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Toxicity of CuO nanoparticles and Cu ions to tight epithelial cells from *Xenopus laevis* (A6): Effects on proliferation, cell cycle progression and cell death

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ABSTRACT

Nanoparticles (NPs) have unique chemical and physical properties caused by their small size (1–100 nm) and high surface to volume ratio. This means that the NPs are potentially more toxic than their bulk counterparts. In the present study a cultured epithelial cell line from *Xenopus laevis* (A6) was used to investigate toxicity of copper (Cu) in 3 different forms; Cu ions (Cu^{2+}), CuO NPs (6 nm) and poly-dispersed CuO NPs (100 nm, poly-CuO). Continuous exposures at concentrations of 143–200 µM demonstrated that cytotoxicity differed among the 3 Cu forms tested and that the effects depend on cell state (dividing or differentiated). Dividing cells treated with poly-CuO, CuO NPs (6 nm) or Cu^{2+} showed cell cycle arrest and caused significant increase in cell death via apoptosis after 48 h, 6 and 7 days of treatment, respectively. Treatment with either CuO NPs (6 nm) or Cu^{2+} caused significant decrease in cell proliferation. Treatments of differentiated cells, revealed the same patterns of toxicity for Cu forms tested, but after shorter exposure periods.

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1. Introduction

Copper (Cu) is essential to animals and higher plants in low amounts, as it serves as a co-factor for a variety of enzymes (e.g., cytocrome c oxidase and superoxide dismutase) (Zhou and Gitschier, 1997). However, Cu is highly toxic in excess amounts because it can interfere with homeostasis of other metals, bind to DNA causing DNA damage, and has the ability to generate Reactive Oxygen Species (ROS) that can adversely modify proteins, lipids and nucleic acids (Aruoma et al., 1991; Banci et al., 2010; Halliwell and Gutteridge, 1984). Cu particles in the nanoscale (1–100 nm) (BSI 2007) are widely used (e.g., in inks, cosmetics and textiles for biocidal effects (Cioffi et al., 2005)). The use of these particles will in all probability persist in the future, resulting in increased human and environmental exposure. The small size of nanoparticles (NPs) means that the surface to volume ratio is very large (Nel et al., 2006) and since reactions take place at the interface, between a particle and its surrounding environment, it is now being recognized to cause NPs to elicit toxicological effects different from their bulk counterparts (Borm et al., 2006; Hood, 2004; Oberdorster et al., 2005; Roduner, 2006). Furthermore, as engineered metal NPs, such as CuO NPs, have not been encountered by living organisms during the course of evolution there will have been no or little selection pressure for protection against uptake and toxic effects of these particles (Moore, 2006). In contrast cells have highly developed processes for cellular internalization of particles in the nanoscale, i.e., endocytosis. CuO NPs have previously been reported to be highly toxic, both in comparison to other metal oxide NPs (Karlsson et al., 2008) and in comparison to bulk Cu (Heinlaan et al., 2008). The mechanisms causing the toxic effects of NPs is still relatively unknown (Griffitt et al., 2008) and the extent to which the mechanisms causing the toxic effects of CuO NPs are the same as for Cu ions remains to be elucidated.

In this study the epithelial A6 cell line is used to examine the toxic effects of Cu ions (Cu^{2+}), CuO NPs (6 nm) and polydispersed CuO particles (100 nm, poly-CuO). A6 cells are commonly used as a model for mammalian cells (Faurskov and Bjerregaard, 2000) and for tight epithelia in general (Bjerregaard, 1995; Perkins and Handler, 1981; Saribansohraby et al., 1983). In suspension the cells are spherical with a non-polarized membrane (Handler, 1983; Rodriguezboulan and Nelson, 1989; Sztul et al., 1987). When attached to a substrate differentiation of the cells occurs which includes insertion of tight junctions which seals adjacent cells tightly together and asymmetrically distribution of proteins, many which are common to non-polarized cells (Rodriguezboulan and Nelson, 1989). A6 cells proliferate as single cells in the G1, S, G2 and M phase of the cell cycle until cell–cell contact is established.

Abbreviations: NP, nanoparticle; Cu, copper; CuO, copper oxide; poly-CuO, polydispersed CuO particles; h, hours; d, days; ROS, Reactive Oxygen Species.

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Thereafter, a confluent monolayer is formed and quiescent cells in G0 will differentiate into tight epithelia that actively transport ions. In the present study continuous exposures were conducted to investigate whether cytotoxicity of Cu depend on form and particle size. Both dividing and non-dividing, differentiated cells were used to assess the effects of cell state on toxicity.

2. Materials and methods

2.1. Chemicals

CuO NPs (6 nm) was delivered by Natural History museum (NHM, UK) in an aqueous suspension as monodispersed spherical particles. The size of particles was determined by Transmission Electron Microscopy (6 ± 1 nm) and the size distribution in de-ionized water was determined by Dynamic Light Scattering (ranged roughly from 9 to 40 nm with the majority around 19 nm). Additional Characterization data is presented in (Pang et al., 2012; Pang et al., Submitted for publication). Poly-CuO were produced as nanopowder by Intrinsiq Materials Ltd (UK) and characterized by NHM. The particle size was determined by TEM (100 nm). Particles were highly polydispersed and polyhedral with sizes ranging from 40 to 500 nm in de-ionized water with a majority around 100 nm, and hence contains particles both in and outside the defined nanoscale. Additional characterization data can be found in (Pang et al., 2012; Pang et al., Submitted for publication). Cu^{2+} was administered as solid crystalline copper chloride dihydrate ($Cu(II)Cl_2$ $2H_2O$) (Merck).

2.2. Stock- and test solutions

A stock of poly-CuO in autoclaved MilliQ water (MQW), was made. This was done immediately before use to limit aggregation and precipitation of CuO particles in the suspension. To avoid inhalation of NPs the transfer was carried out in a glove-box attached to a fume hood. A Cu^{2+} stock solution was made by transferring the needed amount of $Cu(II)Cl_2$ $2H_2O$ into a known volume of autoclaved MQW. Test solutions were made by simple dilution into growth medium using a saturated pipette (suspension is sucked up and down 3 times in the pipette before use). Solutions were vortexed for 60 s between each transfer and before use.

2.3. Cell culture

A6 cells originally isolated from the distal kidney tubulus of the aquatic toad *Xenopus laevis* (Rafferty, 1969) was purchased from American Type Culture Collection (Rockville, MD, USA) at serial passage 67. Cells were cultured in falcon T25 culture flasks (BD bioscience, San Jose, CA) with 10 ml growth medium at 26 °C in humidified atmosphere of 5% CO_2 in air in a Flow laboratories CO_2 incubator 1500. The growth medium was made from Dulbeccós modified Eaglés medium (Gibco, InVitrogen Corporation, Carlsbad, CA, USA) diluted 25%, with autoclaved MQW to adjust to amphibian osmolarity. Additionally the growth medium contained 2% penicillin/streptomycin (5000 units/ml Penicillin + 5000 μg/ml Streptomycin) (Gibco, InVitrogen Corporation, Carlsbad, CA, USA) and 10% Fetal Bovine Serum (Biochrom AG, Berlin, Germany).

2.4. Maintenance of A6 cells

Cells were seeded at a density of 5×10^4 cells/cm^2 in falcon T25 culture flasks and fed weekly by replacing the growth medium. Five to ten days after seeding cells had differentiated and formed a monolayer mimicking epithelium with an apical and a basolateral side. At this point transcellular transport of water to the basolateral side of the cells resulted in pockets of liquid underneath the cell layer and the formation of domes. Cells were sub-cultivated weekly after confluence had been reached, to avoid senescence caused by prolonged high cell density, and to allow seeding in micro titter plates for experiments. For sub-cultivation old growth medium was removed and the cells were suspended by trypsination with trypsin_EDTA 0.05% (InVitrogen Corporation, Carlsbad, CA, USA) as described by Bjerregaard (1995). Cells were seeded by distributing the cell-suspension into 5 new culture flasks and adding fresh growth medium.

2.5. Epithelial cell proliferation and cytotoxicity

For cytotoxicity assessment via visual inspection cells were seeded in 24-well microtiter plates in concentrations of $5–10 \times 10^3$ cells/cm^2 depending on whether dividing cells or non-dividing, differentiated cells were required. After 24 h of cultivation the growth medium was replaced with 1 ml experimental medium containing Cu^{2+}, CuO NPs (6 nm), poly-CuO at high concentrations (143–200 μM) or 1 ml fresh growth medium (controls). Cells were observed with a DMIRB/E Leica inverted microscope equipped with a Leica DC 300 F camera, and digital image acquisition was performed with software from Leica Microsystems Ltd (leica Microsystems A/S, Herlev, Denmark). Pictures were taken of 0.6 mm^2 of the midpoint of each well of the culture dish, just before cell treatment was initiated (0 h) and several times during the continuous exposures (after 3, 24, 48 h, 3, 5, 6 and 7 days of treatments). At least 10 replicate test wells were used for each of the different treatments. The digital images were used to determine proliferation (calculated from degree of cell coverage at different time points) and the amount of dead or dying cells. First the digital images were converted into greyscale to increase the contrast. The degree of cell coverage was estimated by calculating the dark area of the pictures as living cells appear dark and the substratum in the background light. Dead or dying cells appear as white rounded vesicles detached from the substratum and the amount of dying or dead cells was estimated by calculating the area of white (dead or dying cells). All Image analysis was carried out using Image J software (developed at the National institutes of health, Bethesda. MD, USA).

2.6. Flow cytometric analysis of cellular DNA content

Cellular DNA content changes during the cell cycle and can be seen as distinct peaks corresponding to cells in different cell cycle stages (G0/G1, S, G2/M), when cells are analyzed with flow cytometry (Fig. 1). A sub-G1 peak is characteristic of a cell population containing apoptotic cells, as DNA fragmentation occurs during early apoptosis and may lead to loss of DNA content. For flow cytometric analysis cells were seeded in falcon T25 culture flasks at a density of 10×10^3 cells/cm^2. After 24 h cultivation cell layers had reached 60–80% confluence and growth medium was replaced with 10 ml experimental medium containing Cu^{2+}, CuO NPs (6 nm), poly-CuO with a concentration of 200 μM or 10 ml fresh growth medium (controls). After 24 and 48 h exposure cells were harvested for analysis. Growth medium containing the detached cells was collected, the cells attached to the flasks were removed by trypsination and the two fractions were pooled. Cells were collected by centrifugation and washed twice with NaCl Ringer's solution. Approximately 5×10^5 cells were washed in NaCl Ringer's solution, permebealized in 2 ml 70% ethanol, and stored at −20 °C. Preceding flow cytometry cells were washed in PBS and stained with the fluorescent nucleic acid stain Propidium Iodide

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