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Zinc oxide nanoparticle induced autophagic cell death and mitochondrial damage via reactive oxygen species generation

Kyeong-Nam Yu [a,1], Tae-Jong Yoon [b,1], Arash Minai-Tehrani [a], Ji-Eun Kim [a,c], Soo Jin Park [d], Min Sook Jeong [d], Shin-Woo Ha [e], Jin-Kyu Lee [f], Jun Sung Kim [d,*], Myung-Haing Cho [a,c,g,h,*]

[a] Laboratory of Toxicology, College of Veterinary Medicine, Seoul National University, Seoul 151-742, Republic of Korea
[b] Department of Applied BioScience, CHA University, Seoul 135-907, Republic of Korea
[c] Department of Nano Fusion Technology, Graduate School of Convergence Science and Technology, Seoul National University, Suwon 443-270, Republic of Korea
[d] R&D Center, Biterials Co., Ltd., Seoul 140-200, Republic of Korea
[e] Division of Endocrinology, Metabolism and Lipids, Emory University School of Medicine, 101 Woodruff Circle, WMRB Room 1327 Atlanta, GA 30322, United States
[f] Department of Chemistry, College of Natural Sciences, Seoul National University, Seoul 151-742, Republic of Korea
[g] Graduate Group of Tumor Biology, Seoul National University, Seoul 151-742, Republic of Korea
[h] Advanced Institute of Convergence Technology, Seoul National University, Suwon 443-270, Republic of Korea

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ABSTRACT

Zinc oxide nanoparticles (ZnO-np) are used in an increasing number of industrial products such as paint, coating and cosmetics, and in other biological applications. There have been many suggestions of a ZnO-np toxicity paradigm but the underlying molecular mechanisms about the toxicity of ZnO-np remain unclear. This study was done to determine the potential toxicity of ZnO-np and to assess the toxicity mechanism in normal skin cells. Synthesized ZnO-np generated reactive oxygen species (ROS), as determined by electron spin resonance. After uptake into cells, ZnO-np induced ROS in a concentration- and time-dependent manner. To demonstrate ZnO-np toxicity mechanism related to ROS, we detected abnormal autophagic vacuoles accumulation and mitochondria dysfunction after ZnO-np treatment. Furthermore mitochondria membrane potential and adenosine-5′-triphosphate (ATP) production are decreased for culture with ZnO-np. We conclude that ZnO-np leads to cell death through autophagic vacuole accumulation and mitochondria damage in normal skin cells via ROS induction. Accordingly, ZnO-np may cause toxicity and the results highlight and need for careful regulation of ZnO-np production and use.

1. Introduction

Zinc oxide nanoparticles (ZnO-np) have become increasingly popular in electronics, catalysis, clothing, paints, sunscreens and as components of various cosmetics owing to their semi-conductive property and white color (Serpone et al., 2007; Yuranova et al., 2007; Wang, 2008). Current interest in ZnO-np is focused on their medicinal use and biological applications, including as a biosensor (Hanley et al., 2008; Ostrovsky et al., 2009; Premanathan et al., 2011). However, these applications increase human and environmental exposure, and the potential risk related to their toxicity. The metallic nature of metal-derived nanoparticles and the presence of transition metals induce the production of reactive oxygen species (ROS) leading to oxidative stress (MacNee and Donaldson, 2003; Jia et al., 2008; Durocher et al., 2009). Elemental metal nanoparticles such as cadmium and silver can induce oxidative stress and apoptosis in various cell types (Kirchner et al., 2005; Arora et al., 2008).

Abbreviations: ZnO-np, zinc oxide nanoparticles; ROS, reactive oxygen species; H_2-DCFDA, 2,7-dichlorodihydrofluorescein diacetate; NAC, N-acetylcysteine; XRD, X-ray diffraction; ESR, Electron spin resonance; EDX, Energy dispersive X-ray spectrometer; TEM, transmission electron microscope; PBS, phosphate buffered saline; CSLM, confocal laser scanning microscopy; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; TEMP, 2,2,6,6-tetramethylpiperidin; ATG5, autophagy related genes 5; BECN1, Beclin 1; *ptf*LC3, mRFP–GFP tandem fluorescent-tagged LC3; HSP60, heat shock protein 60; VDAC, protein voltage-dependent anion channel protein; AIF, apoptosis inducing factor; JC-1, 5,5′,6′,6′-tetrachloro-1,1′,3,3′–tetraethyl benzimidazolylcarbocyanine iodide; ND6, NADH dehydrogenase subunit 6; SDHA, succinate dehydrogenase complex subunit A; COX IV, Cytochrome c oxidase IV; AMPK, AMP-activated protein kinase.

* Corresponding authors. Address: Laboratory of Toxicology, College of Veterinary Medicine, Seoul National University, Seoul 151-742, Republic of Korea. Tel.: +82 2 880 1276; fax: +82 2 873 1268 (M.-H. Cho), tel.: +82 2 792 3776; fax: +82 2 792 3779 (J.S. Kim).

E-mail addresses: lifeisgood@biterilas.com (J.S. Kim), mchotox@snu.ac.kr (M.-H. Cho).

[1] These authors contributed equally.

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Autophagy involves a multi-step lysosomal degradation process in which cells degrade aged proteins and discarded organelles such as mitochondria (Mizushima et al., 2008; Wang et al., 2011). Stress-activated cellular process for adaptation is important for cells to either tolerate adverse conditions or activate cell death mechanisms such as apoptosis to alleviate damage and potentially dangerous cells (Hanahan, 2000). In contrast to the survival-inducing function autophagy, which is supported by significant evidence, activation of autophagic cell death has been reported as a possible tumor suppression mechanism. This led to the observation that cell death can be present related with features of autophagy (Kroemer and Levine, 2008). Prolonged stress and progressive autophagy can also eventually lead to cell death (Mathew et al., 2007). Excessive cellular damage may lead to cell death by overstimulating autophagy and cellular self consumption (Turcotte et al., 2008).

Mitochondria are vital organelles in cells that participate in energy differentiation and supply. There is a close relationship between mitochondria and ROS production. An abnormal cellular ROS balance can be activated by the structural injury of mitochondria. Furthermore, excess ROS production can induce mitochondrial damage. Mitochondria are considered to be the major cell compartment that can be deleteriously affected by nanoparticle toxicity (Unfried et al., 2007). ROS produced as a by-product of respiration as well as exogenous ROS can induce autophagy due to mitochondrial damage. Mitochondria are the main source of ROS for regulation of autophagy (Shiratora et al., 1989; Chen and Gibson, 2008).

In this study, we synthesized ZnO-np and assessed their potential capability in inducing autophagy and mitochondria disruption, as well as their possible association with cytotoxicity in normal skin cells.

2. Materials and methods

2.1. Synthesis of ZnO-np

To synthesize ZnO-np, zinc acetate dihydrate [$Zn(CH_3CO_2)_2{\cdot}2H_2O$; Sigma–Aldrich, USA] was used as a precursor; potassium hydroxide (KOH, Sigma–Aldrich) and methanol (CH_3OH, Duksan, Korea) were used to prepare a basic solution. $Zn(CH_3CO_2)_2{\cdot}2H_2O$ (14.75 g, 67.2 mmol) was completely dissolved in 120 ml methanol at 65 °C. After heating, a basic solution prepared by dissolving potassium hydroxide (7.4 g, 132 mmol) in methanol (30 ml) was added at room temperature (RT), and the resulting solution was refluxed at 60 °C for 5 days. Once the reacted solution had cooled to RT, it was cleared by centrifugation at 3100*g* for 20 min. The resulting sediments were washed with methanol and dried in an oven at 70 °C for 10 h (Pacholski et al., 2002; Song et al., 2008). Some portions of the samples were prepared as a powder and others were re-dissolved in H_2O to make an aqueous solution.

2.2. Particle characterization

The structure property of ZnO-np nanoparticle was characterized by powder X-ray diffraction (XRD) using a Philips-X'ert PW1827 apparatus (Philips, the Netherlands) with Cu Ka radiation (λ = 1.54064 Å); Samples (about 1 g) were placed onto an aluminum holder. The goniometer was motorized and moved through a range of θ–2θ mode scan. The diffractometer was run at 40 kV and 30 mA over a range of 10–80°. Steps were increments of 0.05° and counts were collected for 5 s at each step. For energy dispersive X-ray (EDX) analysis, the fixed samples were coated with carbon and analyzed using a Tecnai F20 apparatus (FEI, USA).

2.3. Transmission electron microscopy (TEM)

After ZnO-np treatment (10 μg/mL) of JB6 41-5a cells (5×10^6), the cells were fixed with a solution of 2.5% glutaraldehyde with 1% osmium tetroxide (OsO4) buffer for 4 h at 4 °C and dehydrated with ethanol. Then, cells were infiltrated in a 1:1 mixture of propylene oxide and Epon, and finally embedded in Epon by polymerization at 70 °C overnight. Ultrathin sections (about 50 mm) were cut and mounted on pioloform-coated copper grids. Sections were stained with lead citrate and uranyl acetate and viewed with a JEM 1010 transmission electron microscope (JEOL, Tokyo, Japan). The morphology and nanostructures of ZnO-np were further characterized by TEM using an accelerating voltage 100 kV for nanostructure. Samples for TEM analysis were prepared by ultrasonically dispersing the products into methanol, placing a drop of this suspension onto a formvar–carbon film on 300-square mesh copper grid, and drying the grid (RT for 1 h).

2.4. Cell culture and viability experiment

JB6 Cl 41-5a mouse skin epidermal normal cells (ATCC No. CRL-2010™) were kindly provide by Dr. N.H. Colburn (Center for Cancer Research, National Cancer Institute). Cells were incubated in Minimum Essential Medium (Eagle, USA) with 2 mM L-glutamine and Earle's basal salts solution adjusted to contain 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 95% 1.0 mM sodium pyruvate and 5% heat-inactivated fetal bovine serum at 37 °C. We observed viability and proliferation capacity of the skin normal cells with ZnO-np, we used xCELLigence (Roche, USA). In electron chamber, we seeded the cells 0.5×10^4 in each cells. After an overnight incubation, ZnO-np was added to the chamber, and cell viability and proliferation were monitored.

2.5. Measurement of ROS

In order to determine ROS formation by ZnO-np itself, we detected electron spin resonance (ESR) with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) and 2,2,6,6-tetramethylpiperidin (TEMP). We comparatively measured hydroxyl radicals from ZnO-np (10 mg/mL), ZnO-np with DMPO (1.5 M), ZnO-np with DMPO and dimethylsulfoxide (DMSO, 10%; as a hydroxyl radical scavenger). Also, to measure the singlet oxygen, we compared ZnO-np (10 mg/mL), ZnO-np with TEMP (4.7 mM) and NaN_3-treated ZnO-np with TEMP. To measure intracellular ROS production, 2,7-dichlorodihydrofluorescein diacetate (H_2-DCFDA) was used with/without N-acetylcysteine (NAC, 10 mM) as ROS scavenger. H_2-DCFDA is a cell-permeant indicator for ROS that is nonfluorescent until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell (Kahn et al., 2005). Briefly, 10 mM H_2-DCFDA stock solution in DMSO was diluted in culture medium to produce a 5 μM working solution. After 1 h incubation at RT, cells were washed twice with pre-warmed phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde. Cell fluorescence was detected by confocal laser scanning microscopy (CSLM) with a TCS SP5 II apparatus (Leica, Germany) and by fluorescence-activated cells sorting (FACS; BD Bioscience, USA).

2.6. Transfection of ptfLC3

To confirm induction of intracellular autophagy by ZnO-np, JB6 Cl 41-5a cells were transfected with *ptf*LC3 (Addgene plasmid 21074) and after 24 h cells were treated with ZnO-np. *ptf*LC3 is a mRFP-green fluorescence protein (GFP) tandem fluorescent-tagged LC3 plasmid that shows a mRFP and GFP signal before the fusion with acidic environments like as lysosome, and subsequently displays the mRFP signal (Kimura et al., 2007). Transfection was