**Accumulation of copper and DNA fragmentation in grass carp larvae after the exposure to copper oxide nanoparticles**

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**Abstract**

Little information is available on the bioavailability and genotoxicity of ENPs ingrass carp *Ctenopharyngodon idella*. Seven days old grass carp larvae have been exposed to 1, 5, 10, 50, 100 mgl-1 copper oxide nanoparticles for a week. There was no difference in mortality between treated groups and control. Whole body copper concentrations increased significantly in all exposure groups except in 10 mgl-1 group. The genotoxicity of copper oxide nanoparticles was evaluated using DNA laddering technique. Copper oxide nanoparticles were found to be genotoxic at a low concentrations followed by a decrease in extent of DNA damage at higher concentrations.

**Keywords:** Embryo; copper oxide nanoparticles; *Ctenopharyngodon idella*; Accumulation; DNA fragmentation

**Introduction**

Manufactured nanomaterials (NMs) are utilized as a part of commercial and industrial applications including fillers, bio- sensors, cosmetics, textiles, pharmaceuticals, environmental remediation, drug carriers, microelectronics, and catalysts (Masciangioli and Zhang, 2003; Shvendova and Castranova, 2003; Aitken et al., 2006; Guzmán et al., 2006; Helland et al., 2007). Copper oxide nanoparticles (CuO NPs) are utilized as catalyst and added to different materials to increase the mechanical properties (Dziembaj et al., 2011; Sunkara et al., 2013).

The release of engineered nanoparticles (ENPs) from commercial products raises worries about their environmental destiny and toxicity (Benn and Westerhoff, 2008; Kaegi et al., 2008). In aquatic systems, ENPs complex with organic debris and adhere to organisms living in the water column (Oberdorster et al., 2006).

Cells normallyundergo apoptosis in response to mildly adverse conditions, whilstexposure to severe conditions will result in necrosis. Apoptosis is induced by extracellular or intracellular signals, which trigger onset of signaling cascade with characteristic biochemical and cytological signatures including nuclear condensation and DNA fragmentation (Gopinath et al., 2010). Nanoparticle induced oxidative stress leads to DNA damage and apoptosis (Ahamed et al., 2011a). Studies have shown that exposureto CuO NPs caused DNA damage, oxidative stress, increased **cell** death (Karlsson et al., 2008; Midander et al., 2009) and inhibited growth of organisms (Baek and An 2011; Kasemets et al., 2008). The present study aimed to evaluate the accumulation and apoptotic DNA fragmentation of copper oxide nanoparticles by grass carp larvae.

Grass Carp (*Ctenopharyngodon idella*) is an herbivore fish mainly depends on aquatic vegetation (algae, phytoplankton and other plants) as food source. It was selected because of its continuously feeding habits; consuming large amount of vegetation daily, leading to very high uptake of toxicants and eventually accumulation, makes this fish pollution indicator of toxicants particularly heavy metals and nanoparticles in any freshwater body (Ahmed et al., 2011).

**Materials and methods**

**Materials:**

The Copper (II) oxide nanoparticles (CuO) were purchased from sigma-Aldrich, UK. The physical characteristics of the particles according to the manufactures data were; size (<50nm), purity (99.5%), trace metal basis, surface area (5.0 m2/g), density (10.49g/cc). The copper (II) oxide nanoparticles (CuO) were weighed and suspended in deionized water to prepare the required stock solution (1000 mgl-1) and then dispersed by ultrasonic vibrations (100 w. 30khz) for 30 min. The stock suspension of copper (II) oxide nanoparticles (CuO) was re-sonicated for 3 to 5 min prior to tests, and then it was diluted with deionized water to make final test concentrations according to the experimental design.

**Experimental setup and sampling**

Two days old larvae of grass carp *(Ctenopharyngodon idella*) wight 0.007g/fish (7 mlg) were obtained commercially from a fish culture station (Ahywa, Sohag Governorate). The larvae were kept in the laboratory, in aquaria (1-L) containing dechlorinated tap water, and maintained under a good aeration condition.

Exposure started 7 days after hatching (7d-PHS). Larvae were divided into six groups: one control and five groups exposed to Copper (II) oxide nanoparticles (CuO) once for 1, 5, 10, 50, and 100 mgl-1 according to (Özel et al., 2014). Exposure took place in 1-L glass beaker, each group (30 fish). Sampling was done after 7 days. 10 embryos were collected and fixed at -80 °C until measurements of DNA fragmentation and other fixed in concentrated nitric acid for whole body copper analysis. The experimental water was changed everyday. Mortality was carefully recorded based on the Karber method ([Yılmaz et al. 2004](#_ENREF_48)).

 **Whole body copper analysis:**

Because of the small size of the embryos, whole body homogenates were used for the measurements of theWhole body copper analysis according to Federici et al. (2007). Whole fish were digested in 5 ml of concentrated nitric acid at 70 C for 24h followed by ashing in a muffle furnace. Each sample was then analyzed for Cu by flame atomic absorption spectrophotometer (model 2380, Perkin Elmer). Analytical grade standards were used throughout, and the acidity and matrix of the standards was matched to the samples.

**DNA fragmentation:**

Whole fish samples were hashed into small pieces. Tissue was lysed by addition of DNA lysis buffer and incubated at 37 ◦C for 1 h, followed by a 2 h incubation at 55 ◦C in the presence of 100 µ/ml proteinase-K, followed by addition of RNase A (10 µg/ml, 1 h at 37 ◦C). DNA was then extracted from fragmented samples, separated on 1.8% (w/v) agarose gels and visualised with ethidium bromide (6 µg/ml) as described by Jones et al. 1989. Gels were illuminated with 300-nm UV light and a photographic record was made in order to detect the qualitative damage to genomic DNA.

**Statistical analyses**

The results were expressed as means± S.E. Data were statistically analysed with the t-test.

**Results**

**Percentage mortality rate:**

There was no significant difference in mortality between treated groups and the control (Table 1).

**Copper accumulation:**

To provide insights into the NPs uptake in embryos exposed to copper (II) oxide nanoparticles, embryos showing no significant phenotypic defects were assayed for metal contents. Whole body copper concentrations increased in fish exposed to copper (II) oxide nanoparticles compared to the control except in 10 mgl-1 exposed group (Fig. 1).

**DNA fragmentation:**

DNA ladder bands are an indicator of acute and chronic chemical stress, loss of cellular function and structure and are observed at different concentrations of various nanomaterial (NMs) (Fig. 2). The DNA damaging effect of copper oxide nanoparticles in grass carp larvae was evaluated qualitatively using DNA laddering. The result obtained can be correlated with that obtained from copper accumulation. While the negative control showed a weakly stained smear pattern upon electrophoresis, with no evidence of DNA-ladder pattern, the highest extent of DNA damage was observed at treatment concentration of 50 mg l-1 exposed group. The gel (Fig. 2) also clearly indicate an initial increase in DNA damage up to 50 mgl-1 exposed group followed by subsequent reduction in extent of DNA damage with increasing treatment concentrations.

**Discussion**

The increasing production, use and consequent release of nanoparticles into the environment make it necessary to assess the environmental and health hazards that these compounds could exert (Lee et al. 2010; Wang et al. 2013). The present study aimed to evaluate the accumulation and apoptotic DNA fragmentation in grass carp larvae exposed to copper oxide nanoparticles.

Whole body copper concentrations increased in fish exposed to copper (II) oxide nanoparticles compared to the control except in 10 mgl-1 exposed group. In similar studies, Wang et al., (2011) observed that zebrafish (*Danio rerio*) ovaries were found to accumulate nTiO2 at concentrations of approximately 2.5 mg kg\_1 and 7.2 mg kg\_1 under 0.1 mg L\_1 and 1.0 mg L\_1 doses, respectively. Furthermore, Farmen et al., (2012) reported that gill Ag levels were elevated in all juvenile atlantic salmon groups, with the exception of the 1 µg/L commercial AgNP suspension thus verifying that Ag-NP or Ag ions were accumulating or being deposited/ adsorbed on the gill epithelial structures. The same result, accumulated levels of whole body titanium increased in zebrafish embryos (50 ng/embryo) but decreased by half in embryos transferred to clean medium for 24 h (Bar-Ilan et al., 2012). Also, Whole body Ti concentrations increased significantly in fish exposed to both the 1.0 mg l−1 TiO2 NP and bulk TiO2 compared to controls, but concentrations returned to the control levels by the end of the recovery period (Ramsden et al., 2013). In addition, Faria (2014) observed accumulated levels of whole body titanium of nano-particle suspensions measured in 8 dpf zebrafish embryos (80 ng/embryo) were two- to three-fold higher than in those that were allowed to clean for 1 day. A dose-dependent increase in Zn content occurred in zebrafish embryos and eleuthero-embryos after exposure to nZnO (Brun, 2014). In contrast, Chen et al. (2011) showed low accumulation of titanium (5–100 ng g dw−1) in internal organs of zebrafish exposed to 1–7 mg l−1 of TiO2 NM during 6 months.

Genotoxic effects as measured through the DNA strand breakage are of significant interest in ecotoxicology because they can potentially cause long-term inheritable disorders; affecting the genetic population structure in the aquatic environment (Depledge, 1996). Therefore, the responses at molecular level serve as an indicator of both exposure and toxic effects of NMs and underlines the noticeable changes in a given population or community structure (Stegeman et al. 2001).

In the present study, the control set showed a very weakly stained smear pattern upon electrophoresis, with no evidence of DNA-ladder pattern, the highest extent of DNA damage was observed at treatment concentration of 50 mg l-1 exposed group. The gel also clearly indicated of an initial increase in DNA damage up to 50 mgl-1 exposed group followed by subsequent reduction in extent of DNA damage with increasing Cuo NPs concentrations.

Copper may induce apoptosis through pore formation in the mitochondrial membrane. This would give rise to increased amounts of mitochondrial protein. Among these proteins, there is the Endo G, a mitochondrion-specific nuclease that translocates to the nucleus during cell cycle arrest and apoptosis. Once released from mitochondria, Endo G cleaves chromatin DNA into nucleosomal fragments (independently of caspases) (Huang et al., 2006; Mitra et al., 2012). Arora et al. ( 2008) reported that silver nanoparticles (SNP) could induce DNA fragmentation (ladder formation) at concentrations in the range of 1.56–6.25µg/mL in case of A431 cells. For HT-1080 cells this range changed to 0.78–6.25µg/mL.

Also, the percent tail DNA in *N. tabacum*, showed an initial increase in genotoxicity (2 mM), and followed by a decrease up to the highest treatment dose (10 mM). This could be attributed to a property of nanomaterials to form agglomerates by virtue of which, with increase in treatment concentration the nanoparticles had a tendency to precipitate. The greater interaction of nanoparticles amongst themselves that could have increased owing to increase in treatment concentration might have limited the free TiO2 nanoparticles from interacting with the plant system (Ghosh et al., 2010). Furthermore, nickel nanoparticles and nickel ferrite nanoparticles induced reactive oxygen species (ROS) mediated apoptosis in human lung epithelial cells (Ahamed et al., 2011; Ahamed, 2011). In addition, NiO NPs induced apoptosis in HEp-2 and MCF-7 cells and activity of caspase-3 enzyme was higher along with the fragmentation of DNA in NiO NPs exposed cells (Siddiqui et al., 2012). Besides, the control, 10 nm HAp NPs and 23 nm Zn-doped Hydroxyapatite nanoparticles (HAp NPs) showed the presence of undamaged genomic DNA represented by a thick band on the agarose gel, the highest extent of DNA damage was observed for 14 nm HAp NPs, 27 nm Zn-doped HAp NPs, and 3 nm TiO2 NPs. At 3 nm of TiO2 and 14 nm of Hap NPs, large number of fragments, less than 1 kb was observed when compared to control the group (Venkatasubbu, 2012). Moreover, Ramesh (2013) found that DNA from control tissues of zebrafish (*Danio rerio*) was intact, whereas the tissues treated with SiO2 were all fragmented. Also, there was dose-dependent increase in the DNA Fragmentation in buffalo (*Bubalus bubalis*) sperm cells after exposure to TiO2 NPs (Pawar and Kaul 2014).

**Table 1.** Effect of different concentrations of copper (II) oxide nanoparticles on the percentage of mortality rate grass carp larvae.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Control | 1 mgl-1 | 5 mgl-1 | 10 mgl-1 | 50 mgl-1 | 100 mgl-1 |
| 10% | 7% | 10% | 10% | 7% | 10% |

**Figure 1. Total copper concentration (nmol/g wet weight) in whole body of grass carp larvae after the exposure to different copper oxide nanoparticles concentration for 7 days. \* indicates significant difference**



5

4

3

1

2

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**Figure 2:** DNA fragmentation induced in whole body of grass carp larvae after the exposure to copper oxide nanoparticles concentration for 7 days. Lane (1) control group; lane (2) 1 mgl-1 group; lane (3) 5 mgl-1 group; lane (4) 10 mgl-1 group; lane (5) 50 mgl-1 group; (6) 100 mgl-1 group**.**

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**تراكم النحاس وتكسير الحمض النووي في يرقات مبروك الحشائش بعد التعرض للجزيئات أكسيد النحاس النانومترية**

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معلومات قليلة متوفرة عن التراكم الحيوى والسمية الوراثية للجزيئات النانومترية في اسماك المناطق المدارية مثل مبروك الحشائش. تعرضت يرقات مبروك الحشائش إلى 1، 5، 10، 50، 100 مليجرام /لتر جزيئات أكسيد النحاس النانومترية لمدة أسبوع. لم يكن هناك اختلاف معنوى في نسبة الوفيات بين المجموعات المعالجة والمجموعة الضابطة. ازدادت تركيزات النحاس فى أجسام اليرقات بشكل معنوى في جميع المجموعات المعالجة ما عدا فى المجموعة المعرضة ل 10 مليجرام /لتر مقارنة بالمجموعة الضابطة. وجد أن تركيزات جزيئات أكسيد النحاس النانومترية المنخفضة تسبب تكسير الحمض النووي بدرجة كبيرة عكس التركيزات العالية التى تسبب تكسير اقل.