mortality data were subjected to probit values and plotted against log dose concentrations resulting in a straight line (Fig. 2). The LC_{50} value for 24, 48, 72 and 96 hrs of exposure periods were estimated as 20, 25, 30, 35, 40, 45, 50 ppm respectively (Table 1). Results thus clearly indicate the relationship between log concentrations and expected probit which is found to be positive and linear and the percentage mortality has been increased with an increase in toxicant concentrations and also with the increased exposure periods.

3.3. Biochemical changes

Exposure of *Cirrhinus mrigala* to sub lethal concentration of nickel chloride had induced various biochemical alterations. These alterations were manifested variously in different tissues. The prominent biochemical changes studied in the present work may be summarized as alterations in the total protein, reduced glutathione content along with lipid peroxidation content and glutathione peroxidase content of the selected tissues viz., gill, liver, kidney and muscle. In general, the alterations observed in the biochemical parameters of the tissues studied are listed. Histopathological observations are shown in Plates 1–3.

3.3.1. Impact of nickel chloride toxicity on total protein content

Sub lethal toxicity of nickel has a profound effect on the total protein of all the tissues studied. In the control group of *Cirrhinus mrigala* the level of protein content in gill, liver, kidney and muscle were 14.15 ± 0.52, 79.30 ± 2.60, 40.55 ± 2.54 and 73.10 ± 1.31 mg/g wet wt. of tissues respectively (Table 2).
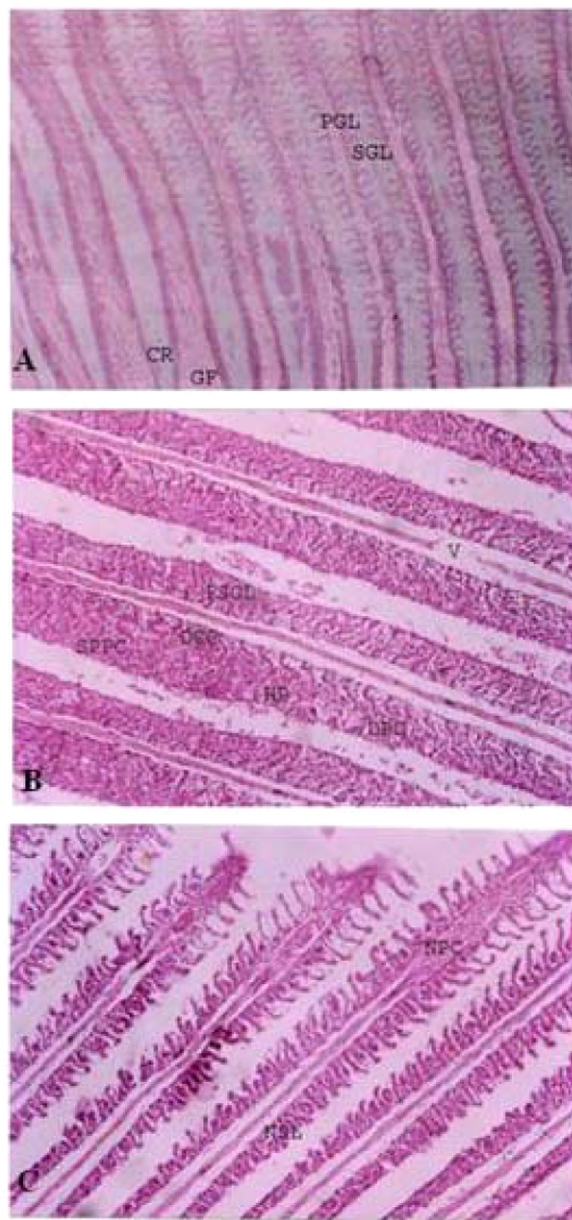

Plate 1. Histopathological studies. GILL. A. Control gill of *Cirrhinus mrigala*. CR – Cartilaginous Rod, GF – Gill Filament, PGA – Primary Gill Arch, PGL – Primary Gill Lamellae, SGL – Secondary Gill Lamellae. B. Gill of *Cirrhinus mrigala* exposed to sub lethal concentration of Nickel chloride. DCC – Disintegrated Chloride Cells, DPC – Disintegrated Pillar Cells, FSGL – Fusion of Secondary Gill Lamellae, HP – Hyperplasia, SPPC – Swollen and Pycontic Pillar Cells, V – Vacuoles. C. Gill of *Cirrhinus mrigala* exposed to sub lethal concentration of Nickel chloride and $CaNa_2$ EDTA. NSL – Normal Secondary Lamellae, NPC – Normal Pillar Cells.

Gill: At sub lethal concentration of nickel chloride-treated group the level of protein content was significantly ($P < 0.01$) reduced to 12.02 ± 0.52 mg/g wet
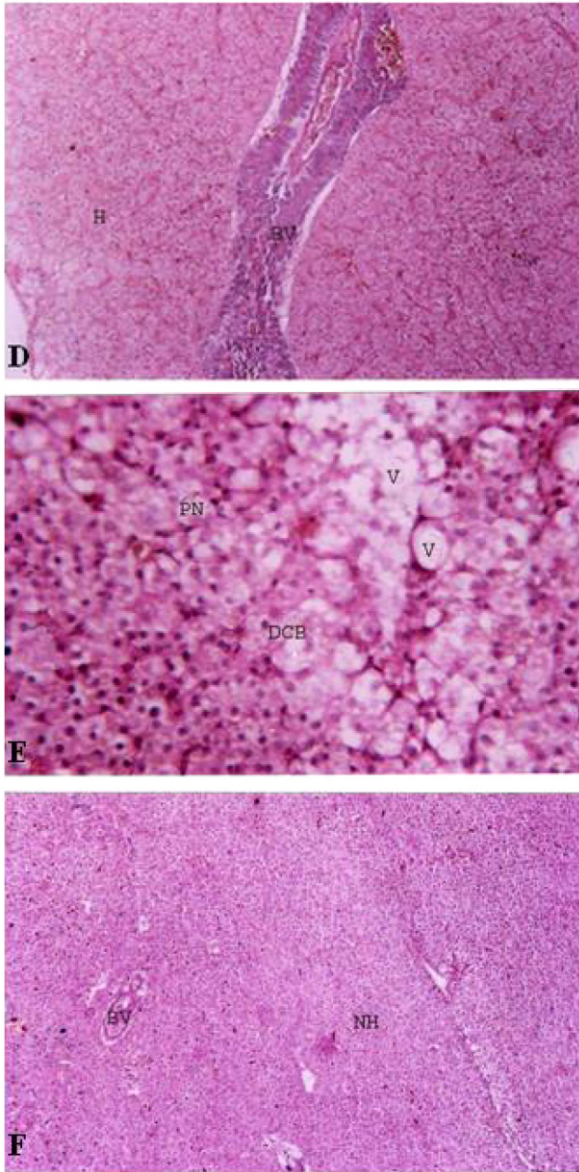
Plate 2. Histopathological studies. LIVER. D. Control Liver of *Cirrhinus mrigala*. BV – Blood Vessels, H – Hepatocytes. E. Liver of *Cirrhinus mrigala* exposed to sub lethal concentration of Nickel chloride. DCB – Disintegrated Cell Boundary, PN – Periphery Nucleus, V – Vacuoles. F. Liver of *Cirrhinus mrigala* exposed to sub lethal concentration of Nickel chloride and CaNa$_2$ EDTA. BV – Blood Vessels, NH – Normal Hepatocytes.
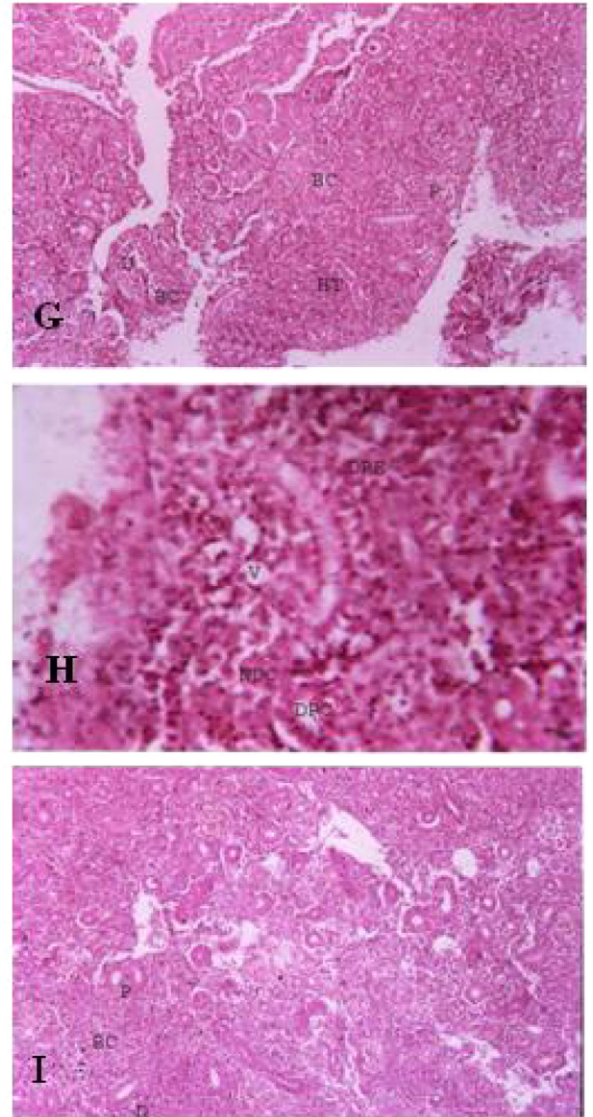
Plate 3. Histopathological studies. KIDNEY. G. Control Kidney of *Cirrhinus mrigala*. BC – Bowman's Capsule, D – Distal Convoluted Tubule, P – Proximal Convoluted Tubule, HT – Hemopoietic Tissue. H. Kidney of *Cirrhinus mrigala* exposed to sub lethal concentration of Nickel chloride. DPC – Disintegrated Proximal Convoluted Tubule, DRE – Desquamated Renal Epithelial Cells, NDC – Necrotic Distal Convoluted Tubule, V – Vacuoles. I. Kidney of *Cirrhinus mrigala* exposed to sub lethal concentration of Nickel chloride and CaNa$_2$ EDTA. BC – Bowman's Capsule, D – Distal Convoluted Tubule, P – Proximal Convoluted Tubule.

wt. in the gill tissues of *Cirrhinus mrigala* when compared with the control. During the nickel chloride and EDTA treatment the level of protein content was significantly ($P < 0.001$) increased to 13.15 ± 0.01 mg/g wet wt. when compared with nickel chloride group.

Liver: At the sub lethal concentration of nickel chloride treated group the level of protein content in the liver was

significantly ($P < 0.01$) reduced to 58.32 ± 0.27 mg/g wet wt. when compared with the control. But in the group with nickel chloride followed by EDTA treatment, the level of protein content was significantly ($P < 0.001$) increased to 77.87 ± 0.05 mg/g wet wt. when compared with nickel chloride group.

Table 2
Level of protein content in different tissues of *Cirrhinus mrigala* exposed to Nickel chloride and Nickel chloride + EDTA.

Tissues	Control (Group I)	Nickel chloride (Group II)	Nickel chloride + EDTA (Group III)
Gill	14.15 ± 0.52	12.02 ± 0.52[**]	13.15 ± 0.01[***]
Liver	79.30 ± 2.61	58.32 ± 0.27[***]	77.87 ± 0.51[***]
Kidney	40.55 ± 2.54	27.38 ± 0.26[***]	38.15 ± 0.24[***]
Muscle	73.10 ± 1.31	52.15 ± 0.57[***]	70.32 ± 0.25

Values expressed in mg/g wet wt. of tissues. Mean $\pm$ S.E of six individual observations. Comparisons were made between Group I vs. Group II, Group II vs. Group III.

[**] $P < 0.01$. [***] $P < 0.001$.

Table 3
Level of reduced glutathione content in different tissues of *Cirrhinus mrigala* exposed to Nickel chloride and Nickel chloride + EDTA.

Tissues	Control (Group I)	Nickel chloride (Group II)	Nickel chloride + EDTA (Group III)
Gill	1.28 ± 0.06	0.85 ± 0.02[*]	1.06 ± 0.02[***]
Liver	2.20 ± 0.24	1.49 ± 0.18[*]	2.02 ± 0.15
Kidney	1.90 ± 0.16	1.28 ± 0.05	1.79 ± 0.18[***]
Muscle	1.70 ± 0.16	1.16 ± 0.02	1.49 ± 0.17

Values expressed in µg/mg protein of tissues. Mean $\pm$ S.E of six individual observations. Comparisons were made between Group I vs. Group II, Group II vs. Group III.

[*] $P < 0.05$. [***] $P < 0.001$.

Kidney: At the sub lethal concentration of nickel chloride-treated group the level of protein content was significantly ($P < 0.001$) reduced to 27.38 ± 0.26 mg/g wet wt. when compared with the control. But in the group with nickel chloride followed by EDTA treatment the level of protein content was significantly ($P < 0.001$) increased to 38.15 ± 0.24 mg/g wet wt. when compared with nickel chloride group.

Muscle: At the sub lethal concentration of nickel chloride treated group the level of protein content was significantly ($P < 0.001$) reduced to 52.15 ± 0.57 mg/g wet wt. when compared with the control. But in the group with nickel chloride followed by EDTA treatment, the level of protein content was significantly ($P < 0.001$) increased to 70.32 ± 0.25 mg/g wet wt. when compared with nickel chloride group.

3.3.2. Impact of nickel chloride toxicity on reduced glutathione content

The GSH level in different tissues viz., gill, liver, kidney, and muscle of control and nickel-exposed fish and the antidote EDTA fish under sub lethal concentration of exposure is shown in Table 3. In control fish, the mean GSH level observed in different tissues were 1.28 ± 0.06, 2.20 ± 0.29, 1.90 ± 0.15 and 1.70 ± 0.15 mg/g wet wt. of tissues. When the fishes were treated with the sub lethal concentration of nickel chloride, they showed a decreasing trend in the GSH level in all the selected tissues of fish after 28 days exposure.

Gill: In the fish exposed to sub lethal concentration of nickel chloride, the GSH level was ($P < 0.05$) reduced to 0.85 ± 0.02 mg/g wet wt. of tissues in gill when compared with control. In the case of nickel chloride followed by EDTA the level of GSH content was significantly ($P < 0.001$) increased to $1.06 \pm 0.0.02$ mg/g wet wt. when compared with nickel chloride group.

Liver: In the fish exposed to sub lethal concentration of nickel chloride, the GSH level was ($P < 0.05$) reduced to 1.49 ± 0.18 mg/g wet wt. of tissues in liver when compared with control. In the case of nickel chloride followed by EDTA the level of GSH content was significantly ($P < 0.005$) increased to 2.02 ± 0.15 mg/g wet wt. when compared with nickel chloride group.

Kidney: In the fish exposed to sub lethal concentration of nickel, the GSH level was reduced to (1.28 ± 0.05) mg/g wet wt. of tissues in kidney when compared to control. In the case of nickel chloride followed by EDTA treatment the level of GSH content was significantly ($P < 0.001$) increased to 1.79 ± 0.18 mg/g wet wt. of tissues when compared with nickel chloride group.

Muscle: In the fish exposed to sub lethal concentration of nickel, the GSH level was reduced to 1.16 ± 0.02 mg/g wet wt. of tissues in muscle when compared to control. In the case of nickel chloride followed by EDTA treatment the level of GSH content was significantly ($P < 0.05$) increased to 1.49 ± 0.17 mg/g wet wt. of tissue when compared with nickel chloride group.

3.3.3. Impact of nickel chloride toxicity on Glutathione peroxidase

The GPx level in different tissues viz., gill, liver, kidney and muscle of control, nickel chloride-treated and nickel chloride and EDTA-treated fish under sub lethal concentration of exposure in shown in Table 4. In control fish, the mean GPx level observed in different tissues were 0.84 ± 0.03, 1.40 ± 0.02, 0.15 ± 0.02 and 0.53 ± 0.03 mg/g wet wt. of tissues. When the fishes were treated in the sub lethal concentration of nickel, they showed a decreasing trend in GPx level in all selected tissues of fish after 28 days exposure.

Gill: When *Cirrhinus mrigala* was intoxicated with sub lethal concentration of nickel chloride, the GPx level

Table 4

Level of glutathione peroxidase content in different tissues of *Cirrhinus mrigala* exposed to Nickel chloride and Nickel chloride + EDTA.

Tissues	Control (Group I)	Nickel chloride (Group II)	Nickel chloride + EDTA (Group III)
Gill	0.84 ± 0.03	$0.28 \pm 0.02^*$	$0.56 \pm 0.18^*$
Liver	1.40 ± 0.02	$0.54 \pm 0.02^*$	1.12 ± 0.01
Kidney	1.15 ± 0.01	$0.59 \pm 0.02^*$	$0.86 \pm 0.026^{***}$
Muscle	0.53 ± 0.02	$0.30 \pm 0.02^*$	$0.58 \pm 0.02^{**}$

Values expressed in µg of GSH consumed/minute/mg protein. Mean ± S.E of six individual observations. Comparisons were made between Group I vs. Group II, Group II vs. Group III.

   * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

Table 5

Level of lipid peroxidation content in different tissues of *Cirrhinus mrigala* exposed to Nickel chloride and Nickel chloride + EDTA.

Tissues	Control (Group I)	Nickel chloride (Group II)	Nickel chloride + EDTA (Group III)
Gill	0.396 ± 0.03	$0.786 \pm 0.03^{***}$	$0.384 \pm 0.02^{***}$
Liver	0.534 ± 0.03	$0.813 \pm 0.026^{***}$	$0.530 \pm 0.02^{**}$
Kidney	0.438 ± 0.06	$0.783 \pm 2.60^{***}$	$0.429 \pm 0.02^{***}$
Muscle	0.423 ± 0.03	$0.762 \pm 0.05^*$	$0.411 \pm 0.02^{***}$

Values expressed in µmol of MDA/g wet wt. of tissues. Mean ± S.E of six individual observations. Comparisons were made between Group I vs. Group II, Group II vs. Group III.

   * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

was decreased to 0.28 ± 0.02 mg/g wet wt. of tissues in gill when compared to control. In the case of nickel chloride followed by EDTA treatment the level of GPx content was significantly ($P < 0.05$) increased to 0.56 ± 0.18 mg/g wet wt. of tissue when compared to nickel chloride group.

Liver: When *Cirrhinus mrigala* was intoxicated with sub lethal concentration of nickel chloride, the GPx level was decreased to 0.54 ± 0.02 mg/g wet wt. of tissues in liver when compared to control. In the case of nickel chloride followed by EDTA treatment the level of GPx content was significantly increased to 1.12 ± 0.01 mg/g wet wt. of tissues when compared to nickel chloride group.

Kidney: When *Cirrhinus mrigala* was intoxicated with sub lethal concentration of nickel chloride the GPx level was ($P < 0.05$) decreased to 0.59 ± 0.01 mg/g wet wt. of tissues in kidney when compared to control. In the case of nickel chloride followed by EDTA treatment the level of GPx content was significantly ($P < 0.001$) increased to 0.86 ± 0.03 mg/g wet wt. of tissues respectively when compared to nickel chloride group.

Muscle: When *Cirrhinus mrigala* was intoxicated with sub lethal concentration of nickel chloride the GPx level was ($P < 0.05$) decreased to 0.30 ± 0.02 mg/g wet weight of tissues in muscle when compared to control. In the case of nickel chloride followed by EDTA treatment the level of GPx content was significantly ($P < 0.01$) increased to 0.58 ± 0.02 mg/g wet weight of tissues when compared to nickel chloride group.

3.3.4. Impact of nickel chloride toxicity on lipid peroxidation content

In the present experimental study the activity of lipid peroxidation has increased significantly after 28 days when *Cirrhinus mrigala* was treated with sub lethal concentration of nickel chloride. In the control fish of *Cirrhinus mrigala* the level of lipid peroxidation content in gill, liver, kidney and muscle were 0.39 ± 0.03, 0.53 ± 0.02, 0.43 ± 0.06 and 0.42 ± 0.03 mg/g wet wt. of tissues respectively (Table 5).

Gill: The mean LPO level in the gill of fish treated with sub lethal dose of nickel chloride significantly ($P < 0.001$) increased to 0.786 ± 0.03 mg/g wet weight of tissues in gills when compared to control. In fish treated with nickel chloride and EDTA the level of LPO was found to be remarkably ($P < 0.001$) reduced to 0.384 ± 0.03 mg/g wet weight of tissues when compared to nickel chloride group.

Liver: The mean LPO level in the liver of fish treated with sub lethal dose of nickel chloride, significantly ($P < 0.001$) increased to 0.81 ± 0.03 mg/g wet weight of tissues in liver when compared to control. In fish treated with nickel chloride and EDTA the level of LPO was found to be remarkably ($P < 0.01$) reduced to 0.530 ± 0.02 mg/g wet weight of tissues when compared to nickel chloride group.

Kidney: The mean LPO level in the kidney of fish treated with sub lethal dose of nickel chloride, significantly ($P < 0.001$) increased to 0.783 ± 2.60 mg/g wet weight of tissues when compared to control. When compared to the nickel chloride-treated group, the level of LPO was found to be remarkably ($P < 0.001$) reduced to 0.429 ± 0.02 mg/g wet weight of tissues in fish treated with nickel chloride and EDTA.

Muscle: The mean LPO level in the kidney of fish treated with sub lethal dose of nickel chloride significantly ($P < 0.05$) increased to 0.762 ± 0.05 mg/g wet weight of tissues when compared to control. When compared to the nickel chloride treated group, the level of LPO was found to be remarkably ($P < 0.001$) reduced to 0.411 ± 0.02 mg/g wet weight in fish treated with nickel chloride and EDTA.

4. Discussion

Despite progress made in environmental waste management, heavy metals still pose immense health hazards to humans and biota. Metals are present in very low concentrations in natural aquatic ecosystems [29], usually at the nanogram to microgram per liter level. Recently the occurrence of especially heavy metals in excess of natural loads in the environment has become an increasing concern [30] for aquatic ecosystem 'health' and is growing at an alarming rate.

The mechanisms of nickel-induced damage include the production of free radicals that alter mitochondrial activity and genetic information. The intake and excretion of heavy metals like nickel, cadmium, mercury and lead depend on the presence of antioxidants and thiols that aid methylation and metallothionein-binding. Antioxidants have specific roles in the mitigation of heavy metal toxicity. Certain metals when used in conjunction with standard chelating agents can improve the mobilization and excretion of heavy metals.

Acute toxicity is defined as the adverse effect occurring within a short time of administration of a harmful substance. It is useful to know the levels below which it may be considered safe for the occurrence of a particular toxicant in the environment [31].

During the present study the *Cirrhinus mrigala* exposed to different concentrations of nickel chloride for varying periods of time showed considerable variations in the degree of the toxicant as evidenced from the occurrence of mortality at varying rates (Table 4). It is evident that about 50% of mortality of *Cirrhinus mrigala* up to 96 hrs was caused by 35 ppm of nickel chloride exposure and is denoted as LC_{50}/96 hrs doses.

The present result indicates that the percentage mortality of the fish for any fixed period increases with increasing concentration of the toxicant and for a particular concentration with an increase in exposure time indicating the regular mode of action of toxicant, which may be due to accumulation and subsequent magnification. Death of the fish may be due to the toxic effect of nickel chloride on the biochemical processes related to cellular metabolic pathways and to other inclusions [32].

A close and careful comparison of LC_{50} values of the present study with earlier reports can bring to light that LC_{50} values also depend upon the type of chemical used and the physical and physiological nature of the treated fish. From the present study it is concluded that the reaction and survival of a treated fish depends not only on the biological state of the fish and physiochemical characteristics of test water but also on the effective concentration levels and time of exposure to nickel toxicants.

Thus knowledge of LC_{50} values obtained for a series of time intervals is highly useful for the final evaluation of the extent of pollution in an aquatic environment caused by heavy metals, especially nickel. The LC_{50} values obtained are also useful in establishing the limits and the levels of acceptability of toxic substances by the aquatic life.

Proteins are the building units of the body and are also the most abundant macromolecules in the cells constituting half of their dry weight [33]. They regulate and integrate various physiological and metabolic processes [34] in the body. Further, the protein content of the fish body mainly determines the quality and food value of its flesh. The maximum concentration of protein in the liver clearly shows the importance of liver in the body metabolism as well as its role as the major storehouse of various metabolites [35]. Further, a higher content of the protein in the liver may be attributed to the fact that liver synthesizes most of the plasma protein [36].

The reduction in the protein content after exposure to nickel chloride may be due to protein synthesis, which is considered the primary biochemical parameter for early indication of stress. This synthesizing is influenced by a large number of exogenous substances. They reduce the protein synthesizing capacity of the endoplasmic reticulum in the cell. In the present investigation, a sub lethal concentration of nickel-exposed fish, *Cirrhinus mrigala* showed a decrease in the protein content of gill, liver, kidney and muscle at the end of 28 days. This may be due to proteolysis, lack of protein biosynthesis or inhibition of translation. A significant decrease in protein content of gill, brain, intestine, liver, kidney and muscle was observed in nickel-treated fish [37]. A study by Jayakumar [38] on exposure to sub lethal concentration of cadmium reveals that the protein content of all the tissues analyzed showed a decreasing trend with variations in the percentage of decrease and a maximum decrease in the tissue of the liver and the gill.

The higher rate of GSH reduction recorded in the gill, liver, kidney and muscle of fish exposed to the sub lethal concentration of nickel chloride indicates primarily the higher amount of nickel chloride accumulated and metabolized by the liver. The gill shows minimum reduction of GSH level when compared to liver, kidney and muscle. The maximum reduction of reduced glutathione was recorded in all tissues after 28 days of exposure. Such reduction in GSH may result in the variability of alteration in membrane integrity, which is evidenced in the present histopathological observation.

Glutathione the most abundant non-protein thiol synthesized in the liver acts as a substrate for the enzyme glutathione peroxidase and also serves as a scavenger of different free radicals [39,40]. Kosower and Kosower [41] stated that the reduced glutathione (GSH) plays an important role in peroxidation (LPO) and provides a defense mechanism for tissues against the reactive oxygen species (ROS). The depletion of reduced glutathione (GSH) in the tissues was induced by heavy metals and it increases the metal toxicity in the tissues by increasing the free radical formation. The increase in free radicals such as H_2O_2 and HCHO through their metabolic reactions results in cell and tissue injury [42,43].

Activity of GSH-Px was significantly decreased in the liver and kidney of cadmium treated fish and this decrease was not prevented by concomitant selenium administration [43]. Glutathione (GSH) an important antioxidant and donor substrate for GSH-Px is also involved in the detoxification of heavy metals soon after they enter the cell. Jamall and Sporwis [44] have also reported the inhibition of heart cytosolic GSH-Px by Ca^{2+} in rats receiving selenium supplements. The therapeutic agents may protect against acute organ dysfunction and cellular injury thereby preventing the leakage of enzymes. The alteration in the permeability of the cell membrane and the inhibition of the ion-transporting system results in disturbances in the fluid movement. Thus the antioxidants administered prove their efficacy in inhibiting their efflux in the blood. Analysis of glutathione-related enzymes indicated lowering of the activity of Glutathione peroxidase and Glutathione reductase in the liver of rats. The reduced GPx activity is perhaps due to a lowered GSH content in liver and could be an adaptive mechanism attempting though unsuccessfully to maintain GSH level in liver. The decline of GPx activity leads to inefficient disposal of peroxides and reduced formation of oxidized glutathione [45]. By way of conclusion, the reduction in the enzyme glutathione peroxidase can be taken as a meaningful biochemical index of nickel chloride toxicity.

Sub chronic $CdCl_2$ exposure for 30 days in fish *Clarias batrachus* showed decrease in Triiodothyronine, Thyroxine concentration and enhanced lipid peroxidation [46]. Cadmium treatment induced lipid peroxidation in the liver and in kidney in both *Channa punctatus* and *Clarias batrachus* [47]. Isamah et al. [48] have reported that the high level of iron in the fish species of the river Warri might impose stress on *Malapterurus electricus*, *Tilapia mariae* and *Thysia consorgii* due to an enhanced level of lipid peroxidation. The results of the present study suggest that lipid peroxidation may be a more appropriate early marker of oxidative stress in liver of this species.

The increased lipid peroxidation activity of liver indicated tissue injury and failure of antioxidant defense mechanisms which prevent the formation of excess free radicals [49]. Significant increase in the lipid peroxidation and decrease in the antioxidant enzyme activities have been reported in *Cyprinus carpiomorpha* exposed to copper sulphate and zinc sulphate in different concentrations for 24 hours [50]. Changes in the cellular defense system observed in the present study indicated that nickel chloride may pose more of a risk to the aquatic environment. The changes in the activities of some antioxidant enzymes and the level of lipid peroxidation are considered as indices of pollution of fish from Warri and Ethiopia rivers [48]. The results of the present investigation demonstrated that fish from polluted water are subjected to oxidative stress of multifarious dimensions.

5. Conclusion

It can be concluded from our present results that chelation therapy with $CaNa_2$ EDTA proved significantly better results in restoring biochemical variables and pathological features immediately after a sub lethal exposure of nickel chloride in fish. Studies regarding their recovery could offer a solution to save fish living in an ecologically damaged habitat. The present investigation shows that the sub lethal levels can bring about changes in the structural and biological functions of the test animals. These changes indicate the healthy status of fish as well as the purity of the water in a particular environment. However, further work is needed before a final recommendation could be made. The findings suggest that a more detailed study of $CaNa_2$ EDTA efficacy is warranted to conduct a randomized-controlled trial of chelation therapy in nickel-induced toxicity. Until such studies are performed, it would be premature to advocate chelation treatment on the basis of this study alone.

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