Factors influencing the cytotoxicity of zinc oxide nanoparticles: particle size and surface charge

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Abstract. Zinc oxide (ZnO) nanoparticle is one of the most important materials in diverse applications, since it has UV light absorption, antimicrobial, catalytic, semi-conducting, and magnetic properties. However, there is little information about the toxicological effects of ZnO nanoparticles with respect to physicochemical properties. The aim of this study was, therefore, to evaluate the relationships between cytotoxicity and physicochemical properties of ZnO nanoparticle such as particle size and surface charge in human lung cells. Two different sizes of ZnO nanoparticles (20 and 70 nm) were prepared with positive (+) or negative (-) charge, and then, cytotoxicity of different ZnO nanoparticles was evaluated by measuring cell proliferation in short-term and long-term, membrane integrity, and generation of reactive oxygen species (ROS). The results demonstrated that smaller particles exhibited high cytotoxic effects compared to larger particles in terms of inhibition of cell proliferation, membrane damage, and ROS generation. In addition, positively charged ZnO showed greater ROS production than ZnO with negative charge. These findings suggest that the cytotoxicity of ZnO nanoparticles are strongly affected by their particle size and surface charge, highlighting the role of the physicochemical properties of nanoparticles to understand and predict their potential adverse effects on human.

1. Introduction
ZnO nanoparticle is one of the most widely used engineered nanomaterials in commercial products due to its UV light absorption, antimicrobial, catalytic, semi-conducting, and magnetic properties [1]. ZnO nanoparticle is, therefore, widely applied to personal care products, sunscreen, paints, electronic materials, rubber manufacture, food additives, and medicine [2-4]. In particular, nano-sized ZnO exhibits unique features that may completely differ from bulk-sized ZnO. As the particle size of ZnO decreases, its transparency to visible light and chemical reactivity increase, contrary to micro-sized ZnO having opacity and low reactivity. These unique characteristics are related to high proportion of atoms on the surface of nano-sized materials compared to bulk-sized ones. Thus, ZnO nanoparticles have been extensively applied to diverse products where transparency or great reactivity is required. On the other hand, the high reactivity of ZnO nanoparticles gives rise to increase biological responses such as cellular uptake and delivery efficiency, and thereby raising concern about their toxicity potential on biological systems. Therefore, the safety aspect of ZnO nanoparticles should be assumed to further expand their industrial applications with safe levels. Many studies on the toxicity of ZnO nanoparticles were performed in cell lines [5-6] as well as in animal models [7-8], but some contradictory results were reported, in particular, in terms of physicochemical parameters affecting...
their toxicity. Thus, toxicity of ZnO nanoparticles still remains to be elucidated, needing more vigorous toxicological evaluation by applying several methods. In this study, we evaluated the cytotoxicity of ZnO nanoparticles of two different sizes (20 and 70 nm) and different charges (positive and negative), respectively, in human lung A549 cells to determine the correlation between physicochemical properties of nanoparticles and their cytotoxicity.

2. Experimental methods

2.1. Materials
ZnO nanoparticles of two different sizes (20 and 70 nm) were purchased from Sumitomo (Japan) and American Elements (U.S.A.), respectively. For surface modification of ZnO nanoparticles with negative charge, ZnO (10 g) was suspended in 20 mM HEPES buffer (pH 7.0) containing 1% sodium citrate. The particle size and surface charge (zeta potential) of ZnO nanoparticles were determined by transmission electron microscopy (TEM: JEM-1010, JEOL) and a zeta potentiometer (Zetasizer Nano ZS system, Malvern Instruments), respectively.

2.2. Cell culture
Human lung epithelial cells (A549) were purchased from the Korean Cell Line Bank and cultured in RPMI1640 medium supplemented with 10% heat inactivated fetal bovine serum (Welgene, Ltd., South Korea), 100 units/ml penicillin, and 100 µg/ml streptomycin, under a humidified atmosphere (5% CO₂ plus 95% air).

2.3. Cell proliferation and viability
Cells (2 × 10³ cells/100 µl) were seeded onto 96-well plates and incubated overnight at 37°C under a 5% CO₂ atmosphere. The medium in the wells was then replaced with fresh medium containing nanoparticles (0.5~1000 µg/ml) and incubation continued for 48 h. The effect of the nanoparticles on cell proliferation and viability was determined by WST-1 assay (Roche). Briefly, 10 µl of WST-1 solution (Roche) was added to each well and the plates were further incubated. After 4 h, the absorbance was measured with a plate reader at 440 nm. Cells incubated without nanoparticles were used as a control.

2.4. Lactate dehydrogenase (LDH) leakage assay
The release of LDH was monitored with the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega). Cells (2 × 10⁴ cells/ml) grown on 24-well plates were incubated with nanoparticles (0.5~1000 µg/ml) for 48 h. The plates were centrifuged, and aliquots (50 µl) of cell culture medium were collected from each well and placed in new microplates. Finally, 50 µl of substrate solution was added to each well and the plates were further incubated for 30 min at room temperature. The absorbance at 490 nm was measured with a microplate reader. Cytotoxicity is expressed relative to the basal LDH release by untreated control cells.

2.5. ROS production
The generation of intracellular ROS was measured with a cell-permeant fluorescent probe, carboxy-2',7'-dichlorofluorescein diacetate (carboxy-H₂DCFDA) (Molecular Probes) according to the manufacturer’s guideline. Cells (2 × 10³ cells/ 100 µl) were exposed to nanoparticles (0.5~1000 µg/ml) for 48 h, washed several times with phosphate-buffered saline (PBS) and incubated with 20 µM carboxy-H₂DCFDA for 60 min at 37°C. After washing, DCF fluorescence was immediately measured with a fluorescence microplate reader (SpectraMas M3, Molecular Devices). Basal ROS generation in cells treated without nanoparticles was used as a control.
2.6. Clonogenic assay
Cells were seeded in 35 mm dish at a density of $5 \times 10^2$ and incubated overnight under a standard condition. The medium in the dish (2 ml) was then replaced with fresh medium containing nanoparticles (5, 50, and 125 µg/ml) and incubation continued for 9 days. For colonies counting, cells were washed with PBS, fixed with 90% methanol for 30 min at 4°C and stained with 0.5% crystal violet solution (in 20% methanol, Sigma) for 1 h. After cells were washed with deionized water and air-dried, colonies consisted of more than 50 cells were counted. Each experiment was done in triplicate and colony number in the absence of nanoparticles was used as a control.

2.7. Statistical analysis
Statistical analyses were performed using Student's t test for unpaired data and $p$ values of less than 0.05 were considered significant. All data are presented as mean ± standard error of the mean (S.E.M.).

3. Results and discussion

3.1. Characterization of ZnO nanoparticles
Particle size and surface charge of ZnO nanoparticles were measured by TEM and zeta potentiometer, respectively. As shown in Table 1, net charge of ZnO nanoparticles in aqueous solution was determined to be positive, about 43 mV, thus surface modification to obtain negatively charged ZnO nanoparticles was performed with citrates. Citric acid or citrates are widely used capping agents for inorganic nanoparticles, giving rise to negative surface coating. This is based on the fact that citrates are important biological ligands for metal ions to form strong metal complexes [9]. The measured particle size of 20 nm was well distinguished from that of 70 nm and the surface charge for positively or negatively charged ZnO was well prepared for comparative cytotoxicity study in the next step.

Table 1. Particle size and surface charge of ZnO nanoparticles as measured by TEM and zeta potentiometer, respectively.

| Sample        | Particle size [nm] | Zeta potential [mV] |
|---------------|--------------------|---------------------|
| 20 nm         | Positive charge    | 24 ± 5.57           | 43.8 ± 0.5 |
|               | Negative charge    |                     | -44.6 ± 0.7 |
| 70 nm         | Positive charge    | 67 ± 12.41          | 44.1 ± 0.6 |
|               | Negative charge    |                     | -45.2 ± 0.8 |

3.2. Effect of particle size and surface charge on cell proliferation and viability
Cytotoxicity of different-sized ZnO nanoparticles with different charges were tested in human lung cancer A549 cells by measuring inhibition of cell proliferation and viability with the WST-1 assay (Figure 1). This colorimetric assay is based on the conversion of tetrazolium salt WST-1 into soluble colored formazan by cellular enzymes like mitochondrial dehydrogenase. Thus, an increase in the amount of formazan products is directly related to the number of metabolically active cells in cell culture system. Figure 1 showed that all the ZnO nanoparticles greatly inhibited cell proliferation and viability after 48 h incubation in a concentration-dependent manner. But, slight difference in cytotoxicity between nanoparticles with different sizes and charges was found; 20 nm (+) exhibited the highest toxicity compared to 20 nm (-) and larger particle 70 nm. IC$_{50}$ (inhibition concentration 50%) values for 20 nm (+), 20 nm (-), 70 nm (-), and 70 nm (+) were about 68.47, 77.48, 94.67, 150.78 µg/ml, respectively.
3.3. Effect of particle size and surface charge on LDH leakage

LDH is an intracellular enzyme normally presented in the cytoplasm and released into the culture medium after cell membrane damage, thus increased LDH levels in the extracellular medium reflect reduced integrity of the plasma membrane. As shown in Figure 2, all the ZnO nanoparticles remarkably induced LDH leakage from A549 cells after 48 h incubation, in particular, at concentration above 50 μg/ml. Particle size and surface charge of ZnO nanoparticles highly affected cytotoxicity; the highest LDH was released from the cells incubated with 20 nm (+), which is consistent with the WST-1 result (Figure 1). In terms of particle size, 20 nm was determined to be more toxic than 70 nm, suggesting size-dependent toxicity of ZnO nanoparticles.

3.4. Effect of particle size and surface charge on colony formation

According to the WST-1 assay, ZnO nanoparticles greatly affect cell proliferation and viability of A549 cells at the concentration above 5 μg/ml. However, it is possible that an exposure to even low...
concentration of ZnO nanoparticles could cause cytotoxicity after incubation for long time. Thus, the long-term cytotoxicity of different-sized or charge ZnO nanoparticles was evaluated by employing clonogenic assay, a method used to evaluate the ability of a single cell to grow into a colony. Figure 3 showed that all the ZnO nanoparticles tested completely inhibited cell proliferation of A549 cells at concentration above 50 μg/ml. But, interestingly, low concentration of 5 μg/ml of ZnO nanoparticles did not affect the colony formation of the cells even after long-term exposure for 9 days. This result is highly consistent the WST-1 assay as shown in Figure 1. It is worthy to note here that size or charge-dependent cytotoxicity of ZnO nanoparticles was not found, indicating exposure concentration and time to ZnO are more important factor affecting the long-term cytotoxicity rather than their physicochemical properties.

Figure 3. Clonogenic assay of A549 cells exposed to ZnO nanoparticles for 48 h exposure. * represents a significant difference from the control (p < 0.05).

3.5. Effect of particle size and surface charge on ROS generation
Several researches reported that oxidative stress is one of the main toxicological effects caused by ZnO nanoparticles in cell culture systems [10-14]. Thus, intracellular production of ROS was evaluated with a non-fluorescent dye, carboxy-H2DCFDA until it is converted to green fluorescent dichlorofluorescein (DCF) upon oxidation within cells. The result represented that all different ZnO nanoparticles could generate ROS in a concentration dependent-manner (Figure 4). Interestingly, particle size and surface charge of ZnO nanoparticle were strongly associated with ROS production; 20 nm (+) generated the highest ROS at concentration range of 250 – 1000 μg/ml with a following order of 70 nm (+) > 20 nm (-) > 70 nm (-). In terms of ROS generation, it seems that surface charge of ZnO nanoparticles is more critical parameter than its particle size. In addition, size-dependent cytotoxicity was also measured, indicating high toxicity of 20 nm compared to 70 nm.
4. Conclusion
In this work, toxicological effects of ZnO nanoparticles with respect to physicochemical properties such as particle size and surface charge were evaluated in cell culture systems by measuring inhibition of cell proliferation and viability in short-term as well as in long-term, membrane damage, and ROS generation. Among ZnO nanoparticles tested with different sizes and charges, 20 nm (+) exhibited the highest cytotoxicity in terms of inhibition of short-term cell proliferation/viability, membrane damage, and ROS production. Small-sized ZnO nanoparticles, 20 nm, were more toxic than larger sized ones, 70 nm, and moreover, positively charged ZnO nanoparticles caused more marked ROS production compared to negatively charged ones. It seems that positively charged ZnO nanoparticles of small size strongly interact with the negatively charged plasma membrane, and thereby induce more toxicological effects. However, it should be noted here that size- or charge-dependent cytotoxicity was not always found and differed with the assessing methods used, indicating essential necessity of several methodological evaluations to conclude the toxicity of nanoparticles. The result demonstrated that the cytotoxic effects of ZnO nanoparticles are highly related to particle size and surface charge, suggesting an important role of the physicochemical parameters of nanoparticles to understand and possibly minimize their toxicity potential on human.

Acknowledgement
This research was supported by a grant (10182KFDA991) from Korea Food & Drug Administration in 2010.

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