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Effect of Particle Size of Zinc Oxides on Cytotoxicity and Cell Permeability in Caco-2 Cells

- Research Note -

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Abstract

The cell permeability and cytotoxic effects of different-sized zinc oxide (ZnO) particles were investigated using a human colorectal adenocarcinoma cell line called Caco-2. Morphological observation by scanning electron microscopy revealed that three zinc oxides with different mean particle sizes (ZnO-1, 20 nm; ZnO-2, 90～200 nm; ZnO-3, 1～5 μm) tended to aggregate, particularly in the case of ZnO-1. When cytotoxicities of all three sizes of zinc oxide particles were measured at concentration ranges of 1～1000 μg/mL, significant decreases in cell viability were observed at concentrations of 50 μg/mL and higher. Among the three zinc oxides, ZnO-1 showed the lowest viability at 50 μg/mL in Caco-2 cells, followed by ZnO-2 and ZnO-3. The permeate concentration of ZnO-1 from the apical to the basolateral side in the Caco-2 model system after four hours was about three-fold higher than that of either ZnO-2 or ZnO-3. These results demonstrated that ZnO-1, with a 20 nm mean particle size, had poorer viability and better permeability in Caco-2 cells than ZnO-2 and ZnO-3.

Key words: zinc oxide, Caco-2, cytotoxicity, cell permeability, particle size effect

INTRODUCTION

Nanotechnology involves functional applications of nanosized biological or nonbiological structures, as well as the manipulation of matter at the nanoscale level, roughly 1 to 100 nanometers (1). The application of nanotechnology to food and agricultural products continues to grow, and global impact of the products in which nanotechnology plays an important role is expected to be approximately $1 trillion annually by 2015 (2). The potential uses of nanotechnology for food and agriculture include: improving food safety, increasing biosecurity and product traceability, increasing the efficacy of functional foods, increasing the efficiency of agricultural production, and creating intelligent packaging materials (2).

Interestingly, the possible toxicological impacts of nanomaterials have drawn much less attention, although, in recent years, toxicokinetics and toxicological effects of nanomaterials have been reported (3,4). Toxicokinetic data indicated that the characteristics of nanoparticles such as size, surface charge, and functional groups are likely to influence the absorption, distribution, metabolism, and excretion (ADME) (3). It was reported that certain nanoparticles were distributed throughout the brain, blood, spleen, and liver, and the lungs, and showed potential passage into cellular barriers, such as the blood-brain barrier and the placental barrier (5). In terms of *in vitro* studies, various nanoparticles can trigger the release of reactive oxygen species, and cause oxidative stress and subsequent inflammation (4). Also, *in vivo* toxicity studies for oral exposure showed acute toxicity at high doses (3). No information on the toxicity after chronic or acute oral exposure at low dose is available. Although there were some reports that describe how properties of nanoparticles such as size, shape and dispersed state are essential for determining their toxicological effects in the physiological environment (6), there is little data and information on the relationship between physicochemical properties of nanoparticles and toxicity, and further, on toxicological effects through *in vitro*, *in vivo* study and human trials.

Zinc oxide (ZnO) is one of the most important trace elements in the mammalian organism, and is involved in homeostasis, immune response, oxidative stress, apoptosis and aging (7). ZnO is added to many food products as a source of zinc, a necessary nutrient, and is on the FDA's generally recognized as safe (GRAS) substance list (8). Nanosized ZnO is also commercially produced, used for plastic wrap as a food packaging material and applied to food as an additive (9).

The Caco-2 cell monolayer used in this study has been widely used as an *in vitro* model and a valuable tool to evaluate the intestinal trans/paracellular permeability

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of macromolecules (10). The cell permeability of biomaterials is important for determining oral bioavailability, and may have substantial effects on biokinetics such as distribution, metabolism and excretion (3,4,11). The research on biokinetics of nanoparticles is essential for understanding their safety for humans and animals. Nevertheless, there are only a few studies on the bioavailability and biokinetics of nanoparticles (3), and there was no report on the size-dependent permeability of zinc oxides, including nanosized particles as a model compound.

In this study, we investigated the cytotoxicity and permeability of different sizes of ZnO particles in a human colorectal adenocarcinoma cell line called Caco-2.

MATERIALS AND METHODS

Materials

Zinc oxides with three different mean particle sizes (ZnO-1, 20 nm; ZnO-2, 90～200 nm; ZnO-3, 1～5 μM) were purchased from American elements (Los Angeles, CA, USA). Caco-2 cells (ATCC no. HTB-37) were obtained from the American Type Culture Collection (Manassas, VA, USA). Transwell inserts (cat. no. 3460) were obtained from Corning Costar Inc. (Cambridge, MA, USA). Fetal bovine serum was obtained from HyClone (Logan, IL, USA), and minimum essential media (MEM), Hank's balanced salt solution (HBSS), and penicillin/streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). All other chemicals and reagents were of the highest purity available.

Microscopic characterization of ZnO particles

Zinc oxide particles were observed using scanning electron microscopy (SEM) (12). An FE-SEM (Philips XL30 ESEM, 3.5 nm, 10 kV) with silicon wafers was used as a support for SEM. A small drop of the ZnO particles (0.1 mg/mL of 10 μL dispersed in ethanol) was deposited on silicon wafers for SEM observation.

Caco-2 cell culture

The human colon adenocarcinoma cell line, Caco-2, was cultured in Dulbecco's modified Eagles' minimal essential medium supplemented with 1% non-essential amino acids, 1% L-glutamine, 20% fetal bovine serum, 100 U/mL of penicillin and 0.1 mg/mL of streptomycin. Cells were grown in a humidified atmosphere of 5% CO_2 in air at 37°C. Cells were subcultured at 80% confluence.

In vitro cytotoxicity assay

Caco-2 cells were seeded into 96-well plate at a density of 2×10^4 cells/well, and then treated with varying concentration of zinc oxide nano/microparticles suspended in PBS for 96 hr. Carboxymethyl cellulose (CMC, Sigma, St. Louis, MO, USA, 0.5%), PEG-40 hydrogenated castor oil (HCO-40, KAO corp., Tochigi, Japan, 0.1%), and polysorbate 80 (Tween-80, Sigma, 0.1%) as a stabilizer or surfactant were used with or without ZnO. The concentrations of stabilizer or surfactant used in this study showed no cytotoxicity. Cell viabilities were determined by MTT assay using an ELISA reader (Molecular Devices, Sunnyvale, CA, USA) at 550 nm (13,14).

Transepithelial electrical resistance (TEER) measurements during growth

Caco-2 cells were seeded on the tissue-culture treated with polycarbonate filter (pore size 3 μm, diameter 12 mm, growth area 1.12 cm^2) in Costar Transwell (12 wells) at a density of 4×10^4 cells/insert. The culture medium was added to both apical and basolateral compartments, and replaced every 48 hr for the first 6 days and every 24 hr thereafter. The cultures were kept in an atmosphere of 95% air and 5% CO_2 at 37°C, and were used for the permeability experiments 18～21 days after seeding, when displayed TEER values of 400～550 Ω cm^2. TEER values of the Caco-2 cell monolayers were monitored every 2～3 days with a Millicell-Electrical Resistance System (Millipore Corp., Bedford, MA, USA) connected to a pair of chopstick electrodes (13,15,16)

In vitro Caco-2 permeability assay

For *in vitro* Caco-2 permeability assay, the method of Markowska et al. (17) was used, which is widely used for measuring cell permeable properties of particles. To initiate the transport experiment, the culture media in the apical and the basolateral compartments were aspirated, and the cells were rinsed twice with pre-warmed Hank's balanced salt solution (HBSS) at pH 7.4. Zinc oxide particles suspended in PBS were added to apical chambers (total of 0.5 mL) to produce the initial concentration at 10 μg/mL (123 μM). Basolateral chambers were filled with 1.5 mL prewarmed HBSS. 500 μL of samples were collected from basolateral chambers at 0, 1, 2, 3, 4 hr, and then were immediately analyzed by a spectrophotometric method. Transwells without seeded cells were run concurrently as a control to measure the permeation rate of zinc oxide particles (17,18).

Statistical analysis

Results of the experiment performed in six replicates are presented as means±SE. The statistical significance among treatments was analyzed by a Student *t*-test or one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. p values$<$0.05 were considered to be statistically significant.

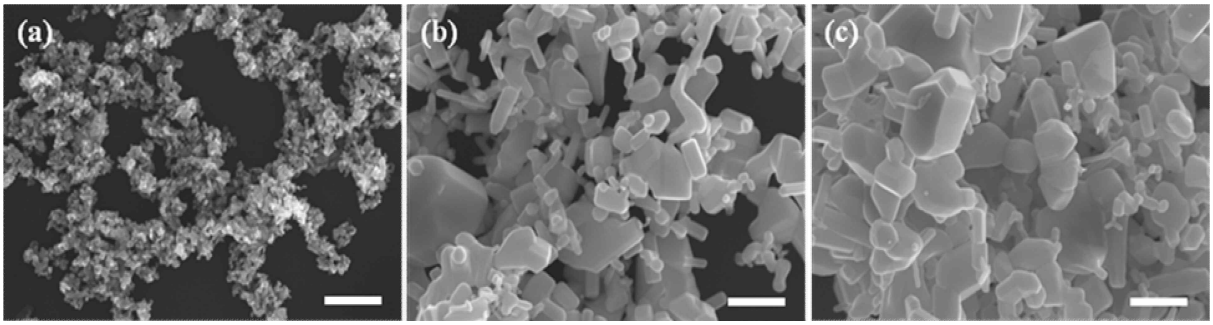

Fig. 1. Scanning electron microscopy images of three zinc oxide particles with different mean particle sizes, 20 nm {ZnO-1, (a)}, 90～200 nm {ZnO-2, (b)} and 1～5 μm {ZnO-3, (c)}. The scale bars correspond to 500 nm.

RESULTS AND DISCUSSION

Microscopic characterization of ZnO particles

ZnO particles with different mean particle sizes were examined by SEM in terms of morphological aspects, such as particle size and shape, and dispersed state. As shown in Fig. 1, the pictures of ZnO particles with three mean particle sizes of 20 nm (ZnO-1, a) 90～200 nm (ZnO-2, b) and 1～5 μM (ZnO-3, c) were represented. The shape of ZnO particles was observed to be polygonal. It is examined that ZnO particles tend to be aggregated, particularly apparent in ZnO-1.

SEM employs relatively low beam energy in comparison to TEM, which is crucial for accurate characterization of nanoparticles in the dry form (19). In our study, light scattering technique was also used to determine the size in the colloidal suspension in nano- and submicron ranges based on the method of Kato et al. (20). Particle sizes of ZnO-1 and ZnO-2 measured by light scattering were over ten times larger than vendor reported sizes (data not shown). This tendency is in accordance with other reports, and is partly explained by agglomeration of nanoparticles in solution (21,22). Hence, the results from SEM analysis were presented in this study.

In vitro cytotoxicity of ZnO particles

Size-dependent cytotoxic effects of ZnO particles with three different mean particle sizes dispersed in PBS were investigated in Caco-2 cells by MTT assay. As shown in Fig. 2, there was no difference in viability of Caco-2 when ZnO particles of 1 μg/mL and 10 μg/mL were treated, while dramatic reductions in cell viability were shown after ZnO particles were treated with higher concentration than 10 μg/mL. ZnO-1 with the smallest mean particle size of 20 nm showed the lowest viability at 50 μg/mL in Caco-2 cells. The order of viability at 50 μg/mL was ZnO-3>ZnO-2>ZnO-1. The use of stabilizers or surfactants, such as 0.5% CMC, 0.1% HCO 40 and 0.1% Tween 80 in samples did not show any change in Caco-2 cell viability compared to the treatment of ZnO-1 dispersed in PBS only.

The slight elevation in Caco-2 cell viability at 10 μg/mL ZnO compared to that of PBS-treated cells, presented in Fig. 2, has never been reported previously. Only Yang et al. (23) reported a similar elevation in different type

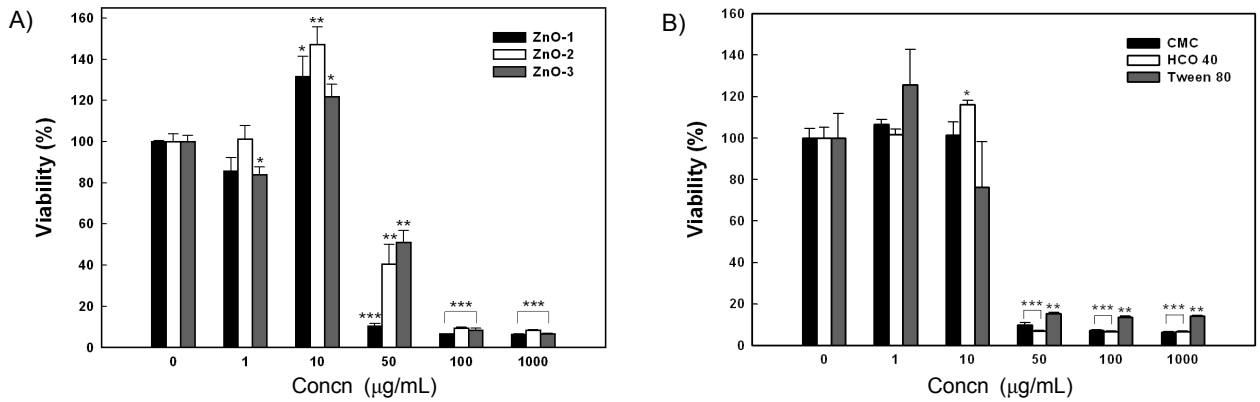

Fig. 2. Viability of Caco-2 cells after treatment of zinc oxide particles. Values are expressed as mean±SE (n=6). $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ compared to the PBS-treated control. A: Three zinc oxides with different mean particle sizes (ZnO-1 of 20 nm, ZnO-2 of 90～200 nm and ZnO-3 of 1～5 μm) dispersed in PBS only; B: ZnO-1 dispersed in PBS containing carboxymethyl cellulose (CMC), HCO 40, and Tween 80.

of cell viability at 10 μg/mL ZnO without significance (23). The reason for showing that phenomena in Caco-2 cell viability at 10 μg/mL ZnO in our study would be explained by the effect of experimental condition rather than concentration-dependent effect. It is considered that returning of ZnO-treated cell viability to that of PBS control after the addition of surfactants or stabilizers was not associated with the effect of surfactants or stabilizers themselves.

The result that ZnO particles displayed a steep decrease in viability above 10 μg/mL ZnO is consistent with the studies of Heng et al. (24) and Hackenberg et al. (25). The other study from Yang et al. (23) on cytotoxic effects of four typical nanoparticles showed that zinc oxide induced much greater cytotoxicity than non-metal nanoparticles. The study also indicated that oxidative stress might be a key route in inducing the cytotoxicity of nanoparticles (24,25), and demonstrated in the end that particle composition probably played a primary role in the cytotoxic effects.

Other groups showed the variability in toxic properties of ZnO nanoparticles depending on the cell type tested. The study of Nair et al. (26) showed that ZnO nanoparticles were more toxic to the cancer cells than the corresponding microparticles, while the study of Colon et al. (27) showed that nanophase ZnO actually not only improved normal osteoblast function but also indicated no toxicity.

The higher cytotoxic action of ZnO-1 than ZnO-2 or ZnO-3 in this study might be explained by one of several possibilities: first, the induction of oxidative stress and inflammation, second, high cellular level of ZnO-1 due to its higher permeable property than ZnO-2 and ZnO-3, and third, the increased surface area as a characteristic of the nanoparticle, acting as a physical barrier to prevent the growth of cells and affect membrane function of the cells. A further study on cytotoxicity of ZnO particles is required to examine the toxicity mechanism.

In vitro Caco-2 permeability of ZnO particles

To investigate the permeability of ZnO particles in the Caco-2 cell model, the integrity of cell monolayer was first checked by measuring TEER value. Transcellular resistance value of Caco-2 monolayer measured at 18～21 days after seeding was 400 to 550 Ω cm^2, whereas the cell free membrane inserts had resistance value of 13 to 17 Ω cm^2. 10 μg/mL ZnO particles were chosen for the experiment based on the results that there were no cytotoxic effects below 10 μg/mL concentration. The permeability of ZnO particles across the Caco-2 monolayer was shown in Fig. 3. The permeability of ZnO particles increased over the four hour time period. The

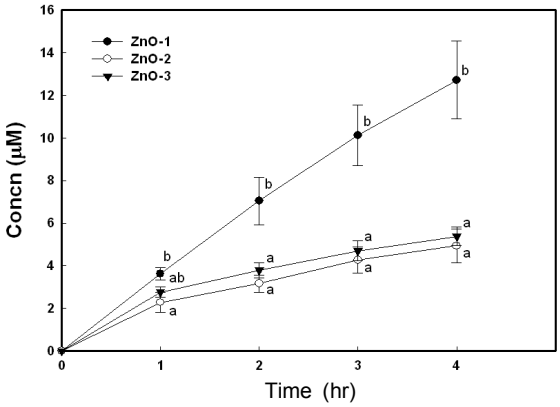

Fig. 3. Cellular permeability of zinc oxide particles across Caco-2 monolayer. Samples were added to the apical side of the cells, and the permeated samples were determined from the basolateral side by the spectrophotometric method. Values are expressed as mean±SE (n=6). Bars with different letters are significantly different at $p<0.05$.

permeate concentration of ZnO-1 was about three-fold higher after four hours than those of ZnO-2 and ZnO-3.

Permeability of orally administered compounds through Caco-2 cell monolayer is well correlated with *in vivo* absorption in human (28). The Caco-2 cell monolayer allows the study of major absorptive mechanisms for drugs, such as passive transcellular and paracellular transport, carrier-mediated influx, and efflux (9). Caco-2 intestinal permeability model has been used to evaluate the bioavailability of nanoparticle formulations. Cell bioavailability is important because it is closely related to translocation and distribution in the body (4).

The factors such as particle size, surface charge, attachment of ligands, and coating with surfactants may influence permeation of nanoparticles in the gastrointestinal tract (5). Among these, the effect of particle size was examined in this study. This study indicates that permeability into Caco-2 cells of ZnO-1 of 20 nm mean particle size was the highest as compared with those of larger sized ZnO-2 and ZnO-3. A similar result was shown by other researchers. According to Jia et al. (29), nanonization of potential drugs, could improve oral bioavailability, showing four times higher permeability than those of microparticles. From those studies, it was found that smaller particles are able to diffuse faster through the mucus layer than larger particles. Although nanoparticles are agglomerated together, they are not tightly bonded, and the nanosized particle could be considered to penetrate into the cell membrane through the transcellular and paracellular routes easily.

In conclusion, our results demonstrated that ZnO-1 of 20 nm mean particle size had poorer viability and better permeability in Caco-2 model compared to those of ZnO-2 and ZnO-3. Further studies on toxicological as-

pects and bioavailability of zinc oxide particles should be continued in order to determine more accurate size effect, especially for various nano-sizes.

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