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Variations in catalase and peroxidase activity in *Cyprinus carpio* in response to copper nanoparticles exposure

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Abstract
Nanoparticles are the essential components of the environment that affect the natural aquatic ecosystem and human beings. Every aerobic organism possesses antioxidant defense system to avoid the oxidative stress. Research was designed to check the changes in catalase and peroxidase activities in different fish organs (liver, gills, kidney, and muscles) of *Cyprinus carpio* after acute exposure of copper nanoparticles. Fish seed for trial was collected from natural breeding ponds. Copper nanoparticles were prepared by co-precipitation method and characterized by different techniques such as Transmission electron microscopy, Scanning electron microscopy, FT-IR, X- ray diffraction method (XRD) and UV-Visible spectroscopy. The LC_{50} of Cu nanoparticle was checked after 96-hr acute exposure by probit analysis method. Copper nanoparticles induced time dependent alterations in the catalase and peroxidase activities, as the time increased the activities of these enzymes also increased. After acute exposure, the catalase activity showed different results in fish organs liver > gills > kidney > muscles, also the results of peroxidase that observed during acute exposure was liver > gills > kidney > muscles. The findings concluded that the toxicity mechanism of Cu nanoparticle, may be due to the oxidative stress caused by these particles. Physio-chemical parameters were also measured. Data was statistically analyzed by ANOVA and correlation.

Keywords: copper nanoparticles, acute toxicity, peroxidase, catalase, common carp

Introduction
Since last century, the applications of nanotechnology are becoming more immense especially in research fields with a variety of materials at nanoscale level. In the wide class of nanoparticles, materials have dimension at least less than 100 nm [1]. These materials can be of different shapes depending on the overall matter [2]. In the field of nanotechnology, applications of nanoparticles in different industries have been rapidly increased [3]. Due to this interest, the risks of nanoparticles ingoing the environment are also increased [4]. These metal particles may accumulate in sediments and bind to nutrients when enter the water bodies [5].
For all living organisms, copper is an essential element because it plays a central role in cell metabolism and as a cofactor for certain enzymes like superoxide dismutase, cytochrome C oxidase and ceruloplasmin. Moreover, the Cu nanoparticles have been used in medicine, in industries also as pesticides and in paints as an antifouling agent [6].
Oxidative stress generally destabilizes the oxidant production and defense systems that damages tissues and biomolecules as radicals by oxidation and causes disease such as neurodegenerative diseases. The tissue destruction that has been caused by metallo proteinases and oxidants have revealed that the optimum copper level play role in restoration, provided it to be applied in adequate levels to improve antioxidant defense system. So, these findings directed to evaluating the addition of copper nanoparticles in diet for immunity and antioxidant capability in common carp. The copper nanoparticles will generate reactive oxygen species and the end point is oxidative, due to the toxic association between the cellular level of these particles [7].
Antioxidant defense mechanisms that consist of enzyme system present in fish tissues mostly in kidney and in liver. Catalase act as antioxidant enzymes that help to maintain the oxidative stress which is produced by metals [8].

Toxicity was greater in gills, but these types of defensive effects were also found in kidney and liver. Enzymes are the biochemically larger molecules which help in controlling the metabolic system of organisms. Due to the changes in activity of enzymes organisms has been severely affected [9].

Catalases and peroxidases are major component of antioxidant system, creating non-radicals penetrating all biological membranes also producing other non-radicals such as ROS, hydrogen peroxide. The harmful effects in fish which were caused by oxidative stress so far, the catalases and peroxidases were considered the biochemical markers [10]. In Eco toxicological fields, oxidative stress has attained signified attention. So, these enzymes classes worked as sensitive bio indicator of oxidative stress before occurrence of hazardous impacts in fish growth and performance [11].

The common carp, *Cyprinus carpio*, is the economically significant fish and extensive species in aquatic ecosystems and show about 1% of annual freshwater aquaculture production worldwide [12]. This fish can give intensive data to measure the quality of aquatic systems [13]. Various studies have suggested that carp can be used as model to measure the nonfatal effects of pollutants because of the supremacy of carp in aquatic systems and their greater capacity for bearing pollutants and their toxic effects than the other laboratory fish, such as Japanese medaka and zebrafish. According to OECD standard carp is the appropriate model for eco toxicological research [14]. Still there are no studies on the Cu requirements particularly in its nanoform, although there is abundant data about the nutritional requirements for this species [15].

The objectives of present research was

- To determine the effect of Cu nanoparticles on catalase and peroxidase activity of common carps
- To compare the effects of copper nanoparticles on different organs of common carps

Material and methods

Experimental Design

The present research was designed to check the "Changes in catalase and peroxidase activity in fish, common carp exposed to copper nanoparticles". Experiment was conducted at University of Agriculture Faisalabad and experimental fish in the present study was common carp collected from university fish farms. The fingerlings having same average weight and length were selected for trial. Fish was acclimatized in aquaria, made of glass with dechlorinated tap water. Water temperature, dissolved oxygen and pH were maintained. Likewise, hardness, Ca, Mg and CO_2 were measured by the methods as described [16]. Fish was fed once daily with commercial pellet food, during the acclimatization.

Synthesis of Cu nanoparticles and suspensions

By co-precipitation method, Cu nanoparticles were synthesized, and this reduction method is better as compared to other methods being cheap and environmentally suitable. The solid metal nanoparticles were having size (1-100nm) in area to volume ratio. By co-precipitation method, copper nanoparticles were synthesized. After that characterization was done by TEM, SEM, XRD, FT-IR and UV-Visible spectrophotometer.

Acute toxicity

To check severe toxicity, in different concentration of Cu (10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 mg/L) nanoparticles, 96-hr exposure to LC_{50} and LC_{99} were applied. During lethal trials and 96-hr LC_{50} fish mortality was observed, dead fish was removed directly from medium.

Determination of antioxidant enzymes

After acute exposure, the organs (gills, liver, kidney, and muscle tissues) were isolated and placed in polythene bags separately and stored frozen for analysis of antioxidant enzymes. Sampling was done after each 24hr. After isolation organs were homogenized.

Catalase Assay

The crude enzyme was subjected to enzyme assay and the catalase activities were measured by described method of Chance and Mehaly [17] with some modifications. Catalase activity was calculated in terms of its ability to decrease the H_2O_2 concentration per minute at 240nm.

$$\text{Catalase (U/mL)} = \frac{\Delta A/\text{min}}{0.04\ \text{mM} - 1\text{cm} - 1\times 0.1\text{mL}} \times 3\text{mL}$$

Peroxidase

For the determination of peroxidase activity, crude enzyme was subjected to enzyme assay according to Civello *et al*. [18]. Activity of peroxidase was determined by measuring its ability to decrease the concentration of H_2O_2 at 470 nm.

$$\text{Peroxidase activity (U/mL)} = \frac{\Delta A/\text{min}}{26.6\times 60\mu\text{l}/3000\mu\text{l}}$$

Statistical Analyses

For the statistical analysis of data, probit method was used for the determination of LC_{50} value. ANOVA, and correlation method was also used to check the difference and interaction between different variables.

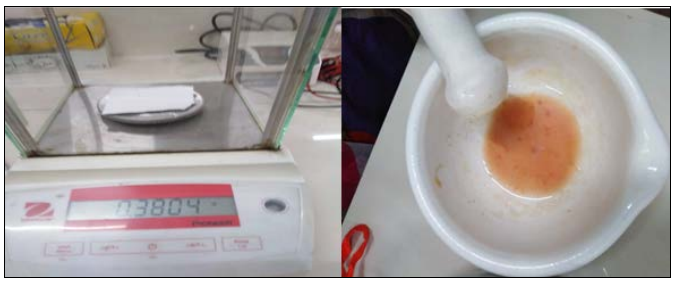

Fig 1: Sartorius Electronic Weighing Balance, BSA 4202S-CW used to measure the exact quantity of Cu-NPs (Left) and Grinding of organs for homogenation (Right)

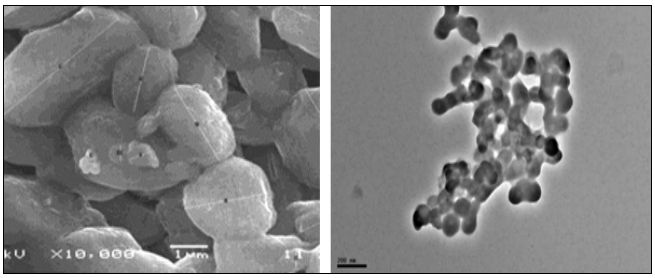

Fig 2: The SEM and TEM images of Cu nanoparticles

Results

Probit Analysis

By various concentrations of copper nanoparticles, the fish mortality was observed in the three replicate groups. The percent mortality of common carp against different concentration of copper nanoparticle which starting from

10.00mgL^{-1} increased up to 110.00mgL^{-1} that was analyzed during acute toxicity trial. In first replicate, LC_{50} of copper nanoparticle was estimated as 74.63±4.71mgL^{-1} while lethal concentration of copper nanoparticles was calculated as 136.863±11.27 mgL^{-1}. Second replicate, LC_{50} was calculated as 76.78±5.10mgL^{-1} and lethal concentration was estimated as 144.10±13.62mgL^{-1}. In the third replicate LC_{50} of copper nanoparticle was estimated as 73.80±5.094 mgL^{-1} while lethal concentration was calculated as 142.26±13.21 mgL^{-1}.

Catalase activity

The analysis of variance for the catalase activity was performed which gave the results, catalase activity showed significant variation for different organs of fish as compared to control group.

In present research the different organs have highly significant values of catalase activity in fish followed the order; liver 239.04±33.63UmL^{-1} > gills 191.36±24.79 UmL^{-1} > kidney 148.66±19.08 UmL^{-1} > muscles 165.99±22.58 UmL^{-1} was recorded.

Peroxidase activity

The analysis of variance for the peroxidase activity was performed which gave the results that showed highly significant variation in peroxidase activity for different organs of fish as compared to control group.

In present research the peroxidase activity in different organs have highly significant value in fish and followed the order: liver 2.63±2.10 UmL^{-1}> gills 1.93±1.79 UmL^{-1} > kidney 1.76±1.67 UmL^{-1} > muscles 1.13±1.13 was recorded.

Table 1: Percent mortality data of Common carp exposed to copper nanoparticles during acute exposure

Concentration	Replication 1	Replication 2	Replication 3	Means
10	-	10	-	
20	10	20	20	13.33
30	20	20	30	23.33
40	30	20	30	26.66
50	40	30	40	36.66
60	50	50	50	50.00
70	60	60	50	56.66
80	70	60	70	66.66
90	80	70	80	76.66
100	90	80	90	86.66
110	100	100	100	100

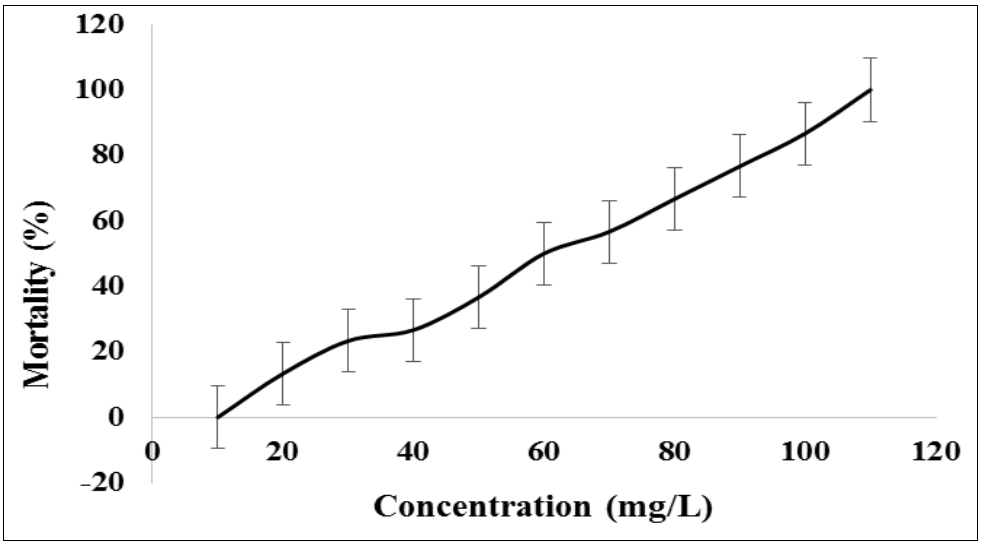

Fig 3: The percent mortality of common carp at different concentrations of Cu nanoparticles during acute toxicity tests

Table 2: Means of catalase activity (UmL^{-1}) in body organs of common carp during 96-hr LC_{50} exposure

Treatments	Exposure Duration	Gills	Organs Liver	Kidney	Muscles	Mean
Control	24	169.10±1.60b	226.11±2.30b	125.05±1.35d	187.45±2.15b	176.92±36.33C
	48	169.01±1.90c	169.01±1.90d	130.27±1.70c	185.50± 2.15d	163.44±20.30D
	72	168.21± 1.94d	225.30± 2.55c	135.50± 1.84b	187.56± 2.13a	179.14±32.50B
	96	169.26± 1.96a	226.38±2.69a	135.55± 1.80a	185.55± 2.15c	179.18±32.67A
Treated	24	192.70±11.61d	250.01±11.40d	150.30±11.19d	155.51±10.09b	187.13±39.81D
	48	209.31±11.78c	260.85±12.01c	161.55±11.16c	161.55±11.16a	198.31±41.03C
	72	220.11±11.44b	270.25±12.40b	170.16±11.55b	134.11±11.09c	198.65±51.39B
	96	233.19±12.60a	284.45±11.45a	180.92±11.15a	130.70±11.16d	207.31±57.41A
Mean		191.36±24.79B	239.04±33.63A	148.66±19.08D	165.99±22.58C	

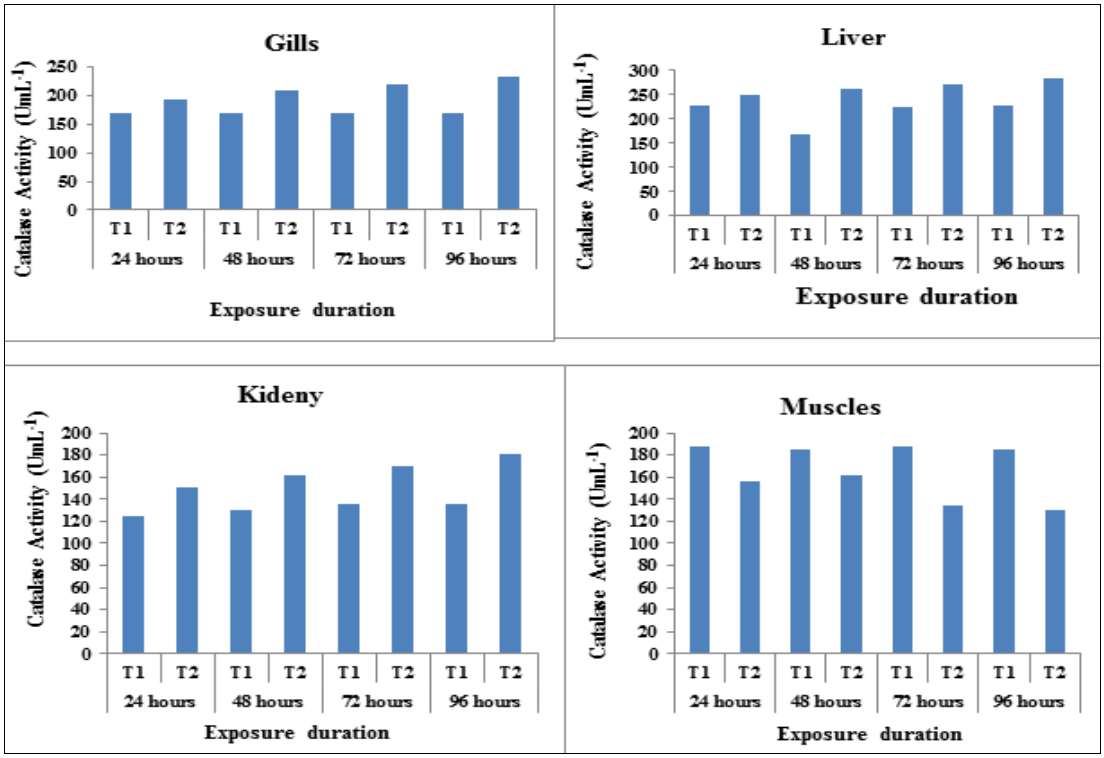

Fig 4: Catalase activity (UmL^{-1}) of copper nanoparticle in organs of common carp during acute exposure

Table 3: Means of peroxidase activity (UmL^{-1}) in body organs of common carp during 96-hr LC_{50} exposure

Treatments	Exposure Durations	Gills	Organs Liver	Kidney	Muscles	Means
Control	24	0.32±0.02c	0.74± 0.04c	0.21± 0.03c	0.06±0.01d	0.33±0.25C
	48	0.02±0.02d	0.76±0.04a	0.20±0.02d	0.07± 0.02c	0.26±0.29D
	72	0.34±0.01b	0.75±0.01b	0.22± 0.04a	0.07±0.06b	0.34±0.25A
	96	0.36±0.07a	0.7± 0.11d	0.22±0.01b	0.09±0.07a	0.34±0.22B
Treated	24	2.25±0.02d	2.56±0.36d	2.00±0.04d	1.59±0.01d	2.1±0.35D
	48	3.50±0.18c	4.10±0.01c	3.22±0.05c	1.85±0.02c	3.16±0.82C
	72	3.95±0.01b	5.35±0.04b	3.69±0.01b	2.25±0.07b	3.81±1.09B
	96	4.72±0.15a	6.01±0.20a	4.39±0.01a	3.11±0.04a	4.55±1.03A
Means		1.93±1.79B	2.62±2.10A	1.76±1.67C	1.13±1.13D	

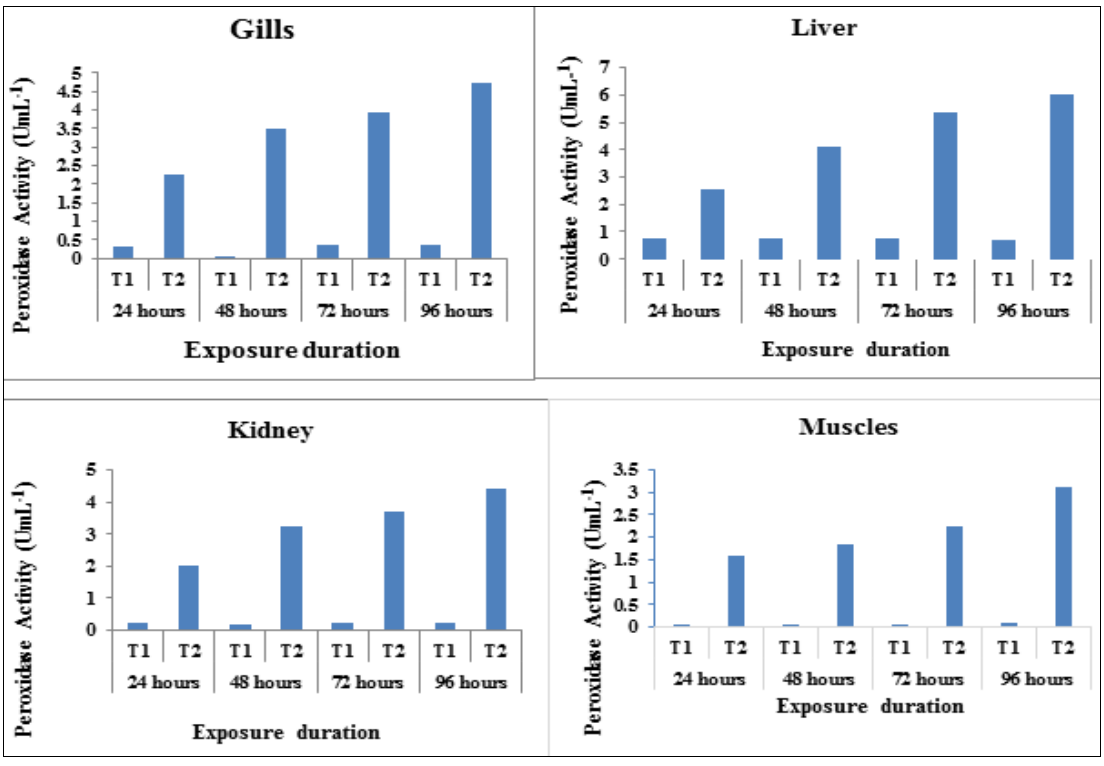

Fig 5: Peroxidase activity (UmL^{-1}) of copper nanoparticle in organs of common carp during acute exposure

Discussion

Organic and inorganic wastes are responsible for metallic ions pollution and are toxic to the aquatic ecosystems due to their nondegradable nature [19]. Copper has no biological function in animals and causes the neurological and physiological dysfunction in fish even at low concentrations due to its toxic nature [20]. Oxidative substances in cells may lead to elevation in antioxidants of both enzymatic and non-enzymatic components as a defense mechanism [21]. Antioxidant enzyme activities are found broadly distributed in tissues of aquatic organisms, with higher activity in the digestive gland of invertebrate organisms.

Catalase and peroxidase are antioxidant enzymes that provide the first line of defense against reactive oxygen species and used as a biomarker of oxidative stress. In the present study, catalase and peroxidase activities were increased in common carp after exposure to Cu NPs. Such increases in CAT activities may be explained as a response to the increased H_2O_2 levels and superoxide anions [22]. The increment of CAT and POD activities may be an adaptive mechanism to prevent the accumulation of toxic reactive oxygen [23]. The elevated enzymatic activities might reflect the possibility of better protection against toxicity of metal-induced lipid peroxidation [24]. In the same trends Mustafa *et al.,* [25] reported that peroxidase activity increased in liver exposed to Cu NPs and attributed this increase to its protective role against damages induced by oxy radical.

During present research work, it was found that the peroxidase activity of enzymes in the kindly of copper treated fish, increased significantly as compared to the control fish. The present results are also in conformity with the findings of Doherty *et al*., [26] who concluded that exposure of copper nanoparticles caused an increase in peroxidase activity compared to control fish group. Eroglu *et al*., [27] reported that sub-lethal exposure of copper nanoparticles resulted into increased peroxidase activity in common carp. In relation to the copper exposed fish, the control fish showed lower values for peroxidase activity during the whole experimental period.

The study suggested that exposure to copper nanoparticles provoked significant oxidative stress and differential tissue specific antioxidant response in carp. The present results are in contrast with the findings of Mary *et al*., [28] who studied that peroxidase activities in the liver, muscles and gills of common carp were decreased with increasing concentration of copper nanoparticles in water. Decreased activities of peroxidase may indicate disturbance in the cell organelles. The results of Awoyemi *et al*., [29] are also against the present findings that copper nanoparticles caused inhibition of peroxidase enzyme activity in the liver of fish. Hence, the enzyme activity was higher in control fish group as compared to the stressed group.

In conclusion, copper nanoparticles were found to cause a moderate toxicity to the common carp. After exposure to acute concentrations of these nanoparticles, they were accumulated progressively inside the body organs over exposure period and resulted in significant alterations. These changes which may represent adaptive mechanisms to this stressful situation may be potentially disruptive for the survival of fish in contaminated aquatic ecosystems. Moreover, the present study revealed the utility of common carp to be used as a bio indicator for copper nanoparticles toxicity.

Conclusions

1. The synthesis of copper nanoparticles was done by co-precipitation method and characterized by different techniques such as SEM, TEM, FT-IR and UV-Visible spectroscopy. The size of copper nanoparticles was about the 15±2nm.
2. Exposure of copper nanoparticles to the experimental fish at 96-hr LC_{50} caused significant variability ($p<0.01$) in enzyme activity.
3. Copper nanoparticles exposure fish had more pronounced effects as it caused significantly ($p<0.01$) increased level of toxicity in liver as compared to other organs but the control fish species there was not shown any type of response.
4. The catalase activity at 96-hr LC_{50} concentration was as followed Kidney> gills> liver> muscles. At lethal concentration same pattern was observed as Kidney> gills> liver> muscles.
5. For peroxidase activity at 96-hr LC_{50} concentration was as followed liver> gills> kidney> muscles. Same in lethal concentration was observed as followed liver> gills> kidney> muscles.
6. All physico-chemical parameters showed significant correlation with different concentration copper nanoparticles.

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