Biomphalaria alexandrina Snails as a Bio-Monitor for Water Pollution using Genotoxic’s Effect of Cuprous Oxide Nanoparticles and Copper Sulphate

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ABSTRACT

Numerous nanoparticles have in recent years come into use for a variety of purposes and may be released into the environment. The genotoxicity effect of Biomphalaria alexandrina snails as a bio-monitor for the detection of copper water pollution was investigated for cuprous oxide nanoparticles (Cu2ONPs) compared with dissolved Cu2+ ions of copper sulphate (CuSO4). Comet assay (single cell gel electrophoresis assay, SCGA) and micronucleus test (MN) were used as standard methods to monitor the genotoxicity of B. alexandrina snails. Genotoxicity, induced by chronic exposure of B. alexandrina to Cu NPs and Cu2+ of CuSO4, has significantly (P<0.01) increased than the control. Cu2ONPs are more toxic than free Cu2+ ions of CuSO4. The present result confirms the genotoxic and mutagenic potential of CuNPs and Cu2+ free ions on the haemocytes of B. alexandrina snails, and emphasizes the efficiency of B. alexandrina snails in the aquatic ecosystem as bio-monitor for water pollution.

Key words: Copper oxide, copper sulphate, Biomphalaria alexandrina, bio-monitor, genotoxicity, water pollution.

Introduction

Nanotechnology is used to modify material at the nano-scale (<100 nm) to create novel properties. Changes in the physicochemical and structural properties of materials caused by the decrease in particle size can lead to new and sometimes unexpected biological effects. Therefore, engineered nanoparticles (NPs) need to be evaluated in terms of their potential to pose risks to human health and the environment (Handy et al. 2008; Nowack, 2009). Natural NPs, including nano-sized particles of metal oxides, exist in all ecosystems and play important roles in biogeochemical processes (Wigginton et al., 2007).

Numerous NPs have in recent years come into use for a variety of purposes and may be released into the environment (Nowack and Bucheli, 2007; Klaine et al., 2008; Nowack et al., 2012). Although copper (Cu NPs) are not listed under the most abundant nanomaterials in consumer products, they are presently under development for various uses (Anyaugu et al., 2008). CuNPs have properties of interest as conductive materials (e.g. as coatings, inks, pastes, electronic slurries), as catalysts for chemical reactions (US Research Nanomaterials, Inc, see www.us-nano.com, accessed 15 September 2015), and as bactericides and fungicides. The use of CuNPs may thus increase in the next few years and lead to increased release of these NPs into aquatic systems. Dissolution of CuNPs to copper ions may occur under conditions found in natural waters (Mudunkotuwa et al., 2012; Odzak et al., 2014).

Copper is well known to be both an essential and a toxic element for aquatic organisms, with a strong dependence on its concentration and its speciation (Sunda and Guillard, 1976; Sunda and Huntsman, 1995; Morel and Price, 2003).

Toxicity experiments have mostly been conducted with copper oxide NPs (Manusadzianas et al., 2012; Bondarenko et al., 2013; Perreault et al., 2014), whereas the toxicity of elemental copper NPs has only been examined in few cases. Effects of CuNPs on mammalian and fish cell lines have been attributed to a combination of the effects of Cu ions and of specific processes induced by CuNPs, e.g. production of reactive oxygen species (ROS) (Song et al., 2012).

Gastropod mollusks are ubiquitous in the aquatic ecosystem and are considered good bioindicators of contaminants in view of their wide geographic distribution, and their easy availability. Moreover, most of the ecotoxicity studies on snails has been conducted using organism level end-points; such as mortality and growth (Cranef et al., 2002), with only a few studies have been performed that include genotoxic endpoints. In snails, pollutants are transferred by blood cells to the digestive gland, which is one of the major target tissues of

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accumulation. *Biomphalaria alexandrina* is an aquatic pulmonate gastropod mollusk belongs to the family Planorbidae.

Comet assay is considered one of the most promising genotoxicity biomarkers to detect a broad spectrum of DNA lesions with a very high sensitivity in aquatic species (Jha, 2008; Frenzilli *et al.*, 2009). The micronucleus assay due to its potentiality to be applied in any proliferating cell population regardless to the karyotype was successfully applied in aquatic organisms (Hayashi 1998; Bolognesi and Hayashi, 2011).

The present study aims at use *B. alexandrina* snails as a bio-monitor for detection of genotoxicity toxic effects of CuNPs and free Cu\(^{2+}\) ions by using micronucleus test (MN) and comet assay (SCGA).

**Materials and Methods**

**Cuprous oxide nanoparticles (Cu\(_2\)O NPs):**

Nanoparticles were prepared with copper sulphate (CuSO\(_4\)) 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 into 10 mL of CuSO\(_4\), 5H\(_2\)O aqueous solution (0.5 mol/L) (El-Gomhouria Chemical Company, Egypt) with stirring at (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 2000 rpm for 30 min. The product was washed by distilled water and absolute ethanol. The final product was dried in vacuum at 60 °C for 8 h (Selim *et al.*, 2015).

**Characterization of Cu\(_2\)O NPs:**

The surface morphology of Cu\(_2\)O NPs was studied using scanning electron microscopy (SEM) (JEOL JSM-5600). The particles size of the resulting Cu\(_2\)O NPs was analyzed using transmission electron microscope (TEM) (EM 208S Philips, Netherlands) connected to a high resolution imaging system. Samples for TEM studies were prepared by placing drops of nanoparticles solutions on carbon-coated TEM copper grids.

**Snails:**

*Biomphalaria alexandrina* snails were obtained from Malacology Department, Theodor Bilharz Institute, Egypt. Snails were maintained, as stock cultures, in a well-prepared snail room, under suitable environmental conditions, in aquaria containing dechlorinated tap water in a density of 10 snails/L. The snails were fed on fresh lettuce leaves, supplemented with tetramine and chalk, after careful selection on the basis of size and age snails were selected for investigation.

**Toxicity test:**

The present experiment was carried out by preparing three replicates of gradual concentrations from each stock solution compared with control group. Ten snails (8-10mm shell width) were used in each replicate and exposed to the tested concentrations for 48 hours, then removed from the experimental concentration, washed with tap water and kept in 1 liter of dechlorinated tap water for next 24 hours for recovery (25±1°C). Unexposed snails (control) were assayed side by side with the treated groups (WHO, 1965 b). Dead snails were recorded as the average of the three replicates and control. Death of snails was distinguished by immersion of snails in a small amount of 15–20% sodium hydroxide solution (Nolan *et al.*, 1953); if bubbles and blood come out of snail, it is recorded as alive and if not, it is recorded as dead. The effectiveness of these components as a molluscicide has been expressed in terms of LC\(_{50}\) and LC\(_{90}\) according to the procedure of Litchfield \& Wilcoxon (1949).

**Hemolymph:**

Snail hemolymph was collected using the techniques described by Michelson (1966). The hemolymph was obtained via a small hole made in the shell into which capillary tube was inserted then it was drawn into tube by capillary suction, collected sample tested fresh after drawn from the snails.

**The micronucleus test (MN):**

The MN test was adapted to the characteristics of the test organism based on protocols described for mussel species (Pavlica *et al.*, 2000). The present study contain three groups, each group contain 20 *B. alexandrina* snails. The first group treated with Cu\(_2\)O NPs in three concentrations and sub divided in to three sub groups: the first was LC\(_{25}\) chronic exposure 9 days continuously to (1.13 PPM) with the exchange of solution every 3 days; the second sub group was exposed to LC\(_{50}\) (1.53 PPM) one day and then recovery until 6 days of the week and repeat these procedure three times) and the third sub group was exposed one day to LC\(_{90}\) (2.29 PPM) and then recovery until 6 days of the week and repeated these procedure three times, the second group treated with CuSO\(_4\) also in three concentrations, and sub divided in to three sub groups. The first was
LC25 chronic exposure 9 days continuously to (1.51PPM) with the exchange of solution every 3 days and then recovery until 6 days of the week and repeat these procedure three times) and the third sub group was exposed to LC50 (3.41 PPM) one day and then recovery until 6 days of the week and repeat these procedure three times) and the third group represented as a control.

Hemolymph was collected from tested and control snails then fixed with an equal volume of sodium citrate (1%) for 5 min and with 40% formalin solution (4%) for 10 min. After fixation, hemolymph was centrifuged for 8 min at 1000 rpm. The supernatant was removed, leaving a sufficient quantity to re-suspend the cells. These were gently placed on the slide to prevent membrane damage. After overnight drying at room temperature, slides were stained for 15 min with Giemsa at 5%. One thousand cells with complete cytoplasm were analyzed in each slide, and the presence or absence of MN was recorded. Each slide was produced from a group of 5 snails, and four slides were prepared per concentration. Micronucleus were identified according to the following criteria: spherical cytoplasmic inclusions with a defined contour, diameter smaller than approximately 1/3 of the main nucleus, color and texture similar to the nucleus, and absence of contact with the nucleus (Pavlica et al., 2000).

The comet assay (single cell gel electrophoresis assay, SCGA):

Cell suspensions are harvested by centrifugation and resuspend cells at 1 x 10⁵ cells/ml in ice cold 1X PBS (phosphate buffer saline). This suspension was stirred for 5 min and filtered. Cell suspension (100 μl) was mixed with 600 μl of low-melting agarose (0.8% in PBS). 100 μl of this mixture was spread on pre-coated slides. The coated slides were immersed in lyses buffer (0.045 M TBE (Tris/Borate/EDTA), pH 8.4, containing 2.5% SDS (sodium dodecyl sulphate)) for 15 min. The slides were placed in the electrophoresis chamber containing the same TBE buffer, but devoid of SDS. The electrophoresis conditions were 2 V/cm for 2 min and 100 mA, staining with ethidium bromide 20μg/ml at 4°C. The DNA fragment migration patterns of 100 cells for each dose level were evaluated with a fluorescence microscope at 510nm. The comets tails lengths were measured from the middle of the nucleus to the end of the tail with 40x increase for the count and measure the size of the comet. For visualization of DNA damage, observations are made of EtBr-stained (Ethidium Bromide Staining) DNA using a 40x objective on a fluorescent microscope (Singh et al., 1988).

Statistical analysis:

Data were analyzed applying the Chi Square and T-test, and ratio test to achieve these statistical Tests, Minitab software (MINITAB® Release 14.1) was used.

Results

The current work was carried out to evaluate the genotoxicity caused in hemocytes of adult B. alexandrina snails that had been chronically exposed to each of the tested compounds: Cu2+ (copper sulphate) and Cu2O NPs (cuprous oxide nanoparticles). This genotoxicity is determined by comet assay (or single cell gel electrophoresis assay, SCGA) and micronucleus (MN) techniques.

The morphology of the produced Cu oxide NPs was examined by SEM. Fig. 1a depicts the SEM images of Cu2O NPs. It shows that the copper oxide NPs are flower shaped. The main size of particles using TEM image be 22.1 nm (Fig. 1b).

![Fig. 1: a) SEM and b) TEM of cuprous oxide nanoparticles (Cu2O NPs).](image-url)
Toxicity test:
This experiment was planned to elucidate the molluscidal properties of CuSO₄ and Cu₂O NPs against adult B. alexandrina snails after 48 hours (hrs) of exposure followed by another 24 hrs for recovery. From the present data (Table 1), it was noticed that, on the basis of LC₉₀ values of the tested CuSO₄ and Cu₂O NPs (LC₉₀= 7.02 and 2.29 ppm, respectively). It is also seen that, Cu₂O NPs was more toxic than copper sulphate(CuSO₄) to B. alexandrina snails under investigation. Thus, on the basis of LC₉₀ values, CuSO₄ toxicity against the snails was about 2.23 times that of Cu₂O NPs (3.41 and 1.53 PPM with slope 4.41 and 1.54 respectively).

Table 1: Molluscidal activity of copper sulphate (CuSO₄) and cuprous oxide nanoparticles (Cu₂O NPs) against adult Biomphalaria alexandrina snails (48 hours exposure)

<table>
<thead>
<tr>
<th>Lethal conc.</th>
<th>LC₅₀ Ppm</th>
<th>LC₁₀ Ppm</th>
<th>LC₂₅ Ppm</th>
<th>LC₅₀ Ppm</th>
<th>LC₉₀ Ppm</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuSO₄</td>
<td>0.25</td>
<td>0.65</td>
<td>1.51</td>
<td>3.41</td>
<td>7.02</td>
<td>4.41</td>
</tr>
<tr>
<td>Cu₂O NPs</td>
<td>0.16</td>
<td>0.52</td>
<td>1.13</td>
<td>1.53</td>
<td>2.29</td>
<td>1.54</td>
</tr>
</tbody>
</table>

Comet assay:
The present results in Table 2 and Fig.2 shows that there is a high significant increase in the tail moment (TM) of B. alexandrina snails chronically, 9 days, that exposed to LC₂₅ of CuSO₄ (P<0.01) being 5.85±0.7, where there has been no significant increase in tail length (2.31±0.25). Moreover, a high significant increasing was recorded in the tail moment (P<0.01) and a significant increasing in the tail length DNA of B. alexandrina snails chronically, 9 days, that exposed to LC₂₅ of Cu oxide NPs (P<0.05) being 6.70±0.6 and 2.7±0.04 respectively.

Table 2: Comet assay test for Biomphalaria alexandrina exposed to chronic sub lethal doses (LC₂₅) of CuSO₄, CuO NPs and control.

<table>
<thead>
<tr>
<th>Exposure</th>
<th>% Tailed</th>
<th>% Untailed</th>
<th>µm Tail length</th>
<th>Tail DNA %</th>
<th>UNIT Tail moment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.00±0.91</td>
<td>94.00±2.90</td>
<td>1.52±0.35</td>
<td>1.64±0.28</td>
<td>2.49±0.80</td>
</tr>
<tr>
<td>CuSO₄ LC₂₅</td>
<td>13.25±1.30</td>
<td>86.75±3.00</td>
<td>2.31±0.25</td>
<td>2.52±0.20</td>
<td>5.85±0.70**</td>
</tr>
<tr>
<td>Cu₂O NPs LC₂₅</td>
<td>14.00±1.80</td>
<td>86.00±4.70</td>
<td>2.70±0.04*</td>
<td>2.65±0.25</td>
<td>6.70±0.60**</td>
</tr>
</tbody>
</table>

*P< 0.05, Data expressed as Mean± Standard Error

Fig. 2: Comet assay test of haemolymph of B. alexandrina snails exposed to 1) control, 2) CuSO₄, 3) Cu₂O NPs.

Micronucleus test:
For snail groups of Cu oxide NPs, there is a high significant increase (P<0.01) in the MN cells of B. alexandrina snails that exposed to NPs for all three concentrations (LC₂₅, LC₅₀ and LC₉₀) compared to the control. Meanwhile, there is also a significant increase (P<0.05) in the MN cells of snail exposed to LC₉₀ and LC₅₀ concentrations of Cu NPs compared to that exposed to LC₂₅ (Table 3, Fig.3).

Table 3: Micronucleus test for Biomphalaria alexandrina exposed to LC₂₅, LC₅₀ and LC₉₀ of Cu₂O NPs.

<table>
<thead>
<tr>
<th>LC of Cu₂O NPs</th>
<th>Mean No. of MN/Slide</th>
<th>P Value to control</th>
<th>P Value to LC₂₅</th>
<th>P Value to LC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.50±0.65</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>LC₂₅</td>
<td>7.25±1.10</td>
<td>0.01**</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>LC₅₀</td>
<td>17.25±3.97</td>
<td>0.008**</td>
<td>0.013*</td>
<td>--</td>
</tr>
<tr>
<td>LC₉₀</td>
<td>17.50±2.60</td>
<td>0.008**</td>
<td>0.023*</td>
<td>0.910</td>
</tr>
</tbody>
</table>

*P< 0.05, **P< 0.01, Data expressed as Mean± Standard Error
For CuSO₄ group, high significant increasing \((P<0.01)\) was revealed in the MN cells of *B. alexandrina* snails that exposed to CuSO₄ for all three concentrations (LC₂₅, LC₅₀ and LC₉₀) compared to the control. Moreover, there is a significant increase \((P<0.05)\) in the MN cells of snail exposed to LC₅₀ and LC₉₀ concentrations compared to that exposed to LC₂₅ concentration. But, there is no significant increase between the snails exposure to LC₅₀ and LC₉₀ concentrations (Table 4, Fig 3).

**Table 4:** Micronucleus test for *Biomphalaria alexandrina* exposed to LC₂₅, LC₅₀ and LC₉₀ of CuSO₄.

<table>
<thead>
<tr>
<th>LC of CuSO₄</th>
<th>Mean No. of MN/slide</th>
<th>(P) Value to control</th>
<th>(P) Value to LC₂₅</th>
<th>(P) Value to LC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.5±0.65</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>LC₂₅</td>
<td>9.5±0.86</td>
<td>0.008**</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>LC₅₀</td>
<td>19.25±2.10</td>
<td>0.005**</td>
<td>0.024*</td>
<td>--</td>
</tr>
<tr>
<td>LC₉₀</td>
<td>19.75±2.30</td>
<td>0.005**</td>
<td>0.025*</td>
<td>0.879</td>
</tr>
</tbody>
</table>

\* \(P<0.05\), ** \(P<0.01\) and Mean± Standard Error

Comparison between the results of MN test for hemocytes of *B. alexandrina* snails exposed to LC₂₅, LC₅₀ and LC₉₀ of Cu₂O NPs and CuSO₄ showed that there are no significant increase in the MN cells in all three lethal and sub lethal doses (Table 5, Fig 3).

**Table 5:** Comparison between MN tests for *Biomphalaria alexandrina* exposed to LC₂₅, LC₅₀ and LC₉₀ of Cu₂O NPs and CuSO₄.

<table>
<thead>
<tr>
<th>LC of Cu₂O NPs</th>
<th>Mean No. of MN/slide</th>
<th>(P) Value to LC₂₅</th>
<th>(P) Value to LC₅₀</th>
<th>(P) Value to LC₉₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC₂₅(1.13 PPM)</td>
<td>7.25±1.10</td>
<td>0.170</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LC₅₀(1.53 PPM)</td>
<td>17.25±1.03</td>
<td></td>
<td>0.570</td>
<td></td>
</tr>
<tr>
<td>LC₉₀(2.29 PPM)</td>
<td>17.50±2.60</td>
<td></td>
<td></td>
<td>0.546</td>
</tr>
</tbody>
</table>

Data expressed as Mean± Standard Error

![Fig. 3: Micronucleus test of a) control haemocyte, b) Haemocyte with equal size MN, c) Haemocyte with variable size MN, d) Haemocyte with start budding new cell and e) Haemocyte with full budding and migration of MN.](image)

**Discussion**

Copper-based compounds have been intentionally introduced into the environmental components as molluscicides, fungicides, algicides, and herbicides. The increasing use of these compounds is producing worldwide pollution risks (Halliwell and Gutteridge, 2007). Copper sulphate (CuSO₄) is often used as a snail poison against certain pulmonate species that are considered pest organisms on horticulture in Egypt (El-Wakil...
and Mesbah, 1995) and around the world (Godan, 1983). On the other hand, recent advances in the synthesis and development of nanoparticles (NPs) for wide applications have led to a serious threat to both human and environmental health. NPs are highly reactive and catalytic in nature compared to their ions or bulk counterparts and thus applicable in various fields including drug delivery, electronics, optics, and therapeutics. Due to these applications, many varieties of NPs in massive amounts are being industrially produced. These NPs are discharged into the environment and thus providing a path to enter into food chain via microorganisms and eventually disturbs the ecological balance (Niazí and Gu, 2009).

Current study has shown that, comet assay directly correlates single strand breaks (SSBs) with the tail moment. The SSBs is defined by Kumaravel and Jha (2006) as the product of the distance between the head and the center of gravity of DNA in the tail and the percentage of DNA in the comet tail. The current results of alkaline comet assay have demonstrated that the level of SSBs induced by CuNPs was significantly higher than that in control group.

The NPs exhibit toxicity to living organisms mainly because of their small size (<100nm), large surface-to-volume ratio and highly reactive facets. Before these NPs enterin to the food chain, it is imperative to evaluate the toxicity associated with NPs in microorganisms. Toxicities associated with NPs in microorganisms are mainly related to their nano-size that cause membrane disorganization, generation of reactive oxygen species (ROS) and in some cases, oxidative DNA damage (Niazí and Gu, 2009).

The current work has also shown that genotoxicity induced by CuSO₄ was also significantly higher than that in control group. The same results obtained by (Trivedi et al., 2012) explaining that when copper sulphatepentoxide (CuSP) dissolves in the pond, it breaks down into copper ions that are presumed to be toxic to algae, parasites and fish. In ponds that have high pH and alkalinity, copper ions quickly react with ions stay in water for such a long period that it is difficult to kill algae and parasites without killing the fish. Copper sulfate is toxic to aquatic invertebrates, such as crab, shrimp, and oysters. The 96-hour LC5₀ of copper sulfate to pond snails is 0.39 mg/L at 20°C. Also, Hinton et al.(1990) stated that copper is present as a cofactor in various enzymes (e.g. Cu, Zn-superoxide dismutase, cytochrome oxidase etc.) but as a toxic substance it can able to bind the cysteine side chains of proteins and to form multidentate complexes with histidine and tryptophan side chains of proteins which is responsible for its toxicity. Also, Segner et al. (1998) stated that Cu is known to induce the formation of reactive form of oxygen which can produce enzymatic deactivation, lipid peroxidation and DNA damage. Copper has a great capacity to alter membrane structural lipids and could provoke membranous disruption (Mirkovic et al., 2015 and RED, 2009). These authors have also stated that the copper ions will link to various chemical groups (imidazoles, phosphates, sulfhydryls, hydroxyls) presented in many proteins and disrupt the function of these proteins and enzymes, resulting cell damage and membrane leakage. Thus, the mode of action of copper hydroxide (or any other copper derivatives) is the nonspecific denaturation (disruption) of cellular proteins. Also, Gaetke and Chow (2003) stated that excessive amounts of Cu can oxidize important biomolecules, such as lipids, proteins, and DNA, mainly through the Fenton reaction. Free Cu ions can be involved in ROS generation. Both cupric (Cu²⁺) and cuprous (Cu⁺) ions can participate in oxidation and reduction reactions to form hydroxyl radicals via the Haber-Weiss reaction as explained by Bremner (1998):

\[
\begin{align*}
    \text{O}_2^- + \text{Cu}^{2+} & \rightarrow \text{O}_2 + \text{Cu}^+ \\
    \text{Cu}^+ + \text{H}_2\text{O}_2 & \rightarrow \text{Cu}^{2+} + \text{OH}^- + \text{HO}^-
\end{align*}
\]

Generation of hydroxyl radicals has been confirmed by analysis of the products of DNA damage (Gaetke and Chow, 2003). Copper binds readily to DNA to form adducts. The endogenous DNA-associated copper could promote local production of hydroxyl radicals and hence oxidative damage to DNA. The fact that copper accumulates within the nucleus at copper overload obviously enhances the likelihood of such reactions occurring (Sagripanti et al., 1991) In this case, formed complex Cu-DNA promotes hydroxyl radical-dependent DNA fragmentation.

The current results of the SCGA and MN test demonstrated increasing in DNA damage for hemocytes of B. alexandrina snail exposed to Cu NPs and CuSO₄. Another study has also shown that the significant increase in DNA damage observed in golden mussel hemocytes by the SCGA at the same concentrations that induced a significant increase in MN frequency (3.75 and 7.50 µg/ml), indicates that the damage caused by copper is equally detected by both tests (Villela et al., 2006). These results imply that CuSO₄ is responsible for clastogenic damage. Guecheva et al. (2001), using the SCGA, found similar genotoxic effects of CuSO₄ in plankanian individuals. Bolognesi et al. (1999), using the alkaline elution method, and analyzing the frequency of MN in mussel gills (Mytilus galloprovincialis), also recorded an increase in the number of DNA strand breaks induced by copper chloride.

Although the mechanisms of nanoparticle toxicity are not well understood, the findings to date suggest that both ionic copper and nano-particulate copper are responsible for the toxicity that is produced. Copper NPs can induce toxicity by mechanisms that are different from those of soluble ions. Iron and copper can act via the redox cycle to produce reactive oxygen species (ROS), which may cause DNA strand breaks (Cheeseman et al., 1993). The genotoxic agents increase oxygen consumption in exposed animals. This is caused by reduced control of the mitochondrial respiratory chain, due to oxidative phosphorylation uncoupling (Pavlina et al., 2000).
High oxygen consumption may cause higher ROS production (Roszell et al., 1996). Oxidative DNA damage generated by ROS, such as those that inevitably appear during respiration, has been attributed to increased damage of gill cell’s DNA of Mytilus edulis (Wilson et al., 1998). The main lesions caused by oxidative stress are simple breaks, the change of apurinic bases and sites. These lesions are predominantly repaired by base excision (Villela et al., 2006).

The induction of SCGA and MN in molluscs exposed to these samples (Cu\(^{2+}\) and Cu NPs) confirms not only the presence of genotoxicity, but also of mutagenicity. Current data show that while genotoxicity of Cu NPs and Cu\(^{2+}\) of CuSO\(_4\) was significantly increased, the LC\(_{50}\) of Cu NPs concentrations was lower than LC\(_{50}\) of CuSO\(_4\) which indicates that Cu NPs has genotoxicity more than the CuSO\(_4\). Existing studies indicate that copper toxicity strongly depends on particle size. As particle size decreases, toxicity increases. In the studies that have been performed, there is a 15- to 65-fold increase in toxicity when nano-sized copper particles are used. In most studies, the increase in nano-copper toxicity is attributed to an increase in solubility and, consequently, bioavailability (Aruoja et al., 2009; Heinilaan et al., 2008; Mortimer et al., 2010). However, increased solubility does not always explain increased nano-copper toxicity. When exposed to equivalent bio-available amounts of nano- and soluble metal-forms, gill copper uptake was identical in zebra fish. However, nano-copper caused greater damage to the gill. Nano-copper produced different morphological effects and global gene expression patterns in the gill than did soluble copper ions alone (Griffitt et al., 2007, 2008, and 2009). Similarly, (Kasemets et al., 2009) reported that soluble copper ions explained 50% of nano-copper toxicity in yeast. In vitro studies provided evidence to show that copper NPs have the ability to cause mitochondrial (Karlsson et al., 2009) and DNA damage (Midander et al., 2009).

Different methods have been established to evaluate DNA alterations. The 32P-postlabelling assay is a highly sensitive procedure mainly applied to the detection of bulky aromatic adducts deriving from complex mixture of environmental pollutants. This technique was applied in a number of aquatic species (Dolcetti et al., 2002), but, due to the complexity of the experimental protocol, is not commonly used in large environmental bio-monitoring programs. Alkaline elution is based on the evidence that the rate at which DNA single strand fragments pass through a membrane filter under alkaline conditions is related to the length of the DNA strand itself (Kohn et al., 1976). This assay measures the extent of single and double strand breaks, DNA-DNA and DNA-protein cross-linking and it was successfully applied in fish and invertebrates exposed to chemical compounds in aquatic environment (Bolognesi et al., 2006). However, the SCGA depend on the same bases of the alkaline elution assay. The DNA alkaline unwinding assay was also used to detect DNA damage caused by complex environmental contamination in aquatic test organisms (Oliveira et al., 2010). In this assay, whole cells or crude DNA extracts are subjected to alkaline assay conditions to allow controlled “unwinding” of double-stranded DNA into single-stranded DNA, beginning at each strand break. The method for quantifying strand breaks uses fluorescent dyes, binding with a high affinity to the intact double stranded. More recently comet assay was developed for detecting DNA damage at the individual cell level. Its use has spread to a variety of areas, including environmental monitoring and genetic ecotoxicology. This technique is now considered one of the most promising genotoxicity biomarkers to detect a broad spectrum of DNA lesions with a very high sensitivity in aquatic species (Frenzilli and Lyons, 2013).

The current result confirms the genotoxic and mutagenic potential of Cu NPs and CuSO\(_4\) to haemocytes of B. alexandrina snail. However, nanoparticles are more toxic than CuSO\(_4\). This study also emphasizes the efficiency of B. alexandrina snail as an organism to bio monitor genotoxicity. The conditions of the study are limited, but it still allows us to suggest that this mollusc can be used as a bio-monitoring organism for the evaluation of genotoxicity in aquatic environments. These snails are present in contaminated fresh water, in large populations with no sampling restrictions. The sensitivity and applicability of the micronucleus and comet assay tests for the evaluation of genotoxicity was also confirmed.

References


