Toxicology in Vitro 24 (2010) 751–758

Contents lists available at ScienceDirect

Toxicology in Vitro

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

Nano-SiO_2 induces apoptosis via activation of p53 and Bax mediated by oxidative stress in human hepatic cell line

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ARTICLE INFO

Article history:
Received 9 June 2009
Accepted 4 January 2010
Available online 8 January 2010

Keywords:
Nano-SiO_2
Oxidative stress
Apoptosis
p53
Bax
Human hepatic cells

ABSTRACT

Nanoparticles such as nano-SiO_2 are increasingly used in food, cosmetics, diagnosis, imaging and drug delivery. However, toxicological data of nano-SiO_2 on hepatic cells *in vitro* and their detailed molecular mechanisms still remain unclear. In order to assess toxicity of nano-SiO_2, L-02 cells were exposed to 0.2, 0.4 and 0.6 mg/ml of SiO_2 colloids (21, 48 and 86 nm) for 12, 24, 36 and 48 h. Lactate dehydrogenase released from damaged cells were quantified, cellular ultrastructural organization was observed, and the levels of reactive oxygen species (ROS), lipid peroxidation and glutathione were measured. Apoptosis induced by 21 nm SiO_2 was characterized by annexin V-FITC/PI staining and DNA ladder assay. Furthermore, apoptosis related proteins such as p53, Bax and Bcl-2 were analyzed by using western blot analysis. Our data indicated that nano-SiO_2 caused cytotoxicity in size, dose and time dependent manners. Oxidative stress and apoptosis were induced by exposure to 21 nm SiO_2. Moreover, the expression of p53 and Bax was increased in time and dose dependent patterns, whereas the expression of Bcl-2 was not significantly changed. In conclusion, ROS-mediated oxidative stress, the activation of p53 and up-regulation of Bax/Bcl-2 ratio are involved in mechanistic pathways of 21 nm SiO_2 induced apoptosis in L-02 cells.

1. Introduction

Nanoparticles are defined as small objects that range in size between 1 and 100 nm. Nowadays, nanoparticles are increasingly used in food, cosmetics, microelectronics, ceramics and catalysts. Moreover, the application of nanoparticles has been extended to biomedicine fields over recent years, such as cancer diagnosis and therapy (Oh et al., 2009; Phan et al., 2009), design of novel candidate nanoscale constructs for drug development (Prato et al., 2008), nanocarriers for targeted delivery of drugs and genes (Husseini and Pitt, 2008; Jabr-Milane et al., 2008).

As one common type of nanoparticles, silica (SiO_2) particles have been utilized in diagnosis, imaging and drugs (Bottini et al., 2007; Jia et al., 2008; Wang et al., 2009). Hence, ingestion, skin absorption, and injection are potential routes of nanoparticle exposure. Because of quantum size effects and large surface area, nanoparticles show unusual physicochemical properties compared with those bulk particles. For example, some of them readily travel throughout the body, deposit in target organs and penetrate cell membranes (Nel et al., 2006). Studies demonstrated that nanoparticles induced toxicological effects mainly on lung, liver, spleen and kidneys tissues (Chen et al., 2006; Wang et al., 2007a). *In vivo* toxicity studies in Sprague Dawley rats showed that inhaled silver nanoparticles (18 nm) at high dose (3.0×10^6 particle/cm^3, 515 $\mu g/m^3$) elicited chronic alveolar inflammation, and small granulomatous lesions in lungs (Sung et al., 2008). After intravenous injection with silica nanoparticles in BALB/c mice, 70 nm particles induced liver injury at 30 mg/kg, while 300 nm or 1000 nm had no effect even at 100 mg/kg (Nishimori et al., 2009). Chen et al. reported that 14 days after intraperitoneal injection TiO_2 was deposited mainly in spleen and kidney (Chen et al., 2009). Nanoparticles can also selectively translocate into brain via olfactory bulb, and may be associated with neurodegenerative diseases (Oberdörster, 2004; Elder et al., 2006; Tin-Tin-Win-Shwe et al., 2008). Moreover, exposure to alumina nanoparticles may also be a risk for the development of vascular diseases (Oesterling et al., 2008).

The available data supported the toxicity of nanoparticles on organs of different animal species, while there are few studies investigating the hazardous effects of nano-SiO_2 on hepatic cells *in vitro*. In addition, detailed molecular mechanisms of nanoparticle toxicity still remain unclear. The nano-SiO_2 colloids used here were prepared especially for this purpose. Recently, some studies stated that injurious response was more prominent with smaller nano-

Abbreviations: DCFH-DA, 2′,7′-Dichlorofluorescin diacetate; EB, ethidium bromide; GSH, glutathione; CC50, 50% cytotoxicity concentration; LDH, lactate dehydrogenase; MTT, methylthiazolyldiphenyl-tetrazolium bromide; PBS, phosphate buffered solution; PI, propidium iodide; ROS, reactive oxygen species; SiO_2, silica; TEM, transmission electron microscopy.

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doi:10.1016/j.tiv.2010.01.001

material than with the same material in larger form (Oberdörster et al., 2005; Midander et al., 2009). Inoue et al. found that pulmonary exposure of TiO_2 particles at dose of 8 mg/kg caused lung inflammation, which tended to be more severe with the smaller nanomaterials than with the larger ones (Inoue et al., 2008). Moreover, it was reported that inflammation and reactive oxygen species (ROS) mediated oxidative stress played an important role in the ability of nanoparticles to induce toxicity (Dick et al., 2003; Lin et al., 2006; Nel et al., 2006). In this study, evaluation of nano-SiO_2 induced cytotoxic effects on normal human hepatic L-02 cell line was performed with the tetrazolium reduction (methylthiazolyldiphenyl-tetrazolium bromide; MTT) and lactate dehydrogenase (LDH) assays. The changes of cellular ultrastructural organization were observed through transmission electron microscopy (TEM) and the biochemical and physiological indicators such as reactive oxygen species (ROS), lipid peroxidation and glutathione (GSH) were measured to evaluate the levels of oxidative stress. In addition, apoptosis, the process of programmed cell death, have a close relationship with oxidative stress. It was published that oxidative stress induced by cerium oxide nanoparticles (diameters < 45 nm) exerted cytotoxicity by an apoptotic process (Park et al., 2008b). Therefore, the percentage of apoptotic cell was determined and the potential mechanism by which nano-SiO_2 regulated apoptotic pathways was elucidated in the present study.

2. Materials and methods

2.1. Nanoparticles

The nano-SiO_2 colloids (21, 48 and 86 nm) used in this study were supplied by Center of Analysis and Test Research (East China University of Science and Technology, Shanghai, China). The characterizations of nano-SiO_2 colloids are shown in Table 1. These nano-SiO_2 colloids were sterilized by using a 0.2 μm filter (Sartorius, German) and adjusted to pH about 7.0 before each study.

2.2. Cell culture and media

The normal human hepatic cell line (L-02) was purchased, mycoplasma free and source certified, from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). L-02 cells were maintained in RPMI 1640 Medium (Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin, and grown at 37 °C in a 5% CO_2 humidified environment.

2.3. Assessment of cytotoxicity of nano-SiO_2

L-02 cells were plated into a 96-well culture plate at a density of 1.0×10^5 cells/ml and allowed to attach for 12 h. Then, the nano-SiO_2 colloids were diluted to appropriate concentrations and immediately applied to the cells. Dose-toxicity study was set by exposing cells to 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml for 24 h. Time-toxicity study was set by exposing cells to 0.6 mg/ml for 12, 24, 36 and 48 h. Viabilities of cells were evaluated by MTT reduction method. The cells were stained with MTT for 4 h and then dissolved in dimethylsulphoxide. Optical density at 570 nm was detected for monitoring the cell viability. The 50% cytotoxicity concentration (CC50) of nano-SiO_2 colloids was calculated and the cells treated with sterile ultrapure water at same concentrations used as controls.

2.4. LDH cytotoxicity assay

L-02 cells were plated in a 24-well culture plate, and treated with 0.2, 0.4 and 0.6 mg/ml of nano-SiO_2 colloids for 12, 24, 36 and 48 h. LDH released from damaged cells in culture medium were quantified by using LDH assay kit (Genmed Scientifics, USA). The LDH activities were quantitated by reading optical densities at 420 nm using Synergy 2 multi-mode microplate reader (BioTek, USA).

2.5. Transmission electron microscopy (TEM) assay

L-02 cells were plated in 10-cm petri dishes, and treated with 0.6 mg/ml of 21 nm SiO_2 colloids for 24 h. Cells were scraped off, collected, rinsed with phosphate buffered solution (PBS) to remove culture medium, and then fixed in 2% glutaraldehyde in 0.1 M PBS (pH 7.4) for 2 h at 4 °C. Subsequently, they were post-fixed in 1% osmium tetroxide in 0.1 M PBS (pH 7.4) for 2 h at 4 °C. Cell samples were washed in buffer and dehydrated in a series of water/ethanol mixtures to 100% ethanol. Then they were infiltrated in sequentially increasing concentrations of araldite 618 to 100%, embedded in Beam capsules, and placed in a 60 °C oven for 48 h. Using LKB-V ultramicrotome, semi-thin sections (0.5 μm) were stained with lead citrate and rinsed with distilled water. Cell section samples were viewed with Tecnai 12 transmission electron microscope (Philips FEI Co., Holland).

2.6. Intracellular ROS assay

Intracellular ROS was detected by using 2′,7′-dichlorofluorescin diacetate (DCFH-DA), which is an oxidation-sensitive fluorescent probe. After treatment with 0.2, 0.4 and 0.6 mg/ml of nano-SiO_2 colloids for 24 h, cells were washed twice with PBS. Then they were loaded with DCFH-DA (10 μM) (Sigma, China) diluted in serum-free medium and incubated at 37 °C for 30 min. The intensities of fluorescence were detected by means of Synergy 2 multi-mode microplate reader (BioTek), with an excitation wavelength of 485 nm and an emission wavelength of 528 nm.

2.7. Intracellular lipid peroxidation and GSH assays

2.7.1. Sample preparation

L-02 cells were plated in 10-cm petri dishes, and treated with 0.2, 0.4 and 0.6 mg/ml of nano-SiO_2 colloids for 24 h. Cells were scraped off, collected, rinsed with PBS to remove culture medium, and then resuspended in 0.5% cold Triton X-100. Complete disruption of cell suspensions was achieved by freeze–thaw twice and centrifuged (14,000*g* for 15 min at 4 °C). The supernatant was used for the following assays. Protein concentrations were determined by using Bradford method.

Table 1
Characterizations of nano-SiO_2 solutions.

	Size and distribution (nm) (mean ± S.D.)	Concentration (mg/ml)	Surface area (m^2/g)	Crystalline structure
21 nm SiO_2	21.2 ± 2.9	9.8525	225	Amorphous
48 nm SiO_2	48.6 ± 5.1	5.6720	106	Amorphous
86 nm SiO_2	86.4 ± 9.0	9.8167	39	Amorphous