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Reactive oxygen species-induced cytotoxic effects of zinc oxide nanoparticles in rat retinal ganglion cells

Dadong Guo [a], Hongsheng Bi [a,*], Bing Liu [b], Qiuxin Wu [b], Daoguang Wang [b], Yan Cui [a]

[a] Key Laboratory of Integrated Traditional Chinese and Western Medicine for Prevention and Therapy of Ocular Diseases in Universities of Shandong, Jinan 250002, China
[b] Eye Institute of Shandong University of Traditional Chinese Medicine, Jinan 250002, China

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ABSTRACT

Recent studies have proved that zinc oxide (ZnO) nanoparticles can cause toxicity in different cell lines, oxidative stress is often hypothesized to be an important factor in cytotoxicity of ZnO nanoparticles. However, the mechanisms are incompletely understood. The present study aimed to investigate the role of oxidative stress in toxicity and possible involvement of mitochondria in the production of reactive oxygen species (ROS) upon exposure of retinal ganglion cells (RGC-5) to ZnO nanoparticles. In this study, the effects of ZnO nanoparticles on mitochondrial membrane potential and ROS levels involved in hydrogen peroxide and hydroxyl radical production were investigated via inverted fluorescence microscope and hydrogen peroxide and hydroxyl radical assay kits, respectively. Furthermore, the mRNA of caspase-12 and the protein secreted into culture supernatant were also determined by means of real-time quantitative PCR and ELISA techniques. Our studies indicate that ZnO nanoparticles could apparently decrease the mitochondrial membrane potential, increase the production of ROS and lead to the overexpression of caspase-12 in RGC-5 cells, suggesting that ZnO nanoparticle-induced toxicity via ROS overproduction will trigger endoplasmic reticulum stress, lead to the RGC-5 cell damage and finally induce apoptosis/necrosis, the overexpression of caspase-12 may be involved in cell death in RGC-5 cells.

1. Introduction

Nanomaterials are generally in the 1–100 nm range. Currently, nanomaterials are receiving considerable attention because of their great potential in biology and medicine. In addition, industrial applications using nanomaterials have also resulted in a significantly growing demand for nanosized materials. All these increase the possibility of human exposure to those nanomaterials through dermal, inhalation and oral routes. Toxicological studies suggest that nanomaterials may cause adverse health effects. Thus, the interaction of nanomaterials with biological systems including living cells has become one of the most urgent areas of collaborative research in materials science and biology (Nel et al., 2006; Medina et al., 2007), and the safety of nanomaterials has become a worldwide hot topic (Moore, 2006; Handy et al., 2008; Kahru and Dubourguier, 2010).

Zinc oxide (ZnO) nanoparticles have been used for decomposition of some organic compounds under the ultraviolet illumination (Wang et al., 2007; Xu et al., 2007). Studies had indicated that ZnO nanoparticles could exert cytotoxicity on both broncho-alveolar lavage cells and white blood cells in rats via interfering with zinc ion homeostasis (Kao et al., 2012), the liver and kidney cells in mice via induction of oxidative stress, DNA damage and apoptosis (Li et al., 2011), liver cells (Sharma et al., 2011; Sharma et al., 2012), human bronchial epithelial cells (Heng et al., 2010) and *Escherichia coli* via oxidative stress (Brayner et al., 2006; Kumar et al., 2011). In addition, ZnO nanoparticles could induce apoptosis in human dermal fibroblasts via p53 and p38 pathways (Meyer et al., 2011). Our previous studies also demonstrated that ZnO nanoparticles could exert cytotoxicity on sensitive and drug-resistant leukemia cell lines (Guo et al., 2008) and hepatocellular carcinoma SMMC-7721 cell line *in vitro* (Li et al., 2010).

Mitochondria can generate most of the cell's supply of adenosine triphosphate (ATP), used as a source of chemical energy (Campbell et al., 2006). In addition to supplying cellular energy, mitochondria are involved in a range of other processes, such as signaling, cellular differentiation, cell death as well as the control of the cell cycle and cell growth (McBride et al., 2006). Moreover, mitochondria play a critical role in many metabolic systems such as regulation of membrane potential and apoptosis. Apoptosis is a highly regulated process of cell deletion and plays a fundamental role in the maintenance of tissue homeostasis in the normal organism. It is a physiological mode of cell death which is important in normal tissue development and remodeling (Fadeel and Orrenius, 2005). It can be triggered by many different types of cell stresses, and can also transiently store calcium for the cell homeostasis

* Corresponding author. Tel./fax: +86 531 82861167.
E-mail address: azuresky1999@163.com (H. Bi).

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which is primarily driven by mitochondrial membrane potential ($\Delta\psi m$). Mitochondria may leak some amount of high-energy electrons in the respiratory chain to form reactive oxygen species (ROS). ROS, including superoxide anion, hydrogen peroxide and hydroxyl radical, can damage DNA, resulting in mutations (McBride et al., 2006). ROS can also oxidize proteins and lipids, leading to the generation of highly toxic electrophilic species including malondialdehyde and 4-hydroxynonenal, which can initiate inappropriate or altered cellular signal transduction pathways and contribute to toxicity (Halliwell and Gutteridge, 1999). It has been proved that mitochondrial outer membrane proteins, which are regulated by the anti- and pro-apoptotic members of the Bcl-2 family, and proteins released from mitochondria, lead to activation of caspases and subsequent cell death (Birbes et al., 2001). Caspase-12 is one of the key mediators of apoptosis (Nakagawa et al., 2000) and active caspase-12 levels have been used as an indicator of apoptosis (Bajaj and Sharma, 2006). Furthermore, caspase-12 also mediates an endoplasmic reticulum (ER)-specific apoptosis pathway (Nakagawa et al., 2000) and ROS could promote caspase-12 expression (Brezniceanu et al., 2010) and further lead to cell death in an ER stress-induced apoptosis manner.

Oxidative stress conditions enhance the production of ROS resulting from a variety of stimuli and are proposed as one of the most important mechanisms for nanomaterials mediated toxicity (Ahamed et al., 2011; Piao et al., 2011; Thakor et al., 2011; Thounaojam et al., 2011; Ye et al., 2011). It was reported that cells exposure to higher concentrations of ZnO nanoparticles could cause oxidative stress and further induce apoptosis/necrosis in arget cells. However, the effects of ZnO nanoparticles on the rat retinal ganglion cells (RGC-5) are still unclear. Besides, the relationship between the expression of caspase-12 (ER stress-associated caspase) and apoptosis/necrosis mediated by ZnO nanoparticles is still unknown. Hence, in the present study, we have explored the effects of ZnO nanoparticles on the changes in cell viability, cell nucleus, $\Delta\psi m$, hydrogen peroxide and hydroxyl radical levels, apoptosis, expression level for both caspase-12 gene and protein through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, 4′,6-diamidino-2-phenylindole (DAPI) staining, hydrogen peroxide and hydroxyl radical assay kits, flow cytometry, real-time quantitative PCR and enzyme-linked immunosorbent assay (ELISA), respectively. Our results demonstrated that ZnO nanoparticles apparently induce the RGC-5 cell death via the overproduction of ROS, enhance the expression of caspase-12, suggesting that ROS and caspase-12 may play an important role in ZnO nanoparticle-mediated cytotoxicity and cell death in RGC-5 cells.

2. Materials and methods

2.1. ZnO nanoparticles

The ZnO nanoparticles (i.e., ZP6) capped with aminopolysiloxane were purchased from Jiangsu Changtai Nanometer Material Co., Ltd and characterized by a field emission scanning electron microscope (SU8020, Hitachi, Japan). The particle size distribution was determined using a Malvern Zetasizer (Malvern Instruments, Britain) with specialized software (Zetasizer Nano ZS).

2.2. Cell culture and preparation of ZnO-nanoparticle solution

RGC-5 cell line (a generous gift from Eye Center of the Second Bethune Hospital, Jilin University) was used in this study. RGC-5 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Life technologies, USA) containing 1.0 g/L of glucose, 10% fetal bovine serum (HyClone, USA), 100 U/mL penicillin, 100 μg/mL streptomycin. All cells were cultured at 37 °C in water-saturated air supplemented with 5% CO2. Cell numbers were determined using an automated cell counter (TC10, Bio-rad, USA). ZnO nanoparticles were dissolved in DMEM and sonicated for 10 min prior to use.

2.3. MTT assay

The RGC-5 cells were seeded in a 96-well microtiter plate and the initial density was 8000 cells per well. The RGC-5 cells were treated with different concentrations of ZnO nanoparticles (i.e., 10.0, 5.0, 2.5, 1.25 and 0.63 μg/mL) in 200 μL volume and cultured for 24, 48 and 72 h, respectively. Controls (untreated cells) were cultivated under the same conditions without addition of ZnO nanoparticles. Cell viability was tested by the colorimetric MTT assay, and was expressed as follows: cell viability (%) = Atest/Acontrol × 100%, where A represents the relevant absorbance at 490 nm. The results were obtained from three independent experiments performed in triplicate and were represented as mean ± SD (standard deviation). The 50% inhibiting concentration (IC_{50}) was defined as the concentration required for 50% inhibition of cell growth.

2.4. Analysis on mitochondrial membrane potential (JC-1 staining)

The changes of $\Delta\psi m$ were explored using the 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl benzimidazolyl carbocyanine iodide (JC-1) probe (stands for 1st J-aggregate-forming cationic dye). It is commonly used to detect mitochondrial depolarization that occurs in the early apoptosis (Cossarizza et al., 1993; Salvioli et al., 1997). In healthy cells, JC-1 accumulates in the mitochondria as JC-1 aggregates (whose fluorescence is red) and also in the cytoplasm as JC-1 monomers (whose fluorescence is green). During early apoptosis, the $\Delta\psi m$ collapses. As a result, JC-1 aggregates cannot accumulate within the mitochondria and dissipate into JC-1 monomers leading to loss of red fluorescence. Therefore, collapse of the $\Delta\psi m$ is signified by decrease in the ratio of red to green fluorescence. In this study, RGC-5 cells were seeded in a 6-well plate at a density of 5×10^4 cells/well and grown overnight, then incubated with ZnO nanoparticles (0, 2.5, 5.0 and 10.0 μg/mL) in 2 mL volume for 24 h, subsequently the cells were washed with PBS and incubated with JC-1 staining working solution (Beyotime, China) at 37 °C for 20 min. After washing with staining buffer (Beyotime, China), the cells were washed with PBS, then the changes of $\Delta\psi m$ were visualized using an inverted fluorescence microscope (Olympus IX71, Japan), and the typical photographs were captured.

2.5. DAPI staining

To monitor the effect of ZnO nanoparticles on RGC-5 cell nuclei, the DAPI nuclear staining was carried out. The RGC-5 cells were seeded in a 6-well plate at a density of 5×10^4 cells per well and grown overnight, then incubated with different concentrations (i.e., 0, 2.5, 5.0, 10 μg/mL) in 2 mL volume of ZnO nanoparticles for 6 h, subsequently the cells were washed with phosphate buffered saline (PBS). After fixation in 4% polymerisatum for 15 min, the fixed cells were washed with PBS and stained for 30 min with 1 μg/mL DAPI solution (Sigma, USA). The stained cells were examined using an inverted fluorescence microscopy (Olympus IX71, Japan), and the typical photographs were captured.

2.6. Measurement of hydrogen peroxide and hydroxyl radical levels

To obtain further evidence for direct actions of ZnO nanoparticles on RGC-5 cells, we monitored the alterations in hydrogen