https://doi.org/10.48048/tis.2022.2674

Exposure of Copper Nano-Particles (CuNPs) Impairs Developmental Stages and Hatchability in the Fresh Water Snail *Indoplanorbis exustus* (Planorbidae: Pulmonate)

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Received: 27 September 2020, Revised: 30 April 2021, Accepted: 27 May 2021

Abstract

Pollution of water bodies by humans, in our day-to-day activities impairs the quality of water, thereby making the water sources unsuitable for aquatic fauna and also for human health. Various sources of pollutants in excess may negatively affect the diversity of living organisms. The evolving growth of nanotechnology has attracted a great deal of attention due to its concern in assessing their environmental and health safety. The present study was conducted to investigate toxicity of copper nanoparticles (CuNP) on the development of fresh water snail *Indoplanorbis exustus*. Acclimatized snails were exposed to increasing concentration of CuNP for 96 h and acute toxicity experiment was carried out to determine LC_{50}, followed by exposure of snails to sublethal concentrations of CuNP to observe developmental deformities and to estimate percentage hatchability and survivability of young ones. The projected LC_{50} value for 96h of CuNPs was found to be 5.8mg/L, followed by exposure to sub-lethal concentrations of $1/6^{th}$(0.96mg/L), $1/5^{th}$(1.16mg/L), $1/4^{th}$(1.45mg/L), and $1/3^{th}$ (1.93mg/L). The highest concentration of (1.93mg/L;$1/3^{th}$of LC_{50}) retarded hatching and caused morphological deformities in the larvae. However, no significant difference was observed in the weight of embryos in 7 days. These results revealed that CuNP exposure at sub lethal concentrations exerted developmental stress.

Keywords: Copper nanoparticles, Veliger, genotoxic, Hatchability, Survival, Developmental stress

Introduction

Nanotechnology is an emerging field of science, the use of NPs for wound healing ointments, disinfectant in medical fields, for protection of water treatment of plants, food processing because of their antifungal, antibacterial, antiviral properties, disease diagnosis, textiles, agriculture etc. [1-3]. The overgrowing applications of NPs in various fields of life may represent a threat to human population and for aquatic fauna [4-6]. Due to growing technology, the green synthesis of NPs is emerging day by day, and has been paid for the development of engineered nano materials [7]. Harmless substances e.g., plant extract and water in the green synthesis of nanoparticles are biologically synchronized and cost effective. Plant extract contains various bio-compounds such as flavonoids, tannins proteins, terpenoids etc. which work as reducing agents [8-10].

Most of the invertebrate models used in aquatic toxicity are from phylum Mollusca, the gastropods are considered as the best model organisms for NPs toxicity because they are adapted to various environmental disturbances due to their dual habitat, easily available, high fecundity, small size, easy rearing and maintenance in laboratory and mostly they are suitable for developmental observations, because of their transparent egg masses [11-16]

From zygote to hatching, embryonic development is the most crucial process. Any change in the embryonic stage can lead to various abnormalities or death of the embryo. The negative impact of excess use of copper-based compounds in aquatic bodies, and assessment of their toxic impact on development have been proved to be an essential tool for environmental hazard and risk assessment, the gastropod embryos are more susceptible to nanomaterials than adults, so they are considered to be important targets for water quality monitoring [17].The development of snail includes various larval stages (trochophore, veliger and hippo) the toxicity of chemicals depends upon the developmental stage[18]. Based on this we have attempted to explore the toxicity of CuNPs on embryonic development and its adverse effects by considering the following objectives in mind: 1) To examine the developmental malformation; 2) To examine the effect on survival and hatchability.

Materials and methods

Collection and rearing of snail

Fresh water snails were collected and acclamatized for 14 days in laboratory conditions, lettuce leaves served as food and substratum for egg laying purpose. The snails were collected from local pond (18.5488° N, 73.8244° E). The parameters like pH (6.7-6.9), temperature (26-28 °C) and electrical conductivity (0.78mS/cm^2 (millisiemens per cm^2) of water were observed.

Median lethal concentration (LC_{50}) of CuNPs on *Indoplanorbis exustus*

Acute copper nanoparticle toxicity tests were performed by exposing freshly laid eggs masses to different concentrations, the treatment was given in glass perti-plates containing 25mL declorinated water, each treatment contained around 32 egg masses. Water quality was monitored and LC_{50} values and 95% confidence intervals were calculated by the regression probit analysis method by using Finney table [19].

Exposure of snails to CuNPs

After determination of LC50 value of CuNPs on *Indoplanorbis exustus*, 4 sub lethal concentration groups were selected (0.96, 1.16, 1.45 and 1.93 mg/L), represented as 1/6, 1/5, 1/4 and 1/3 of the 96 h LC_{50} value respectively, a parallel control group with only water was kept along with experimental groups. The experiment was replicated 4 times with around 16 egg masses each time.

Preparation of the sample

The egg clutches were collected every day in a petridish in declorinated tap water around 5:30-6:30am. On an average 12-17 eggs were found in each egg mass, the egg masses were placed in glass plates (size-90mm diameter×15mm height). The sub-lethal concentration of copper nanoparticles was tested in 4 replications. The different developmental changes from ovi-position till the hatching stage were examined daily till hatching.

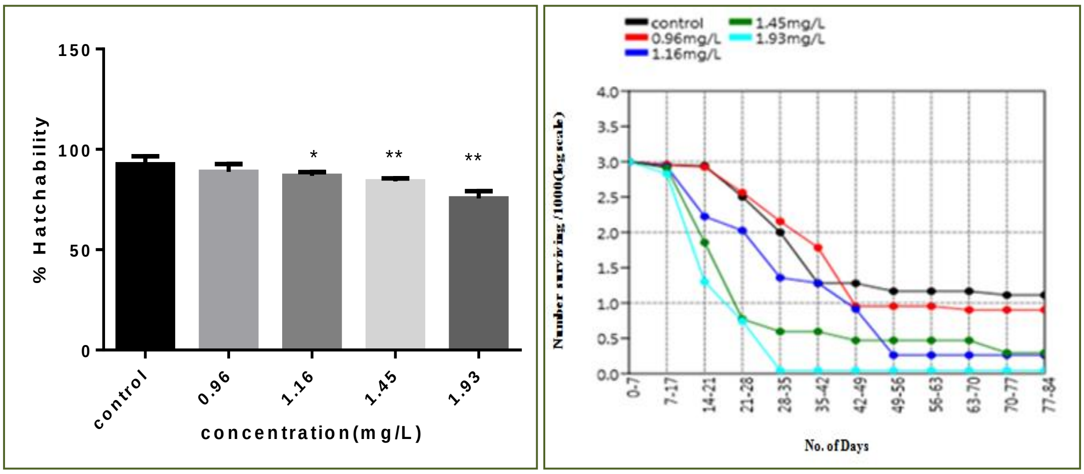
Statistical analysis

Statistical analysis was carried out by using the SPSS. The graphs were plotted by prism 6 and past3 software. The data is presented as mean ± standard deviation (SD), and subjected to one-way analysis of variance (ANOVA) and the non-parametric, Mann Whitney test was performed. The LC_{50} values were calculated by probit analysis.

Results and discussion

Survival rate and hatchability

The survival rate of hatched ones was noticed till the young ones started laying eggs of F1 generation, it took on an average of 84±3 days for the newly hatched snails to reach maturity, the average length and breadth of the snails at the time of egg laying was observed in between 0.9±1.1mm and 0.5±0.7mm. The embryos showed significant decline in hatchability and survival rate (**Figures 1(a)** and **1(b)**. After 14 days there was abrupt decline in survival rate in control and treated groups, and the newly hatched embryos could not survive after 28 days at the highest (1.93 mg/L) concentration. The survival rate of embryos was determined by taking the log fraction.

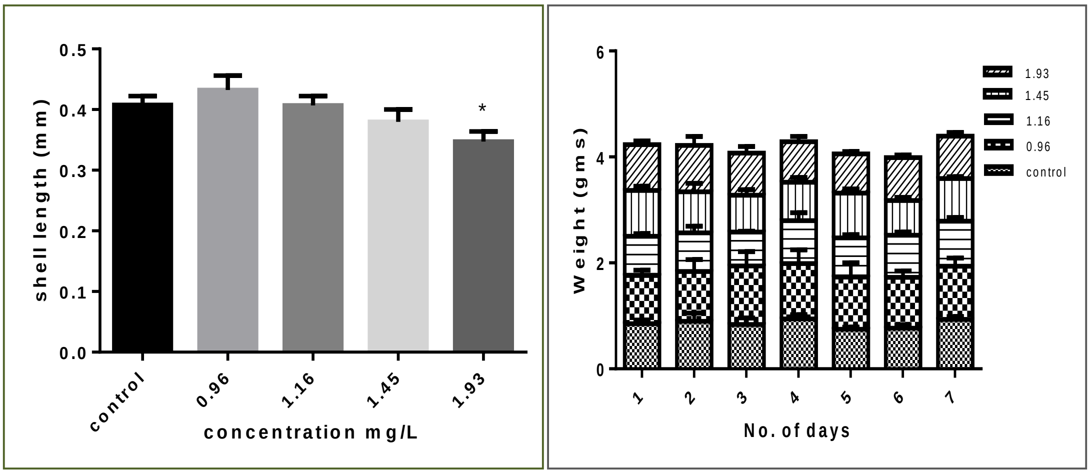


(a) (b)

Figure 1 (a) Graph above represents % hatchability * represents $p \leq 0.05$ and $^{**}p \leq 0.01$, values are expressed as mean ± standard deviation n=5; (b) Survival rate of control as well exposed young ones, vertical axis represents log scale.

Effect on the shell formation and body weight

The embryos showed marked decline in shell length determined at 7th day after CuNPs exposure. The significant decrease in shell length was observed only at the highest concentration of CuNP 1.93mg/L (**Figure2(a)**). The average shell length of control, 0.96, 1.16, 1.45 and 1.93 mg/L at sub lethal concentrations were 0.423, 0.410, 0.405, 0.365 and 0.346 mm, respectively. At 1/3rd (1.93 mg/L) concentration the shell appeared transparent, small and thin. The weight of egg masses was noted every day at 7:00 am for each group till hatching (7 days). However, no significant difference in weight was observed between the control and treated groups (**Figure2(b)**), so CuNPs did not exert any effect on weight of the embryos. The embryos showed delayed shell formation and due to this reason, the hatching was delayed (**Figure 3(b)**).



(a) (b)

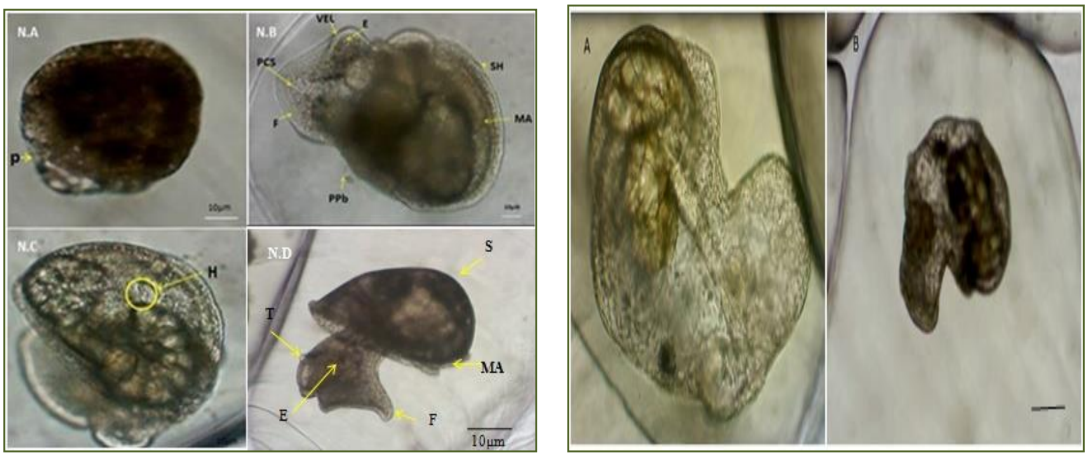
Figure 2 (a) Effect of CuNPs on shell length (*$p \leq\leq 0.05$, n=5) values are expressed as mean±standard deviation. (b) Effect of CuNPs on the weight of the embryos $p \leq\leq 0.05$ was considered significant, n=4, values are expressed as mean±standard deviation.

Effects on heart rate

The heartbeat of snails was recorded upon exposure to increasing concentrations. Our results revealed that normal heartbeats of snail were 58±64 times per min of control animal. The heartbeats showed variation as the concentration increased, at low concentration the heartbeat recorded were in the range of 58-64 /min; however, it showed declined heart rate (24±53 per min) at higher 1/4thand 1/3th concentrations of CuNPs.

Morphological changes

The normal development is observed in control *Indoplanorbis exustus* (**Figure 3(a)**). Morphological deformities were not observed in exposed snails up to 3 days; from 2-cell stage till late trochophore stage, however the malformation was observed at Veliger stage on 4th and 5th day. The deformities became prominent at higher concentration of CuNPs (1/4 and 1/3). The embryos showed delayed shell formation and deformities were observed in foot, eyes and tentacles (**Figure 3(b)**). The obvious decrease in movement was noted at 1.45 mg/L and deformed body without shell was observed at the highest (1.93 mg/L) concentration. The copper nanoparticles showed lambda (λ) max at 520 nm. The average size of the synthesized particles was found to be in the range of 40-100nm and height of 20±40 nm, this was determined by using, Scanning Probe Image Processor (SPIP) software (**Figure4**).



(a) (b)

Figure 3 (a) N.A- Control Trochophore; N.B- Control early veliger; N.C- Control Late veliger; N.D- Control Normal adult; P- prototroch; PCS-post -trochal ciliate strand; VEL- velum; F-foot; E- eye; PPb- polar bodies; MA-mantle; SH, S-shell; H- heart; T-tentacle;(b) The picture above represents the abnormal embryo of *Indoplanorbis exustus* at (A) 1/4th and (B) 1/3th concentration without, shell gland, eyes, tentacles and foot are poorly developed and shell is not formed.

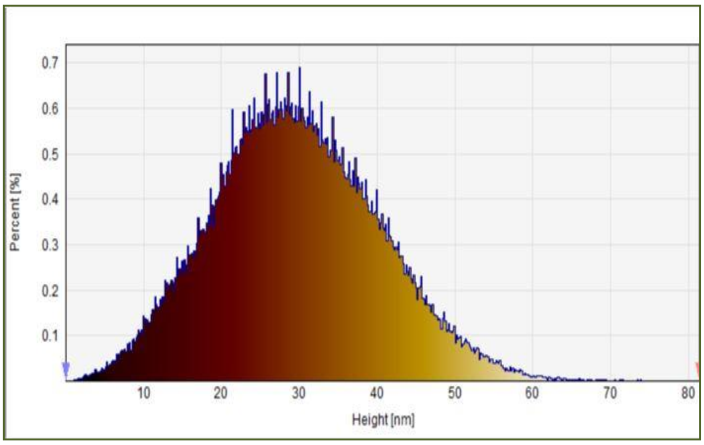


Figure 4 SPIP image above represents the percent height of nanoparticles formed. The height was determined after Atomic Force Microscope (AFM) image of nanoparticles obtained.

Discussion

Copper and its alloy nanoparticles represent a new approach for the treatment of crops from mollusks, fungi and algae. Copper nanoparticles are also employed for water treatment plants, wound healing ointments, bioactive coating, air and liquid filtration because of their antibacterial and antiviral properties. The synthesis of CuNPs was confirmed by change in color of the solution from pale yellow to dark brown after incubation in microwave oven [20].

Nanoparticles should have at least 1 dimension less than 100nm [24]. Snails are the best model organisms for eco-toxicological studies [21]. *Indoplanorbis exustus* is a fresh water snail distributed worldwide in various habitats on the earth. Decline in rate of rotation of trochophore was observed; besides the slow movement no other such abnormality was recorded till 3 days. The delayed development of organs, reduction in shell size, slow movement of embryos was observed on 4th, 5th and 6th day, same was reported by [22,23] nanoparticles led to impairments to many generations, delayed development, death of embryos and reduction in hatching. In an experiment [24] on *C. aspersum*, the size of the egg got reduced; as well the color got changed due to the exposure of Fe_2O_3 NPs. In our result pale pigmentation color of the egg was observed only at the highest concentration. However, there was no significant difference in the weight of embryos up to 7 days in the treated groups as compared to control. As it is experimented after the *Lymnaea luteola* were exposed to 10µg l^{-1} of Cu after 7 and 10 days, the abnormality was observed at the veliger stage [25] and more prominent at hippo stage as reported by [26], so it may be concluded that toxicity induced by nanoparticles is dependent upon the stage of development. The exposure of copper nanoparticles on zebra fish embryos showed retardant hatching, morphological malformation of the larvae, and even killed the embryos [27]. In our study, the embryo hatched on 7th day of exposure, there was significant decrease in percent hatchability, at 1.45and 1.93mg/L concentration, it might be very difficult for them to break the egg membrane; hence young ones could not hatch easily in time. After the exposure of CdTe NPs for 24 hon *B. glabrata*, decreased hatching, morphological alterations and reduced growth rate was observed [28]. The percent hatchability, growth of the foot, tentacles and shell as well as heart development appeared normal at lower concentrations like 0.96and 1.16mg/L concentration of CuNPs. These results showed CuNPs were not harmful for the embryos at lower doses less than 1.45mg/L. It was observed that control and the exposed groups showed dose dependent abrupt decline in survival rate from 14 up to 49 days and from 49 days onwards steady growth was observed.

Conclusions

Dose dependent effect of copper nanoparticles was evident on different parameters such as percent hatchability, survivability and growth of different organs such as foot, shell, eyes, heart and tentacles. The percent hatchability, growth of the foot, tentacles and shell as well as heart development appeared normal at lower concentrations like 0.96and 1.16mg/L concentration of CuNPs. As the concentration of CuNPs increased, significant reduction in shell size, declined heart rate, reduced rate of percentage of hatchability and delayed hatchability was observed. The survival rate of young ones was affected prominently at highest concentration of CuNPs. However, no significant difference in weight between control and exposed groups was observed even at higher concentrations of CuNPs. This study should be further extended to holistically analyze the impact of CuNPs, especially at the molecular level.

Acknowledgements

We are grateful to the Head, the Department of Zoology, Savitribai Phule Pune University, for providing laboratory facilities and Zoological Survey of India (Pune) for identification of the species.

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