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TEM and LM assessment of *Biomphalaria alexandrina* snails' immunotoxicity as a bio-monitor for water pollution by cuprous oxide nanoparticles and copper sulphate

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ABSTRACT

The snail hemolymph is a multi-component mixture comprising various substances with immunological activity that can respond to environmental changes. In previous work that has been done by us, obvious genotoxic effects of cuprous oxide nanoparticles (CuNPs) were shown on *Biomphalaria alexandrina* snails. In this research, the role of *B. alexandrina* snails as a bio-monitor for the detection of water pollution with copper was emphasized. Immunotoxic effect of CuNPs was compared with the effect of dissolved Cu^{2+} ions of copper sulphate ($CuSO_4$) on the hemocytes of the snails. In this investigation CuNPs were more toxic than $CuSO_4$ to *B. alexandrina* snails. The toxicity focuses on the destroying of exposed hemocytes that are involved in the snail's immune response, especially in the biomineralization process. The three defined types of hemocytes: small undifferentiated cells, granulocytes, and hyalinocytes were all affected. Changes in the affected hemocytes as seen under the light microscope included: vacuoles, plenty of granules, an irregular cell membrane, two nuclei (incomplete cell division), some with no nuclei but the presence of nucleus region, and a shrunken nucleus. Whereas changes in the affected hemocytes as seen under the electron microscope included: a large nucleus, cytoplasmic extensions, vacuoles, phagolysosomes, granules, a degenerated outer cell membrane, organelles that were difficult to be identify, irregular nuclear membrane, and the nucleus being shrunk. The present result confirms the molluscicide activity of CuNPs and Cu^{2+} free ions on *B. alexandrina* snails with focus on the effectiveness of nanoparticles that have the strongest effect on hemocytes and emphasizing the efficiency of *B. alexandrina* snails in the aquatic ecosystem as biomonitor for water pollution.

Keywords: Transmission electron microscopy; LM; *Biomphalaria alexandrina*; Bio-monitor; Immunotoxicity; Water pollution; Copper; Nanoparticles

1. Introduction

Anthropogenic activities are increasing the presence of chemical contaminants in aquatic systems, which have the potential to disrupt snail-parasite interactions through acute toxic effects and low-exposure chronic effects on the vital rates and behaviors of snail organisms [1]. Exposure of snails to these pollutants can lead to abnormal physiological responses and cause adverse effects on their development, growth, behavior, and reproduction [2,3]. Epizootic neoplasms have been found in a variety of ectothermic species, such as snails, echinoderms, and fish [4] in association with exposure to specific classes of DNA damaging pollutants [5]. Copper sulphate ($CuSO_4$) is widely used as a pesticide [6] causing genotoxic effects on aquatic organisms and is responsible for clastogenic damage in the golden mussel (*Limnoperna fortunei*) [7]. Shaldoum et al. [8] have also detected the genotoxic effect of cuprous oxide

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nanoparticles (CuNPs) and copper sulphate on *Biomphalaria alexandrina* snails. Frenzilli et al. [7] found similar genotoxic effects of $CuSO_4$ in planarian individuals. Varotto et al. [9] also recorded an increase in the number of DNA strand breaks induced by copper chloride in mussel gills (*Mytilus galloprovincialis*). It is inevitable that nanoparticles and their byproducts end up in the aquatic environment, where they may represent a hazard to aquatic organisms.

Since nanotechnology is progressing at such a fast pace and nanomaterials are being incorporated into new products every day, it is important to fully understand the implications for their ultimate and unavoidable release into the environment [10]. Nanomaterials have unique properties compared with their larger counterparts. Due to the small size and hence higher specific surface area of the nanoparticles, they can easily bind with and transport toxic pollutants. Synthesized metal-NPs have a clear molluscicide effect against snails and could potentially serve as next generation molluscicides [11].

Hemocytes, as multifunctional cellular components of molluscan hemolymph, are also a focus of research [12]. They are responsible for many aspects of molluscan life, including immune response, shell formation, and regeneration processes [13].

The present study was designed to specifically characterize the immunotoxic effects of Cu_2O-NPs on hemocytes of freshwater snail *B. alexandrina* and to confirm the utility of this snail as a bio-indicator for Cu_2O-NPs and copper sulphate in the aquatic environment.

2. Materials and methods

2.1. Chemicals

Copper sulphate pentahydrate ($CuSO_4$+$5H_2O$) From (El-Gomhouria Chemical Company, Egypt) and cuprous oxide nanoparticles (Cu_2O NPs), Cu_2O NPs were prepared with copper sulphate as starting material *via* a simple technique. Exactly 20 mL of NaOH aqueous solution (0.075 mol/L) (El-Gomhouria Chemical Company, Egypt) was added to 10 mL of $CuSO_4$ aqueous solution (0.5 mol/L) (El-Gomhouria Chemical Company, Egypt) with stirring (pH = 10.5). Then, 25 mL of ascorbic acid aqueous solution (0.1 mol/L) (Merck Company, Germany) was added drop wise into the above solution with vigorous stirring. After 1 h a yellow precipitate was obtained (pH = 4–4.5). The particles were separated from the solution by centrifugation at 2,000 rpm for 30 min. The product was washed with distilled water and absolute ethanol. The final product was dried in vacuum at 60°C for 8 h [14].

2.2. Characterization of Cu_2O nanoparticles

Copper oxide nanoparticles was characterized by scanning electron microscopy (SEM), transmission electron microscopy (TEM), and X-ray fluorescence (XRF) [8]. The resulting nanoparticles was analyzed using a TEM (EM 208S Philips, Netherlands) connected to a high-resolution imaging system. Samples for TEM studies were prepared by placing drops of nanoparticle solutions on carbon-coated TEM copper grids; the surface morphology of nanoparticles was characterized by SEM (JEOL JSM-5600, Japan). XRF was performed to learn about the main chemical compositions and elemental analysis of the minerals that are present in nanoparticles. XRF measurements were carried out using the JSX-3222 element analyzer, made in Japan [9]. SEM image provides spherical nanoparticles morphology (Fig. 1a). TEM image shows that the Cu_2O NPs are spherical, and their average diameter is 22.1 nm (Fig. 1b). XRF recorded that 98.5% of the elemental composition of Cu_2O NPs powder is Cu (Fig. 1c).

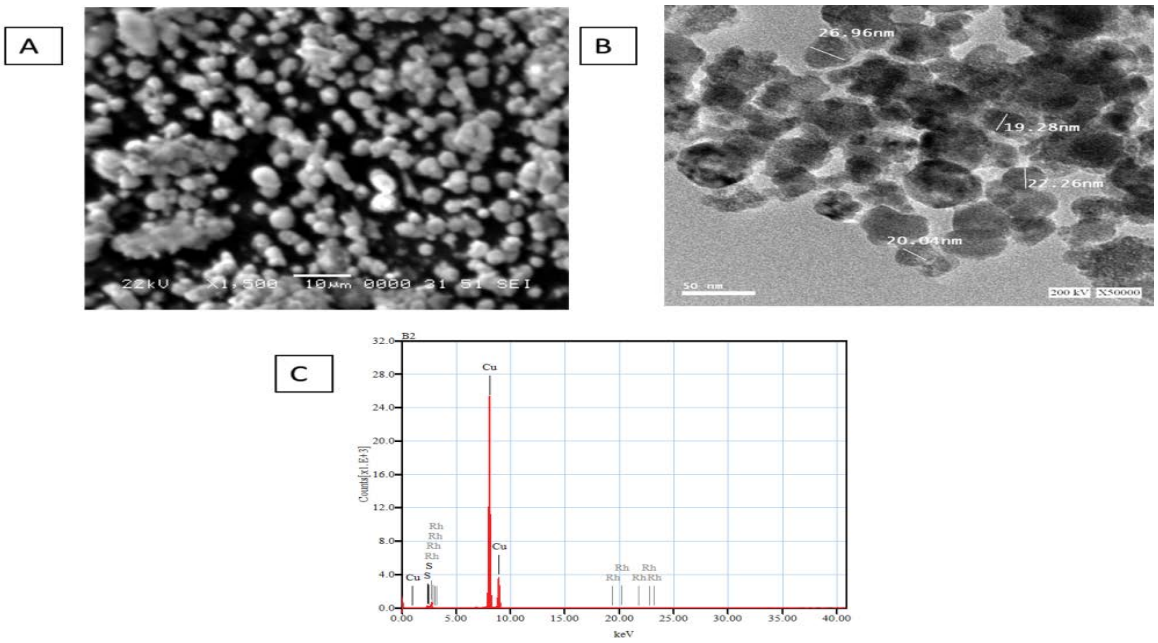

Fig. 1. (a) Scanning electron microscope, (b) transmission electron microscope for cuprous oxide nanoparticles, and (c) X-ray fluorescence of cuprous oxide nanoparticles.

2.3. Animals

B. alexandrina snails were obtained from Egyptian laboratory stock at the Malacology Department, Theodor Bilharz Institute (TBRI), Egypt. Snails were maintained, as stock cultures, in a well-prepared snail room, under suitable environmental conditions, in plastic aquaria (16 cm × 23 cm × 9 cm) containing dechlorinated tap water at a density of 10 snails/L. The snails were fed on oven dried lettuce leaves and blue-green algae (*Nostoc muscorum*) and they were maintained in an air-conditioned room at 24°C and fluorescent light was reflected 30 cm over them during daytime. Lettuce leaves were given daily, and its amount was adjusted as possible to the number and size of the snails and the algae were added weekly. *N. muscorum* algae were originally obtained from Schistosome Biological Supply Center (SBSC) at Theodor Bilharz Research Institute (TBRI) and was then cultivated in Medical Malacology Laboratory. According to Liang et al. [14] water was changed weekly [15,16]. The eggs of these snails were maintained until hatching, growing up and then were used in the experimental tests [16].

2.4. Lethal test

To evaluate the efficacy of the tested copper sulphate and cuprous oxide nanoparticles against adult *B. alexandrina* snails, a stock solution of 1,000 ppm from each compound was prepared according to its active ingredient. A series of concentrations expressed in terms of parts per million (ppm) were prepared from each stock solution that would permit the computation of experimental concentrations LC_{50} and LC_{90} [17]. The LC_0 was estimated at 1/10 of the LC_{50} value [18].

The molluscicide properties of copper sulphate and cuprous oxide nanoparticles against adult *B. alexandrina* snails after 48 h of exposure were followed by another 24 h for recovery.

From the present data (Table 1 and Fig. 2), it was noticed that the LC_{90} values of the tested copper sulphate and cuprous oxide nanoparticles were (LC_{90} = 7.02 and 2.29 ppm, respectively).

It is also seen that cuprous oxide nanoparticles were more toxic than copper sulphate $CuSO_4$ to *B. alexandrina* snails under investigation. Thus, the LC_{50} value of copper sulphate ($CuSO_4$) against the snails was about 2.23 times that of Cu_2O nanoparticles (3.41 and 1.53 ppm, respectively).

2.5. Molluscicide activity of the tested compounds

The present experiment was carried out by preparing three replicates of gradual concentrations from each stock solution. Ten snails (8–10 mm in diameter) were used in each replicate. The snails were exposed to the tested concentrations for 48 h, then removed from the experimental concentration, washed with tap water and kept in 1 L of dechlorinated tap water for another 24 h for recovery (25°C ± 1°C). Unexposed snails (control) were assayed side by side with the treated groups [19]. Dead snails were recorded as the average of the three replicates. Death of snails was distinguished by immersion of snails in a small amount of 15%–20% sodium hydroxide solution [19]; if bubbles and blood come out of the snail, it is recorded as alive and if not, it is recorded as dead. The effectiveness of the compounds as a molluscicide has been expressed in terms of LC_{50} and LC_{90} according to the procedure of Saleh et al. [19].

Table 1
Molluscicide activity of copper sulphate and copper Cu_2O nanoparticles against adult *Biomphalaria alexandrina* snails (48 h exposure)

Lethal conc.	LC_0 (ppm)	LC_{10} (ppm)	LC_{25} (ppm)	LC_{50} (ppm)	LC_{90} (ppm)
Copper sulphate	0.25	0.65	1.51	3.41	7.02
Cuprous oxide NPs	0.16	0.52	1.13	1.53	2.29

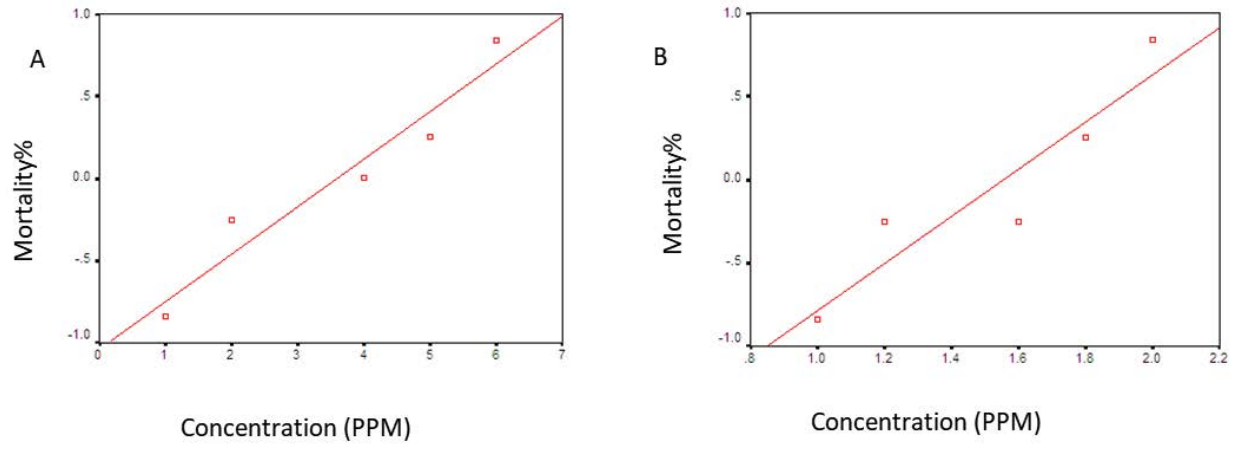

Fig. 2. Molluscicidal activity of the tested compounds against adult *Biomphalaria alexandrina* snails. (a) Copper sulphate and (b) copper Cu_2O nanoparticles.

2.6. Hemolymph

Snail hemolymph was collected using the techniques described by Ibrahim et al. [20]. The hemolymph was obtained via a small hole made in the shell into which a capillary tube was inserted then it was drawn into tube by capillary suction, collected sample tested fresh after drawn from the snails for hemocytes examination by light and electron microscope.

2.7. Light microscopy

Haemolymph smears were fixed in methanol and stained with Giemsa stain [21]. Differential hemocyte counts were performed according to Audu et al. [22].

2.8. TEM analysis of hemocytes

Immediately after the hemolymph collection, the hemocytes were fixed for 15 min in 0.1 M cacodylate buffer at pH 7.2 containing 1% glutaraldehyde, washed in 0.1 M cacodylate buffer at pH 7.2 and postfixed for 20 min with 1% osmium tetroxide in 0.1 M cacodylate buffer. After dehydration with standard serial ethanol, cells were embedded in Epon–Araldite 812 mixture. Sections were obtained with a "Reichert Ultracut S" ultratome (Leica, Austria). Semithin sections were stained with crystal violet and basic fuchsin and observed with a light microscope (Olympus Corporation, Japan). Thin sections were observed with a "JEOL 1010 EX" electron microscope (JEOL, Japan) [23].

3. Results

3.1. Morphology and activity of different categories of hemocytes in hemolymph of adult B. alexandrina snails

The current work was carried out to evaluate the destroying caused in haemocytes of adult *B. alexandrina* snails that had been acutely exposed to each of the tested compounds: copper sulphate and cuprous oxide nanoparticles. A light microscope and electron microscope were carried out on the hemocytes of *B. alexandrina* snails during normal conditions.

3.1.1. By light microscope

Examination of hemocytes monolayers resulted in observation of three morphologically different cell types (plate1): Normal small undifferentiated cell: these cells are undifferentiated and had a spherical profile (Fig. 3a). Normal granulocyte (spreading hemocytes): these cells were filled with a variable number of basophilic granules and have a double membrane (Fig. 3b). Normal hyalinocytes: these cells had a polymorphic profile, with the nucleus located in an eccentric position and might have two nuclei suggesting an atypical cell division. It may be granular or slightly granular (Fig. 3c).

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Several changes occurred to the hemocytes of exposed *B. alexandrina* snails to Cu_2O NPs such as for small undifferentiated cell showing appearance of vacuoles (Fig. 3d). Granulocytes showing with a plenty of granules of different size, irregular cell membrane. Some granulocytes were divided and had 2 nuclei (incomplete cell division), some had no nuclei with the presence of nucleus region, and some had allots of vacuoles (Fig. 3e). Hyalinocytes showed with large vacuolated cytoplasm, the nucleus had been shrunken, some were divided and had 2 nuclei (incomplete cell division), nucleus had been absent, and irregular cell form (Fig. 3f).

Several changes occurred to the hemocytes of exposed *B. alexandrina* snails to copper sulphate ($CuSO_4$) such as for small undifferentiated cells showing the appearance of vacuoles (Fig. 3g). Granulocytes showed plenty of granules of different size, not continuous outer cell membrane, some granulocytes were divided and had 2 nuclei (incomplete cell division), the cell membrane was irregular in shape, and some had allots of vacuoles (Fig. 3h). Hyalinocytes showed with vacuolated cytoplasm, the nucleus had been shrunken, some were divided and had 2 nuclei (incomplete cell division), and irregular cell form (Fig. 3j).

3.1.2. By electron microscope

Examination of hemocytes using an electron microscope showed three morphologically different cell types, small undifferentiated (Fig. 4a), spreading hemocytes or granulocytes (Fig. 4b) and hyalinocytes (Fig. 4c), which were recorded among the light microscopic study. The effect of the two compounds on the hemocytes of *B. alexandrina* exposed to cuprous oxide (Cu_2O) nanoparticles and copper sulphate ($CuSO_4$) with a sub lethal concentration LC_{25} for the two compounds (1.13 and 1.51 ppm, respectively), was studied using electron microscope.

Effect of Cu_2O NPs (LC_{25}) on small undifferentiated cells presented with large nucleus (N) and intact cell membrane, presence of cytoplasmic extensions (pseudopodia-like extensions) and presence of vacuoles and phagolysosomes in the cytoplasm (Fig. 4d). Granulocytes had activated granulocytes with pseudopodia and the presence of cytoplasmic granules and vacuoles in cytoplasm (Fig. 4e). Hyalinocytes have an intact cell membrane, the nucleus is degenerated, and the cellular organelles are difficult to be identified (Fig. 4f).

Effect of $CuSO_4$ (LC_{25}) on small undifferentiated cells presented with large nucleus, intact cell membrane, some vacuoles are found in the cytoplasm and presence of cytoplasmic extensions pseudopodia-like extensions (Fig. 4g). Granulocytes presented with regular cell membrane which extended to form pseudopodia—like extensions, presence of large nucleus with condensed chromatin and the cytoplasm contains phagolysosomes, granules, and mitochondria (Fig. 4h). Hyalinocytes presented with degenerated

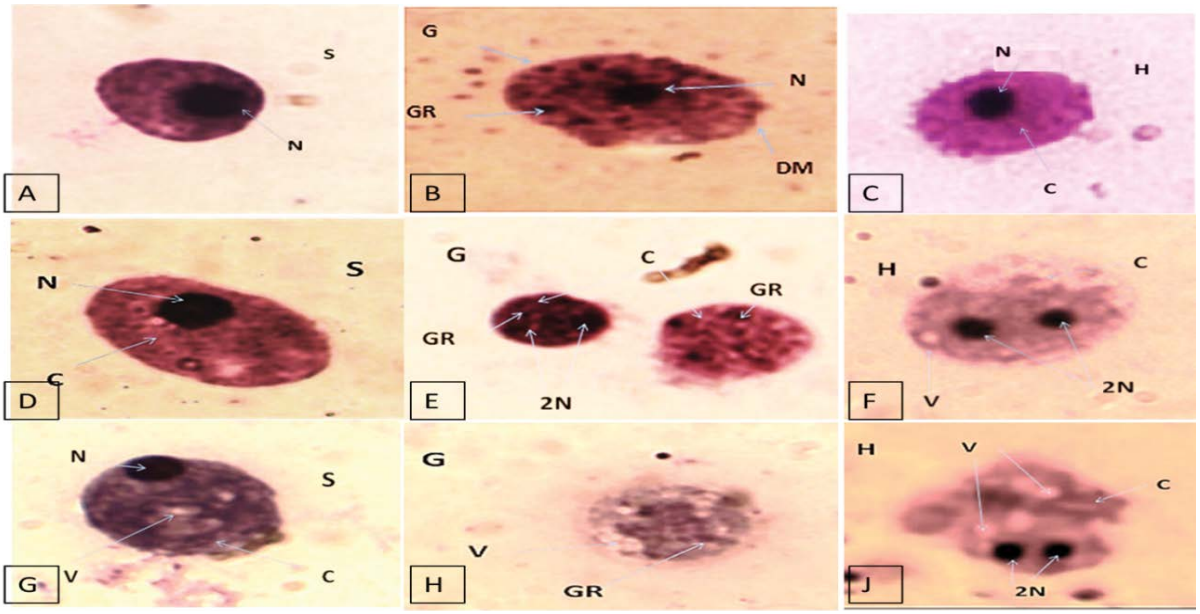

Fig. 3. Light micrograph showing round small (non-differentiated), granulocyte and hyalinocyte of adult *Biomphalaria alexandrina* snails (x40): (a–c) Showing normal round small, granulocyte, and hyalinocyte, respectively. (d–f) Showing round small, granulocyte, and hyalinocyte, respectively exposed to copper nanoparticles. (g–j) Showing round small, granulocyte, and hyalinocyte, respectively exposed to copper sulfate. S: Round small, G: Granulocyte, DM: Double membrane, GR: Granules, N: Nucleus, H: Hyalinocyte, C: Cytoplasm, CM: Cell membrane, NR: Nucleus region, V: Vacuole.

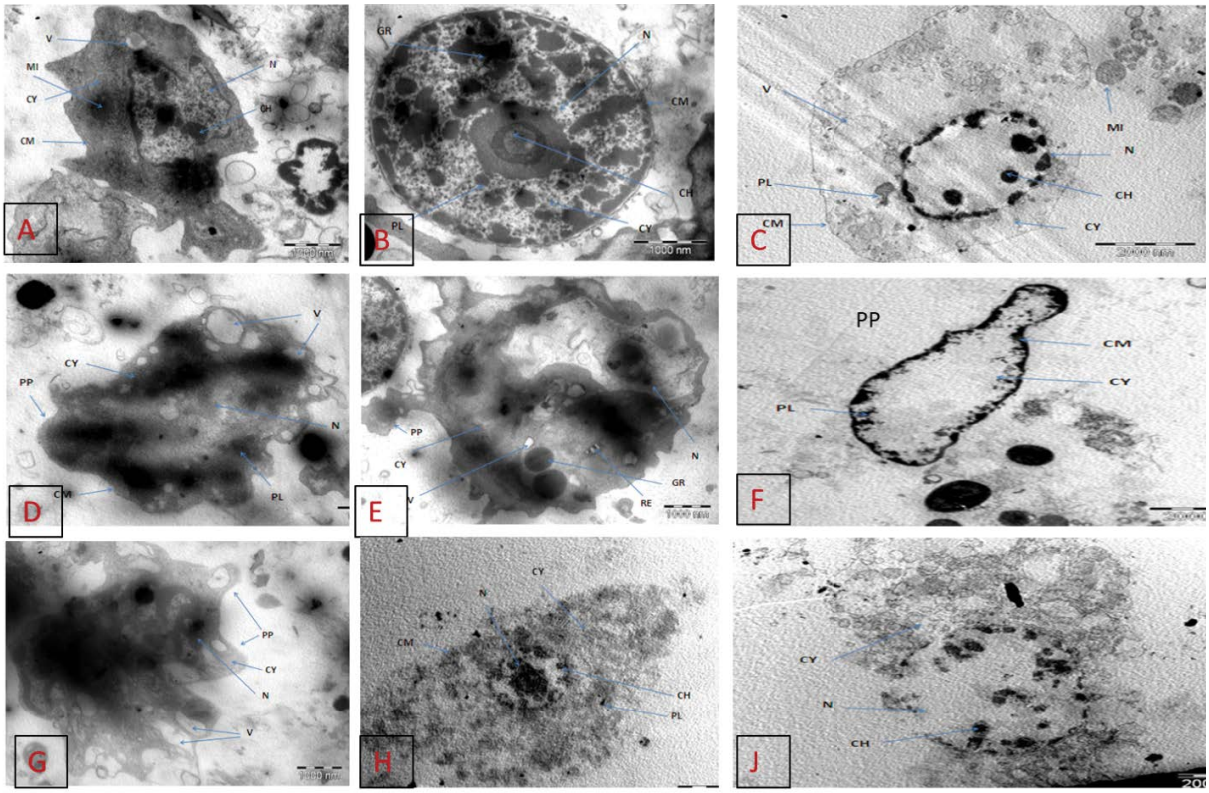

Fig. 4. Electron micrograph showing round small (non-differentiated), granulocyte, and hyalinocyte of adult *Biomphalaria alexandrina* snails (x40): (a–c) Showing normal round small, granulocyte, and hyalinocyte, respectively. (d–f) Showing round small, granulocyte, and hyalinocyte, respectively exposed to copper nanoparticles. (g–j) Showing round small, granulocyte, and hyalinocyte, respectively exposed to copper sulfate. CH: Chromatin, CM: Cell membrane, CY: Cytoplasm, GR: Granules, MI: Mitochondria, N: Nucleus, PL: Phagolysosome, V: VACUOLE, PP: Pseudopodia, RE: Rough endoplasmic reticulum.

outer cell membrane, the internal organelles were difficult to be identified, the nucleus has irregular nuclear membrane, and the nucleus is shrunk in size (Fig. 4j).

4. Discussion

Snails are widely used as indicators of environmental pollution [24]. The freshwater snails are often used to monitor aquatic pollution. The increase in the discharge of chemical contaminants into the aquatic environment has led to an increased urgency for the development of sensitive and reliable methods to assess the impact of these toxic agents on organisms inhabiting lakes, rivers, and seas. The consequences of exposure and the metabolism of chemicals may also be evaluated by investigating biological endpoints, that is adducts and DNA strand breaks [25].

Copper is an essential trace element in all living organisms (bacteria, fungi, plants, and animals), because it participates in different metabolic processes and maintain functions of organisms [26]. The first registration for a copper-containing pesticide, copper sulphate, was issued in 1956, currently 16 copper active ingredients have active food use registrations subject to tolerance reassessment and reregistration review [27].

Copper sulphate is often used as a snail poison against certain pulmonate species that are considered pest organisms on horticulture in Egypt and around the world [28], Copper sulphate was potent molluscicide for many snail species such as, *Biomphalaria glabrata* [29]; *B. alexandrina* [30] and *Lymnaea natalenesis* [31]. Copper sulphate was one of the best molluscicide agents from other sulphate compounds because of the toxicity of copper plus sulphate together. Similar finding was obtained by Rawi et al. [30] who stated that, the tested sulphate salts are descending arranged based on their molluscicide efficiency in the following order $CuSO_4$, $ZnSO_4$, $Fe_2(SO_4)_3$, and finally $(NH_4)_2$ SO_4, respectively, against terrestrial land snail and against aquatic snails including *B. alexandrina*.

Previous work was done to characterize genotoxic effects of Cu_2O-NPs on freshwater snail *B. alexandrina* [7]. The present investigation was designed to specifically emphasize the immunotoxic effect besides the previous proven genotoxic effect of Cu_2O-NPs on freshwater snail *B. alexandrina* and to critically evaluate the utility of this snail as a bio-indicator for Cu_2O-NPs in the aquatic environment.

In the presented study, two type of copper elements were used (ionic and nanoparticles form) $CuSO_4{\cdot}5H_2O$ aqueous solution, and spherical Cu_2O NPs with average diameter 22.1 nm determined by scanning and transmission electron microscope image (SEM, TEM) with determine the consequent of these material by XRF which recorded that 98.5% of elemental composition of Cu_2O NPs powder is Cu. Similar technique (TEM image) was used by Peternela et al. [32].

The presented study supported that ionic and nanoparticles form of copper ($CuSO_4$ and Cu_2O) were toxic to *B. alexandrina* snails and it is also seen that, copper oxide nanoparticles were more toxic than copper sulphate $CuSO_4$ to these snails under investigation. Thus, the LC_{50} value of copper sulphate against the snails was about 2.23 times that of Cu_2O NPs (3.41 and 1.53 ppm with slope 4.41 and 1.54, respectively).

The present study also revealed that survival rates of juvenile *B. alexandrina* snails exposed to the tested compounds were concentration dependent. They were reduced by raising the concentrations of the tested agents from $LC_{0,}$ LC_{10}, and LC_{25} after the exposure period 48 h. However, most of the survived specimens suffered from gradual and continuous death during the recovery period that means the tested agents have a long-lasting toxic effect against these snails. This was previously stated by Ibrahim et al. [18] that *B. glabrata* snails exposed to Colchicine had not recovered by several weeks after the end of the exposure (during the recovery period), and eventually died. Thus, Colchicine appeared to cause irreversible damage that resulted in snails' delayed death. The present findings were also previously reported by Shaldoum et al. [8] using $CuSO_4$ and Cu_2O NPs and by Fayez et al. [33] on the herbicide dithiopyridine, Ibrahim et al. [34] on the insecticides Regent and Mimic, and Abdel-Ghaffar et al. [35] on the pesticides Vertemic and Match against *B. alexandrina* juvenile snails. Vandenberg et al. [36] stated that the relationship between the degree of response of test organisms and the quantity of exposure to the chemical assumes a concentration-response form.

The internal defense system of snails consists of both cellular and humoral components. Circulating hemocytes are the principal line of cellular defense. They can be bound to and kill trematode larva by phagocytosing the syncytial tegument or releasing cytotoxic compounds or both [37].

The present results have shown that the hemocytes, that is responsible for the snails' immune system, were affected by both $CuSO_4{\cdot}5H_2O$ and Cu_2O-NPs. Both light and electron microscopy recorded noticeable abnormalities in small undifferentiated cells, granulocytes, and hyalinocytes. Changes in the affected hemocytes were degenerating or irregular cell membrane sometimes with cytoplasmic extensions, presence of vacuoles and plenty of granules and phagolysosomes within the cytoplasm, organelles were difficult to be identified, and abnormalities in the nucleus and nuclear membrane as having 2 nuclei (incomplete cell division), some had no nuclei with the presence of nucleus region, irregular nuclear membrane, and shrunken nucleus. These abnormal morphological changes will affect the immune function of the snail. Monte et al. [38] have exhibited similar results in their study that herbicide exposure greatly increased the number of dead hemocytes, that may impair the immune system of *B. glabrata* to be more susceptible to parasitic infections. Abaza et al. [39] have also agreed with the present study findings where hemocytes of vulnerable snails appeared by SEM, rounded with smooth or slightly rough surface. However, that of the nonvulnerable snails appeared irregular in shaped with corrugated surface.

It is concluded that, copper oxide nanoparticles (Cu_2O NPs) and copper sulphate ($CuSO_4$) have a considerable molluscicide properties against *B. alexandrina* snails and Cu_2O NPs was more toxic than $CuSO_4$ to these snails under investigation. Moreover, the sub-lethal concentrations of the tested compounds have adverse and deleterious effects on the snails' hematological aspects. Therefore, comprehensive studies are needed to define the proper technique (s) for application of such tested agents in parasites

control aiming to minimize water pollution and saving the non-target organisms in the treated water ecosystem.

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