Section A-Research paper
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Toxicity assessment of spherical Copper oxide nanoparticles in Hydra model

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Abstract

Extensive use of nanomaterials has greatly augmented their discharge into the environment and increased the potential risk to human and animal health by affecting the quality of the environment. Copper oxide nanostructures are extensively employed as antimicrobials, catalysts, surfactants, capacitors, and in other industrial and domestic applications. Copper oxide based NMs pose an environmental hazard due to their high production volumes and increasing use. Copper oxide nanostructures are used as antimicrobials, catalysts, surfactants, capacitors, and in other industrial and domestic applications. Due to their widespread production, copper oxide-based NMs pose an environmental risk. Environmental toxicity of NMs can differ dramatically depending on their physicochemical properties, i.e., dissolution, aggregation, and ROS generation, which are governed by the shape, size, capping agent, and stability. This study aimed to examine the potential toxicity of spherical copper oxide nanomaterials (CuO NMs) on the simple hydra model. *Hydra magnipapillata* 105, exposed to CuO NMs, displayed manifestation of toxicity in a dose and duration-dependent manner. Hydra, in the light of its ease of culture and testing and its sensitivity to contaminants, can be employed effectively as a model organism for risk assessment of chemicals and nanomaterials in aquatic environments.

KEYWORDS: Hydra, *in vivo*, acute toxicity, copper oxide, ROS

1. Introduction

Nanomaterials (NMs) are the building blocks of the rapidly emerging field of nanotechnology. Nanotechnology, in the broadest sense, means understanding and manipulating the properties of matter at a size ranging from 1 to 100 nanometres. Nanoparticles (NPs) of metal oxide are produced for large-scale industrial and household applications. Transition metal oxides are an important class of semiconductors with extensive applications in biomedicine and drug delivery (Daraee, *et al*., 2014), solar energy transformation (Zhang *et al*., 2013), electronics (Contreras *et al*., 2017), catalysis (Zhang *et al*., 2019), etc. Copper oxide (CuO) has garnered interest among transition metal oxides due to its fascinating properties, such as being the premise for high critical temperature (Tc) superconductors (Shakhray *et al*., 2016). CuO nanomaterials are used in antimicrobials (Halbus *et al*., 2019), gas sensors (Wang *et al*., 2016), supercapacitors (Yuan *et al*., 2020), and thermal conductivity devices (Moghadassi *et al*., 2010). These NPs can pass through the membranes of cells easily and participate in intracellular metabolism, and inflict toxicity (Guarnieri *et al*., 2014).

Nanotoxicity refers to the exposure of nanoparticles in an *in vivo* system that can cause morphological, developmental, and physiological changes. The potential toxicity of nanoparticles depends on their shapes, size, concentration, functionalization, and surface charges (Sukhanova *et al*., 2018).

CuO NP toxicity studies conducted on cell lines and aquatic organisms revealed NPs-induced toxicity through oxidative stress and DNA damage, which leads to apoptotic cell death (Ganesan *et al*., 2015) (Shafagh *et al*., 2015) (Wu *et al*., 2017) (Che *et al*., 2018) (Mansano *et al*., 2018) (Aksakal *et al*., 2019).

Hydra is known for its astonishing regeneration ability and belongs to phylum Cnidaria and class Hydrozoa,. It has low senescence and unlimited budding capability all through its life (Gierer *et al*., 1972) (Bosch, 2007). All hydra cells are in contact with the animal's aqueous environment, which facilitates the

absorption of toxic substances (Beach *et al*., 1998). Hydra has been projected as a model organism for ecotoxicity testing in freshwater. Ease of culture, availability of large clonal populations, sensitivity towards xenobiotics, and metal contaminants present in the medium, low cost, and ease of testing make it a useful tool for toxicological risk assessment. Hydra undergoes various morphological changes when exposed to toxic compounds. Zeeshan *et al.* (2016) studied elemental copper toxicity, whereas Murugadas *et al.*, (2016) studied CuO nanorods toxicity on Hydra. This is the first study to investigate the toxicity of spherical CuO NPs on the Hydra model.

2. Material and Methods

CuO nanospheres (NS) were synthesized via inductively coupled plasma obtained from Tekna (France) and characterized using a UV–Vis spectrophotometer (Thermo Scientific Multiskan GO, Finland) with a wavelength range of 300–700 nm & powder X-ray diffraction (Empyrean, Malvern Panalytical, UK) with CuKα radiation (40 kV, 30 mA). TEM images were obtained using a 200 kV JEOL/JEM-2100 (Japan) Transmission electron microscope.

2.1. Hydra Maintenance & Culture

Hydra was maintained at 18 ± 1 °C under 12-hour dark-light cycle in a standard medium according to Lenhoff & Brown, 1970. Hydras were fed trice a week with *Artemia nauplii*. The culture medium was changed every day, a few hours after the feeding,

2.2. Acute Toxicity

Different concentrations of CuO NS (150 µg/L-3300 µg/L) were prepared in the culture medium. Twenty-five polyps were placed in each well of a cell culture plate containing different concentrations of CuO nanospheres and were incubated upto 96 h, adopting the conditions mentioned above. Polyps unexposed to CuO were used as a negative control. For each time point, the median lethal concentration (LC50) of CuO nano was determined using Probit analysis. After every 24 hours, morphological changes were recorded and compared on a scale of 10-0 defined by Wilby (1988), where score 10 refers to a healthy Hydra, whereas 0 is disintegrated.

2.3. Hydra Regeneration assay

A medical scalpel was used to bisect 15 polyps just above the hypostome, and basal body columns were allowed to regenerate in the presence of 150 µg/L and 900 µg/L doses of CuO nanoparticle in 8 ml of Hydra medium for 96 hours. The medium was replenished every 24 h until 96 h was reached. A dissecting microscope, was employed to observe and score polyps undergoing regeneration according to Ambrosone et al., (2012) with slight modifications. Score 0 (no regeneration), Score 1 (presence of tentacle buds), Score 2 (new tentacles emerging) & Score 3 (Disintegration/ Lethal).

2.4. Determination of Reactive Oxygen Species (ROS)

2.4.1. H_2-DCFDA Staining

Generation of ROS was perceived using H_2-DCFDA dye according to Jantzen *et al*. (1998) at the whole animal level. After the treatment period, polyps were rinsed with hydra medium and observed immediately (relaxed in 2% urethane) in the fluorescent microscope (Carl Zeiss).

2.4.2. Intracellular ROS Quantification

ROS was measured according to Keston *et al*. (1965). Hydras were washed with hydra medium after the treatment and macerated in PBS using a micropipette. The protein concentration of the prepared cell lysates was estimated by the Bradford assay (Bradford, 1976), followed by incubation in H2-DCFDA dye (10 µM) for 30 min in the dark. A fluorometer was employed to measure the fluorescence (Fluoroskan, Thermofisher, Finland).

2.5. Statistical analysis

All the experiments were performed in triplicate, and the results are given as the mean ± SE of three separate tests. Two-way ANOVA and Dunnett's multiple comparison test were used to analyse data from the acute toxicity test and ROS generation using Graphpad Prism9.

3. Results and Discussion

3.1. Characterization

CuO NS were dispersed in deionized water and scanned with a UV-Vis Spectrophotometer in a 200 to 700 nm range, exhibiting a broad absorption peak at 365 nm (Fig. 1.A). The XRD pattern indicated the orientation and crystalline nature of CuO NS, which concur well with powder CuO data from the ICDD® (JCPDS-89-5895), validating the formation of a monoclinic crystal structure (Fig. 1.B). CuO NS were well-dispersed spherical particles, with a size distribution of 15-120 nm (61.84 0.54 nm) as determined by transmission electron microscopy (Fig. 1.C).

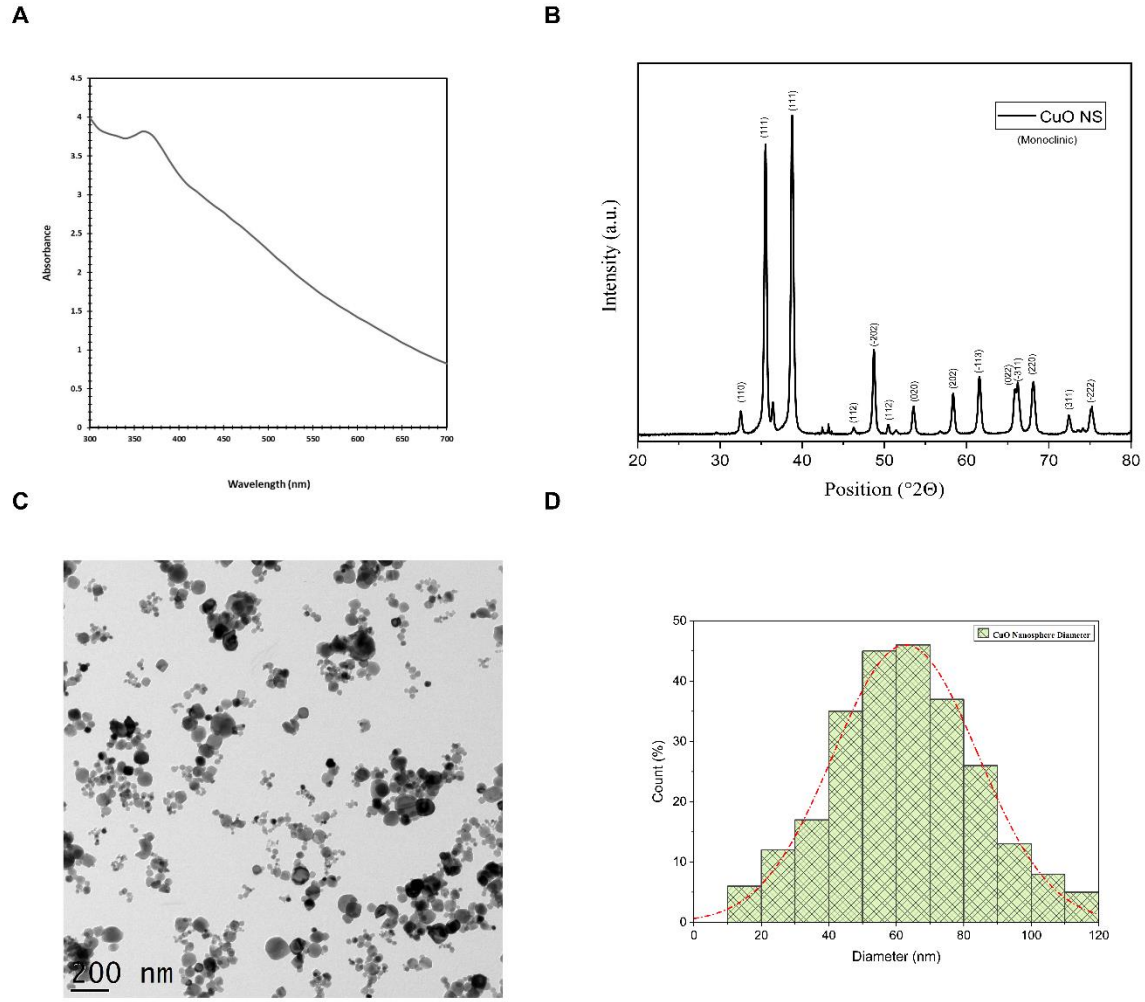


Fig.1. (A) UV–Vis spectrum of CuO NS. (B) XRD pattern of the CuO NS. (C) TEM image of the CuO NS. (D) CuO NS Size distribution

3.2. Acute Toxicity and Determination of Median Lethal Concentration (LC50)

Hydras were subjected to various concentrations of CuO NS and kept under observation for 96 h. The changes in the morphology of hydra (Fig.2) were measured in the Wilby scale, which revealed that CuO NS induced dose- and time-dependent toxicity. The median score of groups of twenty-five hydras was determined for each concentration and time point. (Fig.3). These median scores were used to determine the LC50 using probit

analysis (Table 1). The LC50 value enabled us to determine the sublethal dosage for subsequent experiments:150 µg/L and 900 µg/L were established as the lower and upper sub-lethal doses.

Fig. 2. Morphological changes observed in *Hydra magnipapillata* exposed to CuO NS. (Scale bar: 200 µm)

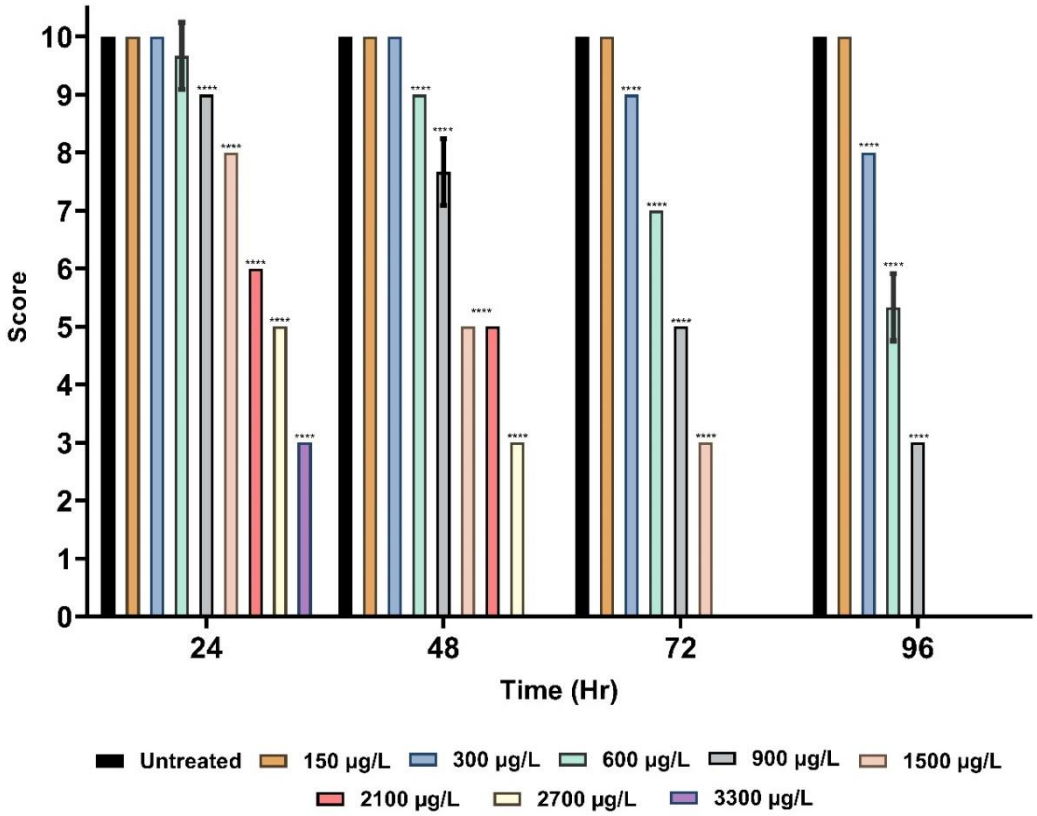


Fig. 3. The median score of hydras subjected to increasing CuO NS concentrations.

Time (h)	95% confidence limits for concentrations (µg/L)		
	Estimate	Lower bound	Upper bound
24	2181.560	1942.655	2413.862

48	1228.960	1154.726	1303.890
72	713.600	673.257	754.337
96	535.049	508.805	563.114

Table 1. For each time point, the LC50 was determined using probit analysis.

3.3. Regeneration assay

According to Tino et al., 2011, an experiment was conducted to determine the impact of CuO NS on Hydra regeneration. 15 Hydras were allocated in three groups, bisected just underneath hypostome, and allowed to regenerate in the presence of hydra medium or 150 µg/ml & 900 µg/ml concentration of CuO NS. Animals' viability and regeneration were evaluated 48 hours, 72 hours, and 96 hours after amputation. Stages: score 0 denotes no tentacles visible; score 1 denotes tentacle buds present; score 2 denotes new emerging tentacles, whereas score 3 indicates complete inhibition of regeneration/death

100% of Hydras in the Control and 150 µg/L treated group showed head regeneration at 48 h, while complete regeneration was observed in the same groups at 72-96 h. Whereas in another group of Hydras treated with higher dose, 60% Hydras showed no recovery, only 9% showed head regeneration, and around 31% degenerated at 48 h. Mortality further increased to 86% at 72 H and 100% at 96 H. These results strongly suggest that CuO NS affects the regeneration ability of Hydra in a dose-dependent manner. To ascertain the mechanism by which CuO nanospheres interfere with this regeneration process, additional research is required.

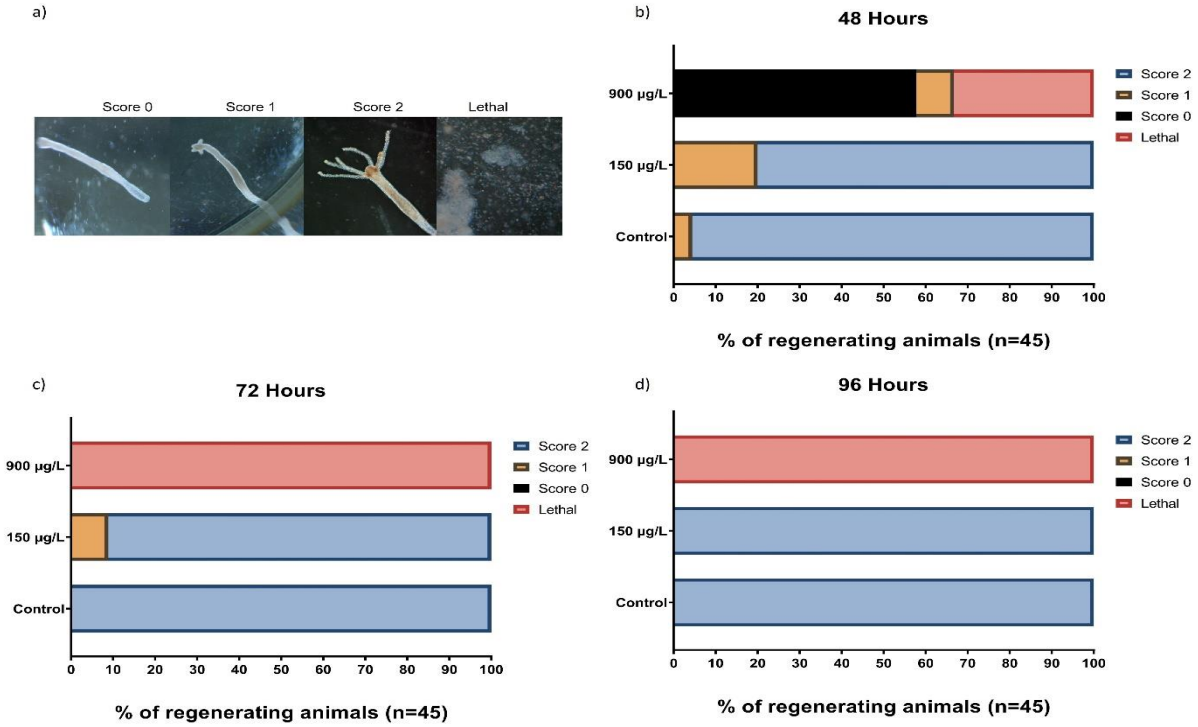


Fig. 4. Various stages of Hydra regeneration. a) Score 0 shows no regeneration, score 1 reveals the tentacles buds are present, score 2 reveals the emergence of new tentacles, and score 3 indicates Disintegration/ Lethal effect of Copper oxide nanospheres on hydra regeneration. (b) 48-hour regeneration after amputation; (c) 72-hour regeneration after amputation; (d) 96-hour regeneration after amputation

3.4.1. H_2-DCFDA Staining

The ability of Copper oxide nano spherical to cause oxidative stress was observed using H_2-DCFDA. The ROS levels increased with increasing CuO NS concentrations, as seen from the fluorescent punctate. The Control group displayed minimal DCF fluorescence compared to the lower and higher dose treatment groups.

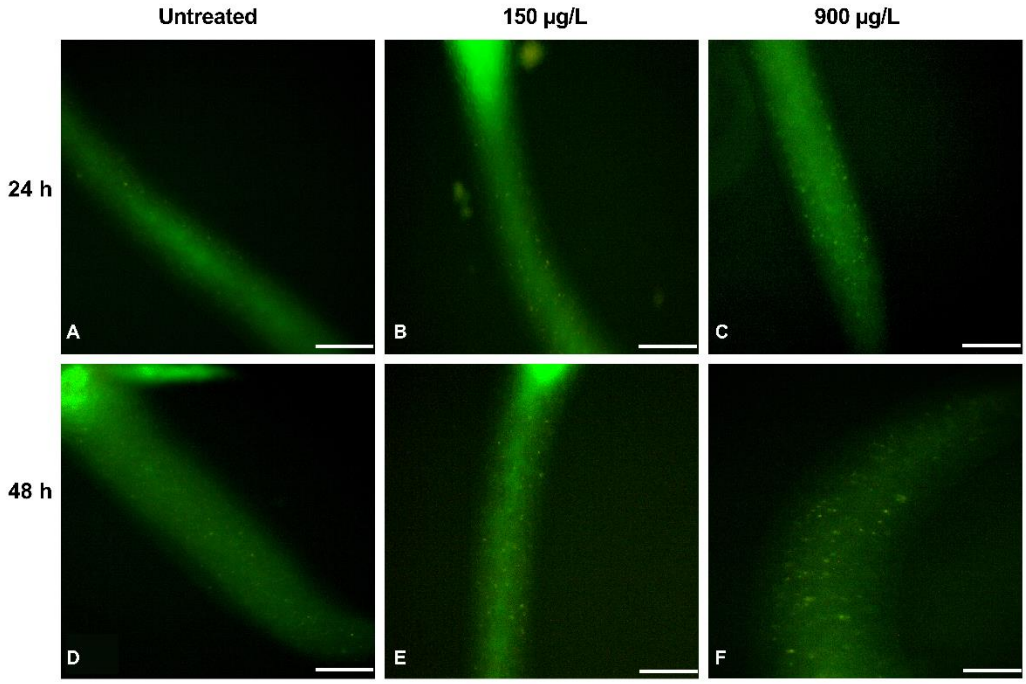
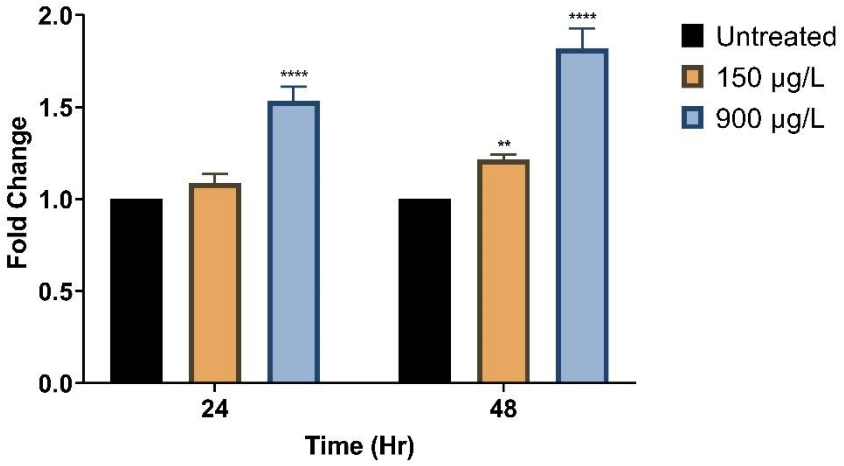


Fig. 4. Representative images of Reactive Oxygen Species generation in hydra following exposure to CuO NS. The punctate of green fluorescence shows the induction of ROS generation. Untreated hydras (A,D) displayed minimal fluorescence, whereas hydras treated with lower (B,E) and higher sublethal dose (C,F) At 24 to 48 hours intervals, the ectodermal layer reveals a punctuated increase in fluorescence. (Scale bar: A-F, 100 µm)

3.4.2 ROS Quantitative Assay

Fig. 5. Determination of intracellular Reactive Oxygen Species generated in hydra in response to CuO NS



exposure. The significance of results between the untreated and treatment groups was determined using a two-way ANOVA and Dunnett's multiple comparison test. (**$p < 0.01$, ****$p < 0.0001$).

The increase in Reactive Oxygen Species production was further confirmed by measuring the fluorescence from DCF in cell lysate of hydra homogenised in PBS. The level of Reactive Oxygen Species is expressed as a fold

change in fluorescence between treatment and control groups. A significant change was observed in hydras treated with a higher sublethal dose, which was 1.535 and 1.821 times greater after 24 & 48 hours compared to control group.

CuO nanoparticles have been proven to be hazardous to aquatic animals like daphnia, urchins, and fish in a number of ecotoxicological studies during the past decade (Wu *et al.,* 2017; Gallo *et al.,* 2018; Mansano *et al.,* 2018). CuO NS can penetrate through the cell membrane and induce ROS production and oxidative stress once inside the cell. (Gupta *et al*., 2016). CuO nanoparticle toxicity in fish has been linked in part to its ability to generate ROS (Srikanth *et al*., 2016). CuO induces oxidative stress through the surface catalytic production of ROS. (Siddiqui *et al*., 2015). *Oreochromis mossambicus* exhibited respiratory distress when exposed to CuO NPs as a result of oxidative stress, antioxidant defence mechanism failure, and genotoxicity. (Shahzad *et al*., 2018).

Metal oxide nanoparticle exposure may disrupt lysosomal membranes, leading to apoptosis and necrosis in cells via leakage of the hydrolytic enzymes (Strauch *et al*., 2020) (Mukherjee *et al*., 2021). CuO NPs-induced toxicity resulting in apoptotic cell death in *Hyphessobrycon eques* and *Ceriodaphnia silvestrii* (Mansano *et al*., 2018). CuO nano induced apoptosis via mitochondrial pathway and ROS generation in the K562 cell line. (Shafagh *et al*. 2015). Thus, ROS production due to CuO NS exposure in the present setting may be the ultimate cause of cell death in hydra leading to DNA damage. CuO NS exposure can enhance the generation of ROS, inducing oxidative stress, DNA damage, and unregulated cell signalling, eventually leading to apoptosis (Alarifi *et al*., 2013) (Kumari *et al*., 2017) (Mani *et al*., 2019).

Further research with side-by-side comparative analysis can help determine the influential role of nanoparticles' multi-molecular and physicochemical parameters (size, shape, surface charge) impact on their toxicity.

4. Conclusion

The research indicates that CuO NS produce detrimental effects in hydra in terms of morphology and ROS generation, as observed in fluorescent punctate in H_2DCFDA staining and quantitative assay. From the study, it can be concluded that CuO NS is dose- and time-dependently deleterious to hydra, as it would be to other organisms. Further, this study confirms that hydra is an ideal organism for assessing the environmental risk of nanomaterials in freshwater environments.

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Credit author statement

Ankit Dilaware: conceptualization, investigation, writing original draft. Klewos Synshiang: Editing, Thamaraiselvi Kaliannan: Supervision, Review & Editing, Suja P Devipriya: Supervision, Review & Editing.

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