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Genotoxicity of copper oxide and silver nanoparticles in the mussel *Mytilus galloprovincialis*

Tânia Gomes, Olinda Araújo, Rita Pereira, Ana C. Almeida, Alexandra Cravo, Maria João Bebianno*

CIMA, Faculty of Science and Technology, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

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ABSTRACT

Though there is some information on cytotoxicity of copper nanoparticles and silver nanoparticles on human cell lines, there is no information on their genotoxic and cytotoxic behaviour in bivalve molluscs. The aim of this study was to investigate the genotoxic impact of copper oxide and silver nanoparticles using mussels *Mytilus galloprovincialis*. Mussels were exposed to 10 μg L^{-1} of CuO nanoparticles and Cu^{2+} and Ag nanoparticles and Ag^{+} for 15 days to assess genotoxic effects in hemocytes using the comet assay. The results obtained indicated that copper and silver forms (nanoparticles and ionic) induced DNA damage in hemolymph cells and a time-response effect was evident when compared to unexposed mussels. Ionic forms presented higher genotoxicity than nanoparticles, suggesting different mechanisms of action that may be mediated through oxidative stress. DNA strand breaks proved to be a useful biomarker of exposure to genotoxic effects of CuO and Ag nanoparticles in marine molluscs.

1. Introduction

Nanoparticles exhibit novel electrical, catalytic, chemical and magnetic properties that can be applied in several commercial and industrial applications that have led to its increased production and use. Inevitably these particles will end up in the environment, accumulate and interact with the biota and their potential genotoxic effects could have short and long-term consequences in aquatic organisms (Handy et al., 2008; Moore, 2006; Singh et al., 2009).

Copper nanoparticles (CuO NPs) are commonly used in many industrial and consumer applications mostly because of its antimicrobial properties, as well as for its elevated thermal and electrical conductive efficiency. These nanoparticles are used in air and liquid filtration, in coatings on integrated circuits and batteries, catalysts, microelectronics and cosmetics, among others (Ahamed et al., 2010; Fahmy and Cormier, 2009; Griffitt et al., 2007; Karlsson et al., 2008). The use of silver nanoparticles (Ag NPs) incorporated in consumer products has become common in the last years because of the antimicrobial and antibacterial effect of the silver ion (e.g. Luoma, 2008). Industry makes use of this new technology in food contact applications, in building materials, wound dressings, socks, and other textiles, air filters, medical equipment and textiles, toothpaste, baby products, vacuum cleaners and washing machines (www.nanoproject.org). Accordingly, these NPs may already exist in the environment, but at present there is no information regarding its levels in the aquatic compartment.

Overproduction of reactive oxygen species (ROS) is commonly considered the major source of genotoxicity that has been observed after exposure to many types of NPs that have been found to cause DNA-strand breaks, point mutations, oxidative DNA adducts, and chromosomal fragmentation (Bhatt and Tripathi, 2011; Handy et al., 2008; Karlsson, 2010; Singh et al., 2009). CuO NPs possess redox cycling properties with the capacity to generate intra- and extracellular generation of reactive oxygen species (ROS) due to a combination between the particle effect and the dissociation of copper ions from the NPs (Fahmy and Cormier, 2009; Gomes et al., 2011; Griffitt et al., 2009). Ag NPs also generates ROS resulting from the release of silver ions, NPs properties (e.g. size and/or shape) or a combination of both (Asharani et al., 2008; Fabrega et al., 2011; Griffitt et al., 2009). Oxidative stress is a significant mechanism of toxicity of CuO and Ag NPs, but their underlying mechanism of action are still uncertain (Ahamed et al., 2010; Asharani et al., 2009; Fahmy and Cormier, 2009; Gomes et al., 2011). Organisms have developed enzymatic and non-enzymatic antioxidant defence mechanisms to prevent and intercept ROS, as well as repair systems for oxidized components. When the rate of ROS production exceeds the antioxidant defences and repair mechanisms, oxidative stress occurs leading to oxidation of key cellular components as proteins,

* Corresponding author. Tel.: +351 289800100; fax: +351 289800069.
E-mail address: mbebian@ualg.pt (M.J. Bebianno).

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DNA, lipids and carbohydrates (Halliwell and Gutteridge, 1984; Valavanidis et al., 2006). In mussel gills and digestive glands, oxidative stress after long-term exposure to CuO NPs and Ag NPs was evidenced by induction or reduction of the antioxidant defence system, lipid peroxidation, as well as metallothionein induction (Gomes et al., 2011, 2012; Gomes et al., own data). Given the capacity of both CuO and Ag NPs to generate oxidative stress and oxidative damage, it becomes imperative to address the genotoxic potential of these particles, principally in bivalve species where scarce information exists. Though there is some information on cytotoxicity of Cu and Ag NPs on human cell lines, the information on genotoxic and cytotoxic behaviour of these nanoparticles in aquatic organisms is scarce (e.g. Ahamed et al., 2010; Asharani et al., 2009; Fahmy and Cormier, 2009; Park and Choi, 2010). Only Gagné et al. (2008) and Kádar et al. (2011) addressed DNA damage in the mussels *Elliptio complanata* and *Mytilus galloprovincialis* after exposure to cadmium telluride quantum dots and nano iron, respectively. DNA strand breaks (single and double) represent one of the major oxidative damage to DNA via oxidative stress that is generally assessed by the Comet assay. This methodology has been extensively used to evaluate the genotoxic effects of contaminants (as metals) in bivalves' hemocytes (e.g. Almeida et al., 2011; Al-Subiai et al., 2011; Steinert, 1995; Valavanidis et al., 2006) that are a potential target for nanoparticles genotoxicity (Canesi et al., 2010, 2012; Gagné et al., 2008; Kádar et al., 2011; Moore et al., 2009).

The main goal of the current work was to assess of the genotoxic potential of CuO NPs and Ag NPs in the mussel *M. galloprovincialis* exposed to 10 $\mu g\ L^{-1}$ either in nano or in the ionic form. The comet assay was applied to detect DNA damage (single, double strand breaks and alkali labile sites) in hemolymph cells (hemocytes) of mussels.

2. Materials and methods

2.1. Nanoparticles preparation and characterization

CuO and Ag nanoparticles were obtained from Sigma–Aldrich (Germany) with the particle size specified as <50 nm and <100 nm, respectively. NPs stock solutions (CuO NPs and Ag NPs) were prepared in bi-distilled water (10 $\mu g\ L^{-1}$), sonicated for 30 min (Ultrasonic bath VWR International, 230 V, 200 W, 45 kHz frequency) and kept in constant shaking. Ionic stock solution (Cu^{2+} and Ag^{+}; 10 $\mu g\ L^{-1}$) were prepared identically but not sonicated. NPs were characterized in terms of particle size, aggregation behaviour in natural seawater using transmission electron microscopy (TEM) (JEOL JEM-1230) equipped with a digital camera (model 785 ES1000W) and by dynamic light scattering (DLS) using an ALV apparatus with Ar ion lased (514.5 nm). More details on the characterization methods are described in Gomes et al. (2011).

2.2. Experimental design

Mussels *M. galloprovincialis* (Cu exposure: 61.7 ± 8.4 mm and Ag exposure: 63.2 ± 5.8 mm) were collected in the Ria Formosa Lagoon (South of Portugal) and acclimated for 7 days in natural seawater at constant temperature and aeration. Fifty mussels were placed in 25 L aquaria, filled with 20 L of natural seawater (around 2.5 mussels/L) in a triplicate design (3 tanks per treatment) and exposed to 10 $\mu g\ L^{-1}$ of CuO NPs and Ag NPs and corresponding ionic form, along with a control group for a period of 15 days. The Cu and Ag concentrations chosen are environmentally relevant and reported for several aquatic systems (Bryan and Langston, 1992; Luoma, 2008). Water, copper and silver solutions were renewed every 12 h to avoid nanoparticles aggregation. Before each renewal, CuO and Ag NPs solutions were sonicated for 30 min (Ultrasonic bath VWR International, 230 V, 200 W, 45 kHz frequency) to break down the size of aggregates. Physico-chemical parameters were measured daily for Cu exposure: temperature (17.8 ± 1.1 °C), salinity (36.3 ± 0.2), percentage of oxygen saturation (97.8 ± 4.9%) and pH (7.8 ± 0.07); and Ag exposure: temperature (17.6 ± 0.3 °C), salinity (36.3 ± 0.1), percentage of oxygen saturation (96.9 ± 3.3%) and pH (7.8 ± 0.05). Multiple sampling was performed (3, 7 and 15 days) where mussels were collected and biotic parameters measured. Mussels were not fed and no mortality was detected during the exposure period.

2.3. Metal analysis

Copper and silver were analysed in water samples collected 12 h before water renewal and re-dosing from the NPs and ionic exposure groups, as previously described in Gomes et al. (2011). Briefly, total metal concentrations from both exposures were determined after acid digestion with 2% nitric acid (HNO_3), while dissolved Cu and Ag from CuO and Ag NPs exposures were determined after water filtration (0.02 μm filter, Anotop 25, Whatman) and acid digestion (Griffitt et al., 2009). Copper and silver concentrations in total edible tissues of five mussels were determined on dried samples (80 °C) after wet digestion with nitric acid by atomic absorption spectrophotometry (AAS Analyst 800, Perkin-Elmer).

2.4. Condition index

To assess the physiological status of control and exposed mussels to CuO and Ag NPs and Cu^{2+} and Ag^{+} during the course of the experiment, the soft tissues and shells of ten individuals were weighted and the condition index (CI) estimated as the percentage (%) of the ratio between drained weight of the soft tissues (g) and total weight (g).

2.5. DNA damage using the comet assay

Hemolymph of ten mussels collected after 0, 3, 7 and 15 days of exposure to CuO and Ag NPs and corresponding ionic forms was extracted from the posterior adductor muscle with a sterile hypodermic syringe. Cell viability was assessed by staining a 100 μl subsample from each experimental condition with 100 μl of trypan blue and measuring the percentage of live cells, by randomly counting 100 cells. DNA damage was estimated using the comet assay in a slightly modified version of that by Singh et al. (1988) and described in Almeida et al. (2011). Briefly, microscopic slides were coated with 0.65% normal melting point agarose (NMA) in Tris-acetate EDTA. After collection, hemolymph cells for each mussel were centrifuged at 3000 *rpm* for 3 min (4 °C) and the pellets with isolated cells suspended in 0.65% low melting point agarose (LMA, in Kenny's salt solution) and casted on the microscope slides. Afterwards, the slides with the embedded cells were immersed in a lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, 10% Dimethylsulfoxide, 1% Sarcosil, pH 10, 4 °C) for 1 h, for the diffusion of cellular components and DNA immobilization in agarose. Following the lysis step, slides were gently placed in an electrophoresis chamber containing electrophoresis buffer (300 mM NaOH, 1 mM EDTA, adjusted at pH 13, 4 °C). The slides were gently submerged and left in this solution for 15 min to permit DNA unwinding. The electrophoresis was carried out for 5 min at 25 V and 300 mA. Once the electrophoresis concluded, the slides were removed and immersed in neutralizing solution (0.4 mM Tris, pH 7.5), rinsed with bi-distilled water and left to dry overnight. Afterwards, slides were stained with 4,6-diamidino-2-phenylindole (DAPI, 1 $\mu g\ mL^{-1}$) and the presence of comets was