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ZnO nanoparticle tracking from uptake to genotoxic damage in human colon carcinoma cells

Maria Condello [a,b,1], Barbara De Berardis [a,1], Maria Grazia Ammendolia [a], Flavia Barone [c], Giancarlo Condello [d], Paolo Degan [e], Stefania Meschini [a,*]

[a] Department of Technology and Health, Italian National Institute of Health, Rome, Italy
[b] Institute of Chemical Methodologies, National Research Council (CNR), Rome, Italy
[c] Department of Environment and Primary Prevention, Italian National Institute of Health, Rome, Italy
[d] University of Rome Foro Italico, Department of Movement, Human and Health Sciences, Rome, Italy
[e] S.C. Mutagenesis, IRCCS AOU San Martino - IST (Istituto Nazionale per la Ricerca sul Cancro), CBA Torre A2, L.go R. Benzi 10, Genova, Italy

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ABSTRACT

Zinc Oxide (ZnO) nanoparticles are widely used both in the industry and in biomedical applications for their chemical and physical nanomaterial properties. It is therefore essential to go in depth into the cytotoxicity mechanisms and interactions between nanomaterials and cells. The aim of this work was to evaluate the dissolution of ZnO nanoparticles and their uptake, from a few minutes after treatments up to 24 h. ZnO nanoparticles routes of entry into the human colon carcinoma cells (LoVo) were followed at different times by a thorough ultrastructural investigation and semiquantitative analysis. The intracellular release of Zn^{2+} ions by Zinquin fluorescent dye, and phosphorylated histone H2AX (γ-H2AX) expression were evaluated. The genotoxic potential of ZnO nanoparticles was also investigated by determining the levels of 8-hydroxyl-2′-deoxyguanosine (8-oxodG). The experimental data show that ZnO nanoparticles entered LoVo cells by either passive diffusion or endocytosis or both, depending on the agglomeration state of the nanomaterial. ZnO nanoparticles coming into contact with acid pH of lysosomes altered organelles structure, resulting in the release of Zn^{2+} ions. The simultaneous presence of ZnO nanoparticles and Zn^{2+} ions in the LoVo cells determined the formation of reactive oxygen species at the mitochondrial and nuclear level, inducing severe DNA damage.

1. Introduction

The growing development of engineered nanomaterials offers great possibilities for industrial, commercial and biomedical applications, thanks to their unique physico-chemical properties, strongly related to their nanostructure. ZnO nanoparticles are an important example of nanomaterial, widely used in consumer and industrial products, due to their higher chemical reactivity, stronger oxidation and corrosion resistance, photocatalysis, absorption and UV-ray shielding capacity, antimicrobial, antifungal properties as compared with larger micro-sized counterparts (Madhumitha et al., 2016). Moreover, recent studies have shown ZnO nanoparticles to be promising candidates for biomedical applications and therapeutic interventions, and also successful as drug carrier and in targeted gene delivery (Peng et al., 2015; Velmurugan et al., 2015). They have also been shown to exert inherent anti-cancer cytotoxicity action (Rasmussen et al., 2010). An increasing amount of data on ZnO nanoparticles toxicity in a wide array of organisms such as bacteria, macroalgae, zebrafish, protozoa, and numerous cell lines, representative of different exposure routes to nanomaterials, are found in the literature (Kasemets et al., 2009; Mortimer et al., 2010; Vandebriel and De Jong, 2012; Zhu et al., 2008). Recent studies have shown that ZnO nanoparticles induced DNA damage in different cell lines, *e.g.* epidermal, nasal mucosa, lymphoblastoid, liver, kidney and neuronal cells (Bhattacharya et al., 2014; Guan et al., 2012; Hackenberg et al., 2011; Sharma et al., 2011, 2012; Valdiglesias et al., 2013). Yet, a complete understanding of the cell toxicity mechanism is still to be achieved. Numerous studies have focused on oxidative stress and its key role in the cell damage (Annangi et al., 2015; Saptarshi et al., 2015), but the effective role of nanoparticles dissolution, and which physico-chemical properties of ZnO nanoparticles are responsible for oxidative stress generation is still unclear. Most researchers attribute a key role to ion release from ZnO nanoparticles in oxidative stress

Abbreviations: ROS, reactive oxygen species; TEM, transmission electron microscopy; 8-oxodG, 7,8-dihydro-8-oxo-deoxyguanosine; SEM, scanning electron microscopy; FBS, foetal bovine serum; TB, trypan blue; FSC, forward side scatter; SSC, side scattered light; NAO, 10-nonylacridineorange; HPLC/EC, high-performance liquid chromatography; MFC, mean fluorescence channel; DSBs, double strand breaks; NPC, nuclear pore complex; SSBs, single strand breaks; STS, Staurosporine.

* Corresponding author at: Department of Technology and Health, Italian National Institute of Health, Viale Regina Elena 299, 00161 Rome, Italy.
E-mail address: stefania.meschini@iss.it (S. Meschini).

[1] Both authors contributed equally to this study.

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induction (Buerki-Thurnerr et al., 2013; Song et al., 2010; Xia et al., 2008). Zinc is a trace element essential for cell growth, division, DNA synthesis and RNA transcriptions. Both zinc deficiency and excess entail significant dysfunctions (Jayaraman and Jayaraman, 2011; John et al., 2010; Stefanidou et al., 2006). An increased level of exogenous zinc induces cytotoxicity, mitochondrial dysfunction and membrane depolarization, superoxide anion generation, caspase activation and apoptosis (Dineley et al., 2005; Donadelli et al., 2009; Gazaryan et al., 2007). Conversely, some studies suggest that direct cell-nanoparticle interaction leads to oxidative stress and toxicity in human cancer cells (Lin et al., 2009; Moos et al., 2010). Other researchers suggest that both cell-nanoparticle interaction and ions release contribute to cytotoxic effects (De Angelis et al., 2013; De Berardis et al., 2010; Hackenberg et al., 2011). Moreover, it is still not clear whether the ions leakage occurs in cell culture media or after cellular uptake of nanoparticles with subsequent increase in intracellular release of Zn^{2+}. Mu et al. (2014) have recently claimed that the cyto-genotoxicity induced by ZnO nanoparticles depended on the equilibrium solubility of nanoparticles in culture media and showed the complete dissolution of nanoparticles suspended in DMEM at 37 °C, pH 7.68, at a ZnO nanoparticle concentration up to 5.5 μg/ml. The authors indicate that above this concentration, the morphology and surface coating of ZnO nanoparticles become further factors contributing to toxicity.

In our previous study, we found a significant decrease in cell viability, oxidative stress and apoptosis induction *via* Reactive Oxygen Species (ROS) production and depolarization of inner mitochondrial membranes (De Berardis et al., 2010). We were not able, though, to discriminate between cell-nanoparticle interaction and ions leakage contributions to the toxicological effects observed.

In order to better understand the cytotoxic effect, the aim of this study was to evaluate the intracellular organization and location of ZnO nanoparticles, by following the cellular uptake from a few minutes of exposure up to 24 h of treatment. We carried out a semi-quantitative analysis of cellular uptake and subcellular ZnO nanoparticles localization at multiple time points by transmission electron microscopy (TEM). Literature data show that nanoparticles internalization is not sufficiently characterized, and there are no TEM studies evaluating the uptake of ZnO nanoparticles immediately after exposure (15–30 min) until the end of nanomaterial treatment. Combining high-resolution X-ray spectromicroscopy and high elemental sensitivity X-ray microprobe analyses, researchers determined the intracellular localization and chemical state of these nanoparticles only after one hour of exposure (Gilbert et al., 2012). These authors claim that a complete understanding of ZnO nanoparticles fate requires multiple time point observations. To quantify nanoparticles uptake, flow cytometry has mostly been used (Suzuki et al., 2007; Toduka et al., 2012), while the TEM technique is rarely performed (Belade et al., 2012).

We also evaluated, at the same time points, the intracellular ions release from ZnO nanoparticles by visualizing intracellular free Zn^{2+} ions with Zinquin, a fluorescent zinc-specific dye. In addition, we assessed the genotoxic potential of nanoparticles by determining 7,8-dihydro-8-oxo-deoxyguanosine (8-oxodG) levels, and the expression of phosphorylated histone H2AX (γ-H2AX). And in order to better elucidate their toxicity mechanism, we related nanoparticle uptake, cell-nanoparticle interaction and Zn^{2+} ions release to the genotoxic effect.

2. Material and methods

2.1. Nanoparticles and single nanoparticles characterization in culture medium

ZnO nanoparticles were purchased from Sigma-Aldrich Company Ltd. (Gillingam, Dorset, UK). The information of the ZnO nanoparticles dissolution in culture medium was published in De Berardis et al., 2010. The nominal size of nanoparticles, as indicated by manufactures, was 50–70 nm. Physico–chemical characteristics of nanoparticles (primary size, size distribution, shape, agglomeration status, and chemical composition) were determined by TEM and scanning electron microscope (SEM) equipped with a thin window EDAX system for X-ray microanalysis and a particle analysis system, as shown in a previous study (De Berardis et al., 2010). Briefly, two different morphologies were observed for ZnO nanoparticles: spherules with primary size ranging from 45 to 60 nm, and rod-like particles, showing a width of 70 nm and a length of 170 nm. The size distribution of ZnO nanoparticles in F12 culture medium ranged from 45 nm to 600 nm and the average diameter was 196 nm. Finally we estimated that the purity of ZnO nanoparticles was 98% ± 2%.

2.2. Cell cultures and treatments

The established human colon carcinoma cell line (LoVo) was grown as monolayer in Ham's F12 medium supplemented with 10% foetal bovine serum (FBS, Hyclone, Europe Ltd., Cramlington, UK), with 1% L-glutamine, penicillin (50 U/ml), streptomycin (50 μg/ml) and 1% vitamins at 37 °C in a 5% CO_2 humidified atmosphere.

For experiments, 2×10^5 cells/well were seeded in 6-wells plate. After 72 h, growth medium was removed and cells were treated with ZnO nanoparticles resuspended in Ham's F12 medium without FBS at concentrations and time points specified below. To evaluate the intracellular dissolution of ZnO nanoparticles by Zinquin probe, after 24 h of treatment, the medium with the nanoparticles was removed and replaced with medium free.

2.3. Transmission electron microscopy (TEM)

After treatment with 10 μg/cm^2 of ZnO nanoparticles for 30 min, 1 h, 2 h, 4 h, 6 h, 24 h both detached and adherent cells were collected. After centrifugation, the pellet was fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at room temperature for 40 min. After postfixation with 1% OsO_4 in 0.2 M cacodylate buffer (pH 7.3) at room temperature for 1 h, cells were dehydrated with ascending concentrations of ethanol, and embedded in epoxy resin (TAAB Laboratories Equipment Limited, Aldermarton, UK). Ultrathin sections, obtained with an LKB Ultratome Nova ultramicrotome (LKB, Bromma, Sweden), were examined with a Philips EM208S transmission electron microscope (FEI Company, Eindhoven, The Netherland).

For the semi-quantitative analysis of cellular uptake by TEM, samples were examined at a magnification of 3200 times on 30 fields of view chosen randomly from different grids for each condition of exposure, ranging from 30 min to 24 h. Each field of view was of 33.69 × 25.26 μm. The number of cells observed in each field and the number of them internalizing nanoparticles were determined. In this condition of analysis a number of cells ranging from 50 to 60 were detected and analysed for each time of exposure.

The error on abundance of cells internalizing nanoparticles was estimated assuming a binomial distribution model, accounting for the presence or absence of nanoparticles in a cell. In such case, the relative error RE can be calculated as:

$$RE = [(1-p_c)/(p_c N)]^{1/2}$$

with p_c equal to the estimated abundance of cells internalizing nanoparticles and N being the total number of cells analysed. Statistically significant differences were assessed by Student's paired *t*-test. A p value ≤ 0.05 was considered significant.

2.4. Cellular morphological changes and nanoparticles uptake

The cellular morphological changes and the potential uptake of ZnO nanoparticles were measured by flow cytometric analysis of the physical parameters (Kumar et al., 2011; Zucker et al., 2010). Cells, untreated and treated with 5 and 10 μg/cm^2 ZnO nanoparticles for 2 h or 6 h, were