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Research article

Acute Intoxication of Metals in Cirrhinus mrigala with Special Reference to the Physiological, Biochemical and Molecular Effects

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Abstract

An experiment to assess the changes in hematology, serum biochemistry and DNA integrity in *Cirrhinus mrigala* exposed to metals was conducted. Results showed that the copper exposure to the fish had more pronounced effects as it resulted in significantly lower RBCs, Hb, Hct and higher WBCs, while Zn exposure showed least toxic effect towards hematological parameters as compared to other metals. Among all the exposure durations of metals, the 96-hr exposure caused maximum negative effects on fish. Lower level of serum Na, Cl, Alb and TP were observed in fish under the exposure of Cu as compared to other metals while K, AST and ALT levels were higher. However, least toxic effect on all above-mentioned parameters were noticed in Zn exposed fish. It is also observed that the highest DNA damage in terms of percent genomic DNA template stability (%GTS) was observed in Cu exposed fish while the Zn exposure to fish resulted in lowest DNA damage. The results revealed maximum squared Euclidean distance between Cu treated fish and the control. This study proposed that the occurrence of toxic metals in aquatic environment has strong impact on hematology, serum biochemistry and DNA integrity of fish.

Keywords: Fish, hematology, serum biochemistry, metals, DNA damage

1. Introduction

Freshwater is highly susceptible to pollution since it acts as a direct sink for the consequences of anthropogenic activities which are always accompanied with the danger of criminal negligence or accidental discharges [1]. Currently, new technologies, the legacies of past contamination and the extensive usage of metals continue to intensify the concentration of metals into the aquatic ecosystems [2].

Survival rate of aquatic organisms is affected due to the cadmium exposure and leads to gradual extinction of their generations in polluted water [3]. Copper is one of the most disastrous metals to aquatic organisms and ecosystems. Its toxicity to aquatic organisms had previously been described by several researchers [4,5,6]. Nickel (Ni) is an essential element for living organisms but it is highly toxic at higher concentration [7]. Recently, several reports are available regarding the Ni toxicity in different animal species [8,9,10]. Zinc (Zn) is also an essential micronutrient and is important for various physiological processes of cells [11], and act as a co-factor of various enzymes. If its level exceeds the physiological requirements, it can act as a toxicant [12].

Fishes are the animals that cannot escape from the negative effects of these contaminants and prove as good bioindicators of aquatic pollution [13]. Fish blood is a pathophysiological indicator of the entire body functions, for that reason it is essential in diagnosing the functional and structural status of fish exposed to toxicants. Numerous studies have showed that metals, for instance cadmium, copper, nickel and zinc induce changes in blood parameters of fish [14-16]. Measurements of serum biochemical parameters is valuable to ascertain the toxicity of target organs along with the overall health status of

animals and provides initial warning of potentially detrimental alterations in stressed organisms [17].

Several studies of fish genotoxicity exhibit the role of metals in eliciting damage to DNA [18,19,20]. Fishes prove excellent material for the study of carcinogenic or mutagenic potential of water, as they can metabolize, concentrate and store waterborne contaminants [21]. In modern days, molecular genetics has provided several novel techniques for the measurement of genotoxicity. In recent times, the RAPD technique has been useful in noticing the genotoxic potential of metals in fish [22]. Pakistan is among the countries facing acute freshwater pollution problems where only 1% of industrial water is treated before its discharge into the rivers and streams [23]. Metals existing in aquatic habitats of Pakistan compete for the up taking routs in organisms, as well as lethal target sites, excretion routs within organism and transport mechanisms [24]. Thus, it is imperative to study the acute responses of fish to metallic ion toxicity that could affect the hemato-biochemical parameters and DNA integrity in *Cirrhinus mrigala*.

2. Material and Methods

The present experiment was conducted in the Wet Laboratory of Fisheries Research Farms, Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad in 2018. *Cirrhinus mrigala* of desired weight (30g) were obtained from the Fish Seed Hatchery, Faisalabad. They were brought to the Wet Laboratory and acclimatized to the laboratory conditions for 14 days. Pure chloride compounds of cadmium ($CdCl_2$. H_2O) and copper ($CuCl_2$. $2H_2O$) nickel ($NiCl_2$. $6H_2O$) and zinc ($ZnCl_2$. $2H_2O$) were used in this experiment and stock solutions were prepared for required metal dilution. Fish were exposed for 96-hr to waterborne lethal concentrations (LC_{50}) of metals which were already determined by [25,26]. The Physico-chemical parameters of the test media were monitored on daily basis by following the methods of [27].

2.1 Hematological parameters: The blood samples were taken at different time intervals from metals exposed and control fishes to study the hematological parameters. Specimens with an average body weight of 30 g were used for sample collection. After anaesthetizing the fish with MS-222 (100 mg/L), blood samples were collected under sterile conditions by the puncture of caudal vein with a heparin-coated 23-gauge needle attached to a 2.5 mL syringe. The hematological parameters i.e. red blood cells, hemoglobin, hematocrit and white blood cells were determined by using automated cell counter (Sysmex KX 21).

2.2 Serum biochemical parameters: Blood samples for serum biochemical analysis were collected without an anticoagulant. The blood samples were left for 1-hr on ice and then centrifuged at 3000 rpm for 10 minutes to isolate the serum. Samples were stored at -80°C before the further analysis. Serum biochemical parameters (sodium, potassium, chloride, total protein, aspartate aminotransferase, alanine aminotransferase) were estimated following standard methods using commercially available kits by BioMed Company.

2.3 RAPD Analysis: The fish liver tissues were taken from metal treated and control fish at specific time intervals during acute trial and stored at -20°C. Total genomic DNA was extracted from small amount (20 mg) of frozen tissues by using traditional Proteinase-K digestion and standard phenol/chloroform technique following the [28]. procedure with slight modifications and visualized on 0.8 % high melt agarose gel in TAE buffer. Amplification of genomic DNA was performed in a gradient thermal cycler (Multigene OptimaxLabNet, USA). The PCR reaction was carried out by using 20 ng of genomic DNA as template DNA. All optimized conditions were used to get reproducible and consistent banding pattern from RAPD (PCR). Initially, 20 random decamer primers were screened in order to test amplification profiles for polymorphism and reproducibility. Finally, the present study utilized 6 primers for RAPD-PCR analysis that provided good results, as shown in Table 1.

2.4 Band Scoring

Only RAPD fragments having high concentration and reproducibility were targeted for markers, estimation of size of such fragments being helped using the 200 bp DNA Ladder (TaKaRa). The presence or absence of fragments was scored as

1 or 0, respectively. RAPD patterns were visually analyzed and scored from photographs. For the analysis and comparison of the patterns a set of distinct well separated bands were selected. The genotypes were determined by recording the presence (1) or absence (0) of these bands.

Table 1. Primers used for the amplification of DNA.

Sr. #	Primer	Sequence
P1	OPC-01	TTCGAGCCAG
P2	OPC-11	AAAGCTGCGG
P3	OPB-06	TGCTCTGCCC
P4	OPB-15	GGAGGGTGTT
P5	OPAY-07	GACCGTCTGT
P6	OPAY-09	CCGATCCAAC

2.5 Estimation of genomic DNA template stability

The GTS (%) was calculated as:

GTS % = 1-a/n × 100 Savva *et al.* (1994)

Where a is the average number of the polymorphic bands detected in each treated sample and n is the total number of bands found in the control.

2.6 Statistical Analysis

Data were statistically analyzed through Factorial design under CRD. Means were compared for statistical differences through Tukey's student Newnan-Keul test [29]. Numerical analysis based on banding pattern obtained from metals exposed fish was compared with the untreated samples (control) via hierarchical cluster analysis. Dendrogram was constructed by the between-groups linkage method using squared Euclidean distance measurement. Genotoxicity judgments were made on the basis of the distance between the specimens. All the analyses were performed by using SPSS software.

3. Results and Discussion

Cirrhinus mrigala was exposed to 96-hr LC_{50} of metals. The physiological, biochemical and molecular changes in fish was determined after 24, 48, 72 and 96-hrs of metals exposure.

3.1 Physiological Changes

The hematological parameters studied during the experiment include red blood cells (RBCsC) count, hemoglobin (Hb), hematocrit (Hct) and white blood cells count (WBCsC). The control fish exhibited higher RBCsC, Hb and Hct than those exposed to different metals while higher WBCsC was noted in metals treated fish than control fish. Among metals, Cu exposure to the fish resulted in minimum RBCsC, Hb and Hct while the same was maximum in Zn exposed medium. Among metals, Cu exposure to the fish maximally increased the WBCsC while the same was minimum under the exposure of Zn as compared to other metals. Comparison of means revealed significantly lower RBCsC and Hb after 96-hr exposure duration as compared to 24-hr exposure duration. The Hct content decreased significantly with the progressive increase in exposure duration and maximum decline was noted after 96-hr exposure duration (Figure 1).

Similar results about hematological parameters have been reported by Hedayati et al. [30]. in silver carp exposed to different concentrations of copper sulfate. Al-Ghanim et al. [31] documented the same results as reduction in Hb, Hct and RBCs in the blood of *Cyprinus carpio* after the Ni exposure. Reduction in these indices was also detected in *Oreochromis mykiss* exposed to Pb and Cu [32]. For the Ni metal similar findings were given by the Ololade et al. [33]. in African catfish with the decrease in Hb, Hct and RBCs. Capkin et al. [34] reported decrease in Hct content of dogfish, *Scyhorhinus canicula* after 24-hr exposure to Cd. They attributed this decrease in Hct content to hemodilution. The high concentrations of metals for short-term exposure usually decline the above-mentioned parameters. Immunological activities and defense mechanisms are usually maintained by WBCsC as reported by Abhijith et al. [35]. According to Moraes et al. [36], one of the most basic ways to assess the immune system is to explore the changes in WBCsC. The Ni exposure resulted in progressive and significant increase in total leucocytes count with an increase in the exposure duration [37]. Increase in WBCsC indicates a defensive response to the metal's exposure [38]. High level of WBC count indicates damage due to infection of body tissues and severe physical stress [39].

3.2 Biochemical Changes

The sodium (Na), chloride (Cl) and total protein (TP) level in serum of metals treated fish was less as compared to their respective control. Among metals, comparatively more toxic effect on Na, Cl and TP level in fish serum was observed under the Cu exposure while least toxic effect was noted under the

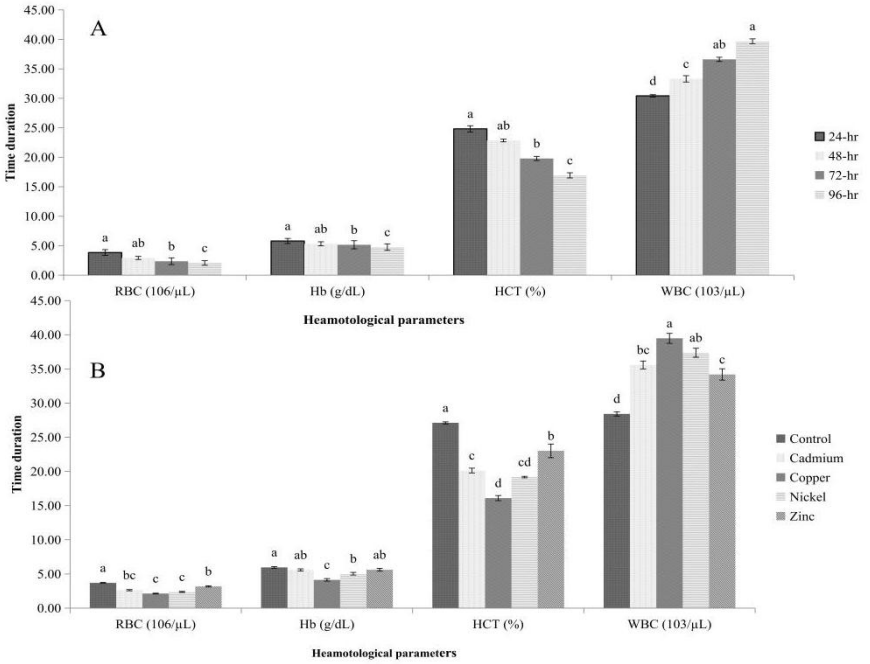

Figure 1: Changes in hematological parameters under the exposure of metals for different time durations

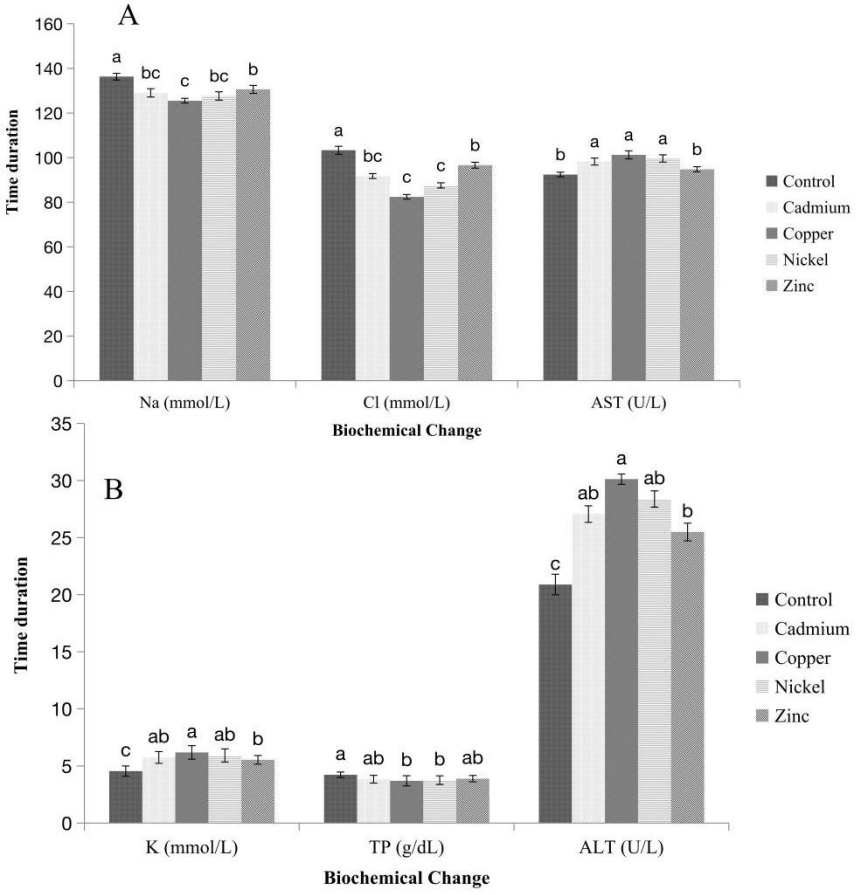

Figure 2: Changes in serum biochemical parameters under the exposure of metals for different time durations.

parameters were recorded after 24-hr of metals exposure and exposure of Zn. The Na, Cl and TP level in control fish was significantly higher than treated. Maximum level of all these *the* same was minimum after 96-hr of exposure (Figure 2).

The present results are in agreement with the findings of Oner et al. [40]. They found that Na and Cl levels decreased in serum of *Oreochromis niloticus* following metals exposure. Similarly, previous studies of Grosell et al. [41] and Firat et al. [42]. showed loss of Na ions after Cu exposure. Levels of Na and Cl decreased in *Oreochromis mossambicus* as studied by Pelgrom et al. [43]. and *Cyprinion maleness* [44]. after exposure to metals. Grosell et l. [45]. reported the toxic effect of metals on gills function which resulted in loss of Na ions. Hypoproteinemia observed in metals treated fishes could be due to liver and kidney damage. This is in agreement with [46]. who stated that every 2-hr analysis of serum total protein level of *Cyprinus carpio* fish showed an initial sharp increase for varying periods from 2- to 20-hr. After this period a steady decline in serum total protein level was observed over a period of 72-hr metals exposure.

The exposure of metals resulted in progressive increase in potassium (K), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) level in fish with increasing exposure durations while it was consistent in control. The fish exposed to Cu and Zn had maximum and minimum K, AST and ALT level, respectively than other metals (Figure 2).

This is in agreement with Vaglio et al. [47]. who observed an increase in liver enzymes activities of fish *Sparus aurata* exposed to Cd. [48]. recorded an increase of liver enzyme activities in stressed *Epinephelus areolatus* fish due to hepatic cells injury or increased synthesis of these enzymes by the liver. Increases in liver enzyme activities in the serum of metals treated fish are assumed to be a result of liver damage by metals [49]. The Cd effect on *Oreochromis mykiss* caused an increase in level of K described by Chowdhury et al. [50].

3.3 Molecular Changes

A total of 59 bands with molecular sizes ranging from 469 to 2654 bp were amplified with six primers using the liver sample of control *C. mrigala*. After exposure to the metals, the amplified products of genomic DNA revealed some differences from fingerprinting patterns of control *C. mrigala*. Present findings are in agreement with those of Galindo et al. [51]. who studied the alterations in RAPD profiles, including appearance and disappearance of bands, after 6-hr, 24-hr and 15-day of Al exposure. These results are consistent with the observations of Zhou et al. [52], who validated DNA damage with RAPD analyses in marine ciliate *Euplotes vannus* exposed to nitrofurazone and found that the damage was dose and exposure time dependent. Oliveira et al. [53]. documented DNA damage in the fish under acute exposure of Cu. [54]. reported Al concentration dependent increase in DNA damage in the lymphocytes of *Cyprinus carpio*.

The percentage of genomic DNA template stability (GTS) in metals treated fish as compared to control at various exposure periods has been presented in Figure 3. It was observed that the percentage of GTS in the fish decreased concomitantly with increase in the exposure duration. However, the minimum of GTS (81.36 %) was observed after 96-hr Cu exposure. The decrease of GTS is considered as the first molecular response toward a toxicant and has been demonstrated being directly related to the extent of DNA damage and/or to the efficiency of DNA repair and replication [55]. The decrease in GTS may be the result of band disappearance and appearance of new bands. The data obtained in the present study on genomic stability are in agreement with the findings of Mohanty et al. [56] who examined *Labeo rohita* fingerlings exposed to furadan at 24-, 48-72- and 96-hr after exposure.

Dendrogram was constructed using “between-groups linkage” method to estimate the level of DNA polymorphism among control and metals treated fish. The squared Euclidean distance between control and metals treated fish at various exposure durations has been given in Figure 4. The farthest squared Euclidean distance (15) from control was recorded for fish exposed to Cu for 96-hr while the nearest squared Euclidean distance (1) was recorded for fish exposed to Ni and Zn for 24-hrs. In the present study, there is an obvious distance between the fingerprinting from fishes treated with metals and control fishes. In a previous study, Zhiyi et al. [57]. demonstrated an

obvious distance between the fingerprinting from *Danio rerio* exposed to the chemicals tested and control. The observed changes in these parameters may provide valuable information concerning the environmental conditions and risk assessment of aquatic organisms.

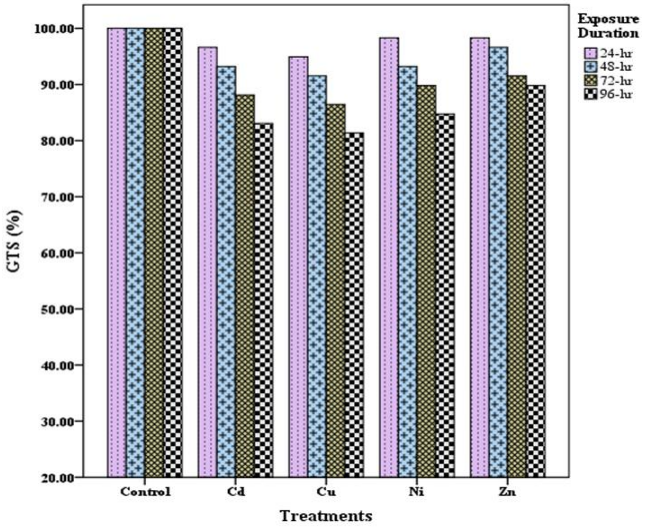

Figure 3: Percentage of genomic DNA template stability (GTS) in metals exposed fish.

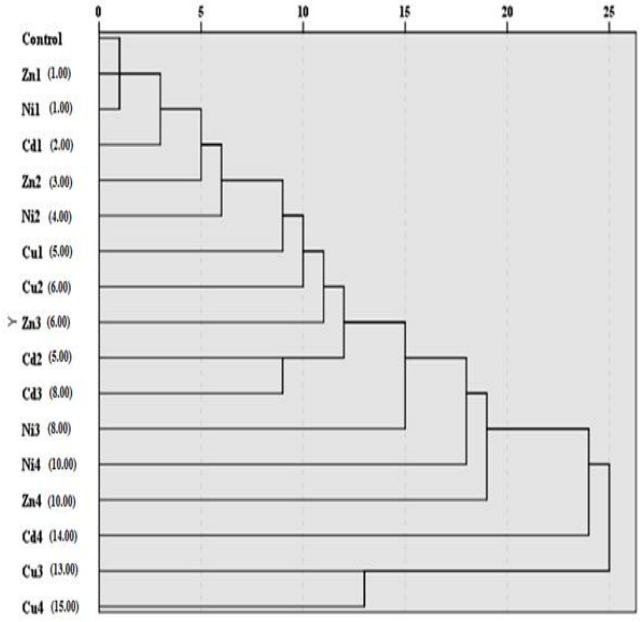

Figure 4: Dendrogram (using average linkage between groups) constructed with control and metals exposed fish for 24 (Cd1, Cu1, Ni1 and Zn1), 48 (Cd2, Cu2, Ni2 and Zn2), 72 (Cd3, Cu3, Ni3 and Zn3) and 96-hr (Cd4, Cu4, Ni4 and Zn4). Numerical values in parenthesis on Y axis denote the squared Euclidean distance from control

4. Conclusions

The present toxicity study on fish revealed the significant effects of metals on fish blood related parameters and DNA damage. This research work will contribute to the applied and basic research needs of aquatic toxicology. Based on results, it appears that human manipulation has a major impact on the fish and the studied parameters are useful tools for detecting this.

Data Availability statement

The data presented in this study are available on request from the corresponding author.

Conflicts of Interest

All authors declare that, they have no conflict of interest.

Author Contributions

Formal analysis, Warda Hassan; Investigations, Dr Sajid Abdullah; Software, Sana Ashraf; Writing – original draft, Warda Hassan; Review & Editing, Shaza Zaheer.

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