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Cytotoxicity and cellular mechanisms of toxicity of CuO NPs in mussel cells *in vitro* and comparative sensitivity with human cells

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ABSTRACT

There is a need to assess human and ecosystem health effects of copper oxide nanoparticles (CuO NPs), extensively used in many industrial products. Here, we aimed to determine the cytotoxicity and cellular mechanisms involved in the toxicity of CuO NPs in mussel cells (hemocytes and gill cells) in parallel with exposures to ionic Cu and bulk CuO, and to compare the sensitivity of mussel primary cells with a well-established human cell line (pulmonary TT1 cells). At similar doses, CuO NPs promoted dose-dependent cytotoxicity and increased reactive oxygen species (ROS) production in mussel and human cells. In mussel cells, ionic Cu was more toxic than CuO NPs and the latter more than bulk CuO. Ionic Cu and CuO NPs increased catalase and acid phosphatase activities in both mussel cells and decreased gill cells Na-K-ATPase activity. All Cu forms produced DNA damage in hemocytes, whereas in gill cells only ionic Cu and CuO NPs were genotoxic. Induction of the MXR transport activity was found in gill cells exposed to all forms of Cu and in hemocytes exposed to ionic Cu and CuO NPs. Phagocytosis increased only in hemocytes exposed to CuO NPs, indicating a nanoparticle-specific immunostimulatory effect. In conclusion, toxicity of CuO NPs is driven by ROS in human and mussel cells. Mussel cells respond to CuO NP exposure by triggering an array of defensive mechanisms.

1. Introduction

Copper oxide nanoparticles (CuO NPs) are one of the most widely used nanomaterials and are applied in a variety of industrial products such as in gas sensors, photovoltaic cells, catalyst applications, heat transfer nanofluids and also in antimicrobial coatings (Zhang et al., 2014). Due to their excellent mending effects, CuO NPs are added into lubricant oils to effectively reduce friction and wear, or to mend a worn surface (Liu et al., 2004). CuO NPs are homogeneously deposited on the surface of graphite to improve its charge–discharge properties (Guo et al., 2002). CuO NPs show antimicrobial properties which make them effective against a large spectrum of gram-positive and negative bacteria (Ren et al., 2009; Baek and An, 2011). CuO NPs are described as one of the most bioreactive metal oxide NPs (Karlsson et al., 2008; Lanone et al., 2009), thus receiving special attention for its safe production and use. The increasing production and consequent disposal of CuO NPs has raised major concerns about their potential toxicity to human and environmental health.

Recent studies *in vitro* in different human cell lines such as cardiac microvascular endothelial cells, kidney cells, neuronal cells, liver and lung epithelial cells have shown that toxicity of CuO NPs is mediated by oxidative stress (Fahmy and Cormier, 2009; Ahamed et al., 2010; Sun et al., 2011; Wang et al., 2011; Piret et al., 2012; Siddiqui et al., 2013; Xu et al., 2013). Among these well-established cell models, human alveolar lung cells are recognized as accurate epithelial cellular models to study NP toxicity (Kemp et al., 2008; Misra et al., 2013; Chen et al., 2013; Sweeney et al., 2014, 2015a, 2015b, 2015c). The respiratory alveolar epithelium is composed of two epithelial cells: type I (ATI) and

Abbreviations: AcP, Acid phosphatase; CAT, catalase; DLS, dynamic light scattering; DMSO, dimethyl sulphoxide; EDTA, ethylenediaminetetraacetic acid; MTT, thiazolyl blue tetrazolium bromide; MXR, multixenobiotic resistance; NP, nanoparticle; NR, neutral red; PBS, phosphate buffered saline solution; Pgp, P-glycoprotein; QDs, quantum dots; ROS, reactive oxygen species; TEM, transmission electron microscopy; TRITC, tetramethylrhodamine B isothiocyanate; TT1, human pulmonary cell line

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type II (ATII). ATI cells are highly attenuated, flattened epithelial cells that provide a large surface area with a thin gas-permeable barrier of approximately 0.2 μm in depth to allow rapid, free gas exchange between the alveolar airspace and the underlying capillary network (Thorley and Tetley, 2007). The ATII cell makes up the majority of the alveolar epithelial cells by number, but only a fraction of the surface area, as only a small projection on the apical side of the cell is apparent between the attenuated ATI cells (Thorley and Tetley, 2007). It is known that ATI cells are easily damaged by environmental challenges from chemical exposures (Thorley and Tetley, 2007). Recently we developed an immortal cell line named transformed type-1 (TT1) cells from human ATII cells (Kemp et al., 2008). In exposures to CuO NPs, TT1 cells showed decreased cell viability and pro-inflammatory responses which depended on the shape and size of NPs (Misra et al., 2013).

Since all contaminants tend to end-up in the aquatic environment, there is a special interest to evaluate the potential risks of NPs to aquatic organisms. Studies have pointed that CuO NPs could represent a risk to the marine environment showing toxic effects on phytoplankton (Bielmyer-Fraser et al., 2014), cnidarians (Siddiqui et al., 2015), polychaetes (Buffet et al., 2013), fishes (Villarreal et al., 2014) and marine bivalves (Gomes et al., 2012, 2013, 2014; Ruiz et al., 2015). Marine bivalves such as mussels have been shown to be sensitive model species to detect the toxicity of NPs both *in vivo* and *in vitro* (Canesi et al., 2012). Few studies with marine bivalves exposed *in vivo* to CuO NPs have been published and most evidence indicates that toxicity of CuO NPs involve oxidative stress and DNA damage (Buffet et al., 2011, 2013; Gomes et al., 2012, 2013; Ruiz et al., 2015). In *Hediste diversicolor* and *Scrobicularia plana*, CuO NPs induced the activity of antioxidant defenses (Buffet et al., 2011, 2013), increased metallothionein (MT) levels and produced DNA damage (Buffet et al., 2013). In the mussel *Mytilus galloprovincialis*, CuO NPs induced catalase and superoxide dismutase activities in digestive glands (Ruiz et al., 2015), increased MT levels, inhibited acetylcholinesterase activity, induced oxidative stress and lipid peroxidation in gills (Gomes et al., 2012) and increased DNA damage in hemocytes (Gomes et al., 2013; Ruiz et al., 2015). Through proteomic analysis, Gomes et al. (2014) showed that CuO NPs can induce mitochondrial and nucleus stress signalling cascades that can lead to apoptosis in *M. galloprovincialis*. In gills of *M. edulis*, it has been observed that CuO NPs strongly altered Cu-Zn superoxide dismutase protein expression (Hu et al., 2014).

Mussel cell cultures have been successfully used to assess the toxicity of NPs (Canesi et al., 2010, 2012; Ciacci et al., 2012; Bruneau et al., 2013; Katsumiti et al., 2014, 2015a, 2015b, 2016). Hemocytes comprise the main internal defense system in mussels. Effects on this cell type could reflect damage on the immune system, which could have consequences at higher levels of biological organization, *i.e.*, individuals and communities. Inflammatory responses were observed in mussel hemocytes after exposure to C60 fullerenes, TiO_2 and SiO_2 NPs (Canesi et al., 2010). Cell-mediated immune responses were observed in hemocytes exposed to C60 fullerenes and metal-bearing NPs (Canesi et al., 2010; Ciacci et al., 2012; Bruneau et al., 2013; Katsumiti et al., 2014, 2015b). Au, ZnO and SiO_2 NPs with different properties were cytotoxic to hemocytes (Katsumiti et al., 2016). Cytotoxicity of TiO_2 NPs to mussel hemocytes varied with NPs properties including size, crystal structure, synthesis method and presence of stabilizers (Katsumiti et al., 2015a). On the other hand, mussel gill cells represent the first target for xenobiotics in the aquatic system (Livingstone and Pipe, 1992), being an interesting epithelial cell model for *in vitro* studies (Gómez-Mendikute et al., 2005). Gills are responsible for transport and uptake of nutrients, gas exchange and ionic osmoregulation (Owen, 1974, 1978; Owen and McCrae, 1976) and could thus be considered to be functionally comparable to human alveolar pulmonary cells. Gill cells have been considered a sensitive model to assess the toxicity of NPs (Cajaraville, 2009; Katsumiti et al., 2014, 2015a, 2015b). To the best of our knowledge, there are no studies addressing the toxicity of CuO NPs in mussel cells *in vitro*.

In this context, this study aimed to assess the cytotoxicity and cellular mechanisms of CuO NPs toxicity in mussel hemocytes and gill cells, and to compare the sensitivity of mussel primary cells with a well-established human epithelial cell line (TT1 cells). Exposures of mussel cells to CuO NPs were performed in parallel with exposures to ionic Cu and bulk CuO.

2. Materials and methods

2.1. CuO NPs, ionic Cu and bulk CuO

CuO NPs were produced by plasma at Intrinsiq Materials Limited (Farnborough, UK) and provided in form of powder. CuO NPs were characterized by different techniques, as previously reported by Buffet et al. (2011, 2013). Briefly, TEM showed NPs with high polydispersed size distribution with particles of 100 nm and below (average size = 29.5 nm) (Buffet et al., 2013). Once suspended in deionized water and natural filtered seawater (25 mg/L), CuO NPs agglomerate with sizes roughly 197 and 810 nm respectively, as indicated by dynamic light scattering (DLS) (Buffet et al., 2011). Zeta potential measurements showed that particles were positively charged (+26.3 mV) and dispersed well in deionized water, while in seawater CuO NPs were negatively charged (−8.2 mV) indicating poor stability (Buffet et al., 2011). DLS measurements were done in deionized water and in seawater, as an example of a medium with a high ionic strength and with the same osmolarity as the culture media used for mussel cells. Regarding solubility, the same CuO NPs used in the present work did not appear to release free Cu ions after 48 h in seawater, according to Buffet et al. (2011).

Bulk CuO (ref. # 203130) was purchased from Sigma Aldrich (St. Louis, USA) and ionic Cu was purchased from Probus (Barcelona, Spain) as $CuCl_2$ (ref. # 047710).

2.2. Obtaining mussel hemocytes and gill cells

Mussels, *Mytilus galloprovincialis* Lmk. (shell length: 3.5–4.5 cm), were sampled in Mundaka, Gulf of Biscay (43°24′16″N; 2°41′43″W), a relatively non-polluted area (Orbea and Cajaraville, 2006; Bellas et al., 2013). After 2 days acclimatization (0.5 L/mussel at 16–18 °C, constant aeration and daily food supply) mussel cells were isolated.

Hemocytes and gill cells were isolated according with the protocol described in Katsumiti et al. (2014). Hemocyte and gill cell suspensions (> 95% viability according with the trypan blue assay) were seeded into 96-well plates in supplemented Basal medium Eagle and Leibovitz L-15 medium, respectively, and kept for 24 h at 18 °C in a Sanyo incubator (Osaka, Japan) in order to establish cell cultures before the exposures.

2.3. Obtaining human TT1 cells

TT1 cells (10^5 cells/mL) were placed in 96-well microplates in DCCM-1 culture medium (Biological Industries, Israel) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 10% newborn calf serum (NCS) and maintained for 24 h to reach cells confluence. Thereafter, cells were left for another 24 h in serum-free media before exposure.

2.4. In vitro exposures to CuO NPs, ionic Cu and bulk CuO

For the preparation of CuO NPs exposure media, powdered NPs were suspended in phosphate buffered solution (PBS), sonicated for 10 min (Ultrasons-H, Barcelona, Spain) and then added to the culture medium at tested concentrations. Samples were vortex just before the exposure. All *in vitro* exposures were performed in serum-free media.

In mussel cell experiments, NPs exposures were performed for 24 h