Effect of copper nanoparticles exposure in the physiology of the common carp (Cyprinus carpio): Biochemical, histological and proteomic approaches

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Abstract
Copper nanoparticles (Cu-NPs) are serious water pollutants but their impact in teleosts performance remains poorly understood. In the present study, we have exposed juvenile carps (Cyprinus carpio), a freshwater teleost edible in India to two different doses (20 and 100 μg/L) of Cu-NPs for seven days. The doses selected were eco-relevant considering the contamination levels of certain water resources. The results indicated that the activity oxidative stress enzymes catalase, superoxide dismutase, and glutathione-S-transferase were significantly increased in the kidney, liver and gills of the treated groups when compared to control. Histological analysis revealed that after exposure, disruption of the secondary lamellae of gills, liver damage with pyknotic nuclei and structural disarray of the kidney occurred. Proteomic analysis of the liver showed down-regulation of several proteins including the ferritin heavy chain, rho guanine nucleotide exchange factor 17-like, cytoglobin-1 and up-regulation of diphospho- mevalonate decarboxylase and selenide & water dikinase-1. Taken together, the results of suggest that short-term exposure of juvenile carp to Cu-NPs causes oxidative stress and impart serious deleterious effects in the tissues which may affect fish growth and development.

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1. Introduction

Nanoparticle (NP) is a microscopic object that behaves as a whole unit with respect to its transport and properties and differs largely from bulk materials of the same composition. The properties of NPs vary with size, shape and chemical environment (Murray, Kagan, & Bawendi, 2000) and despite their widespread applications; NPs associated toxicity has gained significant importance in the last decade (De-Jong & Borm, 2008). Owing to its increased usage, there is an inevitable discharge of nano-materials and of their byproducts from the industries in the aquatic environment, which causes adverse effects in the organisms (Mueller et al., 2012; Theron, Walker, & Cloete, 2008). Knowledge on the interaction of NPs with biotic and abiotic components is scarce and currently there is no reliable method to assess nanomaterial toxicity (Arora, Rajwade, & Paknikar, 2012; Handy, Henry, Scown, Johnston, & Tyler, 2008). However, it has been suggested that the bioavailability of metal NPs by aquatic pollution and subsequent accumulation in fish constitute a substantial risk to human health and to the environment (Shaw & Handy, 2011).

Metal oxides NPs such as copper (Cu), silver, titanium have garnered significant attention due to their negative ecological effects (Klaine et al., 2008). In fact, reports of Cu contamination were reported in several rivers and lakes of India such as Godavari river (Ghorade, Lamture, & Patil, 2014; Lokhande & Keikar, 2000). Though the contamination is minimal, separate pockets of lakes and ponds isolated from the main river stream have a high level of metallic contamination (Ghorade et al., 2014). A wide range of NPs is being used as biosensor immobilizers for greater specificity (Vigneshwar, Sudhakumari, Senthilkumaran, & Prakash, 2016). Due to their low preparation cost and prospective

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applications, copper nanoparticles (Cu-NPs) are intensely used in the industry (Wang, von-dem-Bussche, Kabadi, Kane, & Hurt, 2013). Besides, Cu-NPs are also used as one of the main constituents of fungicides, algaecide and herbicides however they can cause genotoxicity and oxidative DNA damage at cellular level (Song, Li, Kasai, & Kawai, 2012). Cu-NPs have the ability to cross the plasma membrane, cause oxidative stress via interacting with subcellular organelles (Fahmy & Cormier, 2009; Melegari, Perreault, Costa, Popovic, & Matias, 2013; Wang, Li, Zhao, & Xing, 2011; Gómez, Martínez-A, Gonzalez, & Rebollo, 1998) and can accumulate in the tissues such as liver and gills of fish (Wang et al., 2013; Griffitt et al., 2007). In liver, Cu-NPs were shown to induce necrosis and to alter sinusoidal spaces in the gills (Al-Bairuty, Shaw, Handy, & Henry, 2013; Griffitt et al., 2007). Cu-NPs disrupt normal bronchial ionoregulatory homeostasis causing efflux of electrolytes from the blood by the gill epithelium and can lead to death due to a subsequent cardiovascular collapse (Handy, 2003). In this respect, it is important to study the effect of Cu-NPs using an edible fish as the animal model.

The common carp, Cyprinus carpio is a freshwater teleost cultivated in various parts of the world, including India owing to its low cost of production, high muscle content and easy rearing. Despite its economic importance, this teleost is referred as an ideal experimental animal model for studying ecology, developmental biology, and evolution (Bongers, Sukkel, Gort, Komen, & Richter, 1998). Common carp belonged to the Cyprinidae family with an annual global production of 3.4 million tons, accounting for nearly 14% of the freshwater aquaculture production worldwide (Ji et al., 2012). To keep up with global demand, carps are reared in artificial feed-based intensive monocultures system in cages, irrigation reservoirs and running water ponds and tanks, or in recirculation systems and thus are more likely to be exposed to various kinds of pollutants due to farming strategies. Hence, common carp research has received more attention among researchers in recent years as a model to analyze toxicity.

The present study aims to examine the effect of Cu-NPs toxicity in the physiology of the common carp. To this end, three months old juvenile common carps were exposed to a low (20 µg/L) and higher (100 µg/L) doses of Cu-NPs during seven days. Recently, Zhao et al. (2011) investigated the potential toxicity of Cu-NPs (100 mg/L) in juvenile carp and found it significantly inhibited growth. However, the dosage used in our experiments were lower than other studies. The effect of exposure of (0.68 ± 0.15), (0.28 ± 0.04) and (0.22 ± 0.08) mg/L of Cu-NPs had been analyzed in rainbow trout, fathead minnow, and zebrafish, respectively (Song, Vijver, Peijnburg, Galloway, & Tyler, 2015) and also considering contamination of Cu pollution in certain underwater reservoirs or rivers in India (Ghorade et al., 2014) and other water sources in the world (ATSDR, 2002; Şeker & Kütlu, 2014). The present study intended to analyze the toxic effects of Cu-NPs at cellular level in the kidney, liver and gills of juvenile common carp using biochemical, histological and two-dimensional (2D) proteomic analysis to investigate oxidative stress. The effect of metallic Cu was not considered in this study as previously described by others (Song et al., 2015; Wang et al., 2013).

2. Materials and methods

2.1. Animal maintenance and treatments

Juvenile carps (~3 months) reared in Karnataka Veterinary Animal and Fisheries Sciences University, Bangalore, India were procured and acclimated for a fortnight. Laboratory acclimated or farmed fish in hatcheries are better models to understand the impact of eco-relevant doses than wild caught fish. Stock animals were daily fed with commercial carp food ad libitum and reared in glass tanks (50 L) with recirculating filtered water under normal photoperiod and ambient temperature conditions. Fish weighing (9.0 ± 0.1) g (n = 20) were separated into three experimental tanks (20 fish/tank) 24 h prior to the experiment. Commercially available copper (II) oxide nanopowder <50 nm particle size (Cat. No: 544868, Sigma, St. Louis, MO, USA), was used to prepare fresh Cu-NPs 100 mM stock solution in distilled water and sonicated for 5–6 h. Due to the commercial purchase of Cu-NPs with data sheet from Sigma, characterization of physiochemical properties is redundant for the present study. Fish were exposed either to 20 or 100 µg/L of Cu-NPs for 7 days (as short term exposure) under a semi-static water flow condition (80% water change from all experimental tanks with new water every day before the 20 and 100 µg/L re-dosing of Cu-NPs). These doses were selected based on a pilot study and considering two different doses eco-relevant in certain underwater reservoirs or rivers in India (Ghorade et al., 2014). A third group was the control. Cu was not measured as the replenishment of Cu-NPs as per dose was done daily. After exposure the kidney, liver and gills were collected for further analysis. Experiments were conducted in accordance with the guidelines of Institutional Animal Ethics Committee, University of Hyderabad.

2.2. Sample collection

At the end of the treatments, the weights of whole fish as well as of the kidney, liver and gill were measured (Table 1). Tissues were dissected out and used for biochemical, histological and proteomic analysis. For histology, portion of tissues were fixed in freshly prepared Bouin’s fixative (15:5:1; saturated picric acid, formalin, and glacial acetic acid) for 12–14 h at room temperature. For biochemical and proteomics analysis, tissues were snap frozen in liquid nitrogen and stored at −80 °C until use. Tissues from two fishes were pooled to obtain one biological sample and a total of five samples were used for all experiments. For the proteome analysis, samples were obtained from three individuals to obtain one biological sample for larger tissue volume and for tissue replicates similar procedure are followed.

2.3. Histology

Bouin’s fixed tissues were processed with graded alcohol series, followed by xylene and finally embedded in paraplast (Cat. No: P3683, Sigma). Sections of 5 µm thickness were cut using a rotatory microtome (Wetzlar-1512, Leitz, Germany) rehydrated and stained using hematoxylin-eosin. Later, the sections were dehydrated and

| Weight of fish (g) | Tissue weight (g) |
|-------------------|-------------------|
|                   | Liver             | Kidney            | Gills              |
| Control           | 8.03 ± 0.297      | 0.145 ± 0.014     | 0.142 ± 0.006     | 0.383 ± 0.028    |
| Lower dose (20 µg/L) | 8.672 ± 0.315*   | 0.159 ± 0.012*    | 0.160 ± 0.008*    | 0.482 ± 0.029*   |
| Higher dose (100 µg/L) | 10.41 ± 0.786**  | 0.220 ± 0.024**   | 0.181 ± 0.010**   | 0.585 ± 0.039**  |

*P < 0.01; ANOVA followed by Student-Newman–Keuls’ post hoc test.

Table 1: Effect of Cu-NPs on the body and tissue (Liver, Kidney and Gills) weight of control and treated groups of common carp. Data (n = 10) were expressed as mean ± SEM. (*P < 0.05; **P < 0.01; ANOVA followed by Student-Newman–Keuls’ post hoc test).
finally mounted using DPX mountant. The slides were observed, and all microphotographs were taken using an Olympus CX41 microscope (Olympus Corporation, Japan) fitted with Mp3 Micro-Publisher 3.3 RTV (Q-imaging, BC, Canada).

2.4. Measurement of oxidative stress

Tissues were homogenized using a micro-pestle in 1.5 mL microcentrifuge tubes containing equal volumes of 50 mM sodium phosphate buffer (pH 7.4), centrifuged 10,000 × g for 10 min at 4 °C and the supernatants were collected and used for the following assays:

2.4.1. Catalase (CAT) assay

The measurement of catalase activity was performed according to the method proposed by Beers and Sizer (1952). In brief, H2O2 was used as a substrate and the decomposition of H2O2 by the catalase enzyme was observed using UV-vis spectrophotometer (UV-1601, Shimadzu, Germany) by measuring the decrease in the absorbance at 240 nm for 5 min. The assay mixture contained 20 μg of protein along with 3% v/v of H2O2 in a phosphate buffer (50 mM, pH 7.4) for a final volume of 1 mL. The results were expressed as μM of H2O2 consumed/min/mg of protein.

2.4.2. Superoxide dismutase (SOD) assay

SOD activity was measured by the method described by Kostyuk and Potapovich (1989). SOD present in the homogenate blocks the autoxidation of quercetin (Cat No.: Q4951, Sigma). Quercetin oxidizes at pH 10 and this is a free radical chain reaction involving superoxide and hence usable for SOD and thus the function of SOD was directly correlated with the degree of inhibition of quercetin oxidation. The total reaction mixture contains 1 mL of 0.016 M phosphate buffer, N,N,N’,N’-tetramethylethylenediamine buffered with 0.08 mM EDTA. The reaction was initiated by the addition of 0.1 mL of 0.015% quercetin solution. For the assay, the supernatant of tissue homogenates, containing 20 μg of protein was added to the mixture to a final volume of 1 mL and monitored using UV-vis spectrophotometer (Shimadzu). Inhibition of auto-oxidation of quercetin was monitored by a decrease in the absorbance at 406 nm. The results were expressed as U/mg protein.

2.4.3. Glutathione-S-transferase (GST) assay

Jakob, Habig and Jakoby (1980) protocol was followed for measuring GST activity. GST catalyzes the conjugation of GSH to CDNB through the thiol group of the glutathione and making CDNB-GSH adduct and this CDNB-GSH adduct was used to measure GST activity. In brief, 20 μg protein was added to a solution containing 0.2 M potassium phosphate buffer (pH 7.2), 10 mM l-Glutathione reduced (GSH, Cat. No.: G4251, Sigma) and 0.1 M 1-chloro-2,4-dinitrobenzene (CDNB, Cat. No.: 237329, Sigma). GST catalyzes the conjugation of GSH to CDNB through the thiol group of the glutathione and making CDNB-GSH adduct. The absorbance of the resultant adduct of CDNB (S=2, 4-dinitrophenyl glutathione) was measured using UV-vis spectrophotometer (Shimadzu) at 340 nm. The enzyme activity was calculated based on molar coefficient 9 m/M/cm. Results were expressed as μM of adduct formed/min/mg of protein.

2.5. Two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) analysis

2.5.1. Sample preparation

Liver tissue was homogenized in lysis buffer containing 7 M urea (Cat. No.: U6504, Sigma), 2 M thiourea (Cat. No.: T8656, Sigma), 4% w/v 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, Cat. No.: C9426, Sigma), 40 mM tris-HCl buffer, 1% dithiothreitol (DTT, Cat. No.: D9163, Sigma) and protease inhibitor cocktail (Cat. No.: P8340, Sigma) and centrifuged at 12,000 × g for 15 min at 4 °C. The supernatant was carefully taken, precipitated using a 2D-cleanup kit (GE Healthcare, 80648451, United Kingdom) according to manufacturers instructions and the precipitant was dissolved in rehydration buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 2% ampholytes (Cat. No.: P1522, Sigma) of pH 3−10, 30 mM DTT and 0.002% of bromophenol blue. Protein concentration was measured by Bradford assay method (Bio-Rad, Cat. #500-0006, CA, USA) as described by Bradford (1976).

2.5.2. Isoelectric focusing (IEF) and 2D electrophoresis

The procedures followed for IEF and 2D electrophoresis are based on Laidinsangi et al. (2014). IEP strips (Immobiline Drystrip, 3−10 pH, linear, 18 cm; GE Healthcare, 17-1234-01, United Kingdom) were rehydrated with 800 μg of the protein solution for 20 h under low viscosity mineral oil (BIO-RAD; 163-2129, United States). Isoelectric focusing was performed in Ettan IPGPhor 3 manifold (Cat. No.: 80-0498-38, GE Healthcare) at 20 V using the following IEF protocol, for 1 h at 50 V, ramped 5 h at 500 V, 5 h at 1,000 V, 5 h at 2,000 V, ramped 10 h at 8,000 V, 10 h at 12,000 V, final focusing at 10,000 V until 70,000 Vh. After IEF, the strips were equilibrated twice for 15 min in equilibration buffer (6 M urea, 50 mM tris-Cl (pH 8.8), 30% glycerol and 2% SDS). Subsequently, DTT (1% w/v) and iodoacetamide (4% w/v) were added. Equilibrated strips were placed on 12% polyacrylamide gel and second dimension electrophoresis was performed using Etan Dalton SDS-PAGE gel apparatus (GE Healthcare). Protein spots were visualized on the gel by colloidal Coomassie brilliant blue staining.

2.5.3. Image analysis

The gels were scanned using Image Scanner (GE Healthcare), and the images were analyzed with Image Master 2D Platinum Software 6.0 (GE Healthcare) according to the manufacturer’s instructions. All the gels were analyzed for each sample and differentially expressed spots were chosen for further analysis. Based on presence and absence of spots in control and Cu-NPs treated groups, nearly 30 protein spots were excised and about 5 differentially distinctly regulated spots of low molecular weight were taken for MALDI-TOF/TOF identification.

2.5.4. In-gel digestion, protein identification and database search

The MALDI-TOF-MS analysis method described by Shevchenko, Wilm, Vorm and Mann (1996) with some modifications was adopted using an MALDI-TOF/TOF mass spectrometer (Bruker Autoflex III smartbeam, Bruker Daltonics, Bremen, Germany). Manually excised protein spots were treated with 100 μL of 50% acetonitrile (ACN, Cat. No.: 34967, Sigma) in 25 mM ammonium bicarbonate (NH4HCO3, Cat. No.: 09830, Sigma) 5 times for destaining till the spots are distinctly visible. Then protein spots were incubated in 10 mL DTT in 25 mM NH4HCO3 at 56 °C for 1 h followed by 55 mM iodoacetamide in 25 mM NH4HCO3 for 45 min at room temperature (25 ± 2 °C), washed with 25 mM NH4HCO3 and ACN, dried in speed vac (Labconco, SD, USA) and rehydrated in 20 μL of 25 mM NH4HCO3 solution containing 12.5 ng/μL trypsin (Promega, Cat. No: V5111, Madison, WI, USA). Samples were incubated on ice for 10 min and incubated overnight at 37 °C. After complete digestion, samples were centrifuged for 10 min and supernatant was collected in an eppendorf tube. 50 μL of 1% trifluoroacetic acid (TFA, Cat. No.: 91701, Sigma) and ACN (1:1) was added to the supernatant, vortexed and incubated 15 min at room temperature simultaneously. The supernatant was pooled, dried
using the speed vac and reconstituted in 5 µL of 1:1 ACN and 1% TFA. 1 µL was mixed with 1 µL of freshly prepared π-cyano-4-hydroxycinnamic acid (CHCA, Cat. No.: C8982, Sigma) matrix in 50% ACN and 1% TFA (1:1) and 1 µL was spotted on a target plate. Mass data was acquired using the flexControl 3.0 (Bruker Daltonics, Bremen, Germany) software with automatic switching between MS and MS/MS modes. Mass range of m/z 800—3500 in the reflectron positive ion mode was accumulated from an average of 2,500 laser shots with an acceleration of 19 kV. Fragmentation of precursor ions was achieved using an MS/MS positive mode. Contaminating trypsin and keratin corresponding ions were omitted from the peak lists before database analysis using the flex software. Protein spots identification was done using the MASCOT program (http://www.matrixscience.com) from Biotools software (Bruker Daltonics) by searching the database (Peptide Mass Fingerprinting and MS/MS). Similarity searches for mass values were performed with the matrixscience.com) from Biotools software (Bruker Daltonics) by searching the database (Peptide Mass Fingerprinting and MS/MS). Similarity searches for mass values were performed with the

2.6. Statistical analysis

Data are expressed as mean ± SEM (n = 5). Pair wise comparisons were performed using one-way ANOVA followed by Student–Newman–Keuls' test using SigmaPlot 11.0 (Systat software Inc., Chicago, USA) software. A probability of P < 0.05 was considered statistically significant.

3. Results

3.1. Effect of Cu-NPs on fish body and tissue weight

At the end of the experiment, weight of the whole fish and tissues (kidney, liver and gill) were noted for each group (Table 1) and changes in relation to the control were found. The lower dose treated groups showed significant (P < 0.05) increases in tissue weight and body weight when compared with control. However, the higher dose treated groups showed a more pronounced effect and a significant (P < 0.01) increase of tissue (kidney, liver and gill) and body weight were observed when compared to the control.

3.2. Effect of Cu-NPs on antioxidant enzymes (CAT, SOD and GST) in control and treated groups

The levels of various antioxidant enzymes (CAT, SOD, and GST) were analyzed in the kidney, liver and gills of control and treated groups (Fig. 1) and exposure to low and high dose of Cu-NPs was found to modify the enzyme performance. For the catalase activity (Fig. 1a) the results exhibited a significant (P < 0.05; P < 0.01) increase in the level of substrate utilized in the kidney, liver and gill of both treated (lower and higher) groups. Similarly, in SOD enzyme assay both lower and higher dose treated groups showed significant (P < 0.05; P < 0.01) increase in the function SOD activity in kidney, liver and gills when compared to control group (Fig. 1b). For the GST, the absorbance of the resultant adduct of CDNB-GSH showed a significant (P < 0.05; P < 0.01) elevation in kidney, liver and gill tissues of both treated group (lower and higher dose) when compared to the control (Fig. 1c).

3.3. Histological analysis

3.3.1. Kidney

Kidney of control fish showed normal morphology with parietal epithelium of Bowman’s capsule, glomerulus and proximal and distal tubules (Fig. 2a). Lower dose exposed groups displayed degeneration of renal tubules, a few necrotic cells in the hematopoietic tissue and presence of sinusoidal space (Fig. 2b). Further, the higher dose exposed group exhibited a higher number of degenerate tubules and space in between and glomerulus and Bowman’s capsule increased (Fig. 2c).

3.3.2. Liver

Histological analysis of control liver showed normal hepatocytes with sinusoidal space (Fig. 3a). The lower dose treated groups showed significant changes with increased sinusoidal space, cells with pyknotic nuclei and presence of cytoplasmic vacuoles indicating early stages of necrosis (Fig. 3b). The higher dose treatment showed extensive liver damage as revealed by the presence of hepatocytes with pyknotic nuclei and/or cell with dead nucleus, in addition to aggregation of blood cells and damaged blood vessel (Fig. 3c). As liver is a key organ in the accumulation and excretion of Cu pertaining to metabolism in developing fish, tissue proteome analysis was only performed in this tissue.

3.3.3. Gills

Gill morphology of control fish displayed normal appearance of primary and secondary lamellae (Fig. 4a–b). Exposure to lower

![Fig. 1](image_url) Relative antioxidant enzymatic assays in the kidney, liver and gill of common carp following the exposure to Cu-NPs for 7 days. (a) CAT enzyme assay, (b) SOD enzyme assay, and (c) GST enzyme assay. Data (n = 10) were expressed as mean ± SEM. (*, P < 0.05; ANOVA followed by Student-Newman–Keuls’ post hoc test).
Fig. 2. Histological analysis of the kidney following in control and exposed groups to Cu-NPs for 7 days. (a) control, (b) 20 mM Cu-NPs, (c) 100 mM Cu-NPs. Kidney of control fish showed normal morphology with parietal epithelium of Bowman's capsule (BC), glomerulus (G) and proximal (P) and distal (D) tubules. Treated groups exhibited sinusoidal spaces (SE), a higher number of degenerate tubules (Dg) and enlarged the Bowman's space (BSI).

Fig. 3. Histological analysis of the liver in control and Cu-NPs exposed groups. (a) control, (b) 20 mM Cu-NPs, (c) 100 mM Cu-NPs. The liver of control fish showed normal cells (Nc), normal sinusoid space (S) and normal blood vessel (Bv). Treated groups showed increased sinusoid space (InS), pyknotic nuclei (Pn), vacuole formation (V), necrosis (N), oedema in the tissue (Oe) and aggregation of blood cell (AB).
dose of Cu-NPs revealed hyperplasia at the base of the secondary lamellae, oedema of the gill epithelium, lamellar fusion, clubbed tips, occasional aneurism in the secondary lamellae and swollen mucocytes (Fig. 4c–d). Similar changes in tissue morphology were observed in fishes exposed to higher dose (Fig. 4e–f).

3.4. Differential regulation of the liver proteome after Cu-NPs exposure

Histological analysis of liver showed that both exposures provoked similar effects and hence, the liver from higher dose exposed group was used for proteomic analysis. Two-dimensional electrophoresis of common carp liver between control and treated groups (100 µL/L) showed significant differences in the protein profile (Fig. 5), wherein 30 differential spots were identified. In general, several proteins were down-regulated or completely absent in the fish exposed to Cu-NPs. Few up-regulated or new proteins were also observed in the exposure group (Fig. 6). Out of 30 spots, 19 spots from the treated group were down-regulated in relation to the control, while 11 spots were up-regulation in the treated group.

Out of 30 spots, the spots that showed significant differential expression, 4 spots (DR1, DR2, DR3 and DR4) down regulate in exposed group (Fig. 6a, in control gel) and one (UR1) upregulated in exposed group (Fig. 6b) were selected for the MALDI-TOF/TOF analysis based on low molecular weight. This is essentially due to our aim to select few putative low molecular weight proteins to understand the impact of Cu-NPs as high molecular weight protein spots might pick up vitellogenin or its related peptides, which is predominantly analyzed earlier in many toxicological studies. The spots identified using MALDI-TOF/TOF analysis are listed in Table 2.

4. Discussion

The present study depicted the impact of Cu-NPs exposure in the physiology of the common carp using biochemical, histological and proteome analysis. Analysis of oxidative stress related enzymes such as CAT, SOD and GST in kidney, liver and gill of control and Cu-NPs exposed groups revealed deleterious effects of this compound in a dose-related manner. Tissue histological analysis after the exposure also corroborates present findings. Two-dimensional gel
Electrophoresis of the liver identified several differentially expressed proteins after the exposure. Taken together present results suggest that a short-term exposure to Cu-NPs elicits oxidative stress in the common carp even at an eco-relevant concentration observed in the environment as pollutant.

Oxidative stress is a state of abundance of reactive oxygen species (ROS), which interfere with biological processes by disturbing or damaging homeostasis. This results in an imbalance between the production of ROS and the biological system’s ability to readily detoxify the reactive intermediates or repair the resulting damage (Lin, Zhang, Chen, & Cao, 2007; Mittler, 2002). To overcome the excess ROS response, cells can activate enzymatic and nonenzymatic antioxidant systems (Sies, 1991). Interestingly, Manke, Wang, and Rojanasakul (2013) reported that differences in NPs properties can cause adverse effects on biomolecules, which ultimately leads to ROS generation. NPs react with cells and induce their pro-oxidant effects via intracellular ROS production involving mitochondrial respiration and activation of NADPH-like enzyme systems (Driscoll et al., 2001). In this study, the increased activity of oxidative stress enzyme CAT, SOD, and GST indicate the alteration of normal homeostasis. Cu-NPs are causative molecules for generating oxidative stress and responsible for cell death (Fahmy & Cormier, 2009). Further, Cu has redox property and is involved in several enzymatic reactions such as cytochrome-c oxidase, SOD, quercetin 2, 3-dioxygenase, indole 2, 3-dioxygenase. In addition, Cu-NPs disturbs the normal mitochondrial homeostasis, which further causes oxidative stress to the cell (Federici, Shaw, & Handy, 2007).

In fish, the gills are the first line of defense for any pollutant by secreting mucous (Handy & Maunder, 2009). Subsequently, gills respond through generation of oedema with the lifting of gill lamellar epithelium by binding to Na⁺/K⁺-ATPase and inhibiting toxin entry (Stagg & Shuttleworth, 1982). Cu is a well-known inhibitor of gill respiration and ionoregulation (Grosell, Blanchard, Brix, & Gerdes, 2007; Handy, 2003). Histological analysis of the gills after exposure displayed secretion of mucous that might be due to disturbance of their osmotic permeability. In addition, Bilberg, Malte, Wang, and Baatrup (2010) showed that exposure of silver NPs can reduce blood pO₂ level in fish resulting in systemic hypoxia and hyperplasia. Our results showed similar conditions in gills after exposure.

In Cu-NPs treated groups, histology of the kidney showed necrosis, damage of the epithelial cells of the renal tubules and an increase of the Bowman’s space resulting in most serious renal damage including glomerulosclerosis and tubular necrosis (Camargo & Martinez, 2007). Similar effects were observed in the catfish kidney after exposure to heavy metal mercurial compounds (Kirubagaran & Joy, 1988). The kidneys play a critical role to maintain osmoregulatory mechanism and renal filtration rate. Increased of CAT, SOD and GST activities in kidney might have a direct relation with the production of cytokines, chemokines and oxygen free radicles (Scharsack, Kalbe, Derner, Kurtz, & Milinski, 2004). In the present study, increased level of antioxidant enzymes further correlated with the renal cellular damages observed. The effect of metal NP on renal functions in other species is corroborative to present report (Handy et al., 2011).

The present study also analyzed the liver as a central compartment for Cu metabolism (Grosell, Boetius, Hansen, & Rosenkilde, 1996; Handy, Sims, Giles, Campbell, & Musonda, 1999; Kamunde, Grosell, Higgs, & Wood, 2002). Previous reports documented that fish exposed to Cu-NPs displayed blood accumulation and increase in sinusoid space, which is an indication of liver damage (Arellano, Storch, & Sarasquete, 1999; Shaw & Handy, 2011). In present study, exposure to Cu-NPs even to doses lower than LC₅₀ showed a pronounced increase in the number of pyknotic nucleus indicating dead nuclei that may progress to tissue necrosis. Results on higher dose exposure in the present study, displayed accumulation of lipid droplet in the hepatocytes or forming vacuole and cellular swelling

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**Table 2**

| Sample | Protein name                                      | Score | Molecular weight in kDa | Calculated pl |
|--------|--------------------------------------------------|-------|--------------------------|---------------|
| DR1    | ferritin heavy chain                              | 87    | 20,450                   | 5.26          |
| DR2    | PREDICTED: rho guanine nucleotide exchange factor 17-like | 43    | 146,858                  | 6.03          |
| DR3    | Cytoglobin-1                                      | 37    | 20,010                   | 5.22          |
| DR4    | Diphosphomevalonate decarboxylase                 | 29    | 45,084                   | 5.97          |
| UR1    | Selenide, water dikinase 1                        | 37    | 43,408                   | 5.65          |

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Fig. 6. Representative two-dimensional electrophoresis gels of common carp liver (n = 3) following exposure to Cu-NPs for 7 days. (Note: The spots chosen for MALDI-TOF/TOF) (a) Control and (b) Cu-NPs 100 mM exposed groups. The coomassie stained 2D-gels from control and treated groups were compared with Image Master 2D Platinum (GE-Healthcare) system. Spots indicated by circles were found to be up-regulated (U) and down-regulated (D) across the two groups. Spots were: Selenide, water dikinase 1 (UR1), ferritin heavy chain (DR1), rho guanine nucleotide exchange factor 17-like (DR2), Cytoglobin-1 (DR3) and Diphosphomevalonate decarboxylase (DR4).
with a clear cytoplasm due to the presence of small vacuoles, with indistinct shape. This also limits the normal position of nucleus. In this respect, histological analysis endorsed the altered levels of antioxidant enzymes in the common carp liver.

Comparison of the liver proteome profile displayed differentially expressed protein spots and increased protein expression, appearance and absence of protein spots were observed in the treated group when compared to control. Ferritin heavy chain (FHC) was down-regulated in the exposure sample and this protein plays a crucial role in intracellular ion storage, sequestration and detoxification (Percy et al., 1998). It also acts as a mediator for antioxidant and protective activities of NF-xB (Pham et al., 2004). NF-xB plays an important role in apoptosis by regulating JNK pathway through a series of cascade mechanisms. Hence, FHC acts as an anti-apoptotic gene (Berberat et al., 2003) and can inhibit the accumulation of ROS. Down-regulation of FHC in the treated group might be due to the excessive production of ROS causing desensitization of FHC, which is in agreement with our biochemical results. Guanine nucleotide exchange factors (GEFs) was also down-regulated. This protein belongs to Ras family that are involved in cell proliferation and differentiation, cytoskeletal organization, vesicle trafficking, and nuclear transport. Down-regulation of this protein may be due to excessive ROS production which can cause apoptosis or tumor formation as they are activators of Ras super family proteins (Gómez et al., 1998). Cytochrome-1 (CYGB) is also a ubiquitous enzyme present mostly in all tissues. Its function is not well studied but it has been shown to be involved in oxygen transfer from atrial blood to the brain and protects the cell by scavenging ROS species (Hamdane et al., 2003). In human, deficiency of CYGB can cause liver metastasis via the activation of oxidative stress pathway (Thuy-le et al., 2015). Together with the results of biochemical and histological analysis we suggest that CYGB deficiency may exert oxidative stress in the liver resulting in major alteration in oxidative stress related enzymes leading to cell death. This may be the reason for more numbers of pyknotic nucleus which may ultimately lead to necrosis of hepatocytes. Diphospho-3-decarboxylase catalyzes the final step of the mevalonate pathway involved in the production of cholesterol and other sterols and down-regulation of this enzyme may hinder the cholesterol synthesis which is an important precursor for gonadal steroid biosynthesis. Further, present data supports the adverse effect of Cu-NPs not only by causing cellular damages but also affecting gonadal homeostasis either directly or indirectly. Common carp exposed to Cu-NPs showed up-regulation in selenide, a water dikinase-1 (SEPHS1) enzyme that belongs to the transferases family that are involved in transferring phosphorus-containing groups (phosphotransferases) with paired acceptors (dikinases). The enzyme regulates the seleno-amino acid metabolism. However, the detailed analysis of this enzyme and its mechanism in relation to toxicity is not yet well understood. The enzymes analyzed in this study might not be directly related, but their altered levels indicated that the effect of Cu-NPs in causing oxidative stress. The identified protein spots provide valuable evidence for induction of oxidative stress in fishes after NP contamination in the ecosystem. Based on our study, we suggest that low level of contamination of Cu-NPs can cause major problems by creating oxidative stress in developing carp and this can lead to depletion of fish population in natural pond based culture.

5. Conclusion

Exposure of common carp to Cu-NPs increased the activity of oxidative stress enzymes that might lead to the excessive production of free radicals and disturbance of internal homeostasis indicating that this compound has a profound adverse effect on fish health. Histological analysis confirmed altered morphology and cellular damages of the kidney, liver and gills of the exposed animals. 2D gel electrophoresis analysis showed altered and/or differentially expressed protein associated with oxidative stress and steroid biosynthesis either directly or indirectly. Based on our analysis, we suggest that short-term exposure of Cu-NPs even at a low dose can cause oxidative stress and this may lead to developmental disarray in the common carp.

Conflicts of interest

The authors declare no conflict of interest pertaining to the research report in this manuscript.

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