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Qualitative and quantitative analysis on cellular uptake of cerium oxide nanoparticles in the human lung cancer cells

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ABSTRACT

With the fast development of nanotechnology, the nanomaterials start to cause people's attention for potential toxic effects. In this work, the effects of cerium oxide (CeO_2) nanoparticles on the human lung cancer cells were investigated. As an indicator of membrane damage, lactate dehydrogenase (LDH) was quantitatively assessed. Flow cytometer (FCM) and inductively coupled plasma mass spectrometry (ICP-MS) were used for qualitative and quantitative analysis on cellular uptake of CeO_2 nanoparticles, respectively. The results demonstrated that CeO_2 nanoparticles can enter cells through cell membrane and did not induce significant lysosomal membrane destabilization. The intensity of the side scattered light revealed that the nanoparticles were taken up in the cells dose dependently. The quantitative analysis on cellular uptake of CeO_2 nanoparticles could be detected by ICP-MS. These methods could be used for the initial screening of the uptake potential of nanoparticles as an index of nanotoxicity.

KEYWORDS

Cerium oxide nanoparticles;
Cellular uptake;
Lung cancer cells.

INTRODUCTION

Nanotechnology is a dynamically developing field of scientific interest in the entire world and has already become key R&D priorities in Europe and America[1,2]. The emerging development of nanotechnology has lead to concern related to the manufacturing and use of large quantities of nanoparticles[3-5]. There is a serious lack of information concerning their effects on human health and the environment. Therefore, the potential effects to human health arising from nanoparticles need urgently assessed. One major toxicological concern is that nanomaterials are easily taken up in the human body[6,7]. It is a particular concern that nanomaterials are similar in size to major classes of biologically active materials like DNA, RNA, membranes and microtubules. Notably, nanoparticles are considered to be inhaled and distributed in the body[8,9]. Therefore, evaluations of translocation potential in biological tissues and cells corresponding to particle size are essential for the safe use of manufactured nanoparticles[10].

Cerium oxide (CeO_2) is an important material widely used in various applications such as catalysis, solar cells, fuel cells, luminescence, oxygen pumps and automotive catalytic converters[11-13]. The interactions between CeO_2 nanoparticles and biological targets are

somewhat paradoxical. CeO_2 nanoparticles have been shown to scavenge reactive oxygen species (ROS) and to reduce oxidative stress as free radical scavenger[14-16]. In contrast, the toxicological studies revealed that CeO_2 nanoparticles decreased the viability of some human and rodent cells through generation of ROS[17-19]. The uptake potential of nanomaterials depends on size, surface charge, and behavior. In this study, we developed a method to evaluate the uptake potential of nanoparticles using cultured human lung cancer cells. The analysis using flow cytometric light scatter and inductively coupled plasma-mass spectrometry accurately reflected the change in amounts of CeO_2 taken up into cells. This method would be available for the initial screening of uptake into cells as an index of nanotoxicity.

EXPERIMENTAL

Materials and reagents

Dulbecco's modified Eagle's medium (DMEM) and trypsin were purchased from Gibco. MTT, penicillin, streptomycin and cetylpyridium chloride were from Sigma-Aldrich (St. Louis, USA). Neonatal bovine serum (NBS) was purchased from Hangzhou Sijiqing Organism Engineering Institute. A LDH kit was obtained from the Nanjing Jiancheng Biological Engineering Institute (Jiangsu, China). CeO_2 nanoparticles (Purity > 99.9%) was obtained from Sigma-Aldrich Co (St. Louis, USA).

Characterization of CeO_2 nanoparticles

The morphology and size of CeO_2 nanoparticles were measured by field emission scanning electron microscope (JSM-7500F, JEOL, Japan). A minute drop of nanoparticles solution was cast on to a carbon-coated copper grid and subsequently drying in air before transferring it to the microscope. X-ray powder diffraction was performed on a Bruker D8 Advance X-ray diffractometer employing Cu-Kα radiation with 40 kV and 50 mA (D8 ADVACE, Bruker, Germany). The size distribution of the nanoparticles in medium was evaluated by dynamic light scattering (Delsa Nano C, Beckman, USA). Data were analyzed based on six replicated tests.

Cell culture

The A549 human lung carcinoma epithelial-like cell line was obtained from ATCC. Cells were cultured in DMEM supplemented with L-alanyl–L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml) and 10% heat inactivated new born calf serum. Cells were maintained in a humidified atmosphere at 37 °C and 5% CO_2.

Cell viability assay

A549 cells (2×10^3 cells/100 μl) were seeded onto 96-well plates and incubated overnight at 37 °C under a 5% CO_2 atmosphere. The medium in the wells was then replaced with fresh medium containing nanoparticles (5-40 μg/ml) and incubation continued for 24 and 48 h. The effects of the nanoparticles on cell viability were determined using the MTT assay. Briefly, 10 μl of MTT solution was added to each well and the plates were incubated for 4 h. The supernatant was removed and DMSO (100 μl) was added to solubilize the MTT. The absorbance at 570 nm of each well was measured with a microplate spectrophotometer (BioRad Model 3550, USA). Cells incubated without nanoparticles were used as a control. The cell viability was calculated according to the formula: $A_{sample} / A_{control} \times 100\%$.

LDH measurement

Lactate dehydrogenase (LDH) activity in the cell medium was determined using a commercial LDH Kit. One hundred microliters of cell medium was used for LDH analysis. Absorption was measured using a microplate spectrophotometer (BioRad Model 3550, USA) at 340 nm. Released LDH catalyzed the oxidation of lactate to pyruvate with simultaneous reduction of NAD^+ to NADH. The rate of NAD^+ reduction was measured as an increase in absorbance at 340 nm. The rate of NAD^+ reduction was directly proportional to LDH activity in the cell medium.

Flow cytometric assay

Cells treated with several doses (5, 10, 20, and 40 μg/ml) of CeO_2 nanoparticles for 24h were trypsinized, centrifuged and resuspended in PBS. The amounts of particles taken up by the cells were analyzed using a flow cytometer (FCM) (FACSCalibur, BD, USA). In FCM, the laser beam (488 nm) illuminates cells in the sample stream which go through the sensing area. The laser light scattered at narrow angles to the axis of laser beam is called forward scatter (FSC) light. The laser

light scattered at about a 90° angle to the axis of the laser beam is called side scatter (SSC) light. The intensities of FSC and SSC are proportional to the size of cells and the intracellular density, respectively.

Cerium content analysis

Cells treated with several doses of CeO_2 nanoparticles were trypsinized, digested and analyzed for Ce content. Briefly, the cells were digested in nitric acid overnight and heated at about 160 °C the next day. At the same time, H_2O_2 solution was used to drive off the vapor of nitrogen oxides until the solution was colorless and clear. At last, the remaining solutions were fixed to 3 ml with 2% nitric acid. Inductively coupled plasma-mass spectrometry (ICP-MS, Thermo Elemental X7, Thermo Electron Co.) was used to analyze the Ce concentration in each sample. Indium of 20 ng/ml was chosen as an internal standard element.

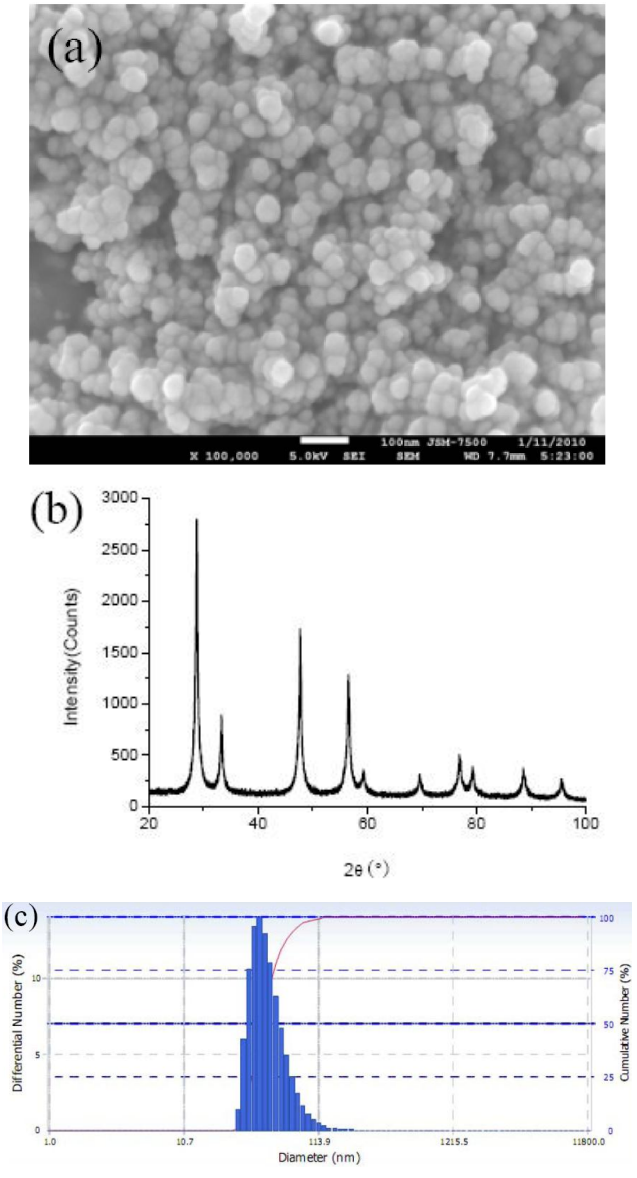
Lysosomal membrane stability

Lysosomal membrane stability in control group and cells incubated with different concentrations of CeO_2 nanoparticles (5, 10, 20 and 40 μg/ml) for 30 min was evaluated by the Neutral Red (NR) Retention time assay as previously described according to Lowe et al[20,21]. Cells in plates were incubated with 30 μl of a NR solution (final concentration 40 μg/ml froma stock solution of NR 40 mg/ml DMSO), after 15 min excess dye was washed out, Control group was run in parallel. Every 15 min cells were examined under an optical microscope and the percentage of cells showing loss of the dye from lysosomes in each field was evaluated. For each time point 10 fields were randomly observed, each containing 8-10 cells. The endpoint of the assay was defined as the time at which 50% of the cells showed sign of lysosomal leaking (the cytosol becoming red and the cells rounded). Triplicate preparations were made for each sample.

RESULTS AND DISCUSSION

Particle characterization

The SEM results showed that CeO_2 nanoparticles were sphere-like with approximate diameters of 40 nm (Figure 1 (a)). The XRD patterns (Figure 1(b)) of CeO_2 nanoparticles indicated that only the CeO_2 phase without any other phases was found and all of diffraction peaks could be assigned according to single cubic crystal phase of CeO_2 which belonging to space group O^5_H-F_{M3M} (JCPDS No.4-0593). It was also revealed that CeO_2 nanoparticles exhibited sharp diffraction peaks, indicating a high crystallinity. The SEM images provided information on the size and shape of nanoparticles, however, it could not provide information on whether the nanoparticles existed in single or aggregated forms in the culture medium. The size distribution in the culture medium, therefore, was investigated using a



(a) SEM images; (b) XRD patterns; (c) Particle size distribution in medium.

Figure 1 : Characteristics of CeO_2 nanoparticles.

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DLS method, which showed that the average size of CeO_2 in the culture medium was 48.1 ± 17.7 nm (Figure 1(c)). The DLS analysis showed that the CeO_2 nanoparticles were homogeneously dispersed in culture medium. After 7 days, homogeneous CeO_2 nanoparticles that were dispersed in the culture medium remained stable. The particle size of the nanoparticles was not noticeably changed.

Effects of CeO_2 nanoparticles on the cell viability

As shown in figure 2, CeO_2 nanoparticles promoted the viability of A549 cells significantly at 24 and 48 h. After cells were exposed to CeO_2 nanoparticles at 10, 20 and 40 μg/ml for 48 h, cell viability increased to 109.8.3%, 111.7% and 113.1%, respectively, compared to the control group. Interestingly, the cytotoxicity of CeO_2 nanoparticles at 24 h was not significantly different from that at 48 h.

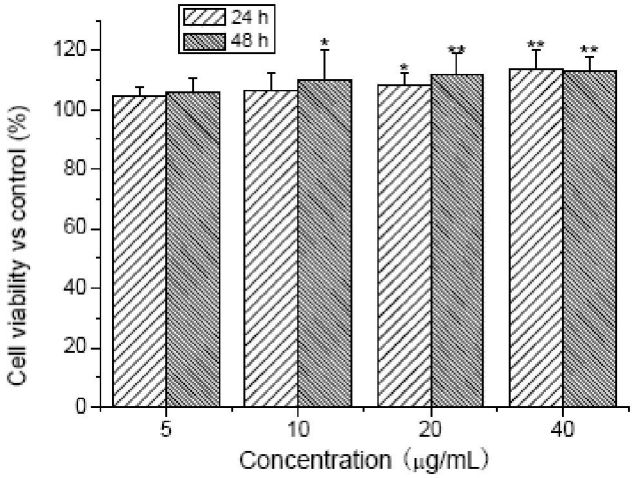


Figure 2 : Viability of A549 cells after exposure to CeO_2 nanoparticles. Values are mean ± SD from three independent experiments. ($^*P < 0.05$, $^{**}P < 0.01$ compared with the corresponding control group, n=6.)

Release of LDH resulted from exposure to CeO_2 nanoparticles

The cell membrane damage was reflected in the elevated LDH levels in the cell medium after cells were exposed to 40 nm CeO_2 nanoparticles for 24 h. The LDH levels in the cell culture were increased in all groups after exposure to CeO_2 nanoparticles for a period of 24 h (Figure 3). The LDH levels were increased by 17.5%, 37.5%, 52.8%, and 59.6% following exposure to 5, 10, 20 and 40 μg/ml of CeO_2 nanoparticles, respectively.

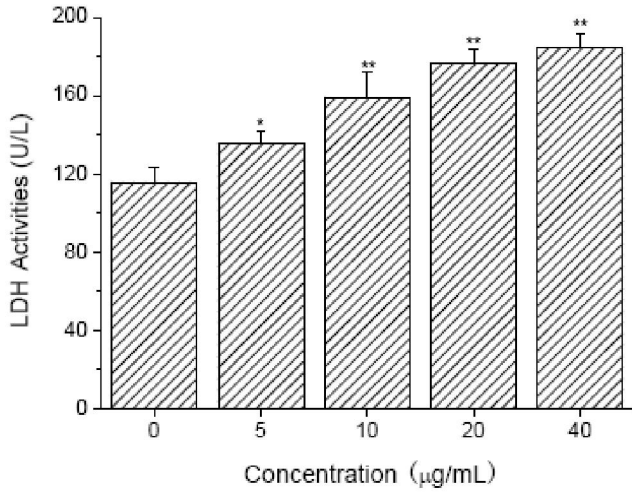


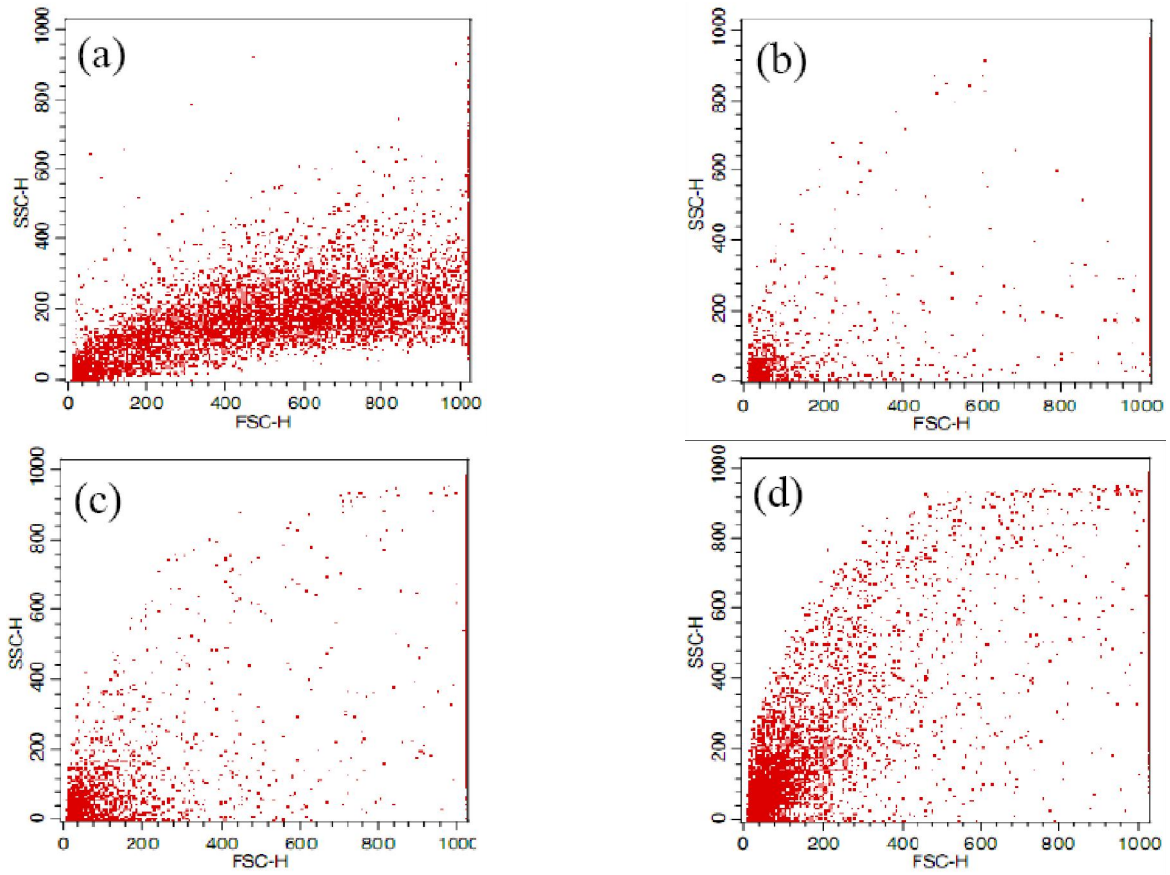
Figure 3 : The LDH activities in the cell culture medium after 24 h exposure to CeO_2 nanoparticles.. Values are mean ± SD from three independent experiments. ($^*P < 0.05$, $^{**}P < 0.01$ compared with the corresponding control group, n=6.)

Qualitative analysis of CeO_2 nanoparticles uptake

Figure 5 shows the FCM light scatter histograms of the cells treated with different doses of CeO_2 nanoparticles. Intensities of FSC and SSC reflect the cell size and inner cell density, respectively. Regardless of treatment with CeO_2 nanoparticles, the value of FSC was constant (Figure 5(a)). On the other hand, higher concentrations of CeO_2 nanoparticles resulted in higher intensities of SSC (Figure 5(b)). That is, the cells which took up higher doses of nanoparticles showed higher intensities of SSC. This result suggested that the determination of SSC is a good way to judge the uptake potential of CeO_2 nanoparticles. Using this experimental approach, a dose-dependent increase in cellular uptake of CeO_2 nanoparticles was detected at doses from 5 to 40 μg/ml after 24 h exposure (Figure 4).

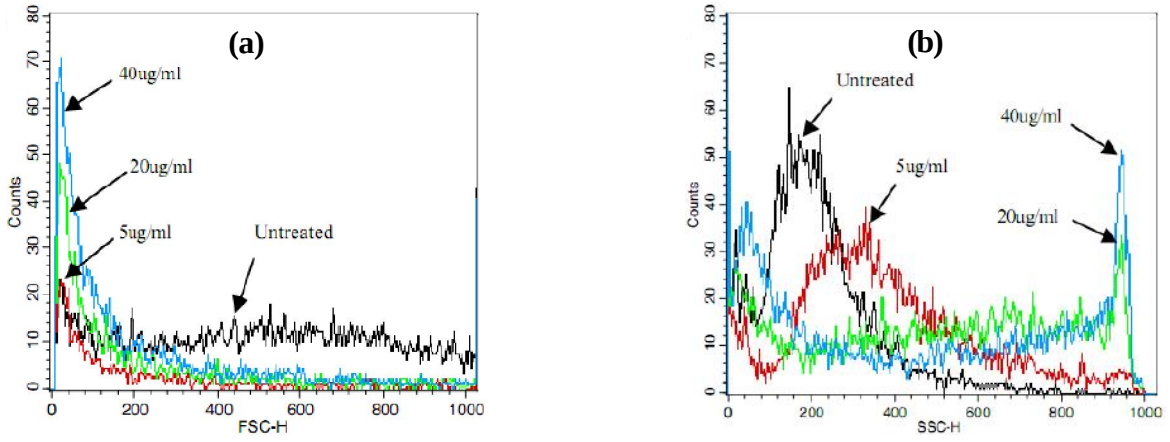
Qualitative analysis of CeO_2 nanoparticles uptake

ICP-MS analyses were employed to further verify the uptake of CeO_2 nanoparticles in A549 cells at different time points and doses. The contents of cerium in cells exposure to CeO_2 nanoparticles are shown in Figure 6. Cerium could not be detected in controls. However, a dose- and time-dependent accumulation of CeO_2 nanoparticles could be measured in A549 cells at 24 and 48 h (Figure 6(b)). After cells were



(a)Untreated (b)5 µg/ml (c)20 µg/ml (d)40 µg/ml

Figure 4 : Analysis of incorporation of CeO_2 nanoparticles by flow cytometric light scatter. Dose-dependent comparison of FSC or SSC intensity.



(a)FSC; (b)SSC

Figure 5 : FCM histograms of FSC and SSC.

exposed to CeO_2 nanoparticles at 10, 20 and 40 µg/ml for 24 h, the cerium content was 6.7 ± 0.1, 18.7 ± 0.1 and 18.6 ± 0.2 ng/mm^2, respectively. When the exposure time extended to 48 h, the cerium content was increased to 12.1 ± 0.1, 24.0 ± 0.1 and 26.0 ± 0.2 ng/mm^2 at the same dose.

Effects of CeO_2 nanoparticles on lysosomal membrane stability

Different concentrations of CeO_2 nanoparticles were first tested for their effects on lysosomal membrane stability (LMS), a common marker of cellular stress, utilising the NR retention time assay. As shown in Fig-

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ure 7, cells exposure with CeO_2 nanoparticles for 24 h did not significantly affect LMS.

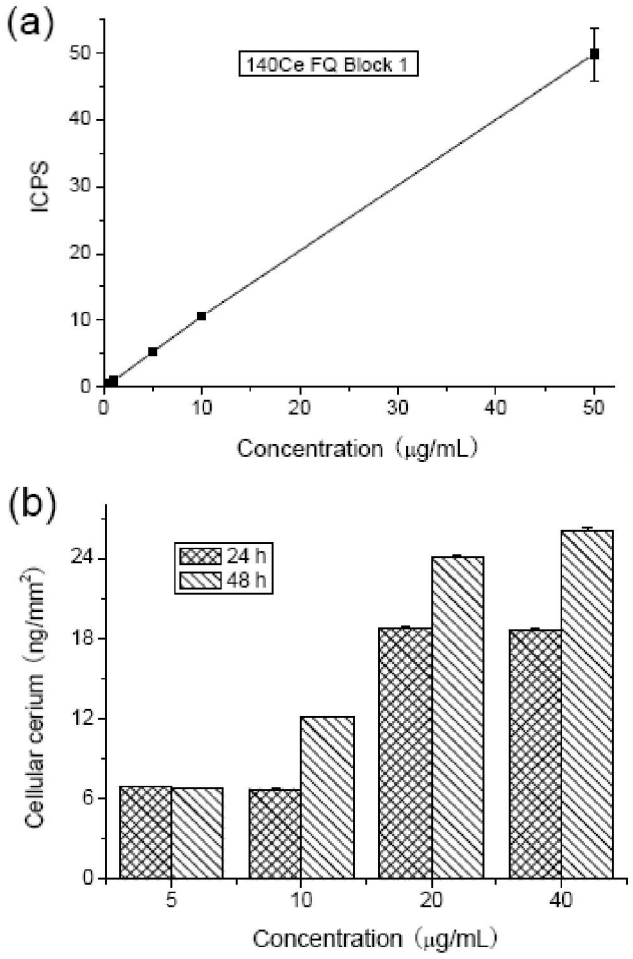

Figure 6 : The contents of cerium in cells were determined by ICP-MS. The data are expressed as mean ± SD of three independent experiments.

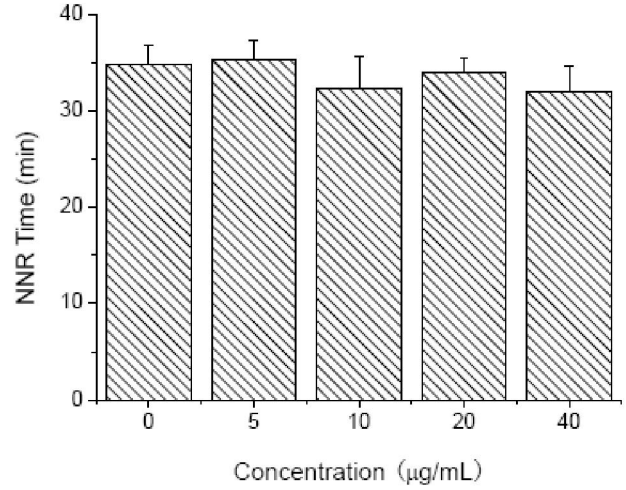

Figure 7 : Effects of CeO_2 nanoparticles on lysosomal membrane stability. The data are expressed as mean ± SD of three independent experiments.

CONCLUSIONS

Usage of nanomaterials will increase with the development of nanotechnology, and assessments of their risks to the environment and human health also will be required.

Academia, industry, and regulatory governmental agencies should seriously consider the view that nanomaterials have new and unique biologic properties and the potential risks are not the same as those of bulk materials of the same chemistry. In this study, we have demonstrated that 40 nm CeO_2 nanoparticles showed no cytotoxic effects towards pulmonary adenocarcinoma A549 cells. CeO_2 nanoparticles can enter cells follow dose-response effect and did not induce significant lysosomal membrane destabilization. At present, accurate, sensitive and cost-effective measurement techniques for characterizing them do not exist. The simple methods introduced in this study were useful for the initial screening of the uptake potential of insoluble nanomaterials in biological tissues and cells.

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