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Short-term effects on antioxidant enzymes and long-term genotoxic and carcinogenic potential of CuO nanoparticles compared to bulk CuO and ionic copper in mussels *Mytilus galloprovincialis*

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ABSTRACT

The aim of this work was to study short-term effects on antioxidant enzyme activities and long-term genotoxic and carcinogenic potential of CuO nanoparticles (NPs) in comparison to bulk CuO and ionic copper in mussels *Mytilus galloprovincialis* after 21 days exposure to 10 μg Cu L^{-1}. Then, mussels were kept for up to 122 days in clean water. Cu accumulation depended on the form of the metal and on the exposure time. CuO NPs were localized in lysosomes of digestive cells, as confirmed by TEM and X ray microanalysis. CuO NPs, bulk CuO and ionic copper produced different effects on antioxidant enzyme activities in digestive glands, overall increasing antioxidant activities. CuO NPs significantly induced catalase and superoxide dismutase activities. Fewer effects were observed in gills. Micronuclei frequency increased significantly in mussels exposed to CuO NPs and one organism treated with CuO NPs showed disseminated neoplasia. However, transcription levels of cancer-related genes did not vary significantly. Thus, short-term exposure to CuO NPs provoked oxidative stress and genotoxicity, but further studies are needed to determine whether these early events can lead to cancer development in mussels.

1. Introduction

During recent years engineered nanoparticles (NPs) are emerging as a potential new type of environmental pollutant due to the extensive development in the field of nanotechnology. NPs are particles less than 100 nm in size in more than one dimension (EPA, 2007). The characteristic size of NPs gives special mechanical, catalytic and optical properties that make them suitable for developing applications in many areas including cosmetics, medicine, food and food packaging, bioremediation, paints, coatings, electronics, fuel catalysts and water treatment (Aitken et al., 2006; Chaudhry et al., 2008; Savage and Diallo, 2005). Those man-made NPs, commonly known as engineered NPs, already include a high number of substances like metals, metal oxides and alloys, carbon-based materials such as fullerenes, silicates and quantum dots as well as polymer composites (Aitken et al., 2006; Chaudhry et al., 2008). Although CuO NPs are currently not as commonly used as other metal or metal-bearing NPs, such as Ag or TiO_2 NPs, they are industrially produced and commercially available in the market place. They show potential to replace noble metal catalysts for carbon monoxide oxidation (Zhou et al., 2006) and to be used as additives in lubricants, polymers/plastics and metallic coating inks.

During past decades, marine invertebrates, and especially bivalve molluscs like mussels, have been extensively used as

Abbreviations: CAT, catalase; dH_2O, deionised water; EDTA, ethylenediamine tetraacetic acid; GADD45α, growth arrest- and DNA damage inducible 45 alpha; GPx, glutathione peroxidase; MN, micronuclei; NM, nanomaterial; NP, nanoparticle; ROS, reactive oxygen species; RQ, relative quantification; SOD, superoxide dismutase.

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sentinel organisms for studying the biological effects of both organic and inorganic pollution (Cajaraville et al., 2000; Zorita et al., 2007). Recent studies have highlighted the utility of marine invertebrates as test organisms for NP ecotoxicity too. Since invertebrates represent about 95% of animal species, they play an important ecological role and participate in transfer of NPs through food chains (Baun et al., 2008). Filter-feeding invertebrates, especially bivalve molluscs, constitute an important target group for NP toxicity due to their highly developed processes for cellular internalisation of nano- and micro-scale particles by endocytosis and phagocytosis, respectively (Moore, 2006). As the final destination of filtered particles within the organism, digestive gland cells are useful to determine NPs fate and effects in mussels (Canesi et al., 2012). Haemocytes (haemolymph cells in charge of the innate immune response in bivalves) are also a major target of NPs (Canesi et al., 2012). During recent years several experiments of exposure to NPs have been carried out both *in vitro* (Canesi et al., 2010; Katsumiti et al., 2014a, 2014b) and *in vivo* (Buffet et al., 2011, 2012; Gagné et al., 2008; Gomes et al., 2011, 2012, 2013, 2014; Tedesco et al., 2008, 2010) using mussels and other bivalves.

Toxicity mechanisms at the cellular level have not been yet completely elucidated for most NPs, but possible mechanisms include disruption of membranes or membrane potential, oxidation of proteins, genotoxicity, interruption of energy transduction, formation of reactive oxygen species (ROS) and release of toxic constituents (Klaine et al., 2008). Oxidative stress has been postulated in several *in vivo* and *in vitro* studies as a primary toxicity mechanism of NPs both in mammalian models (Park and Park, 2009; Ye et al., 2010) and in different aquatic species (reviewed by Klaine et al., 2008 and Fu et al., 2014) including freshwater fish such as zebrafish (Zhu et al., 2008), estuarine fish such as sticklebacks (Sanders et al., 2008) and mussels (Gagné et al., 2008; Tedesco et al., 2008, 2010). Observed toxic effects seem to be mediated through the formation of the very reactive hydroxyl radicals (Reeves et al., 2008). CuO NPs have been shown to be toxic to both vertebrates and invertebrates by increasing intracellular ROS production (Aruoja et al., 2009; Buffet et al., 2011; Chen et al., 2006; Karlsson et al., 2008; Meng et al., 2007).

The capacity of CuO NPs to produce ROS may lead to activation or inhibition of antioxidant enzymes and consequently the alteration of the antioxidant capacity (Ahamed et al., 2010; Buffet et al., 2011; Gomes et al., 2011, 2012; Karlsson et al., 2008). In case antioxidant capacity is overwhelmed, ROS can damage DNA by production of strand breaks, cross links and adducts of nucleotide bases or sugars (Chen et al., 2006; Kang et al., 2012).

In addition to NP induced indirect DNA damage through ROS, NPs can directly interact with DNA due to their small size and high surface area (Singh et al., 2009). Thus, both by direct or indirect mechanisms, NPs can cause DNA damage, as shown by Comet assays or micronuclei (MN) frequency tests. CuO NPs were found to induce DNA fragmentation and MN formation in N2A cells (Perreault et al., 2012). Similar results were observed in murine macrophages RAW 264.7 and in peripheral whole blood from healthy volunteers exposed *in vitro* to CuO NPs of different shapes (Di Bucchianico et al., 2013). In agreement, Gomes et al. (2013) and Rocha et al. (2014) showed that CuO NPs and CdTe quantum dots, respectively, were genotoxic to mussels' haemocytes after 1 and 2 weeks of exposure. Similar results have been reported for other bivalve species, such as the clam *Scrobicularia plana* after 21 days exposure to CuO NPs (Buffet et al., 2013; Mouneyrac et al., 2014).

DNA damage caused by nanomaterials (NMs) can invoke various cellular responses such as cell cycle arrest, apoptosis and DNA repair. When DNA is damaged, a key effector molecule, P53, is activated. This tumour suppressor gene is responsible for arresting the cell cycle and activating transcription of genes that mediate DNA repair, thus preventing the conversion of damage to mutation (Harris and Levine, 2005). However, if the damage is extensive, apoptotic pathways are triggered and elicit cell death (Sancar et al., 2004). Treatment with CdTe quantum dots, TiO_2 NPs and Ag NPs increased P53 expression in zebrafish liver, human lymphocytes and mouse embryonic stem cells and fibroblasts (Ahamed et al., 2008; Cha et al., 2008; Kang et al., 2008). In the case of CuO NPs, exposure of human hepatocellular carcinoma HepG2 cells and human lung epithelial (A549) cells induces the expression of P53 (Siddiqui et al., 2013; Wang et al., 2012). However, exposure of HaCaT human keratinocytes and mouse embryonic fibroblasts to CuO NPs induced decreases in P53 and p-P53 levels, indicating that P53 had not a prodeath function (Luo et al., 2014). In order to maintain genomic stability, DNA repair genes are activated after DNA damage (Hanahan and Weinberg, 2000). Thus, exposure of HepG2 human hepatoma cells to Ag NPs led to up-regulation of DNA repair specific genes, such as *rad51* and *gadd45* (Kawata et al., 2009). Similarly, Ag NPs up-regulated DNA damage repair protein RAD51 in mouse embryonic cells (Ahamed et al., 2008). *ras* oncogene plays a pivotal role in regulating cell growth, differentiation and survival (Patra, 2008) and it is oncogenically activated by mutations in over 25% of all human tumours (Bos, 1989). Mutations of *ras* gene locus were found in the lung of mice exposed to single-walled carbon nanotubes (Shvedova et al., 2008).

Little is known about the potential mutagenic and carcinogenic effects of NMs *in vivo*. It is possible that NMs affect tumour formation through DNA damage, increasing cell proliferation associated with inflammation or by oxidative stress, which is considered as a main non-genotoxic mechanism of carcinogenesis (Klaine et al., 2008; Singh et al., 2009).

Thus, the present work aimed to study the short-term effects on the antioxidant system and the long-term genotoxic and carcinogenic potential of CuO NPs in comparison to effects caused by bulk CuO or ionic copper in mussels. While there are many studies comparing the effects caused by NPs and their counterpart ionic forms, recently Duester et al. (2014) have highlighted the lack of comparative studies with bulk products in order to identify possible nano-specific effects and to assess the need for nano-specific regulations. In order to achieve this objective, an experiment was designed in which mussels were maintained unexposed or exposed to CuO NPs, bulk CuO and ionic copper for 21 days and then kept in clean water for up to 122 days in an attempt to allow possible initiation of tumour lesions. As cancer is a multistage, progressive disease, long-term experiments are needed for carcinogens to induce neoplasia in fish and mammals (Spitsbergen and Kent, 2003; Winslow and Jacks, 2008). In this study, bioaccumulation of copper in mussels exposed to the three copper forms was quantified by chemical analyses and subcellular localization of CuO NPs was addressed by TEM and X-ray microanalysis. Oxidative stress was assessed measuring the activity of the main antioxidant enzymes (catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD)), genotoxicity was studied by the MN frequency assay, and carcinogenic potential was evaluated through the measurement of the transcription level of cancer-related genes *p53*, *ras* and *gadd45α* and through histopathological analysis of mussel tissues. This is the first report on a long-term experiment designed to address potential effects of NPs on cancer-related genes and cancer development.

2. Materials and methods

2.1. Animals and experimental procedure

Adult mussels, *Mytilus galloprovincialis*, of 3.5–4.5 cm shell lengths were obtained from an aquaculture facility in Boiro, A