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Assessment of genotoxicity and nephrotoxicity induced by copper nanoparticles and copper (II) oxide in *Cyprinus carpio*

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Abstract

Nanotechnological research has become a significant priority worldwide, but the adverse effects of these nanoparticles on the organisms have recently drawn much attention. Therefore, the study was aimed to assess the toxic effects of different doses of waterborne copper nanoparticles (Cu-NPs) and copper II oxide (CuO) on the DNA damage and kidney histology in *C. carpio*. For this purpose, the sub-lethal dose (0.5 or 1 or 1.5 mg/l) of Cu-NPs and Cu-BS was given to *C. carpio* for a period of 14 days. At the end of the experiment the toxicity of Cu-NPs and Cu-BS was determined by measuring the micronuclei and histology of kidney. The results revealed that Cu-NPs treated groups showed more DNA damage as compared to CuO treated groups. More nuclear abnormalities were recorded in *C. carpio* exposed to the higher dose of Cu-NPs and CuO. Dose-dependent histological alterations in fish kidney were observed in all treatment groups as compared to control group. Overall, there was non-significant ($p>0.05$) difference between Cu-NPs and CuO treatment, whereas highly significant ($p<0.001$) differences were observed in *C. carpio* exposed to different doses of Cu-NPs and CuO between and within the groups. Further research is suggested to estimate the chronic exposure of Cu-NPs and CuO.

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Introduction

Many engineered nano-sized materials have been increasingly used in consumer's products. But the adverse effects of these nanoparticles on the environment and organisms have recently drawn much attention (Auffan *et al.,* 2009; Avalos *et al.,* 2014; Ahamed *et al.,* 2015). The most common application of nanotechnology is as a substitute energy source, in electronics and medicine as therapeutic (diagnosis), or in the delivery of drug which shows that it contributes to the healthy life. There are growing concerns about the exposure of these nano-sized materials to human and other organisms which directly or indirectly induced toxicological effects on health (Yousefian and Payam, 2012).This technology has vast benefits for human health, but also poses unknown threats to the environment quality and human health by increasing the chance of exposure (Wang *et al.,* 2014a) and they have ability to facilitate the direct generation of harmful radical oxygen species (ROS) inside the cell (Sayes *et al.,* 2005; Brunet *et al.,* 2009; Shinohara *et al.,* 2009; Wang *et al.*, 2014b). These ROS in cells produce injury in the cell which also includes DNA damage. The interactions of these NPs with other metals and organic pollutants have also enhanced the toxicity and bio-accumulation of both NPs and other pollutants (Baun *et al.,* 2008; Brausch *et al.,* 2010; Wang *et al.,* 2014b). So these NPs risk potentials must be adversely affecting the environmental quality and human health.

Copper nanoparticles (Cu-NPs) have been used in biotechnological research to combine Cu-NPs with a polymer to make a compound which accomplished release of metal ions in a precise manner to inhibit the fungal and other pathogenic microorganism growth that might be fought with some disease conditions (Cioffi *et al.,* 2005). The Cu-NPs also enormously used as a germicide in different forms. Moreover, Cu-NPs have been used in marine industries as coatings which act as an antifouling for ships (Noureen and Jabeen, 2015) which might result in the release of Cu-NPs into the aquatic bodies. Moreover, manufactured Cu-NPs used as lubricant (Prabhu *et al.,* 2009), it has also been used in cosmetics and skin products to heal and prevent skin from infection (Midander *et al.,* 2009).

It is obvious from the increasing use of NPs that aquatic species are at risk of NPs exposure, and a body of literature is emerging concerning the chemical behavior of NPs in aquatic systems, including their accumulation and toxicity in aquatic species. NPs tend to accumulate in cells, such as macrophages and hepatocytes (Witasp *et al.,* 2009; Johnston *et al.,* 2009). Moreover, they could be absorbed, causing toxic effects in aquatic organisms such as phytoplankton, mollusks, crustaceans and fish in freshwater and seawater (Ward and Kach 2009; Tao *et al.,* 2009; Ates *et al.,* 2014). Therefore, the current study was designed to investigate the effects of Cu-NPs and CuO on the DNA damage and histology of kidney in *C. carpio*.

Materials and methods

All the experimental trial to assess the toxicity of Cu-NPs and CuO on the DNA damage and histology (kidney) of *C. carpio* was performed in the Department of Zoology, Government College University Faisalabad, Pakistan.

Fish procurement and acclimatization

A fish husbandry was established prior to the start of experiments to maintain the health of fish by maintaining the water quality and environment of stock aquaria. *Cyprinus* (*C*) *carpio* of the same weight (40-45g) were procured from the Fish Seed Hatchery Satiana Road Faisalabad Pakistan, transported in plastic containers with continuous aeration to the laboratory of the Department of Zoology, Government College University Faisalabad, Pakistan and were acclimatized in the tank with 100 L capacity for two weeks prior to the experiment. Un-chlorinated tap water was used for the experiment and physicochemical parameters of water were determined. During acclimatization period water temperature was maintained at 25℃, while dissolved oxygen and pH were 6.6–7.6 mg/l, and 6.9–7.5, respectively.

NH_3 concentration, total hardness and total dissolved solids were 0.4-0.6 ppm, 47-52 ppm and 6.5-7.8 ppt, respectively. Photoperiod was 12 h light: 12 h dark. During the acclimatization period, fish were fed twice daily with commercial fish feed. Water was changed daily and dead fish as well as any fish showing any unusual symptoms were excluded.

Chemicals

The high quality analytical and molecular grade chemicals were used in the study. Engineered Cu-NPs and CuO were purchased from Sigma Aldrich.

Preparation Stock Solutions

The Cu-NPs and CuO used in the study were 60-80 nm and <10 µm, respectively. For preparation of exposure medium, the required amounts of Cu-NPs and Cu salt were weighed into polypropylene tubes and dispersed in deionized water. To achieve maximum dispersion, the suspension was homogenized by vortex (5 minutes at 2000 rpm), exposed to the ultrasound sonication bath for 1.5 hour and immediately transferred into the exposure glass tanks.

Sub-acute toxicity testing

210 *C. carpio* of similar weight (40-45 g) were randomly transferred into twenty one experimental glass aquaria (10 fish/ aquaria) with the same physicochemical parameters as in the acclimatization period and were acclimated for 48 hrs prior to the experiment. Three aquaria per treatment were randomly allocated and fish was exposed in triplicate to one of the following treatments for 14 days. Control (no added Cu-NPs or CuO) or 0.5 or 1 or 1.5 mg/l Cu as Cu-NPs or CuO. The dosing was adopted based on sub lethal doses of 96-hrs LC50 of Cu-NPs and CuO for *C. carpio*. During trial period the water in the aquaria was changed daily and freshly prepared solution was added to maintain the concentration of Cu-NPs and CuO at constant level. During the experiment fish were fed at the rate of 2.5% body weight with commercial fish meal twice daily. Moreover, during the experiment, water was continuously monitored.

Water samples were taken at the start and end of the experiment to assess the pH, temperature, dissolved oxygen, total ammonia and water hardness.

Sampling

At the end of experiment fish sampling was done and sampled fish were anesthetized with 70 mg/l of clove oil in a bucket for 6 minutes (Javahery *et al.,* 2012). Blood was collected from the caudal vein for the assessment of micronucleus. The kidney tissues were collected for histological studies.

Micronucleus Test (MNT)

Blood samples were processed for MNT immediately by following the method of Jorge *et al.* (2014).Briefly the blood samples were smeared on three clean glass slides and were air dried. These slides were fixed with absolute methanol for 10 minutes and stained with Giemsa stain. Micronuclei were scored with the help of light microscope (Nikon Eclipse E200 POL). Each slide was studied thoroughly with at least 20 fields for any abnormality. The frequency of abnormalities was measured by using the following formula:

$$\text{MN Frequency} = \frac{\text{No. of abnormalities}}{1000} \text{X} 100$$

Histological studies

Small pieces of kidney tissues were fixed in sera (absolute alcohol, formaldehyde and glacial acetic acid) for 4-6 hours at room temperature. The fixation was followed by dehydration by 80, 90 and 100% ethanol. After dehydration fixed tissues were transferred to cedar wood oil until they become clear and transparent at room temperature. Then tissues were embed in paraplast. Embedded tissues were then transferred into molted wax in a boat. Bubbles were removed and the wax was allowed to solidify. Paraffin embedded tissues were mounted on wooden blocks and 4-5 µm thin sections were cut using microtome (Leica Biosystems RM2125 RTS, The Essential Microtome). The tissues were then stained with hematoxylin and eosin after hydration. After staining the slides were mounted with Canada balsam. Cover slips were placed on the slides and were placed in an incubator overnight.

Extra Canada balsam was removed by xylene. Slides of all the control and treated groups were studied and photographed by light microscope (Nikon Eclipse E200 POL) at 400 X magnification.

Statistical analysis

The data were analyzed by Minitab17 software using General Liner Model (ANOVA). Data of all selected parameters regarding control, Cu-NPs, Cu-BS were expressed as mean ± SD. LSD multicomparision was applied with the help of Tuckey Test for the comparison of mean from different treatments and control groups. P-value less than 0.05 was considered to be statistically significant.

Results

The experimental fish showed nuclear abnormalities like Bi nucleated (BN), Micronuclei (M), Lobed nucleus (LN), Notched Nucleus (NN), Vaulted nucleus (VN) and Nuclear bud (NB) Fig. 1. More nuclear abnormalities were recorded in *C. carpio* exposed to higher doses of Cu-NPs and Cu-BS (Table 1).

Table 1. Mean (%) nuclear alterations in blood of *C. carpio* among different groups.

Treatment Groups	Bi- nucleated	Micronuclei	Lobed nucleus	Notched Nucleus	Vaulted nucleus	Nuclear bud
GNC	0.67 ± 0.02^{G}	0.92 ± 0.01^{F}	1.25 ± 0.02^{G}	0.05 ± 0.04^{F}	1.44 ± 0.02^{G}	1.17 ± 0.02^{G}
GN1	2.74 ± 0.02^{E}	4.94 ± 0.02^{E}	3.12 ± 0.01^{F}	4.12 ± 0.01^{D}	5.65 ± 0.03^{F}	5.16 ± 0.03^{E}
GN2	3.45 ± 0.02^{D}	4.93 ± 0.02^{E}	5.15 ± 0.02^{D}	4.14 ± 0.02^{D}	5.94 ± 0.03^{E}	5.95 ± 0.03^{D}
GN3	3.95 ± 0.04^{C}	7.76 ± 0.02^{C}	7.46 ± 0.01^{B}	9.82 ± 0.01^{A}	7.36 ± 0.02^{D}	10.02 ± 0.01^{B}
GSC	0.67 ± 0.02^{G}	0.92 ± 0.01^{F}	1.25 ± 0.02^{G}	0.05 ± 0.04^{F}	1.44 ± 0.02^{G}	1.17 ± 0.02^{G}
GS1	2.27 ± 0.02^{F}	6.52 ± 0.01^{D}	3.76 ± 0.02^{E}	2.75 ± 0.04^{E}	8.48 ± 0.01^{C}	3.97 ± 0.02^{F}
GS2	5.17 ± 0.15^{B}	9.73 ± 0.01^{B}	5.76 ± 0.02^{C}	5.26 ± 0.03^{C}	9.96 ± 0.03^{B}	6.59 ± 0.01^{C}
GS3	7.43 ± 0.21^{A}	12.70 ± 0.20^{A}	8.13 ± 0.02^{A}	9.52 ± 0.01^{B}	14.78 ± 0.02^{A}	10.58 ± 0.02^{A}

Means with different letters in the same column differ significantly ($P<0.05$), Nanoparticles= GN, CuO= GS, C=control, 1=0.5mg/l, 2=1.0 mg/l, 3= 1.5 mg/l.

Table 2. Histological alterations in kidney of *C. carpio* exposed to different concentrations Cu-NPs and CuO.

Histological alterations	GNC	GN1	GN2	GN3	GS1	GS2	GS3
Necrosis and tubular degeneration	-	+	++	+++	+	++	+++
Hypertrophy of tubules	-	-	-/+	+	-	-/+	+
Reduced lumen	-	-	-	-/+	-	-/+	-/+
Abnormal glomerulus	-	+	+	++	+	+	++
Shrinked glomerulus	-	-	-/+	+	-	-/+	+
Swollen tubules	-	+	++	+++	+	++	+++
Degenerative tubules	-	+	++	+++	+	++	+++
Complete degeneration	-	-	-/+	+	-	-/+	+

(-) no histological alterations (normal histological structure); (+/-) mild histological alterations; (+) moderate histological alterations; (++) severe histological alterations; (+++) very severe histological alterations in the kidney.

There were non-significant ($p>0.05$) differences in all nuclear abnormalities of *C. carpio* treated with Cu-NPs and CuO except vaulted nuclei in Cu-NPs treatment ($P<0.05$; Table 2). Whereas, highly significant ($p<0.001$) differences were observed in all blood nuclear abnormalities of *C. carpio* exposed to different doses of Cu-NPs and CuO between and within the groups (Table 1).

Multiple comparison of different doses showed significant ($p<0.001$) changes in all studied blood nuclear abnormalities except the non-significant effect of dose 0.5 and 1.0 mg/kg on micronuclei, lobed nucleus and vaulted nucleus (Table 1).

The histological investigation of kidney of *C. carpio* revealed that Cu-NPs and CuO induced significant alteration in the structure and tissues of the kidney which increase with increase in dose when compared to the control. The alteration in kidney including necrosis and tubular degeneration (NTD), hypertrophy of tubules (HT), reduced lumen (RL), abnormal glomerulus (AG), shrunken glomerulus (SG), swollen tubules (ST), complete degeneration (CD) and degenerative tubules (DT) (Fig.2-8). Fig.9 and 10 presents the comparative histology of all treatment groups while the intensity of histological changes is shown in Table 2.

Discussion

The micronucleus test has been used increasingly to evaluate the genotoxicity of many metals and their organic compounds in aquatic ecosystems.

The use of endemic aquatic organisms as biological sentinels has been proved useful in environmental monitoring (Rocha *et al.,* 2011). Micronuclei (MN) are surrogate measures of structural and numerical chromosomal aberrations; it can also be considered bridging biomarkers of genotoxic exposure (Bonassi *et al.,* 2007; Recio *et al.,* 2010). In the current study the more erythrocytes abnormalities were observed at the highest dose of Cu-NPs and non-significant alterations were detected in the Cu-NPs and CuO treatments in term of MN assay. It reflects that NPs can enter into the nucleus and they might interact with DNA during cell division, causing genetic damage altered bases or chromosomal damage. NPs can also reach the nucleus during mitosis and interfere with the microtubules, causing clastogenic effects (Bonassi *et al.,* 2007).

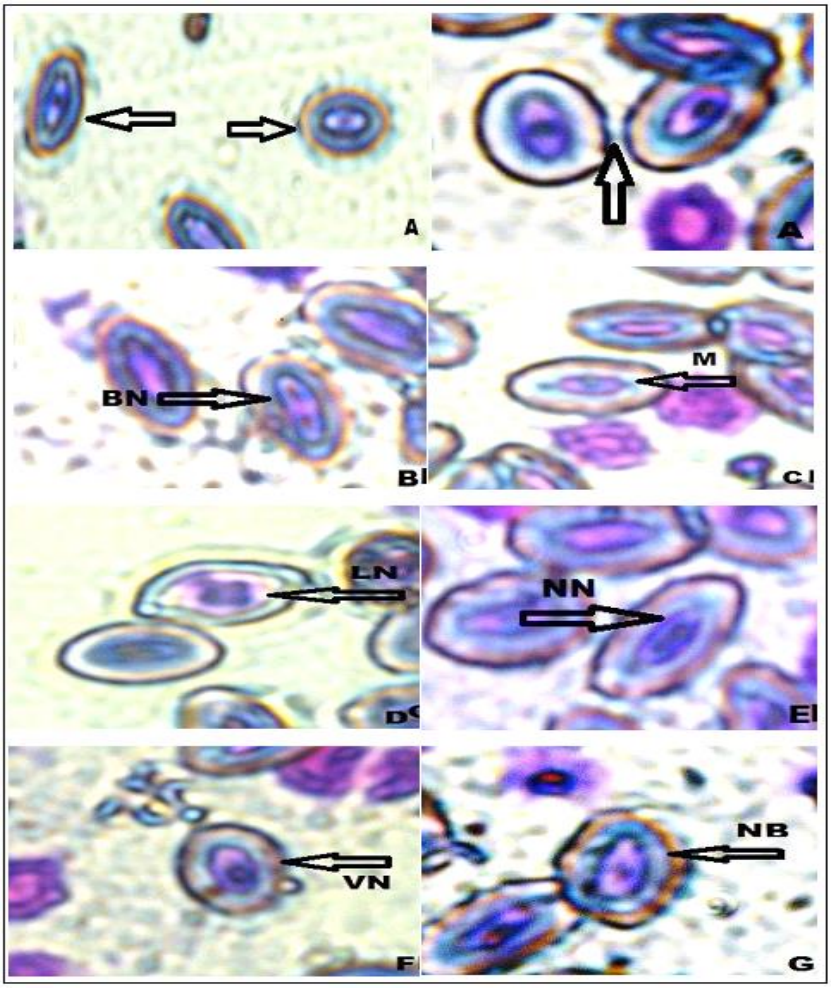

Fig. 1. Photomicrograph (X400) of erythrocytes of control and treated groups. A: Normal, B: Binucleated (BN), C: Micronuclei (M), D: Lobed Nucleus (LN), E: Notched Nucleus (NN), F: Vacuolated Nucleus (VN), G: Nuclear Bud (NB).

All these events may result in pre-mutagenic lesions that can lead to mutations and possibly to cancer and other diseases (Love *et al.,* 2012; Klien and Godnić-Cvar, 2012; Doak *et al.,* 2012; Azqueta and Dusinska, 2015; Bahadar *et al.,* 2016).

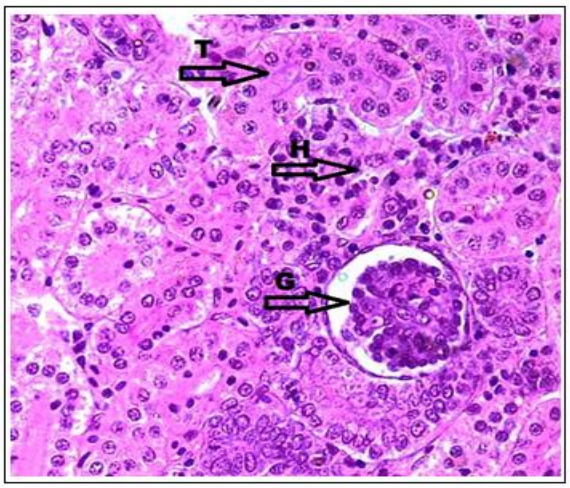

Fig. 2. Photomicrograph (H&E; X 400) of kidney of control *C. carpio* showing normal hematopoietic tissues (H), tubules (T) and glomerulus (G).

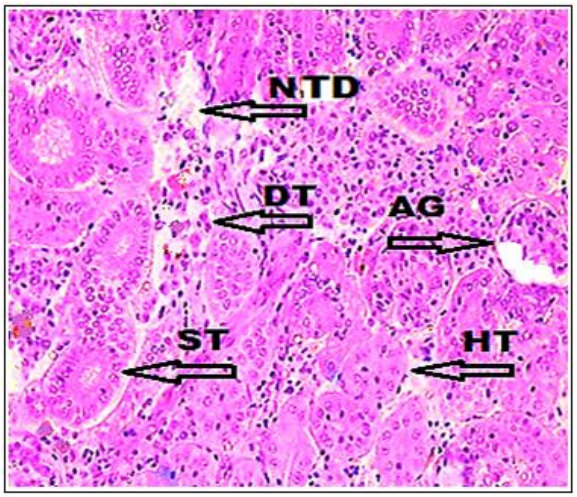

Fig. 3. Photomicrograph (H&E; X400) of kidney of *C. carpio* treated with 0.5 mg/l Cu-NPs (GN1) showing necrosis and tubular degeneration (NTD), hypertrophy of tubules (HT), abnormal glomerulus (AG), swollen tubules (ST), and degenerative tubules (DT).

The previous studies also recorded increased frequency of MN in fish (*C. carpio,* Zebra fish and *Oriochromus niloticus*) with increase in the concentration of toxicant (NaClO, Erythromycin, Lincomycin and rotenone, respectively) (Canistro *et al.,* 2012; Rocco *et al.,* 2016).

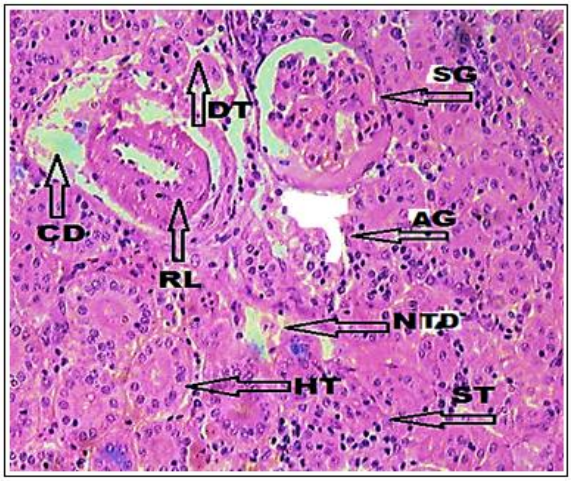

Fig. 4. Photomicrograph (H&E; X400) of kidney of *C. carpio* treated with 1.0 mg/l Cu-NPs (GN2) showing necrosis and tubular degeneration (NTD), hypertrophy of tubules (HT), reduced lumen (RL), abnormal glomerulus (AG), shrinked glomerulus (SG), swollen tubules (ST), complete degeneration (CD) and degenerative tubules (DT).

The kidney is a complex organ made up of thousands of repeating units called nephrons, pressure filtration of blood occurred by the glomerulus, situated at the top of each nephron (Al-Tamimi *et al.,* 2015).

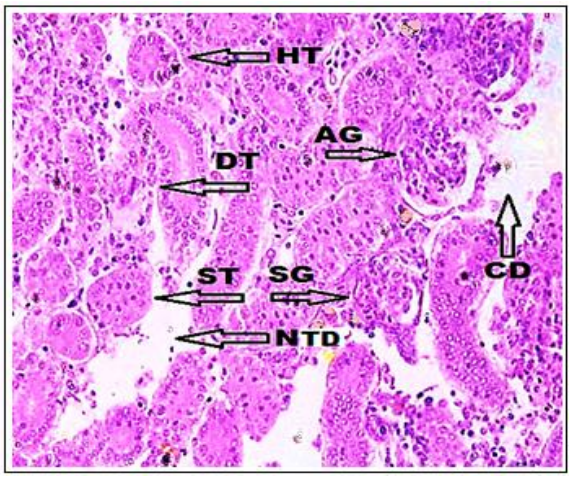

Fig. 5. Photomicrograph (H&E; X400) of kidney of *C. carpio* treated with 1.5 mg/l Cu-NPs (GN3) showing necrosis and tubuler degeneration (NTD), hypertrophy of tubules (HT), abnormal glomerulus (AG), shrinked glomerulus (SG), swollen tubules (ST), complete degeneration (CD) and degenerative tubules (DT).

In the present study, after 14-day of exposure of Cu-NPs and CuO following histological alterations *i.e.*, necrosis of hematopoietic tissues (NHT), hypertrophy of tubules (HT), reduced lumen (RL), abnormal glomerulus (AG), shrunk glomerulus (SG), swollen tubules (ST), complete degeneration (CD) and degenerative tubules (DT) were observed in kidney of *C. carpio*.

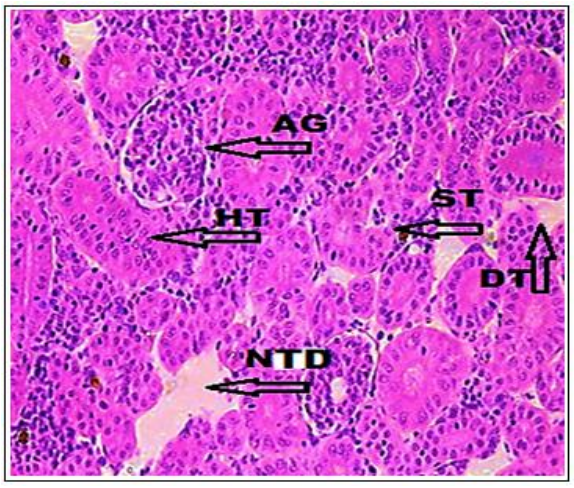

Fig. 6. Photomicrograph (H&E; X400) of kidney of *C. carpio* treated with 0.5 mg/l CuO(GS1) showing necrosis and tubuler degeneration (NTD), hypertrophy of tubules (HT), reduced lumen (RL), abnormal glomerulus (AG), shrinked glomerulus (SG), swollen tubules (ST), complete degeneration (CD) and degenerative tubules (DT).

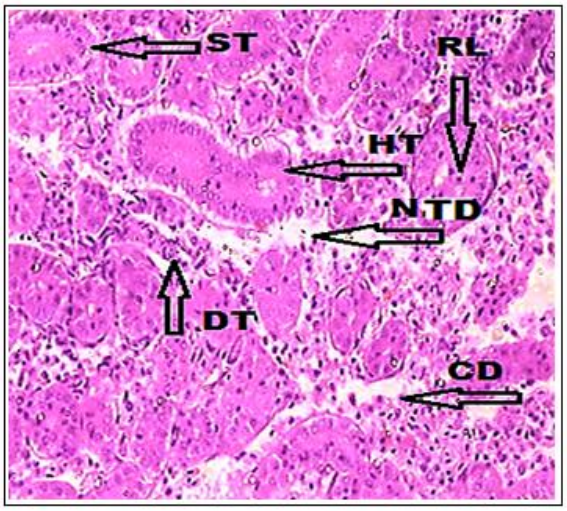

Fig. 7. Photomicrograph (H&E; X400) of kidney of *C. carpio* treated with 1.0 mg/l CuO (GS2) showing necrosis and tubuler degeneration (NTD), hypertrophy of tubules (HT), reduced lumen (RL), swollen tubules (ST), complete degeneration (CD) and degenerative tubules (DT).

It was observed that severity of pathologies increases with an increase in the concentration of CuO and Cu-NPs. The fish exposed to Cu-NPs showed more severe pathologies as compared to CuO, because the kidney is prime organ which is affected by contaminants in the water.

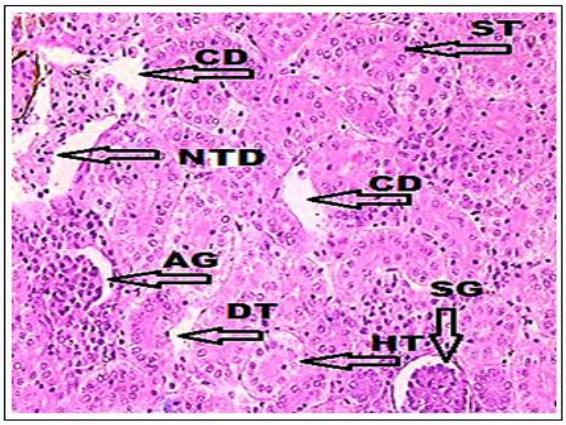

Fig. 8. Photomicrograph (H&E; X400) of kidney of *C. carpio* treated with 1.5 mg/l CuO (GS3) showing necrosis and tubuler degeneration (NTD), hypertrophy of tubules (HT), abnormal glomerulus (AG), shrinked glomerulus (SG), swollen tubules (ST), complete degeneration (CD) and degenerative tubules (DT). presents the comparative histology of all treatment groups while the intensity of histological changes is shown in Table 2.

Disturbance of living processes at the molecular and subcellular levels of biological organization by xenobiotic can lead to cell injury, resulting in degenerative and neoplastic diseases in target organs.

The histology of the controlled kidney tissues exhibited a normal pattern of renal corpuscles and collecting tubules with no abnormalities in any other part of the renal cellular layout. The same findings were reported by Al-Tamimi *et al.* (2015), who assessed the histological changes in kidney of *C. carpio* when exposed to different concentrations of Cu.

The histological alterations in the kidney tissues exposed to toxic agents in fish reported by many researchers. Das and Mukherjee (2000) reported dilation of renal tubules and necrotic changes in *Labeo rohita* exposed to hexachloro-cyclohexane.

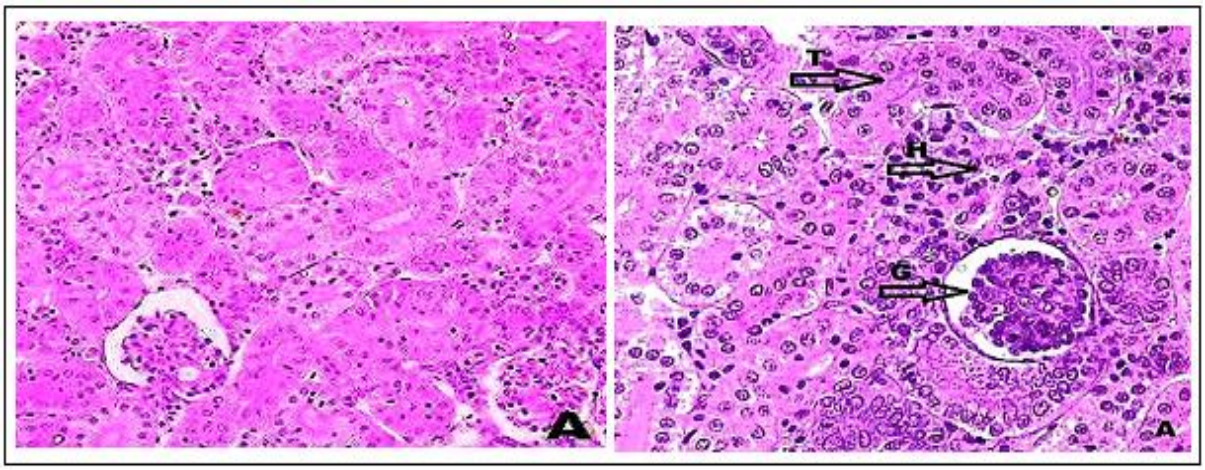

Fig. 9. Photomicrograph of Normal Kidney of Control Group (A) showing normal Histology (H&E; X400).

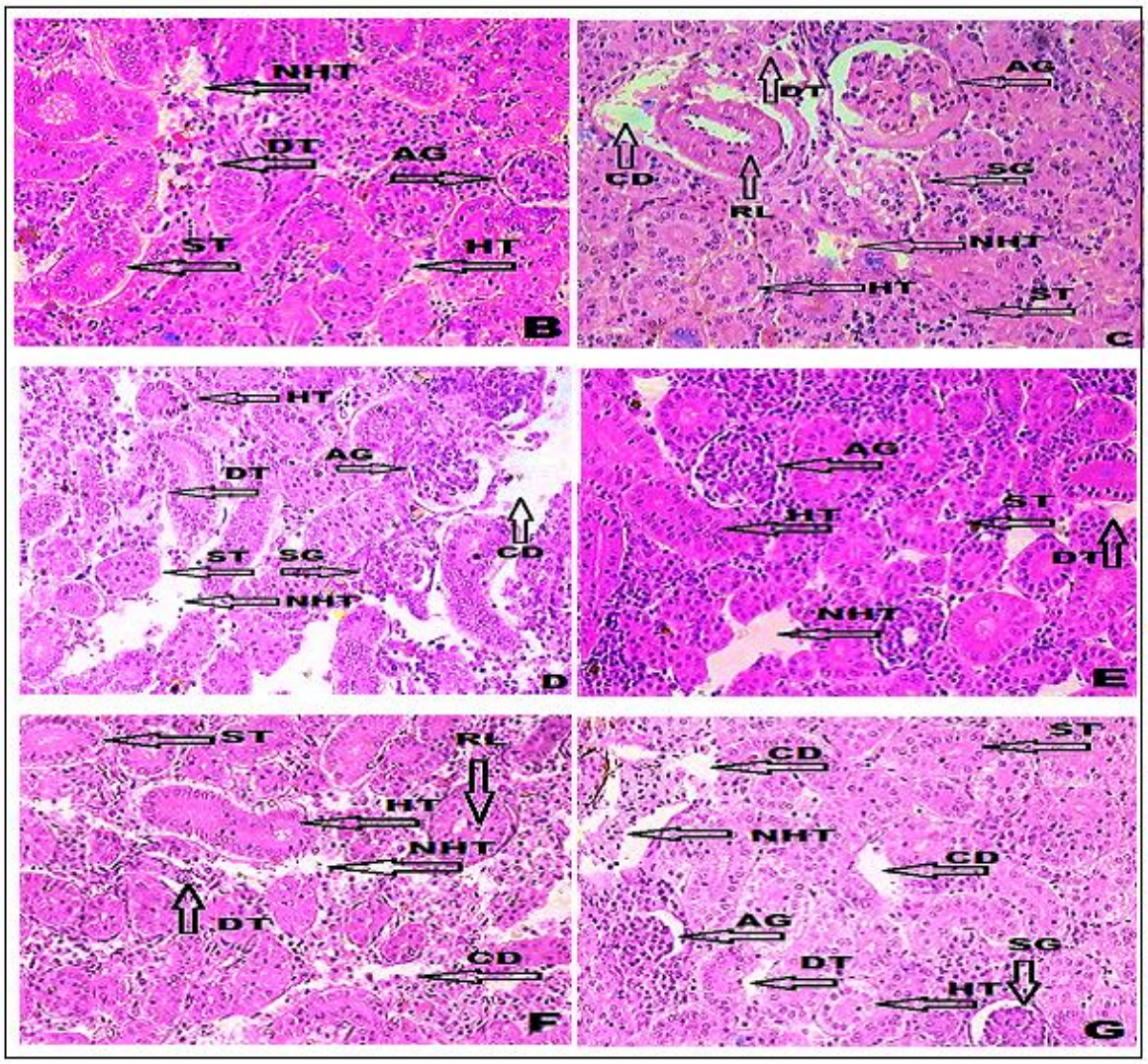

Fig. 10. Comparative histological alterations in kidney of *C. carpio*, exposed to Cu-NPs and Cu-BS: B - 0.5 mg/l Cu-NPs, C –1 mg/l Cu-NPs, D - 1.5 mg/l Cu-NPs, E - 0.5 mg/l CuO, F - 1 mg/l CuO, G - 1.5 mg/l CuO, showing necrosis and tubuler degeneration (NTD), hypertrophy of tubules (HT), reduced lumen (RL), abnormal glomerulus (AG), shrinked glomerulus (SG), swollen tubules (ST), complete degeneration (CD) and degenerative tubules (DT) (H&E; X400).

Tilak *et al.* (2001) noticed severe necrosis, cloudy swelling in the renal tubules, cellular hypertrophy and vacuolization in kidney tissues of *Ctenopharyngodon idella* after exposure to fenvalerate.

In another study Butchiram *et al.* (2009) reported severe degeneration in kidney tissues of *Channa punctatus* when exposed to alachlor. Chloropyrifos damaged the architecture of kidney in *Catla catla* and *Cirrhinus mrigala* (Tilak *et al.,* 2005a, b). The pycnotic nuclei in the hematopoietic tissue, dilation of glomerular capillaries and degenerative glomerulus were observed in the kidney tissues of fish treated with deltamethrin (Cengiz, 2006). Velmurugan *et al.* (2007) reported pyknotic nuclei in tubular epithelium, abnormalities in renal tubules, shrinkage of the glomerulus in the kidney of *Cirrhinus mrigala* treated with monocrotophos. Gill *et al.* (1989) revealed histological changes such as degeneration of renal tubules and crumpling of glomerulus in the kidney of *Puntius conchonius* exposed to cadmium in time dependent manner. Coulibaly *et al.* (2012) reported histological alterations of gills, liver and kidney of Black-Chinned Tilapia *Sarotherodon melanotheron* when exposed to water contaminated by heavy metals including Cu. These findings are in line with the current study.

Conclusion

From the results, it could be concluded that the Cu-NPs were more toxic than their bulk counterpart and potentially induced DNA damage and histological alterations in *C. carpio*.

References

Ahamed M, Akhtar MJ, Alhadlaq H, Alrokayan S. 2015. Assessment of the lung toxicity of copper oxide nanoparticles. Nanomedicine (Lond.) **10,** 2365 -2377.
http://dx.doi.org/10.2217/nnm.15.72.

Al-tamimi AH, Al-azzawi AJ,Al-a'dhmi MA. 2015. Chronic toxicity assessment of histological changes and micronuclei in fish *Cyprinus carpio* L. after exposed to copper. American Scientific Research Journal for Engineering, Technology, and Sciences **13,** 194-210.

Ates M, Dugo MA, Demir V, Arslan Z, Tchounwou PB. 2014. Effect of copper oxide nanoparticles to sheep head minnow (*Cyprinodon variegatus*) at different salinities. Digest Journal of Nanomaterials and Biostructures **9,** 369-377.

Auffan M, Rose J, Bottero J, Iowry GV, Jolivet JP, Wiesner MR. 2009. Towards a definition of inorganic nanoparticles from an environmental, health and safety perspective. Nature Nanotechnology **4,** 634–641.
http://dx.doi.org/10.1038/nnano.2009.242.

Avalos A, Haza AI, Mateo D, Morales P. 2014. Cytotoxicity and ROS production of manufactured silver nanoparticles of different sizes in hepatoma and leukemia cells. Journal of Applied Toxicology **34,** 413-423.
http://dx.doi.org/10.1002/jat.2957

Azqueta A, Dusinska M. 2015. The use of the comet assay for the evaluation of the genotoxicity of nanomaterials. Frontiers in Genetics **6**, 1-4.
http://dx.doi.org/10.3389/fgene.2015.00239

Bahadar H, Maqbool F, Niaz K, Abdollahi M.2016. Toxicity of nanoparticles and an overview of current experimental models. Iranian Biomedical Journal **20,** 1-11.
http://dx.doi.org/10.7508/ibj.2016.01.001

Baun A, Sorensen SN, Rasmussen R, Hartmann NB, Koch CB. 2008. Toxicity and bioaccumulation of xenobiotic organic compounds in the presence of aqueous suspensions of aggregates of nano-C (60). Aquatic Toxicology **86**, 379-87.
http://dx.doi.org/10.1016/j.aquatox.2007.11.019.

Bonassi S, Znaor A, Ceppi M, Lando C, Chang WP, Holland N, Kirsch-Volders M, Zeiger E, Ban S, Barale R, Bigatti MP, Bolognesi C, Cebulska-Wasilewska A, Fabianova E, Fucic A, Hagmar L, Joksic G, Martelli A, Migliore L, Mirkova E, Scarfi M.R, Zijno A, Norppa H, Fenech M.2007. An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. Carcinogenesis**28**, 625–631.
http://dx.doi.org/10.1093/carcin/bgl177

Brausch KA, AndersonTA, Smith PN, Maul JD. 2010. Effects of functionalized fullerenes on bifenthrin and tribufos toxicity to *Daphnia magna*: survival, reproduction, and growth rate. Environmental Toxicology Chemistry **29,** 2600–6. http://dx.doi.org/10.1002/etc.318

Brunet L, Lyon DY, Hotze EM, Alvarez PJ, Wiesner MR.2009. Comparative photoactivity and antibacterial properties of C60 fullerenes and titanium dioxide nanoparticles. Environmental Science and Technology **43**, 4355–60. http://dx.doi.org/10.1021/es803093t.

Butchiram MS, Tilak KS, Raju PW. 2009. Studies on histopathological changes in the gill, liver and kidney of *Channa punctatus* (Bloch) exposed to Alachlor. Journal of Environmental Biology **30,** 303-306.

Canistro D, Melega S, Ranieri D, Sapone A, Gustavino B, Monfrinotti M, Rizzoni M, Paolini M. 2012. Modulation of cytochrome P450 and induction of DNA damage in *Cyprinus carpio* exposed in situ to surface water treated with chlorine or alternative disinfectants in different seasons. Mutation Research **3729**, 81-90. http://dx.doi.org/10.1016/j.mrfmmm.2011.09.008.

Cengiz EI. 2006. Gill and kidney histopathology in the freshwater fish *Cyprinus carpio* after acute exposure to deltamethrin. Environmental Toxicology and Pharmacology **22**, 200-204. http://dx.doi.org/10.1016/j.etap.2006.03.006

Cioffi N, Ditaranto N, Torsi L, Picca RA, Sabbatini L, Valentini A, Novello L, Tantillo G, Bleve-Zacheo T, Zambonin PG.2005. Analytical characterization of bioactive fluoropolymer ultra-thin coatings modified by copper nanoparticles. Analytical and Bioanalytical Chemistry**381**, 607-16.

Coulibaly S, Atsé BC, Kouamélan EP.2012. Histological alterations of gill, liver and kidney of black-chinned tilapia *Sarotherodon melanotheron* contaminated by heavy metals from Bietri Bay in Ebrie Lagoon, Cote d'Ivoire. International Journal of Science and Research**3**,1970-1975.

Das BK, Mukherjee SC. 2000. A histopathological study of carp (*Labeo rohita*) exposed to hexachlorocyclohexane. Veterinarski Arhiv**70**, 169-180.

Doak SH, Manshian B, Jenkins GJS, Singh N.2012. In vitro genotoxicity testing strategy for nanomaterials and the adaptation of current OECD guidelines. Mutation Research **745**, 104-111. http://dx.doi.org/10.1016/j.mrgentox.2011.09.013

Gill TS, Pant SC, Tewari H.1989. Cadmium nephropathy in a freshwater fish *Puntius conchonius* Hamilton. Ecotoxicology and Environmental Safety **18**, 165-172. http://dx.doi.org/10.3923/jfas.2013.553.580

Javahery S, Nekoubin H, MoradluAH.2012. Effect of anaesthesia with clove oil in fish (review). Fish Physiology and Biochemistry **38** 1545-1552. http://dx.doi.org/10.1007/s10695-012-9682-5

Johnston HJ, Semmler-Behnke M, Brown DM, Kreyling W, Tran L, Stone V.2009. Evaluating the uptake and intracellular fate of polystyrene nanoparticles by primary and hepatocyte cell lines in vitro. Toxicolology and Applied Pahrmacology **242**, 66-70. http://dx.doi.org/10.1016/j.taap.2009.09.015

Jorge RE, Robinson RG, Moser D, Tateno A, Crespo-Facorro B, Arndt S. 2014. Major depression following traumatic brain injury. Archives of General Psychiatry Journal **61**, 42–50.

Klien K, Godnic-Cvar J. 2012. Genotoxicity of metal nanoparticles: focus on *in vivo* studies. Archives of Industrial Hygiene and Toxicology **63,** 133-145. http://dx.doi.org/10.1371/journal.pone.0111960

Love SA, Maurer-Jones MA, Thompson JW, Lin YS, Haynes CL. 2012. Assessing nanoparticle toxicity. Annual Review of Analytical Chemistry **5**, 181-205. http://dx.doi.org/10.1146/annurev-anchem-062011-143134

Midander K, Cronholm P, Karlsson HL, Elihn K, Möller L, Leygraf C, Wallinder IO.2009. Surface characteristics, copper release, and toxicity of nano- and micrometer-sized copper and copper (II) oxide particles: a cross-discipinary study", Small **5**, 389-399.
http://dx.doi.org/10.1002/small.200801220

Noureen A, Jabeen F.2015. The toxicity, ways of exposure and effects of Cu nanoparticles and Cu bulk salts on different organisms. International Journal of Biosciences **6**, 147-156.
http://dx.doi.org/10.12692/ijb/6.2.147-156

Prabhu BM, Ali SF, Murdock RC, Hussain SM, Srivatsan M.2009. Copper nanoparticles exert size and concentration dependent toxicity on somato sensory neurons of rat. Nanotoxicology **4**, 150-160.

Recio L, Hobbs C, CasparyW, Witt KL. 2010. Dose-response assessment of four genotoxic chemicals in a combined mouse and rat micronucleus and comet assay protocol. The Journal of Toxicological Sciences **35**, 149-162. PMCID: PMC3520611.

Rocco L, Peluso C, Stingo V. 2016. Micronucleus test and comet assay for the evaluation of Zebra fish genomic damage induced by erythromycin and lincomycin. Environmental Toxicology**4**, 598-604.
http://dx.doi.org/10.1002/tox.20685

Rocha C, Cavalcanti B, Pessoa CÓ, Cunha L, Pinheiro RH, Bahia M, Ribeiro H, Cestari M, Burbano R. 2011. Comet assay and micronucleus test in circulating erythrocytes of Aequidens tetramerus exposed to methylmercury. *In Vivo* **25**, 929-933. PMID: 22021686

Sayes CM, Gobin AM, Ausman KD, Mendez J, West JL, Colvin VL. 2005. Nano-C60 cytotoxicityis due to lipid peroxidation. Biomaterials **26**, 7587-7595. PMID:16005959

Shinohara N, Matsumoto T, Gamo M, Miyauchi A, Endo S, Yonezawa Y.2009. Is lipid peroxidation induced by the aqueous suspension of fullerene C60 nanoparticles in the brains of *Cyprinus carpio*. Environmental Science & Technology**43,** 948-953.

Tao X, Fortner JD, Zhang B, He Y, Chen Y, Huges JB. 2009. Effect of aqueous stablefullerene nancrystals on *Daphnia magna*; evaluation of sublethal reproductive responses and accumulation. Chemosphere **77**, 1482-1487.
http://dx.doi.org/10.1016/j.chemosphere.2009.10.027

Tilak KS, Rao DK, Veeraiah K.2005a. Effects of chloropyrifos on histopathology of the fish *Catla catla*. Journal of Ecotoxicology and Environmental Monitoring **15**, 127-140.

Tilak KS, Veeraiah K, Yacobu K.2001. Studies on histopathological changes in the gill, liver and kidney of *Ctenopharyngodon idellus* (Valenciennes) exposed to technical fenvalerate and EC 20%. Environmental Science and Pollution Research **20,** 387-393.

Tilak KS, Veeraiah K, Rao DK. 2005b. Histopathological changes in the gill, liver, brain and kidney of the Indian major carp *Cirrhinus mrigala* (Hamilton) exposed to chloropyrifos. Environmental Science and Pollution Research **24,** 101-111.

Velmurugan B, Selvanayagam M, Cengiz EI, Unlu E.2007. The effects of monocrtophos to different tissues of freshwater fish *Cirrhinus mrigala*. Bulletin of Environmental Contamination and Toxicology **78**, 450-454.

Wang T, Long X, Cheng Y, Liu Z, Jiangsu SY.2014a. The potential toxicity of copper nanoparticles and copper sulphate onjuvenile *Epinephelus coioides*. Aquatic Toxicology **152**, 96–104.

Wang T, Long X, Cheng Y, Liu Z, Yan S.2014b. The potential toxicity of copper nanoparticles and copper sulphate on juvenile *Epinephelus coioides*. Aquatic Toxicology **152**, 96-104.

Ward JE, Kach DJ.2009. Marine aggregates facilitate ingestion of nanoparticles by suspention feedind bivalves. Marine Environmental Research**68**, 137- 142.
http://dx.doi.org/10.1016/j.marenvres.2009.05.002

Witasp E, Shvedova AA, Kagan VE, Fadeel B.2009. Single-walled carbon nanotubes impair human macrophage engulfment of apoptotic cell corpses. Inhalation Toxicology **21**, 131-136.

Yousefian M, Payam B.2012. Effects of nanochemical particles on some histological parameters of fish (Rewiev). Advances in Environmental Biology **6**, 1209-1215.