Original Article

ZnO, TiO_2, SiO_2, and Al_2O_3 Nanoparticles-induced Toxic Effects on Human Fetal Lung Fibroblasts*

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Abstract

Objective This study aims to investigate and compare the toxic effects of four types of metal oxide (ZnO, TiO_2, SiO_2, and Al_2O_3) nanoparticles with similar primary size (~20 nm) on human fetal lung fibroblasts (HFL1) *in vitro*.

Methods The HFL1 cells were exposed to the nanoparticles, and toxic effects were analyzed by using MTT assay, cellular morphology observation and Hoechst 33 258 staining.

Results The results show that the four types of metal oxide nanoparticles lead to cellular mitochondrial dysfunction, morphological modifications and apoptosis at the concentration range of 0.25-1.50 mg/mL and the toxic effects are obviously displayed in dose-dependent manner. ZnO is the most toxic nanomaterials followed by TiO_2, SiO_2, and Al_2O_3 nanoparticles in a descending order.

Conclusion The results highlight the differential cytotoxicity associated with exposure to ZnO, TiO_2, SiO_2, and Al_2O_3 nanoparticles, and suggest an extreme attention to safety utilization of these nanomaterials.

Key words: Metal oxide nanoparticles; Toxic effects; Fibroblasts

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INTRODUCTION

During the past decade, there has been a rapid progress on research in the areas of nanoscience and nanotechnology. Nanoparticles are defined as particles with a diameter of less than 100 nm in one structural dimension. Compared to the same materials with micrometer scale dimensions, nanomaterials have specific properties, such as small size, large surface area, shape, and special structure[1]. Because of the unique dimensional and morphological properties, nanomaterials can be physically and chemically manipulated and widely used in industrial and biomedical processes[2-7]. Many consumer products may contain nanoparticles[8-10]. Titanium dioxide (TiO_2), silicon dioxide (SiO_2), Aluminum oxide (Al_2O_3), and zinc oxide (ZnO) are common materials with a variety of applications. For example, TiO_2 and ZnO nanoparticles are currently used for protection against UV ray exposure. Many sunscreens contain these nanoparticles which reflect and scatter ultraviolet radiation more efficiently. Nanosized crystalline SiO_2 is commonly used in semiconductor manufacture. Also, Aluminum oxide film inhibits chemical corrosion on the surface of nanoscale

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aluminum-made products.

Despite of these potential benefits, there is concern over the possible adverse effects of nanoparticles on human health and environment. Researches in the recent years have shown that nanoparticles may interact with the biological system and exhibit some toxicity[11-13]. Exposure of cultured cells to TiO_2 nanoparticles may lead to cell apoptosis, IL-8 level increase, reduced glutathione (GSH) decrease and induction of micronuclei[14-16]. Nano-sized SiO_2 particles result in IL-6 increase, DNA damage and apoptosis[17-19]. ZnO, TiO_2, and Al_2O_3 nanoparticles exhibit adverse effects on cell proliferation and cell viability in human lung epithelial cells and A549 carcinoma cells[20].

Many existing nanotoxicity researches have concentrated on evaluation of individual nanomaterials. However, limited comparative toxic information about the nanoparticles with similar physical characteristics (e.g. particle size) is supplied. It is necessary to make a comparison of the toxic effects of such nanoparticles for prediction of their possible hazard. This requires exploration and a more thorough understanding of the potential toxicity of such nanomaterials. The present research was therefore performed to investigate and compare a group of different metal oxides (ZnO, TiO_2, SiO_2, and Al_2O_3) nanoparticles with similar primary size (~20 nm), regarding cell viability, morphology modification and apoptosis. The objectives of this study were to (i) determine whether these metal oxide nanoparticles affected cell activity, (ii) provide information on relative cytotoxicity of these nanoparticles. In this study, well-dispersed suspensions of ZnO, TiO_2, SiO_2, and Al_2O_3 nanoparticles were obtained for the assessment of their cytotoxicity. The cell line used was a fibroblast type derived from the normal human lung tissues. Cell viability, morphology, and apoptosis were examined by methyl thiazolyl tetrazolium (MTT) cytotoxicity assay, phase contrast microscopy and Hoechst 33 258 staining, respectively.

MATERIALS AND METHODS

Chemicals

Titanium dioxide (primary particle size 21 nm, 80% anatase, 20% rutile, specific surface area 50±15 $m^2 \cdot g^{-1}$, Degussa GmbH, Germany), Alumnium oxide (primary particle size 13 nm, specific surface area 100±10 $m^2 \cdot g^{-1}$, Degussa GmbH, Germany), Zinc oxide (primary particle size 20 nm, specific surface area 90 $m^2 \cdot g^{-1}$, Nanjing High Technology of Nano Co., Ltd.), and Silicon dioxide (primary particle size 20 nm, specific surface area 600 $m^2 \cdot g^{-1}$, Nanjing High Technology of Nano Co., Ltd.) nanoparticles were commercially available and used for the experiments. Dulbecco's modified Eagle's medium (DMEM)/Ham's Nutrient Mixture F-12 (1:1) medium and 0.25% Trypsin-EDTA were purchased from invitrogen (Invitrogen Co., Ltd., USA). Fetal bovine serum (FBS) was purchased from Hyclone (Newzealand). Penicillin-streptomycin was purchased from SunShine Biotechnology Co., Ltd. (Nanjing, China). The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Hoechst 33 258 were purchased from Sigma-Aldrich (St. Louis, USA). All other chemicals were of highest quality available.

Dispersion and Characterization of Nanoparticles

The dry powder of nanomaterials (ZnO, TiO_2, SiO_2, and Al_2O_3) was weighed on an analytical mass balance (Sartorius BS210S, Goettingen, Germany), suspended in distilled water at a concentration of 50.0 mg/mL, respectively. This suspension was vibrated by vortex for 2 min (SK-1 Vortex, Hengfeng Instrument Factory, Jintan, China) and sonicated at 33 W (S-4000 Sonicator Ultrasonic Processor, Misonix, Inc., Farmingdale, NY, USA.) for 1 min to aid in preparing a homogeneous suspension. To prepare nanoparticle suspensions (ZnO, TiO_2, SiO_2, and Al_2O_3) at the concentrations of 0.25, 0.50, 0.75, 1.0, 1.50 mg/mL, DMEM/F-12 containing 10% (v/v) fetal bovine serum was added to 50.0 mg/mL nanoparticle suspensions, respectively. Subsequently, all the suspensions were vibrated for 2 min and sonicated for 10 min at a frequency of 20 kHz and an intensity of 33 W[21]. The average sizes of various particles in suspensions were determined by the method of dynamic light scattering (Zetasizer Nano ZS90, Malvern Instruments Ltd., Worcestershire, UK) and analyzed by transmission electron microscopy (JEM-200CX, JEOL Ltd., Tokyo, Japan), respectively.

Cell Culture

Human fetal lung fibroblast (HFL1) cells were purchased from the Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Science, China. The cells were supplied with DMEM/F-12 cell growth medium containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in 25 cm^2 flask (Corning, Lowell, USA) at 37 °C in a 5% CO_2 humidified atmosphere (BB16uv/BB5060uv CO_2 incubator, Heraeus, Germany). After reaching

confluence, monolayer cells were trypsinized for 3 min by using 0.25% Trypsin-EDTA solution and seeded in 96 or 24-well plates (Costar, USA) at a density of 1×10^4 cells/well or 5×10^4 cells/well for subsequent MTT test and cell morphology observation, respectively. For the immunofluorescence microscopy of cell nuclei, cells were trypsinized and seeded at a density of 1×10^5 cells/well in 6-well plates (Costar, USA) containing an 18 mm×18 mm glass coverslip in each well. Before use, the glass coverslips had been prewashed with methanol and PBS and air dried in a sterile ventilated hood.

Cell Viability

MTT assay was used to evaluate the viability of HFL1 cells cultured in medium containing different concentrations of metal oxide nanoparticles. Mitochondrial enzymes in metabolically active cells can decompose the tetrazolium salt to a colored formazan product. Once a confluent monolayer of cells had formed in 96-well plates, cells were treated with a variety of metal oxide suspensions at the concentrations of 0.25, 0.50, 0.75, 1.0, and 1.50 mg/mL for 48 h, respectively. After incubation, the supernatant was discarded and cells were supplied with fresh medium. Subsequently, the HFL1 cells were incubated with MTT (5.0 mg/mL) in the culture medium at 37 °C for 4 h. The medium was aspirated and the formazan product was dissolved in dimethyl sulfoxide and quantified spectrophotometrically at 490 nm. The results were expressed as percentage of untreated control in DMEM/F-12 cultured medium.

Cell Morphology by Phase Contrast Microscopy

Cells were plated into 24-well plates at a density of 5×10^4 cells/well. After a 70% confluent monolayer of cells had formed in 24-well plates, supernatants from the culture plates were aspirated out and freshly prepared suspensions containing metal oxide nanoparticles at the concentrations of 0.25, 0.50, and 1.0 mg/mL were added. After 48 h incubation, cells were washed with PBS and the morphological changes were observed under an inverted phase contrast microscope at 200× magnification.

Cell Apoptosis

To evaluate whether nanoparticles of ZnO, TiO_2, SiO_2, and Al_2O_3 induced apoptosis, the HFL1 cells were cultured on coverslips in 6-well plates. After a 70% confluent monolayer of cells had formed in 6-well plates, HFL1 cells were treated with various prepared suspensions at the concentrations of 0.25, 0.50, and 1.0 mg/mL for 48 h. After treatment, the cells were washed twice with PBS(pH 7.4) and then fixed in ice cold methanol/acetic acid (3:1) for 5 min. Cells were then stained with 5 μg/mL Hoechst 33 258 fluorochrome for 10 min and washed twice in distilled water, followed by an examination under a fluorescence microscope. Apoptotic cells were identified as those with brightly blue staining condensed nuclear chromatin. The quantitative analysis of apoptosis was done by counting apoptotic cells. A total of five hundred cells were counted, and the number of apoptotic cells was expressed as a percentage of the total number of cells.

Statistical Analysis

The data were expressed as mean±SD of three independent experiments. Wherever appropriate, the data were subjected to the statistical analysis by one-way ANOVA test followed by Student-Newman-Keuls test for comparison of all pairs of means. A value of $P<0.05$ was considered to be statistically significant. SPSS 11.5 for Windows software was used for the statistical analysis.

RESULTS

Particle Characterization

The average sizes of ZnO, TiO_2, SiO_2, and Al_2O_3 nanoparticles in suspensions measured by the dynamic light scattering analysis were 327.9±26.0, 163.4±7.0, 113.4±10.8, and 186.8±5.6 nm, respectively. TEM images of the ZnO, TiO_2, SiO_2, and Al_2O_3 nanoparticles were shown in Figure 1. The average size of particles measured by DLS analysis was larger than the particle size determined by TEM for all particle types. These results indicate that the nanoparticles formed agglomerates in the prepared cultured medium suspensions. The agglomerates were about ten times larger than the primary particle sizes.

Cell Viability

The effects of ZnO, TiO_2, SiO_2, and Al_2O_3 nanoparticles on the viability of HFL1 cells were evaluated by using MTT assay (Figure 2). The results showed that exposure to ZnO, TiO_2, SiO_2, and Al_2O_3 nanoparticles for 48 h resulted in dose-dependent increase in mitochondrial dysfunction. There is a statistically significant difference between different metal oxide nanoparticles. Compared to the untreated control in DMEM/F12 cultured medium, ZnO nanoparticles

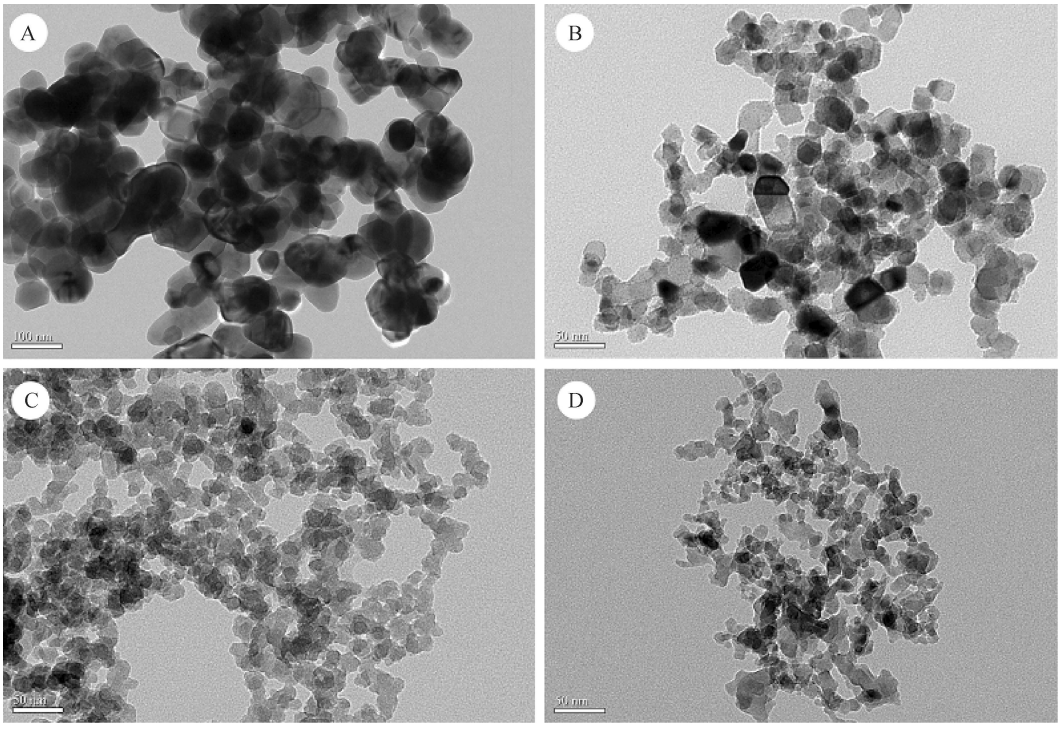

Figure 1. TEM analysis of nanomaterials. (A) ZnO, (B) TiO_2, (C) SiO_2, and (D) Al_2O_3. Original magnification ×120 000.

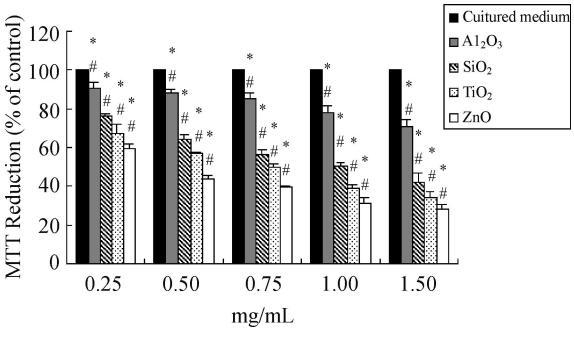

Figure 2. Effects of nanoparticles on HFL1 cell viability. Cells were treated with different concentrations of nanoparticles for 48 h. At the end of the incubation period, the mitochondrial function was determined by the MTT assay. Control cells cultured in nanoparticle-free media were run in parallel to the treatment groups. The OD value of control cells was taken as 100%. The MTT reduction of nanoparticle-exposed cells was calculated as the ratio of OD value of the treatment groups to that of the control cells. The data are expressed as mean±SD of three independent experiments. The (*) mark indicates a statistically significant difference compared to the control at the same dose (P<0.05), while the (#) sign indicates a statistically significant difference compared to other metal oxide nanoparticles at the same dose (P<0.05).

showed a higher cytotoxicity at the concentrations of 0.25, 0.50, 0.75, 1.0, and 1.50 mg/mL. The MTT assay showed ZnO nanoparticles were more toxic to HFL1 cells, followed by TiO_2, SiO_2, and Al_2O_3 nanoparticles in a descending order.

Cell Morphology

The cell morphological changes of the control cells and metal oxide nanoparticle-exposed cells were illustrated in Figure 3. No morphological changes of control cells were observed (Figure 3A). With addition of ZnO, TiO_2, SiO_2, and Al_2O_3 nanoparticles, morphological changes were induced in the nanoparticle-exposed cells and characterized by cell shrinkage and irregular shapes. At the low dose (0.25 mg/mL), the morphology of the cells appeared to be altered (Figure 3B, E, H, K). With increasing doses of metal oxide nanoparticles (0.50 and 1.0 mg/mL), cell morphology was destroyed and nanoparticles were accumulated inside the cells (Figure 3C and D, F and G, I and J, L and M). Among the four types of metal oxide nanoparticles, the morphology change and nanoparticle accumulation were induced remarkably in ZnO nanoparticle-exposed cells (Figure 3L and M), followed by TiO_2, SiO_2, and Al_2O_3 nanoparticles-exposed cells in a descending order (Figure 3I and J, F and G, C and D).

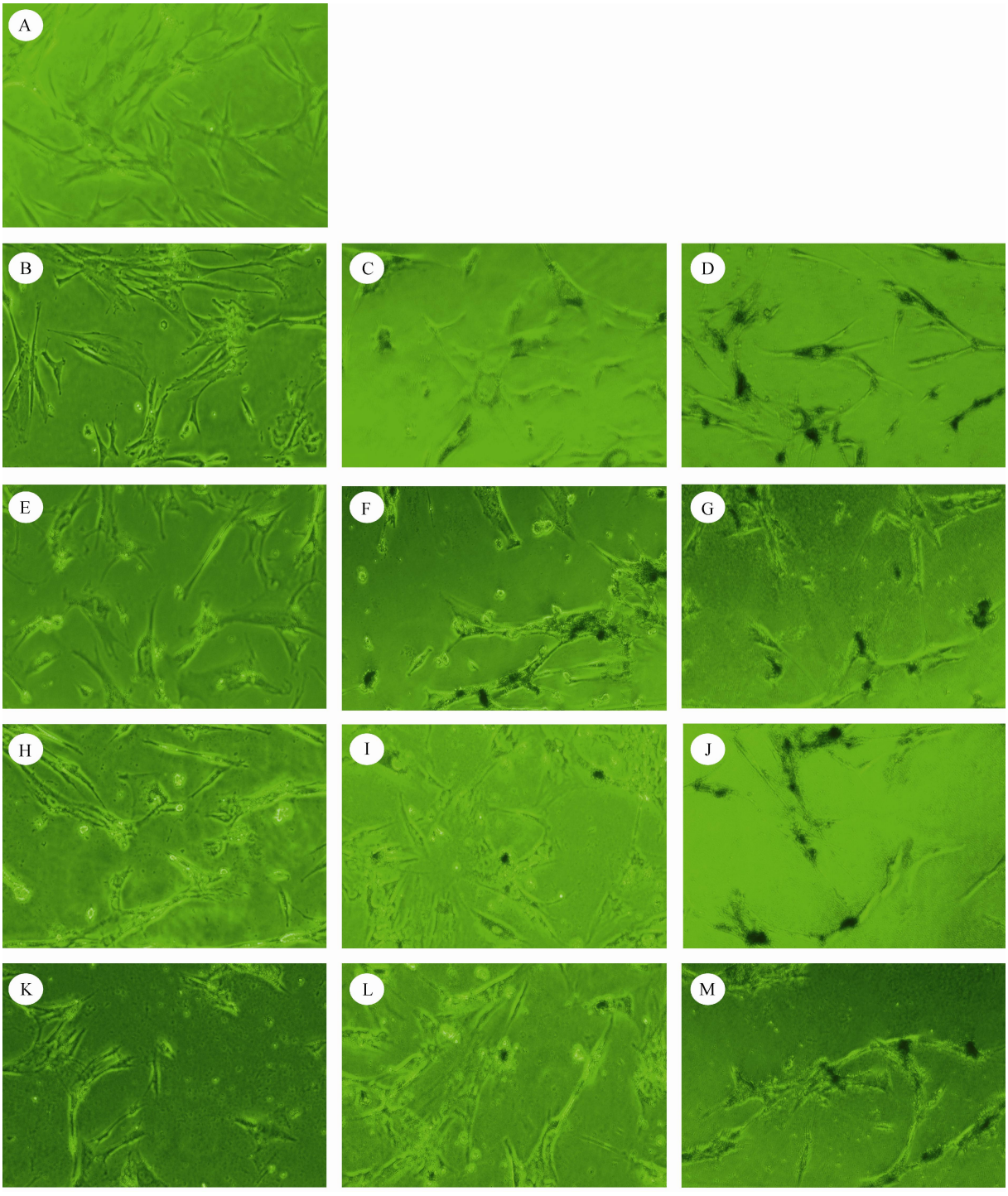

Figure 3. Morphological characterization of human fetal lung fibroblasts (HFL1). Cells were treated with ZnO, TiO_2, SiO_2, and Al_2O_3 nanoparticles at the concentrations of 0.25, 0.50, and 1.0 mg/mL in DMEM/F-12 cultured medium and incubated for 48 h at 37 °C in a 5% CO_2 atmosphere, respectively. After 48 h exposure, cells were washed twice with PBS and visualized by an inverted phase contrast microscope at 200×magnification. Cells were cultured with: (A) DMEM/F-12 growth medium only (control); (B-D) Al_2O_3 nanoparticles; (E-G) SiO_2 nanoparticles; (H-J) TiO_2 nanoparticles; and (K-M) ZnO nanoparticles, respectively.

Cell Apoptosis

The nuclear chromatin of cells was stained with Hoechst 33 258 fluorochrome, followed by observation under a fluorescence microscope (Figure 4). Apoptotic cells were distinguished by the findings of condensed chromatin in bright blue color. These metal oxide nanoparticles showed apoptotic effects on HFL1 cells. Compared with untreated cells (normal blue nuclei, Figure 4A), metal oxide (ZnO,

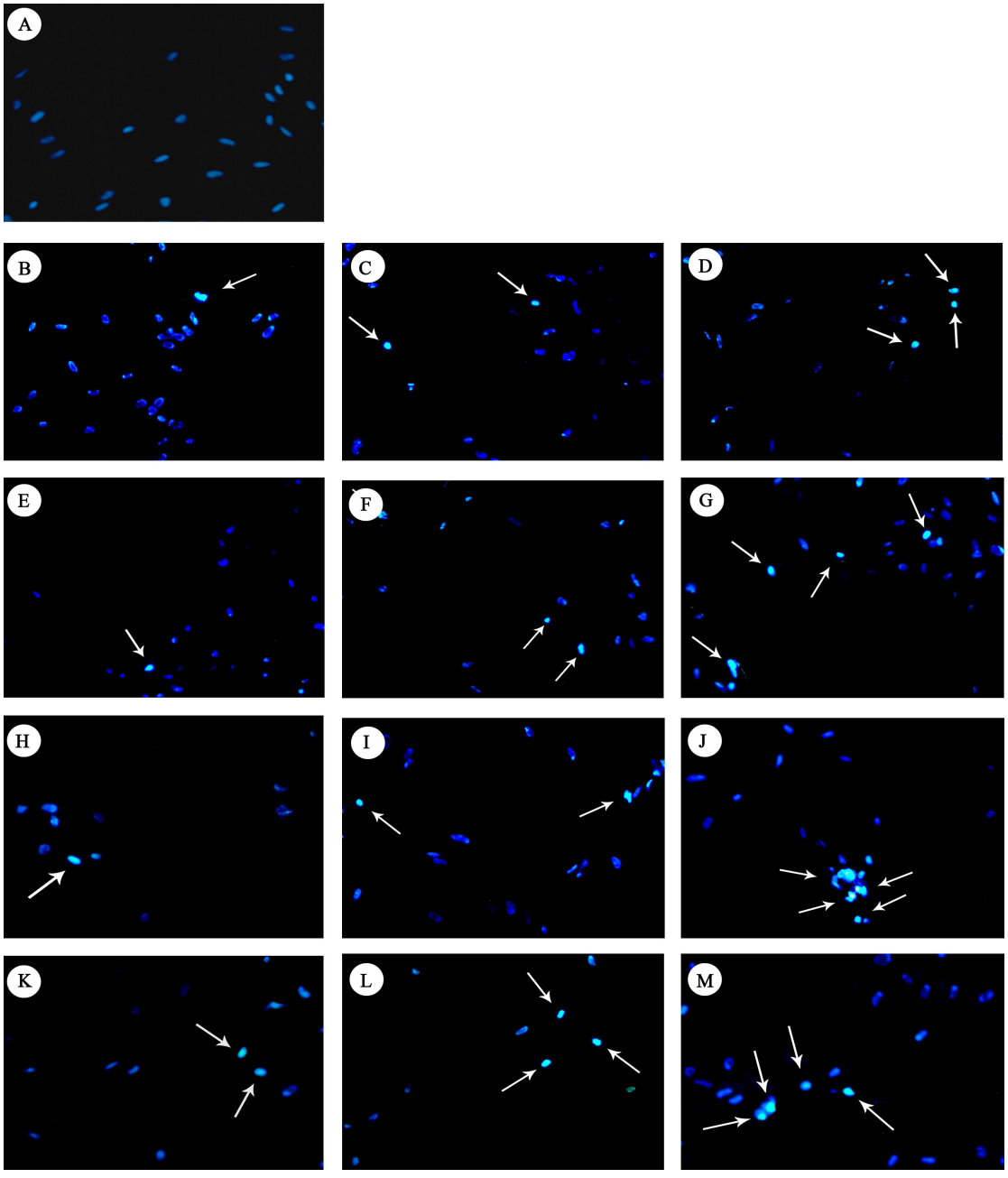

Figure 4. Fluorescent photograph of HFL1 cells stained with Hoechst 33 258. After addition of ZnO, TiO_2, SiO_2, and Al_2O_3 nanoparticles at the concentrations of 0.25, 0.50, and 1.0 mg/mL for 48 h, cells were washed twice, fixed in ice cold methanol/acetic acid and stained with Hoechst 33 258. The brightly blue stained condensed nuclei of apoptotic cells (arrows) can be distinguished from normal diffusely stained nuclei. Cells were cultured with: (A) DMEM/F-12 growth medium only (control); (B-D) Al_2O_3 nanoparticles; (E-G) SiO_2 nanoparticles; (H-J) TiO_2 nanoparticles; and (K-M) ZnO nanoparticles, respectively. The magnification was 200×.

TiO_2, SiO_2, and Al_2O_3) nanoparticles induced nucleic chromatin condensation with visible bright blue color (Figure 4B-M). The formation of apoptotic nuclear was dose-dependent with 0.25 mg/mL being the lowest concentration (Figure 4B, E, H, and K). Further, these metal oxide nanoparticles (0.5 and 1.0 mg/mL) markedly induced the chromatin condensation in HFL1 cells (Figure 4C and D, F and G, I and J, L and M). As shown in Figure 5, the percentages of apoptotic cells significantly increased in metal oxide (ZnO, TiO_2, SiO_2, and Al_2O_3) nanoparticles treated HFL1 cultures. Compared with untreated cells, ZnO nanoparticles evidently induced the apoptosis in HFL1 cells, and TiO_2, SiO_2, and Al_2O_3 nanoparticles-exposed cells showed a cellular apoptosis in a descending order.

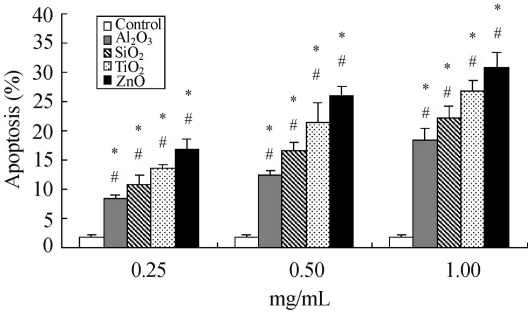

Figure 5. Induction of apoptosis of HFL1 cells by different types of nanoparticles. Cells were treated with ZnO, TiO_2, SiO_2, and Al_2O_3 nanoparticles at the concentrations of 0.25, 0.50, and 1.0 mg/mL in DMEM/F-12 cultured medium for 48 h, respectively. After 48 h exposure, apoptotic cells were detected by using Hoechst 33 258 staining and visualized with fluorescence microscopy. Results were expressed as the percentage of apoptotic cells (apoptotic cells/total cells ×100). The (*) mark indicates a statistically significant difference compared to the control at the same dose (P<0.05), while the (#) sign indicates a statistically significant difference compared to other metal oxide nanoparticles at the same dose (P<0.05).

DISCUSSION

As the increasing use of nanomaterials, it becomes more and more important to investigate their possible adverse effects on the environment and human health[22]. However, safety evaluation of nanomaterials on environment and human health are not enough[23]. To date, there are few studies directly investigating the toxicity of these nanoparticles and making a comparison of these toxic effects subsequently. Cellular effects of nanoparticles are strongly dependent on chemical and structural characteristics, surface/mass ratio, solubility, and shape. Moreover, the actual sizes of the particles in suspension were different from the original primary size. The purpose of this investigation was to evaluate and compare the potential toxicity of four types metal oxide nanoparticles (ZnO, TiO_2, SiO_2, and Al_2O_3) with a similar primary size that were largely used in industrial manufacture.

In vitro, ZnO, TiO_2, SiO_2, and Al_2O_3 nanoparticles were tested for their ability to induce toxicity in human fetal lung fibroblasts. The nanoparticle suspensions were prepared and subjected to the HFL1 cells at the concentrations of 0.25, 0.50, 0.75, 1.0, and 1.50 mg/mL. After 48 h treatment, MTT reduction was calculated for each case. The results indicated that all four types of nanomaterials induced a decrease in cell viability as compared to the cultured medium control. The MTT reduction was gradually decreased by increasing nanoparticles concentrations. These four nanomaterials induced cytotoxicity in a dose-dependent manner over the 48 h treatment period. We then performed the microscopic observation to evaluate the effects of different types of nanomaterials on cell morphology, and our data suggested that these nanomaterials could enter into cells and induce morphologic alternation in a dose-dependent manner. Meanwhile, nanoparticles induced apoptosis was clearly demonstrated in HFL1 cells with nuclear staining.

In agreement with our results, dose-dependent cytotoxicity in nanoparticles-exposed cells was also reported by several other research groups[24-25]. Oxidative DNA lesions were caused by TiO_2 nanoparticles in human bronchial epithelial cells and lymphoblastoid cells[26-27]. The cell viability was markedly reduced by treatment with the media containing different concentrations of TiO_2 nanoparticles[25]. Exposure to TiO_2 nanoparticles in mice caused acute toxicity and lead particles remained in the liver, spleen, kidneys, and lung tissues[28]. Furthermore, Hussain et al. reported that cytotoxic effects of TiO_2 occurred in rat liver cells at higher doses (0.10-0.25 mg/mL)[29]. ZnO nanoparticles also showed toxicity in mammalian cells, leading to the generation of reactive oxygen species (ROS), oxidant injury, excitation of inflammation, and cell death[30-34]. Ye et al. indicated that ROS-mediated oxidative stress and apoptosis were induced by exposure to 21 nm SiO_2 in L-02 cells[35]. Also, exposure to 15, 20, 46, and 50 nm SiO_2

nanoparticles caused cytotoxicity in a dose-dependent manner at the dosage levels between 10 and 100 μg/mL in cultured HEK293 cells and human bronchoalveolar carcinoma-derived cells that were associated with increased oxidative stress[36-37]. Simon-Deckersa et al. confirmed that aluminium oxide nanoparticles were able to rapidly enter into cells, and get distributed in the cytoplasm and intracellular vesicles[38]. Aluminium oxide nanoparticles induced cytotoxicity in a low but significant level, and slightly increased with the concentration in A549 cells. Also, Virgilio et al. reported exposure to titanium oxide and aluminium oxide nanoparticles caused dose-related cytotoxic effects by changes in lysosomal and mitochondrial dehydrogenase activity in CHO-K1 cells[24]. Both of the nanoparticles were found to have formed vesicles inside the cells.

Our data also demonstrates significant differences between different types of nanomaterials at the same concentration. The MTT reduction induced at various concentrations was the highest in ZnO nanoparticles, followed by TiO_2, SiO_2, and Al_2O_3 nanoparticles in a descending order. This result is similar to the report of Hu et al., who hold that ZnO is the most toxic nanoparticle with the lowest LD_{50} value[39]. Compared to the lower concentrations of nanomaterials, the higher concentrations of nanomaterials induced significantly higher percentages of MTT reduction, morphologic alternation, and cell apoptosis. The result of morphologic observation and cell apoptosis induction also indicates that ZnO nanoparticles cause maximal cytotoxicity in HFL1 cells, and Al_2O_3 nanoparticles seem to be less cytotoxic than the other nanoparticles at the same concentration.

Oxidative stress is defined as an abnormal level of reactive oxygen species (ROS) leading to the oxidative damage in a cell, tissue, or organ on a steady state level. This damage can affect a specific molecule and cause toxic effects through the production of peroxides and free radicals. Klaine et al. indicated that the induction of intracellular oxidative stress seemed to be a key event of the toxicity mechanisms of many nanomaterials[40]. Moreover, several research groups confirmed that ZnO, TiO_2, and SiO_2 nanoparticles could induce intracellular oxidative stress and cause adverse biological responses[20,25-26,34-37]. However, the mechanism of cytotoxicity caused by aluminum metal oxide nanoparticles is unclear, and need to be further explored.

In summary, our results show that the four types of metal oxide nanoparticles lead to cellular mitochondrial dysfunction, morphological modification and apoptosis at various concentrations (0.25-1.50 mg/mL) and the toxic effects are obviously shown in a dose-dependent manner. ZnO is a kind of the most toxic nanomaterials, followed by TiO_2, SiO_2, and Al_2O_3 nanoparticles in a descending order. These results highlight the differential cytotoxicity associated with exposure to different types of metal oxide nanoparticles, and suggest an extreme attention to safe use of these nanomaterials.

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