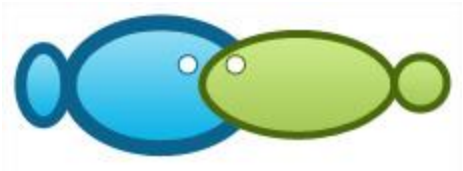

CuO nanoparticles-induced micronuclei and DNA damage in *Cyprinus carpio*

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Abstract. Due to the wide production and application of copper nanoparticles and a subsequent increase in the possibility of their penetrance into the aquatic ecosystems, a growing concern has been emerged about the potential effects of these materials on the health of aquatic organisms. The goal of the present study was to evaluate the genotoxic potential of copper oxide nanoparticles (CuO-NPs) in common carp (*Cyprinus carpio*). For this, the fish were exposed to 0 (control), 2.5, 6.25 and 10 mgL^{-1} of CuO-NPs for 14 days. To evaluate DNA damage through single cell gel electrophoresis, sampling was performed from the tissues of gill and hepatopancreas on the 7^{th} and 14^{th} days of exposure. The blood sampling was also done to determine micronuclei frequency in the erythrocytes. According to the results, CuO-NPs exposure induced micronuclei formation and DNA damage in *C. carpio*, so that the frequency of micronuclei and values of DNA damage parameters including Tail Length, Tail Moment, Tail DNA%, and genetic damage index (GDI) significantly increased in CuO-NPs-exposed treatments as compared to the control ($P \leq 0.05$). The rate of induced damage was dependent on the exposure time and concentration. The hepatopancreas tissue also showed more susceptibility to CuO-NPs genotoxicity, so that the damage level observed in hepatopancreas was higher than that in the gill. Overall, according to the present findings, CuO-NPs could be considered as a potential genotoxic pollutant for *C. carpio*.

Key Words: comet assay, common carp, CuO-NPs, genotoxicity, micronucleus test.

Introduction. Nowadays, metal nanoparticles (NPs) have attracted a lot of attention due to the unique traits such as high surface area and reactivity, so that the different forms of nanoparticles are being applied in various fields of science and industry including the pharmacy and medicine (Liu et al 2008), cosmetics and even environment (Mehndiratta et al 2013). Amongst the divers NPs, copper-based nanoparticles have been widely synthesized due to the high physicochemical stability, easy availability as well as low cost (Mallik et al 2001; Umer et al 2012) and applied in various fields such as electronics, optics, medicine as well as making lubricants, nanofluids, conductive films and antimicrobial agents (Yu et al 1997; Jana et al 2001; Patel et al 2005). However, the growing production and application of NPs increase the probability of human and environmental exposure to these materials. Since, like most of the industrial wastes and products, nanoscale products and by-products tend to end up in aquatic environment (Daughton 2004), a growing concern has emerged regarding the potential consequences of NPs for environmental health. The entry of nanoparticles into the aquatic environments may have various consequences which currently cannot be predicted due to the lack of sufficient information. It may seem that the tendency to accumulation and low solubility of NPs can limit the accessibility of living organisms to these substances, but some laboratory studies have indicated the high accumulation of NPs, especially metal oxide NPs, in aquatic organisms. This is especially important for copper oxide nanoparticles (CuO-NPs), which have relatively high stability in aquatic environments (Kukla et al 2017). Some studies have shown that the fish are susceptible to copper nanoparticles. In this regard, Bai et al (2010) reported that CuO-NPs exposure induced morphological changes and even mortality in the zebrafish (*Danio rerio*)

embryo at the gastrula stage. A recent study by Wang et al (2016) also revealed significant increase in reactive oxygen species (ROS) formation and malondialdehyde level in the primary cell culture of *Epinephelus coioides* under exposure to copper nanoparticles.

Different methods and markers are being used to study the effects of environmental pollutants on aquatic organisms. In this regard, the study of DNA changes in aquatic organisms is an effective method for evaluating the genotoxic effects of contaminants (Scalon et al 2010). Single cell gel electrophoresis (SCGE), or comet assay, is considered as a proper method with high sensitivity, speed and simplicity to assess the induced DNA damage (Dhawan et al 2009). In fact, the comet assay is a reliable method which can even detect low levels of DNA damage (Ullah et al 2016). In this method, the identification of DNA damage is based on the migration of damaged cells from the cell nucleus (Mizuno et al 2015). Today, the comet technique which was used specifically to evaluate DNA damage in mammalian cells, are being widely applied in identifying the genotoxic damages in different types of cell lines and organisms including fish (Rajaguru et al 2001; Lah et al 2008; Fabender & Braunbeck 2013). Micronuclei evaluation is also considered as one of the methods for identifying cytogenetic damages. Micronuclei are chromosomal fragments which are not transmitted to the daughter cells during the anaphase stage of cell division and remained in the mother's cytoplasm (Siu et al 2004) Evaluation of these micronuclei is an effective method for determining the genotoxic effects of compounds with low level of accumulation as well as an indicator of genetic damages induced to the cells (Gravato & Santos 2003). Micronucleus test has been widely used in fish erythrocytes, especially in *Cyprinus carpio* (Al-Sabti 1986; Nepomuceno et al 1997; Gustavino et al 2001; Buschini et al 2004). In the present study, due to the wide production and application of copper nanoparticles in various industries, as well as the lack of sufficient information on the genetic effects of these substances on aquatic organisms, the genotoxic potential of CuO-NPs were evaluated in *C. carpio* using comet assay and micronucleus test.

Material and Method

Preparation and maintenance of fish. A total of 180 *C. carpio* with the average weight of 20±4 g were prepared from Sijeval Bony Fishes Breeding Center (Golestan, Iran) and transported to the laboratory of aquaculture at the Environment and Fisheries Faculty, Gorgan University of Agricultural Sciences and Natural Resources, Iran. The fish were kept in 400-L tanks with mild aeration for 2 weeks to adapt with the laboratory conditions. During this period, the fish were fed daily on commercial feed. Water exchange was also done daily. The physicochemical parameters of water during maintenance period were as follows: temperature: 23±1C^0, pH: 7.38±0.29, dissolved oxygen 7.8±0.34 mg L^{-1}, and hardness: 253±4/9 mg L^{-1}.

Copper oxide nanoparticles (CuO-NPs). Copper oxide nanoparticles (CuO, 99+%, 40 nm, Copper Oxide Nanopowder, US Research, Inc, Nanomaterials) were prepared from Iranian Nanomaterials Pioneers Company. Nanoparticles were homogenized in distilled water (200 mg L^{-1}) using an ultrasound device (Parsonic 7500s, Iran) (20 min, 400 rpm). Since nanoparticles precipitate in aqueous solutions after 24 to 48 hours, daily resuspension was done using a homogenizer (IKA T25 Digital Ultra –Turrax, USA) (15 min, 14,000 rpm). Zeta potential, size and distribution of the particles were determined using Dynamic Light Scattering (ZEN 3600, Germany).

Experimental design. After adaptation period, fish were randomly divided into four groups: one group was considered as the control (without CuO-NPs) and three groups were exposed to 2.5, 6.25 and 10 mg L^{-1} of CuO-NPs. These sub-lethal concentrations were chosen according to a study by Hedayati et al (2015). Three replications were considered for each treatment as well as the control (7 samples for each replication). The exposure

duration was 14 days and the sampling was performed on 7^{th} and 14^{th} days of exposure. For sampling, fish were anesthetized using cloves powder (200 mg L^{-1}).

Single cell gel electrophoresis. To perform single cell gel electrophoresis, comet assay, the PBS buffer as the homogenizer (pH 7.4) containing 0.8 g sodium chloride, 0.02 g potassium chloride, 0.144 g disodium hydrogen phosphate and 0.024 g dihydrogen potassium phosphate, neutral buffer (pH 7.5) containing 4.8 g tris and electrolyte buffer for electrophoresis containing 12 g sodium hydroxide and 0.372 g EDTA were prepared. A lysis buffer (pH 10) with a mixture of 14.1 g sodium chloride, 3.722 g EDTA, 0.12 g tris and 0.8 g sodium hydroxide, was also prepared freshly for each sampling time.

Comet assay was carried out according to the protocol of Singh et al (1988) and Tice et al (2000) with slight modification. Briefly, after separating a part of the gill and hepatopancreas tissues of each sample was washed with phosphate-buffered saline (PBS), a cell suspension was prepared using PBS through scissoring out. After adding Thripsin-EDTA (0.25%) and mixing the contents, FBS was added to prevent the enzymatic digestion. Then, to obtain single cells, centrifuge at 4^0C was done for 10 min at 1,170 rpm. 100 µL of homogenizer buffer was added to the obtained cell precipitation and centrifuge was carried out again (1,130 rpm, 7 min, 4^0C). After adding 10 µL of homogenizer and 100 µL of melted agarose (low melting point agarose) at 37-40^0C, the contents were loaded on the slides coated with normal agarose (1.2%) and placed at the temperature of 4^0C for 1 hour. After treating the slides with lysis buffer (1 hour at 4^0C) and washing with distilled water, electrophoresis was carried out (21 V, 20 min). The slides were treated with neutral buffer for 2 minutes and washed with distilled water slowly. Finally, the slides were stained using Sybr Green and 100 cells were investigated for each sample. The genetic damage index (GDI) was calculated according the formula:

$$GDI=TypeI+2(TypeII)+3(TypeIII)+4(TypeIV)/Type0+TypeI+TypeII+TypeIII+TypeIV$$

Where: Type 0, I, II, III and IV refers to the types without damage and tail, tail length up to 1.5, 1.5-2, 2-2.5 times the diameter of the nucleus and the maximum damage, respectively. The DNA damage parameters including Tail length (TL), Tail Moment (TM), and Tail DNA% (TDNA%) were also analyzed using CASP software version 1.2.2 (Konca et al 2003).

Micronucleus test. Micronucleus test was performed according to the method of Grisolia & Cordeiro (2000) with slight modification. Briefly, blood sampling was done from caudal vein with a heparin syringe and immediately the blood smear was prepared on clean glass slides. The slides were then air dried overnight and fixed with absolute methanol for 15 minutes. The slides were stained using Giemsa (5%) for 20 minutes. Finally, the micronucleus frequency per 1,000 erythrocytes was determined for each sample by a microscope (F-TS100 Eclipse, Nikon, Japan).

Statistical analysis. The data obtained from the comet assay and micronucleus test were analyzed through one-way ANOVA using SPSS 19. The Tukey test was also used to compare mean values ($P \leq 0.05$).

Results

Copper oxide nanoparticles. Zeta potential, size and distribution of copper oxide nanoparticles as well as the SEM image are shown in Figure 1 (a-c). Given the observed range of zeta potential, it could be stated that copper oxide nanoparticles are homogenized and distributed uniformly in the fluid medium. Also, 95% volumetric of particles in the colloidal solution have had a diameter of 85 nm which confirms the nanoscale size of the

particles. According to the results, it could be stated that the copper oxide nanoparticles have not been precipitated at the bottom and they have been uniformly distributed.

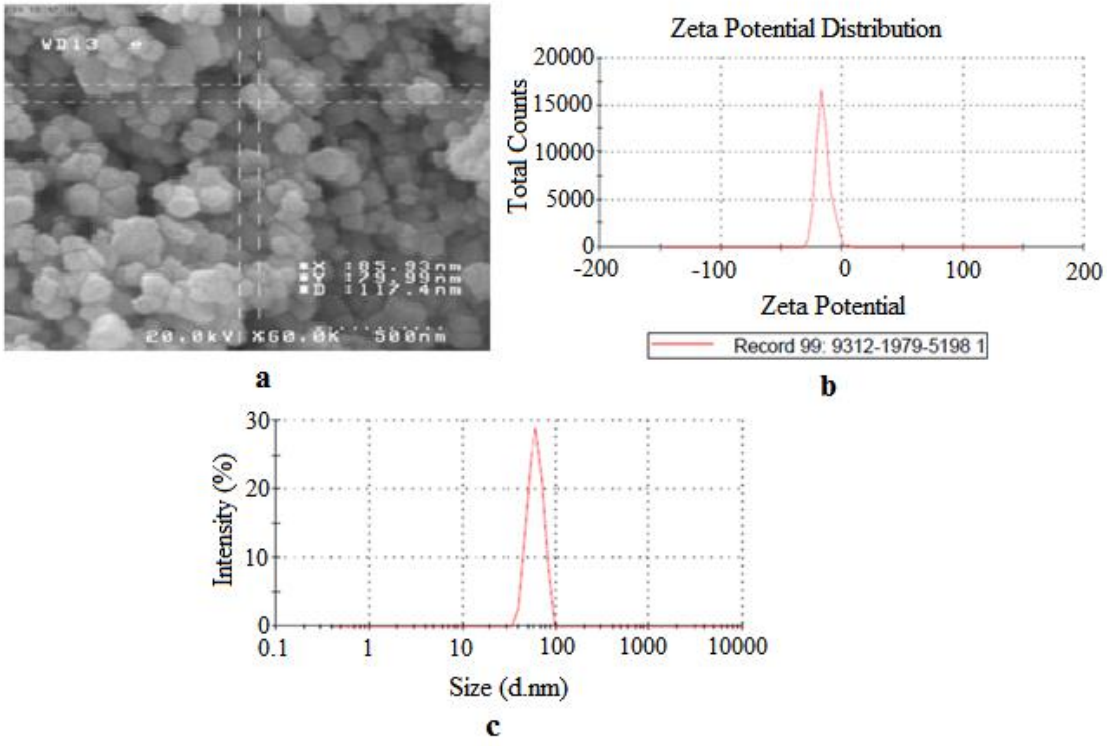

Figure 1. CuO-NPs characteristics applied in the present study; a, b and c represent SEM picture, zeta potential and size distribution of CuO-NPs, respectively.

DNA damage in the gill tissue. The genotoxicity of copper oxide nanoparticles in the tissues of gill and hepatopancreas of *C. carpio* were evaluated using single cell gel electrophoresis. The values of DNA damage parameters including Tail Length, Tail Moment, and Tail DNA% in the cells of gill tissue are presented in Table 1. The comet classes (Type o to 4 of the damage) and genetic damage index (GDI) during different exposure times are also presented in Tables 2 and 3. Our results indicated that CuO-NPs induced DNA damage in the gill cells, so that the values of TL, TM, and TDNA% parameters, as well as the GDI index significantly increased ($P \leq 0.05$) in the treatments exposed to CuO-NPs as compared to the control. An increasing trend in the values of TL and TM parameters was observed with the increase in the CuO-NPs concentration ($P \leq 0.05$). A similar increasing trend was also noticed in the DNA percentage in the comet tail which was significant ($P \leq 0.05$) among all treatments, except the treatments of 6.25 and 10 mg L^{-1} on the day 7. The GDI index also increased by increasing the CuO-NPs concentration from 2.5 to 10 mg L^{-1}, but the change was only significant ($P \leq 0.05$) between the treatments of 2.5 and 10 mg L^{-1} on the 14th day. The highest GDI index was 0.81±0.08 which observed in the treatment exposed to 10 mg L^{-1} of CuO-NPs on the day 14.

In assessing the effect of time on the DNA damage induced in the gill cells, a time dependent increase in the damage parameters was observed, so that the highest level of damage was recorded on the 14th day; TL: 56.93±1.49, TM: 19.23±2.00 and TDNA%: 32.87±2.55. The effect of time on the induced damage was significant ($P<0.05$) in all cases, except the TM and TDNA% parameters in 10 mg L^{-1} and 6.25 mg L^{-1}, respectively. No significant differences in the control treatment were observed over the time ($P>0.05$). Also, the changes observed in genetic damage index from 7th to 14th day were not statistically significant ($P>0.05$).

Table 1

DNA damage parameters in the gill tissue of *Cyprinus carpio* under exposure of CuO-NPs

	Tail length (TL)		*Tail moment (TM)*		*Tail DNA% (TDNA %)*	
	Day 7	Day 14	Day 7	Day 14	Day 7	Day 14
Control	3.86 ± 0.9^{Da}	4.57 ± 0.56^{Da}	0.36 ± 0.10^{Da}	0.42 ± 0.16^{Da}	6.71 ± 1.52^{Ca}	7.09 ± 1.34^{Da}
2.5 mg L^{-1}	14.94 ± 1.23^{Cb}	21.09 ± 0.91^{Ca}	2.64 ± 0.66^{Ca}	5.48 ± 0.46^{Cb}	15.87 ± 2.97^{Ba}	24.38 ± 0.72^{Cb}
6.25 mg L^{-1}	33.65 ± 2.55^{Bb}	48.76 ± 1.51^{Ba}	9.26 ± 1.03^{Ba}	14.91 ± 0.86^{Bb}	25.82 ± 2.16^{Aa}	29.48 ± 0.95^{Ba}
10 mg L^{-1}	43.26 ± 1.52^{Ab}	56.93 ± 1.49^{Aa}	12.45 ± 1.16^{Aa}	19.23 ± 2.00^{Aa}	28.93 ± 1.05^{Ab}	32.87 ± 2.55^{Aa}

Data's are presented as Mean±SD. Different upper and lower case letters show significant differences within the column and row, respectively (P≤0.05).

Table 2

Comet classes and genetic damage index in the gill tissue of *Cyprinus carpio* after 7 days exposure of CuO-NPs

	Type 0	*Type 1*	*Type 2*	*Type 3*	*Type 4*	*GDI*
Control	93.48 ± 6.66^{A}	4.39 ± 0.84^{C}	2.13 ± 0.45^{C}	0^{C}	0^{C}	0.08 ± 0.006^{B}
2.5 mg L^{-1}	74.12 ± 2.93^{B}	8.72 ± 1.40^{B}	11.08 ± 2.56^{A}	5.23 ± 1.03^{B}	0.85 ± 0.13^{C}	0.5 ± 0.12^{A}
6.25 mg L^{-1}	68.37 ± 6.12^{B}	10.28 ± 2.11^{B}	11.43 ± 1.03^{A}	7.22 ± 0.81^{A}	2.70 ± 0.69^{B}	0.65 ± 0.25^{A}
10 mg L^{-1}	64.51 ± 3.91^{B}	16.47 ± 1.80^{A}	5.54 ± 1.25^{B}	9.16 ± 1.61^{A}	4.32 ± 0.99^{A}	0.72 ± 0.20^{A}

Data's are presented as Mean±SD. Different upper case letters show significant differences within the column (P≤0.05).

Table 3

Comet classes and genetic damage index in the gill tissue of *Cyprinus carpio* after 14 days exposure of CuO-NPs

	Type 0	*Type 1*	*Type 2*	*Type 3*	*Type 4*	*GDI*
Control	92.57 ± 2.37^{A}	4.42 ± 0.90^{C}	2.61 ± 0.62^{B}	0.4 ± 0.12^{C}	0^{B}	0.11 ± 0.02^{C}
2.5 mg L^{-1}	72.48 ± 2.04^{B}	9.26 ± 1.10^{B}	14.43 ± 1.62^{A}	2.83 ± 0.98^{B}	0.92 ± 0.26^{B}	0.51 ± 0.16^{B}
6.25 mg L^{-1}	63.17 ± 1.94^{C}	14.76 ± 3.86^{C}	10.48 ± 2.57^{A}	6.97 ± 1.93^{A}	4.62 ± 1.03^{A}	0.75 ± 0.21^{AB}
10 mg L^{-1}	62.05 ± 4.58^{C}	13.41 ± 2.07^{BC}	11.73 ± 3.23^{A}	7.57 ± 1.27^{A}	5.24 ± 1.95^{A}	0.81 ± 0.08^{A}

Data's are presented as Mean±SD. Different upper case letters show significant differences within the column (P≤0.05).

DNA damage in the hepatopacreas tissue. The changes in DNA damage parameters in the cells of hepatopancreatic tissue under exposure to CuO-NPs are shown in Table 4. Tables 5 and 6 also represent the comet classes and GDI changes in the hepatopancreas tissue. According to the results, CuO-NPs had genotoxic effects on the cells of hepatopancreatic tissue. Compared to the gill tissue, the hepatopancreas showed more susceptibility to the genotoxicity of CuO-NPs, so that the level of damage observed in the hepatopancreas was higher than that in the gill. Also, the DNA damage induced to the hepatopancreous cells was completely dependent on the exposure time and concentration. In this regard, the observed changes trend was similar to that in the gills. The highest values of damage parameters in hepatopancreas cells were noticed in the treatment exposed to 10 mg L^{-1} CuO-NPs on the 14^{th} day; TL: 72.43±1.47, TM: 28.67±2.02 and TDNA%: 41.73±2.22. The highest genetic damage index was obtained to be 1.13±0.45 which was observed in the treatment 10 mg L^{-1} on the day 14. In comparing the different exposure concentrations, the observed differences in terms of DNA damage parameters were statistically significant ($P<0.05$) among the different treatments, except TDNA% between the 6.25 and 10 mg L^{-1} treatments on the 7^{th} day. The concentration dependent increase in GDI was only significant among the treatments of 2.5 and 10 mg L^{-1} ($P\leq0.05$). The observed increase in GDI index over the time was not statistically significant ($P>0.05$).

Micronucleus test. Exposure to CuO-NPs led to the micronuclei induction in the erythrocytes of *C. carpio*. The changes in the micronuclei frequency at different times and concentrations are presented in Table 7. According to the results, the micronuclei frequency in all treatments exposed to CuO-NPs increased significantly compared to the control ($P\leq0.05$). The micronuclei formation were also dependent on the exposure concentration, so that the highest level of micronuclei frequency was noticed in the treatment exposed to 10 mg L^{-1} of CuO-NPs; 3.74±0.13 and 4.61±1.23 on the exposure days of 7 and 14, respectively. The concentration dependent changes were only significant among the treatments of 2.5 and 6.25 mg L^{-1} on the 7^{th} day as well as 2.5 and 10 mg L^{-1} on the 7^{th} and 14^{th} days ($P\leq0.05$). Also, the observed increase in micronuclei frequency over the time was not statistically significant ($P>0.05$).

Table 4

DNA damage parameters in the hepatopancreas tissue of *Cyprinus carpio* under exposure of CuO-NPs

	Tail length (TL)		*Tail moment (TM)*		*Tail DNA% (TDNA %)*	
	Day 7	Day 14	Day 7	Day 14	Day 7	Day 14
Control	4.73 ± 0.95^{Da}	5.16 ± 0.77^{Da}	0.51 ± 0.16^{Da}	0.56 ± 0.06^{Da}	8.28 ± 0.94^{Ca}	8.79 ± 0.51^{Da}
2.5 mg L^{-1}	19.47 ± 1.07^{Cb}	30.52 ± 1.20^{Ca}	4.06 ± 0.62^{Cb}	8.37 ± 1.16^{Ca}	19.25 ± 2.26^{Ba}	25.37 ± 2.19^{Cb}
6.25 mg L^{-1}	42.96 ± 2.00^{Ba}	61.08 ± 1.60^{Bb}	11.45 ± 1.40^{Bb}	21.83 ± 1.84^{Ba}	27.26 ± 3.51^{Aa}	35.62 ± 1.85^{Ba}
10 mg L^{-1}	58.74 ± 1.23^{Ab}	72.43 ± 1.47^{Aa}	19.34 ± 1.08^{Ab}	28.67 ± 2.02^{Aa}	32.01 ± 3.22^{Aa}	41.73 ± 2.22^{Aa}

Data's are presented as Mean±SD. Different upper and lower case letters show significant differences within the column and row, respectively (P≤0.05).

Table 5

Comet classes and genetic damage index in the hepatopancreas tissue of *Cyprinus carpio* after 7 days exposure of CuO-NPs

	Type 0	*Type 1*	*Type 2*	*Type 3*	*Type 4*	*GDI*
Control	89.41 ± 2.30^{A}	8.23 ± 0.84^{C}	2.36 ± 1.02^{C}	0^{C}	0^{C}	0.13 ± 0.02^{C}
2.5 mg L^{-1}	73.26 ± 3.34^{B}	9.83 ± 2.25^{BC}	10.28 ± 1.93^{B}	5.42 ± 1.50^{B}	1.56 ± 0.57^{B}	0.51 ± 0.16^{B}
6.25 mg L^{-1}	63.86 ± 1.88^{C}	15.74 ± 2.27^{A}	12.06 ± 1.11^{B}	3.70 ± 0.94^{B}	4.65 ± 1.08^{A}	0.69 ± 0.09^{AB}
10 mg L^{-1}	58.09 ± 1.46^{D}	12.64 ± 1.25^{AB}	16.73 ± 3.76^{A}	8.27 ± 1.21^{A}	3.19 ± 1.01^{A}	0.84 ± 0.11^{A}

Data's are presented as Mean±SD. Different upper case letters show significant differences within the column (P≤0.05).

Table 6

Comet classes and genetic damage index in the hepatopancreas tissue of *Cyprinus carpio* after 14 days exposure of CuO-NPs

	Type 0	*Type 1*	*Type 2*	*Type 3*	*Type 4*	*GDI*
Control	91.16 ± 1.42^{A}	5.76 ± 0.98^{C}	2.45 ± 0.71^{C}	0.72 ± 0.11^{B}	0^{B}	0.13 ± 0.04^{Ca}
2.5 mg L^{-1}	70.68 ± 2.70^{B}	11.24 ± 2.54^{B}	7.41 ± 1.37^{B}	9.07 ± 1.75^{A}	1.91 ± 0.71^{B}	0.60 ± 0.20^{Ba}
6.25 mg L^{-1}	57.43 ± 3.44^{C}	14.73 ± 1.08^{A}	9.91 ± 1.68^{B}	10.98 ± 2.52^{A}	6.44 ± 2.83^{A}	0.93 ± 0.31^{ABa}
10 mg L^{-1}	53.28 ± 2.53^{C}	10.48 ± 1.25^{B}	14.15 ± 2.49^{A}	12.47 ± 2.46^{A}	9.26 ± 2.08^{A}	1.13 ± 0.45^{Aa}

Data's are presented as Mean±SD. Different upper case letters show significant differences within the column (P≤0.05).

Table 7

Micronuclei frequency in the erythrocytes of *Cyprinus carpio* under CuO-NPs exposure

	Micronucleus frequency	
	Day 7	Day 14
Control	0.24 ± 0.06^{Ca}	0.18 ± 0.03^{Ca}
2.5 mg L^{-1}	1.63 ± 0.54^{Ba}	2.08 ± 0.99^{Ba}
6.25 mg L^{-1}	2.91 ± 0.97^{Aa}	3.65 ± 1.16^{ABa}
10 mg L^{-1}	3.74 ± 0.13^{Aa}	4.61 ± 1.23^{Aa}

Data's are presented as Mean±SD. Different upper and lower case letters show significant differences within the column and row, respectively (P≤0.05).

Discussion. In the present study, the results from comet assay showed that exposure to CuO-NPs led to the DNA damage induction in common carp, so that the values of TL, TM, TDNA% parameters as well as the GDI index significantly increased in the treatments exposed to CuO-NPs when compared to the control ($P \leq 0.05$). The hepatopancreas tissue also showed more sensitivity to the toxicity of CuO-NPs as compared to the gill tissue. It was found that different tissues have different susceptibility to genotoxic compounds, which may be due to the differential mechanisms of defense and DNA repair. Additionally, the observed difference among the tissues may be associated with the cellular differences including membrane composition, the number of lysosomes and mitochondria due to their origin and function (Chelomin et al 2017).

The data obtained for the DNA damage of *C. carpio* in this work are in consistent with those reported in previous studies on other organisms, including *Escherichia coli* (Entrobacteriaceae) (Bondarenko et al 2012), *Oncorhynchus mykiss* (Isani et al 2013), *Mytilus trossulus* (Mytilidae) (Chelomin et al 2017) and *M. galloprovincialis* (Mytilidae) (Gomes et al 2013). While there is not enough information about the underlying mechanisms for CuO-NPS genotoxicity in aquatic organisms, several potential models have recognized for mammalians. According to a study by Chelomin et al (2017), the possible genotoxicity mechanisms of copper oxide nanoparticles can be considered as direct (direct effect on the DNA structure or its repair system) and indirect (intermediates induction) mechanisms. Previous studies have shown that due to the small size, metal nanoparticles can reach to the cell nucleus, react with it (Shukla et al 2010; Chen & Mikecz 2005) and induce physical and chemical damages through direct contact with genetic materials (Chelomin et al 2017). In fact, like other metal nanoparticles, copper oxide nanoparticles may directly react with DNA or even with the enzymes of DNA repair system and induce DNA damage (Garnett & Kallinteri 2006). Unfortunately, enough information is not available about the CuO-NPs- induced inactivation of the DNA repair system in aquatic organisms. However, this hypothesis could not be ignored since previous studies on mammalian models have shown the potential of nanoparticles in translocating to the nucleus. In this regard, in the study by Semisch et al (2014) on human cell line of A549 exposed to copper oxide nanoparticles, high accumulation of copper was observed in the nucleus. However, direct effect of copper nanoparticles on DNA repair system could only be considered as a hypothesis and more investigations are needed in this area. In addition to the direct mechanism, copper nanoparticles may cause DNA damage through the induction of intermediates and oxidative stress. Previous studies on human cells have suggested that the induced DNA damage by nanoparticles is mainly resulted from the DNA oxidative damage rather than direct reaction with DNA (Karlsson et al 2008; Midander et al 2009). In fact, based on the available information, oxidative stress could be considered as the main factor in induction of genetic toxicity by nanoparticles (Song et al 2012; Baker et al 2014). In this regard, Ahamed et al (2010) reported DNA damage induced by oxidative stress in A549 cell line exposed to CuO-NPs. Oxidative stress was also suggested as an important factor in inducing DNA damage in the haematocytes of *M. galloprovincialis* (Gomes et al 2013). Under oxidative stress, nanoparticles have a potential to produce ROS which can induce damage to the bimolecules (Chelomin et al 2017). It is known that ROS is capable to react with the DNA molecule and damage to the organic bases of purine and pyrimidine and DNA structure (Martinez et al 2003). Therefore, oxidative stress could be considered as an important factor in DNA damage induction. However, considering the lack of enough information, further studies seem to be important to determine the exact mechanisms involved in CuO-NPs-induced DNA damage in aquatic organisms, especially fish.

In the present study, micronucleus test was also used to evaluate the genotoxic potential of CuO-NPs. Using comet assay, DNA damage could be identified in a short time after exposing to the genotoxic agent while identifying the micronuclei resulted from chromosomal fragmentation or mitotic abnormalities needs the passage through mitosis (Buschini et al 2004). The micronucleus test in fish is mainly carried out using the

erythrocytes (Al-Sabti & Metcalfe 1995; Bony et al 2010; Bottcher et al 2010). The present results indicated that exposure to CuO-NPs resulted in inducing micronuclei formation in erythrocytes of *C. carpio*. The changes observed in micronuclei frequency were not significant over the time ($P>0.05$). Also, despite the observed differences, the micronuclei frequency in the CuO-NPs-exposed treatments was relatively low which was in agreement with the previous findings by Ferraro et al (2004), Bolognesi et al (2006); Andreikenaite et al (2007); Grisolia et al (2009) and Rocha et al (2011). It is shown that the mitotic rate is low in fish erythrocytes (Grisolia & Corderio 2000). In fact, the rate of micronuclei formation and their basal frequency is basically low in fish (Bolognesi et al 2006; Ferraro et al 2004). However, despite the low level of micronuclei formation, their frequency increased with the increase in CuO-NPs concentration that suggests genotoxic effects of CuO-NPs on *C. carpio*. In fact, the micronuclei formation could be considered as a sign of genomic damage induction at the chromosome level or DNA damage (Saleh & Sarhan 2007).

Conclusions. The present findings indicated that exposure to copper oxide nanoparticles induced DNA damage and micronuclei formation in *C. carpio*. Although the level of damage detected by comet assay was more evident compared to the micronucleus test, the results from both methods were in agreement with each other. The level of genetic damage observed in *C. carpio* was dependent on the exposure time and concentration of CuO-NPs. However, based on our results, copper oxide nanoparticles could be considered as a potential genotoxic pollutant for *C. carpio*. In this regard, considering the lack of sufficient information, further research is recommended to identify the exact mechanisms of CuO-NPs genotoxicity in aquatic animals.

References

Ahamed M., Siddiqui M. A., Akhtar M. J., Ahmad I., Pant A. B., Alhadlaq H. A., 2010 Genotoxic potential of copper oxide nanoparticles in human lung epithelial cells. Biochemical and Biophysical Research Communications 396:578-583.

Al-Sabti K., 1986 Comparative micronucleated erythrocyte cell induction in three cyprinids by five carcinogenic-mutagenic chemicals. Cytobios 47:147-154.

Al-Sabti K., Metcalfe C. D., 1995 Fish micronuclei for assessing genotoxicity in water. Mutation Research 343(23):121-135.

Andreikenaite L., Barsiene J., Vosyliene M. Z., 2007 Studies of micronuclei and other nuclear abnormalities in blood of rainbow trout (*Oncorhyncus mykiss*) treated with heavy metal mixture and road maintenance salts. Acta Zoologica Lituanica 17(3):213-219.

Bai W., Tian W., Zhang Z., Chai Z., 2010 Effects of copper nanoparticles on the development of zebrafish embryos. Journal of Nanoscience and Nanotechnology 10(12):8670-6.

Baker T. J., Tyler C. R., Galloway T. S., 2014 Impacts of metal and metal oxide nanoparticles on marine organisms. Environmental Pollution 186:257-271.

Bolognesi C., Perrone E., Roggieri P., Sciutto A., 2006 Bioindicators in monitoring long term genotoxic impact of oil spill: Haven case study. Marine Environmental Research 62:287-291.

Bondarenko O., Ivask A., Kakinen A., Kahru A., 2012 Sub-toxic effects of CuO-nanoparticles on bacteria: Kinetics, role of Cu ions and possible mechanisms of action. Environmental Pollution 169:81-89.

Bony S., Gaillard I., Devaux A., 2010 Genotoxicity assessment of two vineyard pesticides in zebrafish. International Journal of Environmental Analytical Chemistry 90(3-6):421–428.

Bottcher M., Grund S., Keiter S., Kosmehl T., Reifferscheid G., Seitz N., Rocha P. S., Hollert H., Braunbeck T., 2010 Comparison of in vitro and in situ genotoxicity in the Danube

River by means of the comet assay and the micronucleus test. Mutation Research 700:11–17
Buschini A., Martino A., Gustavino B., Monfrinotti M., Poli P., Rossi C., Santoro M., Dorr A. J. M., Rizzoni M., 2004 Comet assay and micronucleus test in circulating erythrocytes of *Cyprinus carpio* specimens exposed in situ to lake waters treated with disinfectants for potabilization. Mutation Research 557:119-129.
Chelomin V. P., Slobodskova V. V., Zakhartsev M., Kukla S., 2017 Genotoxic potential of copper oxide nanoparticles in the bivalve mollusk *Mytilus trossulus.* Journal of Ocean University of China (Oceanic and Coastal Sea Research) 16(2):339-345.
Chen M., Mikecz A., 2005 Formation of nucleoplasmic protein aggregates impairs nuclear function in response to SiO_2 nanoparticles. Experimental Cell Research 305(1):51-62.
Daughton C. G., 2004 Non-regulated water contaminants: emerging research. Environmental Impact Asses Review 24:711-732.
Dhawan A., Bajpayee M., Parmar D., 2009 Comet assay: a reliable tool for the assessment of DNA damage in different models. Cell Biology and Toxicology 25:5-32.
Fabender C., Braunbeck T., 2013 Assessment of genotoxicity in gonads, liver and gills of zebrafish (*Danio rerio*) by use of the comet assay and micronucleus test after in vivo exposure to methyl methanesulfonate. Bulletin of Environmental Contamination and Toxicology 91:89-95
Ferraro M. V. M., Fenocchio S. A., Mantovani M. S., Ribeiro C. O., Cestari M. M., 2004 Mutagenic effects of tributyltin and inorganic lead (Pb II) on the fish *H. malabaricusas* evaluated using the comet assay and the piscine micronucleus and chromosome aberration tests. Genetics and Molecular Biology 27(1):103-107.
Garnett M., Kallinteri P., 2006 Nanomedicines and nanotoxicology: Some physiological principles. Occupational Medicine 56:307-311.
Gomes T., Araujo O., Pereira R., Almeida A. C., Cravo A., Bebianno M. J., 2013 Genotoxicity of copper oxide and silver nanoparticles in the mussel *Mytilus galloprovincialis*. Marine Environmental Research 84:51-59.
Gravato C., Santos M. A., 2003 Genotoxicity biomarkers' association with B(a)P biotransformation in *Dicentrarchus labrax* L. Ecotoxicology and Environmental Safety 55:352-358.
Grisolia C. K., Cordeiro C. M. T., 2000 Variability in micronucleus induction with different mutagens applied to several species of fish. Genetics and Molecular Biology 23:235-239.
Grisolia C. K., Rivero C. L. G., Starling F. L. R. M., Silva I. C. R., Barbosa A. C. B., Dorea J. G., 2009 Profile of micronucleus frequencies and DNA damage in different species of fish in a eutrophic tropical lake. Genetics and Molecular Biology 32(1):138-143.
Gustavino B., Scornajenghi K. A., Minissi S., Ciccotti E., 2001 Micronuclei induced in erythrocytes of *Cyprinus carpio* (Teleostei, Pisces) by X-rays and colchicines. Mutation Research 494:151-159.
Hedayati A., Hoseini S. M., Hoseinifar S. H., 2015 Response of plasma copper, ceruloplasmin, iron and ions in carp, *Cyprinus carpio* to waterborne copper ion and nanoparticle exposure. Comparative Biochemistry and Physiology, Part C 179(2016):87-93.
Isani G., Falcioni M. L., Barucca G., Sekar D., Andreani G., Carpene E., Falcioni G., 2013 Comparative toxicity of CuO nanoparticles and $CuSO_4$ in rainbow trout. Ecotoxicology and Environmental Safety 97:40-46.
Jana N. R., Gearheart L., Murphy C. J., 2001 Evidence for seed-mediated nucleation in the chemical reduction of gold salts to gold nanoparticles. Chemistry of Materials 13(7):2313-22.
Karlsson H., Cronholn P., Gustafsson J., Moller L., 2008 Copper oxide nanoparticles are highly toxic: a comparison between metal oxide nanoparticles and carbon nanotubes. Chemistry Research in Toxicology 21:1726-1732.

Konca K., Lankoff A., Banasik A., Lisowska H., Kuszewski T., Gozdz S., Koza Z., Wojcik A. A., 2003 Cross-platform public domain PC image-analysis program for the comet assay. Mutation Research 534:15-20.
Kukla S. P., Slobodskova V. V., Chelomin V. P., 2017 The Genotoxicity of copper oxide nanoparticles to marine organisms based on the example of the Pacific mussel *Mytilus Trossulus* Gould, 1850 (Bivalvia: Mytilidae). Russian Journal of Marine Biology 43(2):171-175.
Lah B., Vidic T., Glasencnik E., Cepeljnik T., Gorjanc G., Marinsek-Logar R., 2008 Genotoxicity evaluation of water soil leachates by Ames test, comet assay, and preliminary Tradescantia micronucleus assay. Environmental Monitoring and Assessment 139:107-118.
Liu Z., Robinson J. T., Sun X., Dai H., 2008 PEGylated nographene oxide for delivery of water-insoluble cancer drug. Journal of American Chemical Society 130:10876-10877.
Mallik K., Mandal M., Pradhan N., Pal T., 2001 Seed mediated formation of bimetallic nanoparticles by UV irradiation: a photochemical approach for the preparation of "coreshell" type structures. Nano Letters 1(6):319-22.
Martinez G. R., Loureiro A. P., Marques S. A., Miyamoto S., Yamaguchi L. F., Onuki J., Almeida E. A., Garcia C. C., Barbosa L. F., Medeiros M. H., Di Mascio P., 2003 Oxidative and alkylating damage in DNA. Mutation Research 544(2-3):115-127.
Mehndiratta P., Jain A., Srivastava S., Gupta N., 2013 Environmental pollution and nanotechhnology. Environment and Pollution 2(2):49-58.
Midander K., Cronholm P., Karlsson H. L., Elihn K., Moller L., Leygraf C., Wallinder I. O., 2009 Surface characteristics, copper release, and toxicity of nano-and micrometer-sized copper and copper (II) oxide particles: a cross-disciplinary study. Small 5(3):389-399.
Mizuno K., Shinohra N., Miyakoshi J., 2015 *In vitro* evaluation of genotoxic effects under magnetic resonant coupling wireless power transfer. International Journal of Environmental Research and Public Health 12:3853-3863.
Nepomuceno J. C., Ferrari I., Spano M. A., Centeno A. J., 1997 Detection of micronuclei in peripheral erythrocytes of *Cyprinus carpio* exposed to metallic mercury. Environmental and Molecular Mutagenesis 30:293-297.
Patel K., Kapoor S., Dave D., Mukherjee T., 2005 Synthesis of nanosized silver colloids by microwave dielectric heating. Journal of Chemistry Sciences 117(1):53-60.
Rajaguru P., Kalpana R., Hema A., Suba S., Baskarasethupathi B., Kumar P. A., Kalaiselvi K., 2001 Genotoxicity of some sulfur dyes on tadpoles (*Rana hexadactyla*) measured using the comet assay. Environmental and Molecular Mutagenesis 38:316-322.
Rocha C., Cavalcanti B., Pessoa C. O., Cunha L., Pinheiro R. H., 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(6):929-33.
Saleh K., Sarhan M. A. A., 2007 Clastogenic analysis of chicken forms using micronucleus test in peripheral blood. Journal of Applied Sciences Research 3(12):1646-1649.
Scalon M. C. S., Rechenmacher C., Siebel A. M., Kayser M. L., Rodrigues M. T., Maluf S. W., Rodrigues M. A. S., Silva L. B., 2010 Evaluation of Sinos River water genotoxicity using the comet assay in fish. Brazilian Journal of Biology 70(4):1217-1222.
Semisch A., Ohle J., Witt B., Hartwig A., 2014 Cytotoxicity and genotoxicity of nano- and microparticulate copper oxide: Role of solubility and intracellular bioavailability. Particle and Fibre Toxicology 11:10-16.
Shukla R. K., Sharma V., Pandey A. K., Singh S., Sultana S., Dhawan A., 2010 ROS-mediated genotoxicity induced by titanium dioxide nanoparticles in human epidermal cells. Toxicology in Vitro 25(1):231-241.

Singh N. P., McCoy M. T., Tice R. R., Schneider E. L., 1988 A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental Cell Research 175(1):184-191.
Siu W. H. L., Caob J., Jack R. W., Wu R. S. S., Richardson B. J., Xu L., Lam P. K. S., 2004 Application of the comet and micronucleus assays to the detection of B[*a*]P genotoxicity in haemocytes of the green-lipped mussel (*Perna viridis*). Aquatic Toxicology 66:381-392.
Song M. F., Li Y. S. Kasai H., Kawai K., 2012 Metal nanoparticle-induced micronuclei and oxidative DNA damage in mice. Journal of Clinical Biochemistry and Nutrition 50:211-216.
Tice R. R., Agurell E., Anderson D., Burlinson B., Hartmann A., Kobayashi H., Miyamae Y., Rojas E., Ryu J. C., Sasaki Y. F., 2000 Single cellgel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environmental and Molecular Mutagenesis 35(3):206-221.
Ullah S., Begum M., Ahmad S., Dhama K., 2016 Genotoxic effect of endosulfan at sublethal concentrations in Mori (*Cirrhinus mrigala*) fish using single cell gel electrophoresis (comet assay). International Journal of Pharmacology 12:169-176.
Umer A., Naveed S., Ramzan N., 2012 Selection of suitable method for the synthesis of copper nanoparticles. NANO: Brief Reports and Reviews https://doi.org/10.1142/S1793292012300058.
Wang T., Chen X., Long X., Liu Z., Yan S., 2016 Copper nanoparticles and copper sulphate induced cytotoxicity in hepatocyte primary cultures of *Epinephelus coioides.* PLoS ONE 11(2):e0149484.
Yu Y. Y., Chang S. S., Lee C. L., Wang C. C., 1997 Gold nanorods: electrochemical synthesis and optical properties. Journal of Physical Chemistry B 101(34):6661-6664.

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