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"*In vitro* toxicity studies of zinc oxide nano- and microrods on mammalian cells: A comparative analysis"

Elena Zanni [a,b], Silvia De Palma [c], Chandrakanth Reddy Chandraiahgari [b,d], Giovanni De Bellis [b,d], Samantha Cialfi [e], Claudio Talora [e], Claudio Palleschi [a,b], Maria Sabrina Sarto [b,d], Daniela Uccelletti [a,b,*], Patrizia Mancini [c,**]

[a] Department of Biology and Biotechnology C. Darwin, Sapienza University of Rome, Piazzale Aldo Moro 5, Rome, Italy
[b] Research Center on Nanotechnology Applied to Engineering of Sapienza (CNIS), SNNLab, Sapienza University of Rome, Piazzale Aldo Moro 5, Rome, Italy
[c] Department of Experimental Medicine, Sapienza University of Rome, Viale Regina Elena 324, Rome, Italy
[d] Department of Astronautic, Electrical and Energetic Engineering, Sapienza University of Rome, via Eudossiana 18, Rome, Italy
[e] Department of Molecular Medicine, Sapienza University of Rome, Viale Regina Elena 291, Rome, Italy

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ABSTRACT

This study aims at investigating the cytotoxicity of zinc oxide nanorods (ZnO-NR) and microrods (ZnO-MR) in two different human cell lines. The materials were produced by a simple and cost-effective hydrothermal route and induced a slight dose dependent toxic effect on MCF7 and HaCaT cells. Both cell lines showed a very low cytotoxicity when exposed to suspensions containing 1–100 μg/ml of ZnO-NR and ZnO-MR. Moreover, Field-emission scanning electron microscopy (FE-SEM) images of both cell lines exposed to the materials showed no significant effect on cell morphology compared to untreated cells. However, the treatments induced modifications of filopodia, mainly in MCF7 cells exposed to ZnO-NR. In addition, a very slight increase in ROS generation was observed only in ZnO-MR-treated cells. Moreover, an apoptotic effect of ZnO-NR rather than a cell cycle arrest was observed in MCF7 cells after treatment.

Overall the cytotoxicity in *in vitro* systems reflects a biocompatibility of both ZnO-NR and ZnO-MR, representing powerful tools in several applications.

1. Introduction

Currently, over 500 consumer products in the market claim to contain elements of nanoscience and nanotechnology with daily new entries [1]. This market annually requires metric tons of raw nanomaterials, ranging from nano-sized metals and metal oxide particles to carbon nanotubes [2–4]. Metal oxide nanomaterials possess enhanced physico-chemical properties, such as mechanical, thermal, or electrical conductivity that has attracted a great deal of research interest for many potential applications [5,6]. Ever-increasing demand and utilization of these materials ultimately emerge as multiple different sources of their disposal into the environment [6,7]. Therefore, toxicity and risk assessments of nanomaterials have received much attention. Specific interactions of these materials with biological systems originate mainly from their small size, large surface area, and intrinsic reactivity [8,9].

Among metal oxides utilized in the field of nanotechnology, zinc oxide (ZnO) is drawing much research interest due to its unique optical, luminescent, electronic and biocompatible properties.

In recent years, many methods have been used to synthesize ZnO material as one-dimensional (1D) nanostructures with different morphologies including nanowires, nanorods, and other superstructures [10–12]. The high-yield mass production of such nanostructures by catalyst-free methods is a crucial aspect to enable a cost-effective large-scale development of new ZnO-based materials. Recently, a method for the mass-production of high-purity ZnO-nanorods with a uniform size distribution was reported [13]. The same material was previously shown to have antimicrobial properties against Gram-positive bacteria such as *Staphylococcus aureus* [14].

As the uses of nanomaterials are potentially extensive, and new

* Corresponding author at: Department of Biology and Biotechnology C. Darwin, Sapienza University of Rome, Piazzale Aldo Moro 5, Rome, Italy
** Corresponding author. Department of Experimental Medicine, Sapienza University of Rome, Viale Regina Elena 324, Rome, Italy
E-mail addresses: elena.zanni@uniroma1.it (E. Zanni), silvi.adp@live.it (S. De Palma), c.chandraiahgari@uniroma1.it (C.R. Chandraiahgari), giovanni.debellis@uniroma1.it (G. De Bellis), samantha.cialfi@uniroma1.it (S. Cialfi), claudio.talora@uniroma1.it (C. Talora), claudio.palleschi@uniroma1.it (C. Palleschi), mariasabrina.sarto@uniroma1.it (M.S. Sarto), daniela.uccelletti@uniroma1.it (D. Uccelletti), patrizia.mancini@uniroma1.it (P. Mancini).

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applications continue to emerge, in order to benefit of the nanomaterials and their applications it is essential to understand their implication for human safety. Due to the promisingly innovative characteristics of the above cited large scale production procedures, the toxicity evaluation of zinc oxide nanorods and microrods has been compared for the first time against two different human cell lines and here reported.

2. Experimental

2.1. ZnO materials

Reagent grade chemicals: Zinc acetate dihydrate ($Zn(CH_3COO)_2 \cdot 2H_2O$, Sigma), hexamethylenetetramine ($C_6H_{12}N_4$, Fisher Scientific), zinc nitrate hexahydrate ($Zn(NO_3)_2 \cdot 6H_2O$, Acros Organics).

ZnO-MR and ZnO-NR were synthesized through a hydrothermal process and the thermal decomposition method, respectively, and characterized as described in our earlier work [13]. From FE-SEM analysis it resulted that the ZnO-NR, have diameter ranging between 20 nm and 40 nm and lengths up to 4 μm, whereas ZnO-MR, generally aggregated in cross- or star-like clusters, have dimensions ranging between 200 nm and 500 nm in diameter and 2–4 μm in lengths (Fig. S1).

2.2. Cell culture, viability and proliferation

MCF7 and HaCaT cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Euroclone), supplemented with 10% fetal bovine serum and maintained in a humidified incubator with 5% CO_2 at 37 °C.

The suspensions of ZnO-NR and ZnO-MR were freshly prepared (1 mg/ml) in DMEM followed by 30 min sonication, and subsequently diluted.

MCF7 and HaCaT cells were seeded on 24-well plates, cultured overnight, and incubated or not with ZnO-NR or ZnO-MR for 24 h (cell viability) or for 24, 48 and 72 h (cell proliferation) at the indicated concentrations. Cells were detached by 0.05% trypsin-EDTA solution and stained with Trypan blue for 2 min. The number of dead and viable cells was obtained by counting manually using a hemocytometer. Cell viability was expressed as the percentage relative to the untreated control cells. For cell proliferation, the number of cells was compared to that counted before treatment (t0).

2.3. FE-SEM and immunofluorescence analysis

MCF7 and HaCaT cells, grown on coverslips, and treated for 24 h with 20 μg/ml ZnO-NR or ZnO-MR, were fixed, in the case of FE-SEM, with 2% glutaraldehyde for 1 h, post-fixed with 1% osmium tetroxide for 1 h and dehydrated in a series of increasing ethanol concentrations (30%, 50%, 70%, 95% and 100%). The cover glasses were then mounted on the stubs and cells examined with a FE-SEM (Auriga, Zeiss) using accelerating voltages between 1 kV and 3 kV.

For immunofluorescence, cells were fixed with 4% paraformaldehyde in PBS for 30 min, treated with 0.1 M glycine in PBS for 20 min and with 0.1% Triton X-100 in PBS for additional 5 min to allow permeabilization. Cells were then incubated with TRITC-labeled phalloidin (Sigma) in PBS for 45 min and with 10 μg/ml DAPI (Sigma). Coverslips were mounted with Mowiol (Calbiochem) for observation. Fluorescence signal was analyzed using an Axio Observer inverted microscope, equipped with the ApoTome System (Carl Zeiss).

2.4. Detection of intracellular ROS

Cells, grown on glass coverslips, were treated as in FE-SEM analysis, and subsequently incubated with H_2DCF, 75 μM, at 37 °C for 15 min, and observed at microscope. The detection of ROS was assessed by evaluating the number of positive cells for ROS production compared to the total number of counted cells.

2.5. Flow cytometry analysis

MCF7 and HaCaT cells were treated with 20 μg/ml concentration of ZnO-NR or ZnO-MR for 72 h. After harvested the cells adhered on the dishes were washed in 1% PBS, fixed in chilled 70% ethanol, treated with RNase A (50 μg/ml) for 15 min at 37 °C and stained with propidium iodide (PI) (10 μg/ml) in the dark for 30 min at room temperature. Data acquisition and analysis was carried out using a flow cytometry. Data analysis was performed with CellQuest Pro software (BD Biosciences).

3. Results and discussion

In order to assess the effect of ZnO-NR and ZnO-MR on cell viability, we used two human cell lines: the breast cancer cells MCF7 and the immortalized keratinocytes HaCaT, treated with different concentrations of ZnO rods for 24 h. A very low cytotoxic effect was observed when the cells were exposed to ZnO-MR; in fact, in both cell lines the survival was greater than 70% at the highest tested concentration (100 μg/ml) (Fig. 1). HaCaT cells showed a slightly higher cytotoxicity compared to MCF7; the exposure of HaCaT to ZnO-MR showed a low decrease of viability also at the concentration of 50 μg/ml, while no significant reduction of viable cells was observed for MCF7 cells (Fig. 1). Similar effects were found when cells were exposed to ZnO-NR. Indeed, less than 30% of MCF7 or HaCaT cells did not survive after the treatment with the two highest concentrations (Fig. 1).

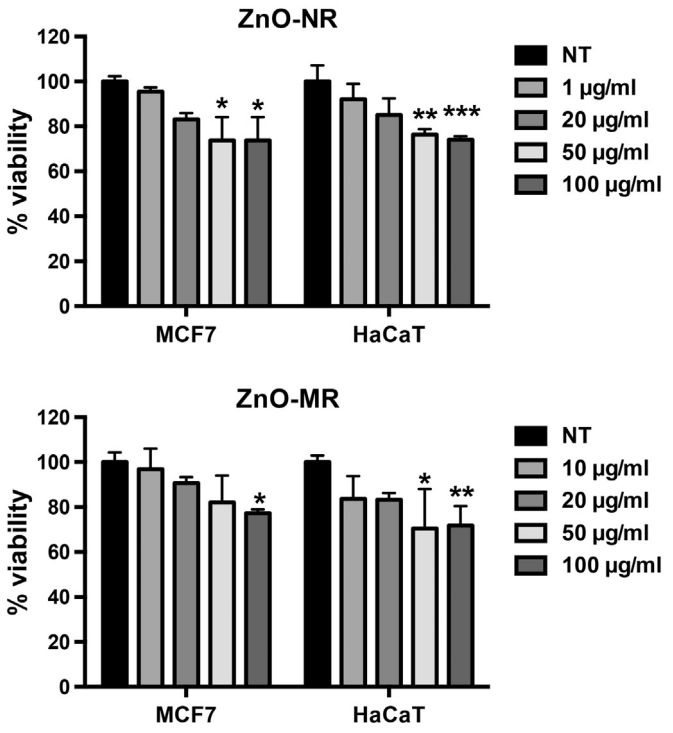

Fig. 1. Viability of MCF7 and HaCaT cells exposed to ZnO-NR or ZnO-MR for 24 h at different concentrations.