Environment International 55 (2013) 92–100

Contents lists available at SciVerse ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Neuronal cytotoxicity and genotoxicity induced by zinc oxide nanoparticles

Vanessa Valdiglesias [a,b,1], Carla Costa [c,*,1], Gözde Kiliç [a,d], Solange Costa [c], Eduardo Pásaro [a], Blanca Laffon [a], João Paulo Teixeira [c]

[a] Toxicology Unit, Department of Psychobiology, University of A Coruña, Edificio de Servicios Centrales de Investigación, Campus Elviña s/n, 15071, A Coruña, Spain
[b] Clinical and Molecular Epidemiology, IRCCS San Raffaele Pisana, Via di Val Cannuta, 247, 00166, Roma, Italy
[c] Department of Environmental Health, Portuguese National Institute of Health, Rua Alexandre Herculano, 321, 4000-055, Porto, Portugal
[d] Department of Cell and Molecular Biology, University of A Coruña, Facultad de Ciencias, Campus A Zapateira s/n, 15071, A Coruña, Spain

ARTICLE INFO

Article history:
Received 24 December 2012
Accepted 28 February 2013
Available online 26 March 2013

Keywords:
Zinc oxide nanoparticles
Cytotoxicity
Genotoxicity
Oxidative damage
SHSY5Y cells
Zn^{+2} ions

ABSTRACT

Zinc oxide nanoparticles (ZnO NPs) are one of the most abundantly used nanomaterials in consumer products and biomedical applications. As a result, human exposure to these NPs is highly frequent and they have become an issue of concern to public health. Although toxicity of ZnO NPs has been extensively studied and they have been shown to affect many different cell types and animal systems, there is a significant lack of toxicological data for ZnO NPs on the nervous system, especially for human neuronal cells and tissues. In this study, the cytotoxic and genotoxic effects of ZnO NPs on human SHSY5Y neuronal cells were investigated under different exposure conditions. Results obtained by flow cytometry showed that ZnO NPs do not enter the neuronal cells, but their presence in the medium induced cytotoxicity, including viability decrease, apoptosis and cell cycle alterations, and genotoxicity, including micronuclei production, H2AX phosphorylation and DNA damage, both primary and oxidative, on human neuronal cells in a dose- and time-dependent manner. Free Zn^{2+} ions released from the ZnO NPs were not responsible for the viability decrease, but their role on other types of cell damage cannot be ruled out. The results obtained in this work contribute to increase the knowledge on the genotoxic and cytotoxic potential of ZnO NPs in general, and specifically on human neuronal cells, but further investigations are required to understand the action mechanism underlying the cytotoxic and genotoxic effects observed.

1. Introduction

Zinc oxide nanoparticles (ZnO NPs) are one of the most abundantly used nanomaterials in consumer products and biomedical applications due to their specific properties, e.g. transparency, high isoelectric point, biocompatibility, and photocatalytic efficiency. They are widely employed in a variety of devices including cosmetics, toothpaste, sunscreens, fillings in medical materials, textiles, wall paints, and other building materials, and they can be also utilized in environmental remediation for elimination or degradation of pollutants in water or air (Qiang, 2001). Furthermore, ZnO NPs have promising applications in the medicine field since they have been proposed as a possible treatment for cancer and/or autoimmune diseases after being found to be selectively toxic towards potential disease-causing cells (Hanley et al., 2008; Premanathan et al., 2011; Akhtar et al., 2012), and they are being considered to be used in fabrication of nerve guidance channels for treatment of nerve injury (Seil and Webster, 2008). As a result of all these uses, human exposure to these NPs is highly frequent. They can enter the organism through different pathways (respiratory tract, digestive system and parenteral routes) and have shown a systemic distribution in in vivo studies (Vandebriel and De Jong, 2012), so they can potentially reach any organ or tissue and involve a risk for human health. Toxicity of ZnO NPs has been extensively studied and they have been shown to affect many different cell types and animal systems (Chiang et al., 2012; De Berardis et al., 2010; Osman et al., 2010; Sharma et al., 2012a, 2012b; Wahab et al., 2011). Commonly, the toxicity of NPs is associated with their small size and high specific surface area and therefore, nanoforms are theoretically expected to be more toxic than their bulk counterparts (Xiong et al., 2011).

In recent years, there have been an increasing number of works reporting that different NPs can reach the brain and cause neurological injuries, being associated even with neurodegenerative diseases (Block et al., 2004; Hu and Gao, 2010; Peters et al., 2006). This translocation can happen both directly, through axonal transport from olfactory epithelium, or indirectly by passing to the bloodstream and crossing the blood brain barrier (Oberdörster et al., 2004). Similarly to other metal oxide NPs, it has been recently found that ZnO NPs reach the brain of experimental animals after oral (Lee et al., 2012) and inhalatory (Kao et al., 2012a) administration. Still, there is a significant lack of toxicological data for ZnO NPs on nervous system, especially for human neuronal cells and tissues.

In vitro data have shown that ZnO NPs induce cytotoxicity in mouse neuroblastoma Neuro-2A cells and neural stem cells (Deng et al., 2009; Jeng and Swanson, 2006), in rat RSC96 Schwann cells and primary

* Corresponding author. Tel.: +351 223401147; fax: +351 223401149.
E-mail address: cstcosta@gmail.com (C. Costa).
[1] These authors contributed equally to this work.

0160-4120/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.envint.2013.02.013

neuronal cells (Chiang et al., 2012; Yin et al., 2012), and in human glioma cells (Ostrovsky et al., 2009). Besides, they were found to enhance the excitability of rat neurons by altering the ion channels (Zhao et al., 2009) and to decrease the adhesion of rat astroglial cells (Seil and Webster, 2008), although in this last case the cells were exposed to composite materials with ZnO NPs.

The only in vivo study describing neurological effects after ZnO NP exposure reported attenuation in spatial learning and memory ability by alteration of synaptic plasticity in rats after intraperitoneal administration (Han et al., 2011). Besides, morphological and histochemical changes in brains of rats fed with bulk ZnO were also described (Kozik et al., 1980).

Given the wide and frequent use of ZnO NPs in many fields closely related to human beings, their promising beneficial applications in medicine, and the scarce knowledge on their potential neurotoxic effects, the main objective of this work was to investigate the cytotoxic and genotoxic effects of ZnO NPs on human SHSY5Y neuronal cells and to explore the underlying mechanisms involved in these effects.

2. Materials and methods

2.1. Chemicals

ZnO NPs (CAS No. 1314-13-2), mytomycin C (MMC) (CAS No. 50-07-7), bleomycin (BLM) (CAS No. 9041-93-4), camptothecin (Campt) (CAS No. 7689-03-4), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (CAS No. 298-93-1), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1) (CAS No. 3520-43-2), neutral red dye (CAS No. 553-24-2), and propidium iodide (PI) were purchased from Sigma-Aldrich Co. (Madrid, Spain). MMC, BLM, and Campt were dissolved in sterile distilled water prior use.

2.2. Nanoparticle suspension: preparation and characterization

A stock suspension of ZnO NPs (final concentration 80 μg/ml) was prepared in either deionized water or complete cell culture medium (with fetal bovine serum, FBS). Prior to each treatment, this suspension was ultrasonicated (Branson Sonifier, USA) at 30 W for 5 min (1.5 min on and 1 min off twice, and 2 min on), and diluted to prepare the different NP concentrations tested. Average hydrodynamic size, size distribution and zeta potential of particles in suspension were determined by Dynamic Light Scattering (DLS) using a Zetasizer Nano-ZS equipped with 4.0 mW, 633 nm laser (Model ZEN 3600, Malvern Instruments Ltd., Malvern, UK).

2.3. Cell culture and treatments

Human neuroblastoma SHSY5Y cell line was obtained from the European Collection of Cell Cultures and cultured in nutrient mixture EMEM/F12 (1:1) medium with 1% non-essential amino acids, 1% antibiotic and antimycotic solution, and supplemented with 10% heat-inactivated fetal bovine serum (FBS), all from Invitrogen (Barcelona, Spain). The cells were incubated in a humidified atmosphere with 5% CO_2 at 37 °C. To carry out the experiments, cells were seeded in 96-well plates (flat bottom) and allowed to adhere for 24 h at 37 °C. Cell densities were approximately in the range of 2–5 × 10^5 cells/well at the beginning of cell culture. For cell treatment, these were incubated at 37 °C for different periods in the presence of a variable number of NP concentrations, depending on the assay performed, or the control solutions. Complete medium was used as negative control in all experiments. The following chemicals were used as positive controls: Campt (10 μM) in apoptosis assessment, cell cycle and H2AX analysis; and MMC (1.5 μM), BLM (1 μg/ml) and H_2O_2 (2 μM for 3 h treatments, and 1 μM for 6 h treatments) in MN test, comet assay, and oxidative DNA damage evaluation, respectively. Besides, TiO_2 NPs (120 μg/ml) and triton X-100 (1%) were employed as positive controls in the cellular uptake and the lactate dehydrogenase (LDH) assays, respectively.

2.4. Cellular viability

MTT assay (according to Mosmann, 1983) and NRU assay (according to Borenfreund and Puerner, 1985) were used to test the potential effects of ZnO NPs on viability of neuronal SHSY5Y cells. In each assay, 8 different NP concentrations (0–80 μg/ml) and 4 exposure times (3, 6, 24, and 48 h) were evaluated. Absorbance was measured at 595 nm (MTT) or 540 nm (NRU) using a Cambrex ELx808 microplate reader (Biotek, KC4). A parallel set of experiments conducted without cells was carried out to exclude the potential interaction of the NPs with the dyes used in MTT and NRU assays. Data obtained demonstrated no interaction between the ZnO NPs tested and the dyes used for cytotoxicity assessment. From the results obtained in the viability experiments, two exposure times (3 and 6 h) and three different concentrations of ZnO NPs (20, 30 and 40 μg/ml) were selected to perform the subsequent experiments.

2.5. Cellular uptake

A flow cytometry methodology (FACSCalibur Flow Cytometer, Becton Dickinson, Madrid, Spain) was employed to assess the potential of the ZnO NPs to enter the cells. The analysis was carried out on the basis of the size and complexity of the cells by measuring the forward scatter (FSC) and the side scatter (SSC) following the protocol described by Suzuki et al. (2007).

2.6. Cell cycle

The cell distribution along the different phases of the cell cycle was examined in cells treated with ZnO NPs or the controls by evaluating the relative cellular DNA content with a flow cytometry technique (FACSCalibur Flow Cytometer, Becton Dickinson, Madrid, Spain) following Valdiglesias et al. (2011a). DNA content was assessed from the PI signal detected by the FL2 detector in a minimum of 10^4 events. Cell Quest Pro software (Becton Dickinson, Madrid, Spain) was used to analyze cell cycle histograms, to calculate the percentage of occupancy of G_0/G_1, S and G_2/M regions.

2.7. Membrane integrity

Lactate dehydrogenase (LDH) activity in cell culture medium was measured by using a commercial kit (Roche Diagnostics Corp) according to the manufacturer's instructions. After exposure, half the amount of the cell culture medium was collected for LDH measurement. Absorption was measured at 490 nm with a reference wavelength of 655 nm using a Cambrex ELx808 microplate reader (Biotek, KC4). Positive control experiments were performed with 0.1% Triton X-100 and set as 100% cytotoxicity. LDH release was calculated by the following equation:

$$\mathrm{LDH}(\%) = \frac{[A]_{\mathrm{sample}} - [A]_{\mathrm{medium}}}{[A]_{\mathrm{positivecontrol}} - [A]_{\mathrm{medium}}} \times 100$$

where $[A]_{\mathrm{sample}}$, $[A]_{\mathrm{medium}}$, and $[A]_{\mathrm{positive\ control}}$ denote the absorbance of the sample, medium negative control and Triton X-100 positive control, respectively.

2.8. Apoptosis

2.8.1. Analysis of apoptosis by PI–annexin V staining

Apoptosis rate was determined by means of annexin V/PI double staining, using the BD Pharmingen™ annexin V–FITC apoptosis detection kit I (Becton Dickinson, Madrid, Spain), following the manufacturer's recommendations. At least 10^4 events were acquired with a FACSCalibur Flow Cytometry (Becton Dickinson, Madrid, Spain). Data from annexin