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Development of a new tool for the long term in vitro ecotoxicity testing of nanomaterials using a rainbow-trout cell line (RTL-W1)

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ABSTRACT

The current wide use of manufactured nanomaterials (MNs) is leading to the release of nanoparticles (NPs) to water bodies. Aquatic organisms, including fish, are exposed to low concentrations of NPs for long periods of time being necessary to develop laboratory toxicity tests reflecting realistic conditions. Additionally, today there is a demand of in vitro assays respecting the 3Rs principle. Thus, the main aim of this work was to stablish an in vitro tool for the assessment of long-term NPs ecotoxicity. Considering the key role of liver in detoxification, a rainbow trout liver cell line, RTL-W1, was used. CuO NPs were chosen to validate this tool taking into account their important production level. Cells were exposed for 21 days to 25 or 100 μg CuO NPs/ml. Every seven days cells were split and one fourth of them transferred to a new plate with appropriate concentrations of NPs in culture medium. Lower concentrations of CuO NPs did not cause any deleterious effect, whereas higher concentrations led to significant mortality after 14 days and to the intracellular accumulation of Cu particles. Identical results were observed in cells exposed to $CuSO_4$ at the same Cu concentrations. Therefore, the observed toxic effects might be mainly due to Cu^{2+} ions.

1. Introduction

Manufactured nanomaterials (MNs) are currently used in a number of consumer products so that they can enter the environment and be spilled into the aquatic compartments, through waste water treatment plants and industrial effluents, agricultural run-offs, accidental spills, etc. As a consequence, in recent years, engineered nanoparticles (NPs) are emerging as a potential new type of environmental pollutant (Ruiz et al., 2015). Hence, the release of NPs into the aquatic environment could be threatening the survival of inhabiting organisms (Farré et al., 2009; Handy and Ramsden, 2011).

Among metal-based NPs, CuO NPs probably show one of the highest production volumes, since they are employed in a number of fields. For instance, they are used as additives in lubricants, polymers and plastics, metallic coating inks, or antimicrobial coatings, and they are also applied in textiles due to their biocidal activity, or in cosmetics. In addition, environmental remediation and electronics take advantage of their particular physico-chemical properties (Nanotech project, 2014; Park et al., 2007; Perelshtein et al., 2009; Cioffi et al., 2005; Ren et al., 2009; Rubilar et al., 2013). The precise concentration of CuO NPs in the aquatic environment is difficult to establish but some modelling studies revealed that they can appear at concentrations of 0.01 mg of Cu/l in marine environments highly contaminated by this metal (Bryan and Langston, 1992).

Although Cu is an essential element for living organisms, taking part in many biological processes, for instance as co-factor for redox active enzymes (like cytochrome *c* oxidase and superoxide dismutase), it results in high toxicity when present in excess amounts because it can interfere with homeostasis of other metals, cause DNA damage, and generate reactive oxygen species (ROS) that can adversely modify proteins, lipids and DNA (Aruoma et al., 1991; Banci et al., 2010; Boyles et al., 2016; Halliwell and Gutteridget, 1984; Xie et al., 2006).

The toxicity of CuO NPs has been extensively studied using both in vivo and in vitro approaches (Dai et al., 2015; Isani et al., 2013; Ivask

Abbreviations: BSA, Bovine serum albumin; CFDA-AM, 5-carboxyfluorescein diacetate, acetoxy methyl ester; CYP, cytochrome P450; DLS, dynamic light scattering; EDTA, ethylenediaminetetraacetic acid; EDX, energy dispersive X-ray spectroscopy; FBS, fetal bovine serum; ITS, intelligent testing strategy; MEM, Minimum Essential Medium; NP, nanoparticle; NRU, neutral red uptake; P/S, penicillin/streptomycin; PBS, phosphate buffer saline; RMANOVA, repeated measurements analysis of variance; ROS, reactive oxygen species; SEM, standard error of the mean; SDS, sodium dodecyl sulphate; TEM, transmission electron microscopy

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et al., 2014; Ivask et al., 2015; Mancuso and Cao, 2014; Pedroso et al., 2013; Rossetto et al., 2014), and it has been reported that these NPs are toxic to both vertebrates and invertebrates (Aruoja et al., 2009; Buffet et al., 2011). Actually, some studies identified CuO NPs as the most cytotoxic among various metal oxide NPs (Karlsson et al., 2008).

In the context of aquatic ecotoxicology, fish are the most widely used vertebrates in risk assessment and regulation (Fent, 2001). Therefore, understanding the actions of NPs on fish is essential for an appropriate evaluation of the health of the aquatic environment. Nevertheless, in vivo testing is extremely time-consuming and costly, requiring much maintenance and a high number of animals, which is ethically debated. Therefore, the European Commission encourages the development and application of alternatives to animal tests (Castaño et al., 2003; Lee et al., 2008; Scholz et al., 2013). The use of in vitro methods with fish cells has been seriously considered for assessing the hazardous effects of chemicals on living systems, since many studies have demonstrated a good in vivo/in vitro correlation and it is generally accepted that the first interaction of a toxicant with an organism occurs at the cellular level (Schirmer, 2006). The changes provoked by NPs in cells can then possibly be translated to higher levels of organization, finally reflecting the effects on the whole organism (Babich et al., 1991; Castaño et al., 2003; Fent, 2001; Rusche and Kohlpoth, 1993; Zahn et al., 1995).

An important gap of in vitro ecotoxicological experiments is that they are mainly conducted for short exposure times, which do not reflect real-life situations with continuous release of products over a long period. Actually, there is a general lack of knowledge about MNs long term toxic effects. In this sense, in vitro systems using long-term exposure protocols, as those described in this article, can be a powerful tool to initially determine the mechanisms underlying the potential cytotoxic effects of NPs after prolonged exposure at levels that are not toxic in acute experiments.

Rainbow trout (*Oncorhynchus mykiss*) is probably the most commonly studied cold water fish species (Wolf and Rumsey, 1985) and it is considered as one of the most sensitive species for acute toxicity testing (Fent, 2001). Therefore, the use of rainbow trout derived cell lines can be of relevance for in vitro studies dealing with pollutants ecotoxicity. In addition, since liver is one of the target organs for NPs toxic action and bioaccumulation (Connolly et al., 2016), we decided to use a rainbow trout cell line of hepatic origin for our studies: RTL-W1 cells, which are probably biliary epithelial cells in origin (Malhão et al., 2013) derived from the normal liver of an adult male rainbow trout. RTL-W1 cells retain important catabolic activities as xenobiotic reductase and glutathione-*S*.transferase activities and those dependent on cytochrome P450 1A (CYP1A), (Lee et al., 1993; Nehls and Segner, 2001; Smeets et al., 1999; Thibaut et al., 2009).

However, the main drawback of using fish cell cultures in ecotoxicological studies is that cell lines are much less sensitive than whole organisms (Bols et al., 1985; Castaño et al., 1996; Segner and Lenz, 1993). Nevertheless, the sensitivity of cytotoxicity tests can be increased by the selection of appropriate cytotoxic endpoints (Castaño et al., 2003). Regarding this aspect, it is important to evaluate changes in cell viability (a cellular general response) with a number as high as possible of assays covering different mechanisms of toxic action. Taking this into account, in the present work we have applied a system normally used in our laboratory based on the utilization of three indicator dyes per sample, reflecting effects on general metabolic activity of the cells, on lysosomal functioning and on plasma membrane integrity, what provides a broad overview of the sensitivity of the cells (Lammel and Navas, 2014). Independently of the limitations of in vitro approaches, the information gained can be used at different levels in an integrated testing strategy (ITS), for instance in order to prioritize chemicals for further testing in higher tier more costly in vivo assays. Actually, data obtained in vitro have demonstrated to be useful for the toxicity ranking of chemicals or MNs (Farcal et al., 2015).

According to what has been said above, we carried out the present work with the main aim of determining the long term (21 days) cytotoxic effects of CuO NPs on RTL-W1 cells. For the selection of the appropriate concentrations to be applied, first 24 h acute cytotoxicity assays were performed. Influence of CuO NPs on RTL-W1 cell viability and subcellular morphology were explored at different time points along the 21 days-experiment. Inasmuch there is general consensus that dissolution of CuO NPs is an important factor that influences their toxicity (Aruoja et al., 2009; Ivask et al., 2014), impacts of CuO NPs on cells were compared with those produced by $CuSO_4$ salt, used as control source of ions and applied at the same Cu concentrations and treatment periods as NPs. Although the system developed in our laboratory focused on testing CuO NPs toxicity on a specific fish cell line, a similar approach could be used to observe the effects of other MNs on a variety of cell lines from different origin.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents were purchased from Sigma-Aldrich (Madrid, Spain), at least than otherwise stated. For cell culture, L-Glutamine (200 mM), penicillin and streptomycin (P/S) (10,000 U/ml each), trypsin-ethylenediaminetetraacetic acid (EDTA) (200 mg/ml) and Leibovitz's (L-15) cell culture medium were purchased from Lonza (Barcelona, Spain). Phenol-red free serum-free Minimum Essential Medium (MEM) was supplied by Gibco (Life Technologies, Madrid, Spain). Among the reactants used for determining cytotoxicity, resazurin and 5-carboxyfluorescein diacetate, acetoxy methyl ester (CFDA-AM) were purchased from Invitrogen (Madrid, Spain). For the electron microscopy analyses, paraformaldehyde (16%) and glutaraldehyde (25%) were supplied by Electron Microscopy Sciences (Hatfiled, UK) and Spurr's resin was provided by TAAB Laboratories Equipment Ltd. (Aldermaston, UK).

2.2. Nanoparticles and nanoparticle suspension preparation

Uncoated spherical CuO NPs, of 15–20 nm in diameter size according to the manufacturer's information, were supplied by PlasmaChem GmbH (Berlin, Germany). Initial CuO NP dispersions at 10 mg/ml were freshly prepared in milli-Q water prior to exposure experiments. The suspensions were sonicated for 20 min in an ice-water bath using a probe sonicator (Vibra cell VCX130, Sonics & Materials Inc., Newtown, CT, USA) at 80% amplitude in continuous mode with a 2 mm microtip. For the exposure experiments, initial dispersions of CuO NPs were mixed at a proportion 3:1 with a solution of bovine serum albumin (BSA, 80 mg/ml in water) used as dispersant agent. This mixture was then diluted into L-15 medium to reach a concentration of 100 μg/ml. This suspension was vortexed for 5 s and thereafter sonicated for 10 min in a water-bath sonicator (S 40H Elmasonic, Elma, Germany) immediately before use. $CuSO_4 \cdot 5H_2O$ was used as a control source of Cu^{+2} ions.

2.3. Physico-chemical characterization of CuO NP suspensions

2.3.1. Dynamic light scattering (DLS)

The hydrodynamic size frequency distributions of CuO NPs in both the initial suspensions in milli-Q water and in culture medium at 100 μg/ml were determined by means of DLS, using a Zetasizer Nano-ZS device (Malvern Instruments Ltd., Malvern, UK). Milli-Q water and L-15 medium with BSA were used as background controls. Since the same initial CuO NP dispersion in water was employed for the preparation of all dispersions during the three long-term experiments (21 days of length), DLS measurements were carried out in fresh CuO NP initial dispersions (time 0), and after 1, 7, 14 and 21 days. In addition, given that CuO NP suspensions in L-15 medium were renewed every 6 or 7 days, those suspensions at 100 μg/ml were analysed by DLS

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