Research Article

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Comparative study of cytotoxicity, oxidative stress and genotoxicity induced by four typical nanomaterials: the role of particle size, shape and composition

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ABSTRACT: Although the biological effects of some nanomaterials have already been assessed, information on toxicity and possible mechanisms of various particle types are insufficient. Moreover, the role of particle properties in the toxic reaction remains to be fully understood. In this paper, we aimed to explore the interrelationship between particle size, shape, chemical composition and toxicological effects of four typical nanomaterials with comparable properties: carbon black (CB), single wall carbon nanotube, silicon dioxide (SiO_2) and zinc dioxide (ZnO) nanoparticles. We investigated the cytotoxicity, genotoxicity and oxidative effects of particles on primary mouse embryo fibroblast cells. As observed in the methyl thiazolyl tetrazolium (MTT) and water-soluble tetrazolium (WST) assays, ZnO induced much greater cytotoxicity than non-metal nanoparticles. This was significantly in accordance with intracellular oxidative stress levels measured by glutathione depletion, malondialdehyde production, superoxide dismutase inhibition as well as reactive oxygen species generation. The results indicated that oxidative stress may be a key route in inducing the cytotoxicity of nanoparticles. Compared with ZnO nanoparticles, carbon nanotubes were moderately cytotoxic but induced more DNA damage determined by the comet assay. CB and SiO_2 seemed to be less effective. The comparative analysis demonstrated that particle composition probably played a primary role in the cytotoxic effects of different nanoparticles. However, the potential genotoxicity might be mostly attributed to particle shape. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: nanomaterials; engineered nanoparticles; cytoxicity; oxidative stress; genotoxicity; reactive oxygen species

Introduction

In recent years, as nanotechnology and materials science have progressed by leaps and bounds, engineered nanomaterials have been mass produced and widely applied. Simultaneously, people are increasingly exposed to various kinds of manufactured nanoparticles. Owing to their unique nano-scale, nanoparticles are provided with many special physicochemical properties, and thereby may yield extraordinary hazards for human health (Donaldson *et al*., 2002; Kipen and Laskin, 2005; Holsapple *et al*., 2005; Nel *et al*., 2006; Borm *et al*., 2006). With increasing interest in its potential toxicity, the adverse effects of engineered nanoparticles are intensively investigated *in vivo* or *in vitro*. To date, animal studies have confirmed an increase in pulmonary inflammation, oxidative stress and distal organ involvement upon respiratory exposure to nanoparticles (Zhou *et al*., 2003; Lam *et al*., 2004; Warheit *et al*., 2004; Oberdorster *et al*., 2005b). *In vitro* studies have also supported the physiological response found in whole animal models and further provided data indicating an increased incidence of oxidative stress in cells exposed to various nanoparticles (Stone *et al*., 2007; Cui *et al*., 2005; Lin *et al*., 2006; Wang *et al*., 2007). However, most researchers have focused on the effects of one single type of particle or several particl types of the same substances, for example, carbon black (CB) nanoparticles and carbon nanotubes (CNTs) as carbonaceous nanomaterials. Rare studies have compared the toxicological effects of different types of nanomaterials, including carbonaceous, siliceous and metal oxide nanoparticles. Therefore, the interrelationship between nanoparticles properties (e.g. size, shape, chemical composition) and their biological effects remains unclear.

Generally, for one type of nanomaterial, the biological activity increases as the particle size decreases (Cassee *et al*., 2002; Oberdorster, 1996; Huang *et al*., 2004). Particle size may be a critical parameter for nanomaterial bioactivity, but it is difficult to ascertain which parameter plays an essential role in the biological effects when concerning various types of nanoparticles with different shapes and compositions. Because we are extremely lacking in epidemiological data on human exposure and health effects of nanomaterials at present, it is probably meaningful to elucidate this question for preventive sanitary control and health supervision during the creation and production of nanomaterials with special parameters. On the other hand, quantitative comparisons of various particle types from the literature may be confounded by differences between studies in the biological model tested, the experimental protocol and endpoints reported (Veranth *et al*., 2007). Therefore, a comparative study on the toxic effects of nanoparticles with varying properties seems to be necessary.

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Table 1. Characterization on particle parameters of four typical nanomaterials

Particles	Supplier	Size	Shape	Composition
CB	Nano-Innovation Co. Ltd, Shenzhen	12.3 ± 4.1 nm	Sphere	C > 99.4%
CNTs	COCC, Chinese Academy of Science, Chengdu	diameters: 8 nm Length: <5 μm	Rope-shaped	C > 99.99%
SiO_2	Runhe Co. Ltd, Shanghai	20.2 ± 6.4 nm	Crystal structure	SiO_2 > 99.0%
ZnO	Nanuo Co. Ltd, Shenzhen	19.6 ± 5.8 nm	Crystal structure	ZnO > 99.9%

In this study, we used a consistent set of *in vitro* experimental protocols to study four different typical nanomaterials that are characterized by particle size, shape and chemical composition: (i) carbon black; (ii) single wall carbon nanotubesl (iii) silicon dioxide (SiO_2); and (iv) zinc oxide (ZnO) nanoparticles. The objectives were to: (1) explore the relationship between the comparable properties with the viability response of primary mouse embryo fibroblasts (PMEF) treated *in vitro* with different manufactured nanoparticles; (2) identify whether particle properties impact cytotoxicity through altering intracellular oxidative conditions; and (3) compare the genotoxicity induced by different nanomaterials at low treatment concentrations. Accordingly, cytotoxicity was sufficiently measured by three forms of viability assays: the MTT assay, the WST assay and lactate dehydrogenase (LDH) assay. We also focused on the oxidative effect induced by nanoparticles; therefore, reactive oxygen species (ROS), the intracellular levels of glutathione (GSH), superoxide dismutase (SOD) activity and malondialdehyde (MDA) were respectively determined. The genotoxic effect was evaluated by DNA damage through comet assay.

Materials and Methods

Particle Preparation

Manufactured nanoparticles of CB, CNTs, SiO_2 and ZnO were purchased from commercial suppliers indicated in Table 1. The particles were sterilized by heating for 4 h at 180 °C in the oven, and then suspended in fetal bovine serum (FBS, Gibco), which was found to be the best dispersing vehicle in our pilot study and also used by other researchers (Lam *et al*., 2004; Leong *et al*., 1998). In order to break the agglomerate and ensure a uniform suspension, all particle samples were sonicated six times intermittently (30 s every 2 min) and characterized using TEM (JEM-100CX, Japan). The size and shape of nanoparticles were summarized in Table 1. The characterization in our study indicated that CB nanoparticles had a sphere shape with an average size of 12.3 nm [Fig. 1(A)]. CNTs were rope-shaped with lengths less than 5 μm and diameters of approximate 8 nm. SiO_2 and ZnO nanoparticles exhibited a crystal structure with an average size of 20.2 and 19.6 nm, respectively [Fig. 1(C, D)]. The chemical

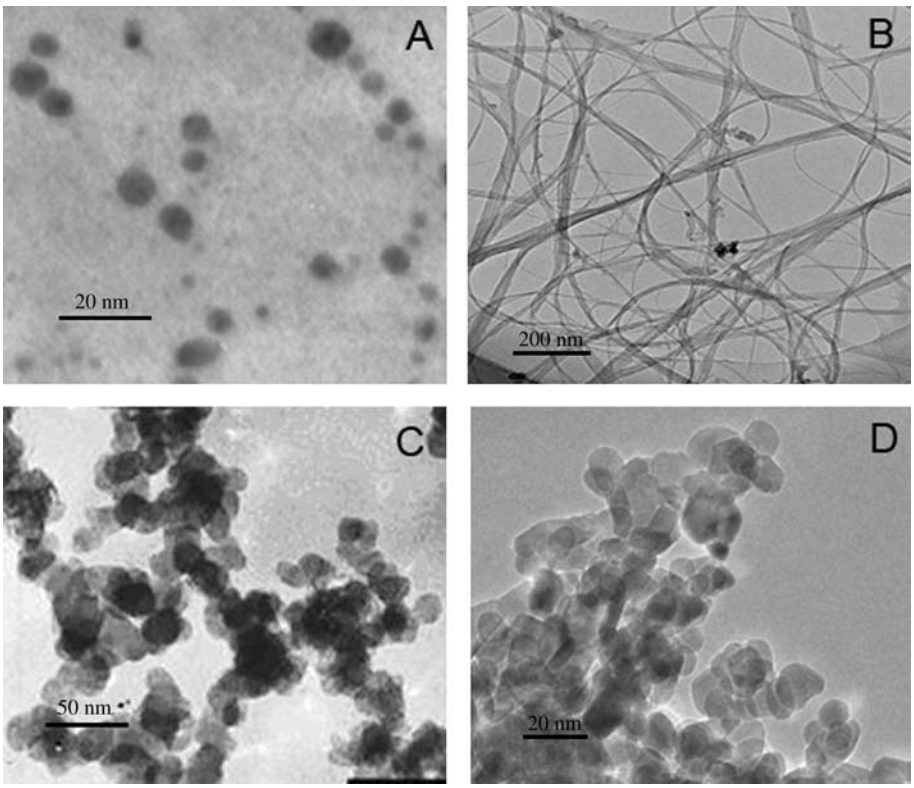

Figure 1. TEM images of engineered nanoparticles (A) CB; (B) CNTs; (C) SiO_2; and (D) ZnO.

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composition was quantitatively analysed by Raman spectroscopic technique and the results show that the purity of four nanomaterials are all more than 99.0%.

Cell Culture and Exposure to Nanoparticles

Primary mouse embryo fibroblasts were freshly derived and used for each experiment. Compared with cell lines, primary cells have many particular strongpoints in toxicological studies and are recommended in the toxicological assessment of nanomaterials *in vitro* (Oberdorster *et al*., 2005a). On the other hand, mouse embryo fibroblast cells (BALB/3T3), serving as differentiated cells, are commonly used in the embryonic stem cell test (EST), which is an alternative *in vitro* method for embryotoxicity testing (Bremer and Hartung, 2004; Andrea *et al*., 2004; Spielmann, 2005). BALB/c mice of about 6 weeks old were provided by Laboratory Animal Centre (Academy of Military Medical Science, Beijing, China). The animals were housed by sex in plastic cages in a 20 ± 2 °C, 50–70% relative humidity room with a 12 h light/dark cycle. After 14 days acclimation, females were caged with males nightly in a 2:1 ratio. Mating was confirmed by observing vaginal plugs in the morning and defined as day 0.5 of gestation. On day 13.5 of gestation, pregnant mice were sacrificed humanly and the embryos were collected sterilely. PMEF cells were prepared as described previously (Hertzog *et al*., 2001). The cells were routinely cultured in Dulbecco's modified Eagle's low glucose medium (DMEM/low, Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS), plus 2 mM L-glutamine, 1% (v/v) penicillin–streptomycin (10 U ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin) and grown at 37 °C in a 5% CO_2 humidified environment. In order to obtain fibroblast cells with high consistency and homogeneity, the impure cells were removed from freshly derived cells mixture by speed-discriminated adherence. When the remaining cells had reached 70% confluence, they were trypsinized (0.25% Trypsin– 0.04% EDTA, Sigma) and passaged (1:3). Cells within three passages were used for experiments.

Particle suspensions were freshly prepared before the cells were exposed, and diluted to appropriate concentrations (5, 10, 20, 50 and 100 μg ml^{-1}) with the culture medium, then immediately applied to the cells. Cells not treated with particles served as controls in each experiment.

Cell Viability Assays

Effects of nanoparticles on the viability of PMEF cells were evaluated using two methods: the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay and the WST [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] assays. The MTT assay was used according to the method of Mosmann *et al*. (1983). PMEF cells were plated into 96-well plates at a density of 2.0×10^4 cells per well in 200 μl culture medium and allowed to attach for 12 h before treatment. Afterward, culture medium in the plates was replaced by 200 μl particle suspension at concentrations of 0–100 μg ml^{-1} and the cells were exposed for 24 h. Then 20 μl MTT (0.5 mg ml^{-1}) was added to each well and incubated at 37 °C for 2 h. Mitochondrial dehydrogenases of viable cells reduce the yellowish water-soluble MTT to water-insoluble formazan crystals, which were solublized with dimethyl sulfoxide (DMSO). The cell culture medium was aspirated cautiously, after which 150 μl DMSO was added to each well and mixed thoroughly. Optical density (OD) was read on an ELISA reader (Wellscans MK3, Thermo Labsystems, Finland) at 570 nm, with 630 nm as a reference wavelength. The results were expressed as percentage viability compared with the untreated controls.

In the WST assay, the reduced tetrazolium salt is water-soluble. Therefore, no DMSO extraction is necessary. After cell exposure, the WST-1 (Beyotime, China) solution was added to each well, incubated for 2 h, and then the coloured supernatants were measured at 450 nm. The results obtained from both assays were given as relative values to the untreated control in percent. All experiments were performed in triplicate.

LDH Measurement

LDH leakage, which is another measure of cytotoxicity on the basis of membrane integrity damage, was determined using a commercial LDH Kit (Jiancheng Bioengineering Co. Ltd, Nanjing, China) according to the manufacturer's protocols. This procedure is based on the method developed by Ulmer *et al*. (1956), optimized for greater sensitivity and linearity. 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. After incubation with nanoparticles for 24 h, the cell culture medium was collected for LDH measurement. An aliquot of 100 μl cell medium was used for LDH activity analysis and the absorption was measured using a UV–visible spectrophotometer (TianMei UV-8500, Shanghai, China) at 340 nm.

Intracellular ROS Measurement

The intracellular reactive oxygen species (ROS) was determined using 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Wan *et al*., 1993). DCFH-DA passively enters the cell where it reacts with ROS to form the highly fluorescent compound dichlorofluorescein (DCF). Briefly, 10 mM DCFH-DA stock solution (in methanol) was diluted 1000-fold in cell culture medium without serum or other additive to yield a 10 μM working solution. After 24 h of exposure to nanoparticles, the cells in the six-well plates were washed twice with PBS and incubated in 2 ml working solution of DCFH-DA at 37 °C for 20 min. Then the cells were washed three times with cell culture medium without serum to eliminate DCFH-DA that did not enter the cells. Cells were collected in suspension, the fluorescence was then determined at 488 nm excitation and 525 nm emission using a fluorospectrophotometer (RF-5301, Shimadzu, Japan).

Oxidative Damage

We employed intracellular GSH, SOD and MDA measurement to indicate the oxidative damage caused by nanoparticles. PMEF cells were plated into six-well plates at a density of 5.0×10^5 cells per well in 2 ml culture medium and allowed to attach for 12 h before exposure. Cells were treated in triplicate with the particle suspensions at concentrations of 5, 10, 20, 50 and 100 μg/ml for 24 h. Then, the cells were rinsed with ice-cold PBS, trypsinized and immediately disrupted by a repeated frozen–thaw process (three times). The cell lysates were centrifugated and frozen at −20 °C for subsequent determination. GSH, MDA and SOD were respectively measured using the reagent kits purchased from Jiancheng Bioengineering Co. Ltd, Nanjing, China, according to the manufacturer's instructions.

Comet Assay

After exposure to nanoparticles at 5 and 10 μg ml^{-1} for 24 h, cells in six-well plates were washed twice with pre-chilled PBS, centrifuged at 78*g* for 5 min and resuspended in PBS. As assessed by Trypan blue dye-exclusion staining, cell viability was over 95% under the tested doses in this study. The alkaline comet assay for assessment of DNA damage was performed according to the method described by Singh *et al.* (1991) with some modifications. Electrophoresis was conducted in the refrigerator at 4 °C for 30 min at 23 V and 300 mA. After electrophoresis, the slides were rinsed with deionized water and immersed in 70% ethanol for 40 min, then drained and 50 μl of propidium iodide, PI (5 μg ml^{-1}) was added. All steps after lysis were carried out under yellow light in the cold room to prevent any induction of additional DNA damage. Slides were scored at 200 magnification using a fluorescence microscope (Olympus BX-50, UK) with an excitation filter of 515–560 nm and barrier filter of 590 nm and photographed with a high-resolution CCD camera (CoolSNAP, Olympus). At least 50 randomly selected images were analyzed from each sample with the CASP software package (Konca *et al.*, 2003). In our study, DNA damage was evaluated by comet length, Olive tail moment and the percentage of DNA in the tail (%Tail DNA), which are considered the most informative and reliable measurements (Olive and Durand, 2005; Kumaravel and Jha, 2006).

Statistical Analysis

The experiments were replicated three independent times and the data are presented as mean ± SEM (standard error of mean). Statistical analysis of the data was carried out using ANOVA, followed by Tukey's HSD *post hoc* test (equal variances) or Dunnett's T3 *post hoc* test (unequal variances). Otherwise, the nonparametric Kruskal–Wallis test was used. In the study of DNA damage by the comet assay, Student's *t*-test for independent samples was also used. These tests were performed using SPSS software, version 11.5. Differences were considered statistically significant when the *P*-value was less than 0.05.

Results

The Dose-dependent Cytotoxicity of Nanoparticles

After 24 h exposure at varying doses of CB, SiO_2 and ZnO nanoparticles, PMEF cell viabilities detected by the MTT assay resulted in explicit dose-dependent reduction (Fig. 2). When cells were treated at lower doses, i.e. 5 and 10 μg ml^{-1}, cytotoxicity of CB nanoparticles was statistically greater ($P < 0.05$) than SiO_2 and ZnO, but parallel with CNTs. After exposure dose increased to 20 μg ml^{-1}, ZnO induced a much greater decrease ($P < 0.01$) of cellular activity than CB and SiO_2. The cell viabilities of CB, SiO_2 and ZnO groups at 20 μg ml^{-1} were respectively inhibited by 41.5, 27.6 and 73.5%, compared with the control group. However, at higher dosage levels, i.e. 50 and 100 μg ml^{-1}, cell viabilities were significantly elevated in CNT groups, which showed a reverse effect to the dose-dependent cytotoxicity revealed at lower dosage levels. The unusual outcome prompted us to analyse the cytotoxicity of nanoparticles with another method: the WST assay.

Unfortunately, the WST assay failed to agree with the MTT assay on the effect of CNTs (Fig. 3). In contrast with MTT assay, the reduction of cell viability induced by CNTs was dose-dependent, for example, the greatest viability loss (25.9%) was found at 100 μg ml^{-1}. According to previous research, the reason for the reversed results possibly was that CNTs attached to the insoluble MTT product formazan and thereby disturbed the test at higher particle concentrations (Worle-Knirsch *et al.*, 2006). Therefore, the MTT assay may be not suitable for inspecting the cytotoxicity of some categories of engineered nanoparticles. In this study, the result based on the MTT assay was similarly suspectable,

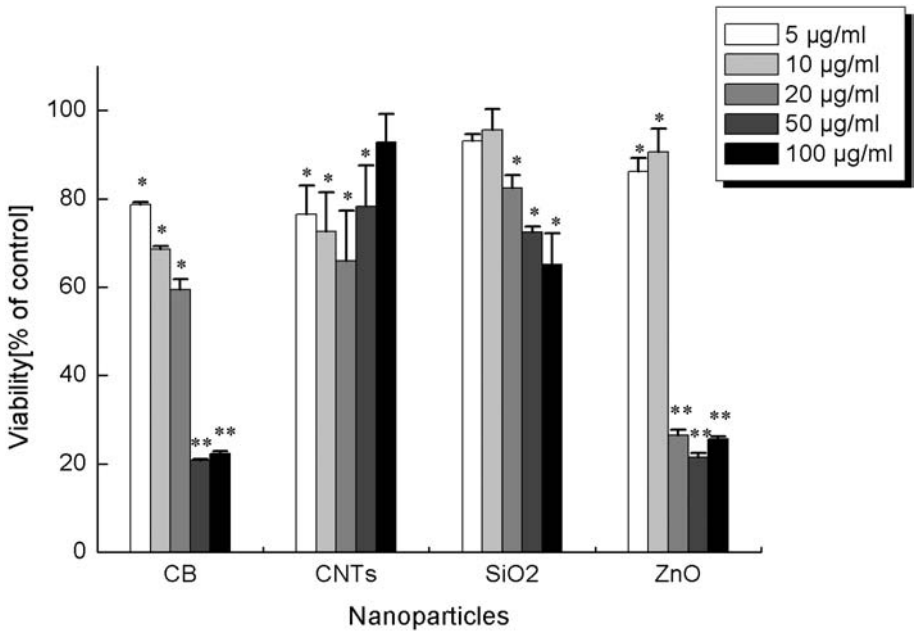

Figure 2. Viability of PMEF cells exposed to nanoparticles with different exposure concentrations determined by the MTT assay. Cells were respectively treated with 5, 10, 20, 50 and 100 μg ml^{-1} of CB, CNT, SiO_2 and ZnO for 24 h. The viability was measured with the MTT assay and results are given in percent related to untreated to controls. Results are the mean ± SEM (vertical bars) of three independent experiments each carried out in triplicate. *$P < 0.05$; **$P < 0.01$ in comparison to untreated controls.

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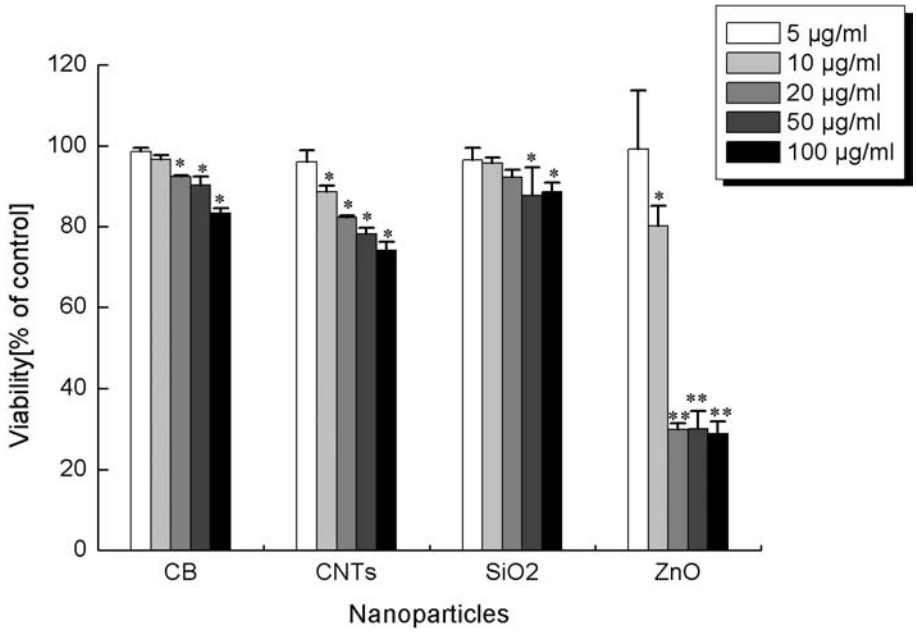

Figure 3. Viability of PMEF cells exposed to nanoparticles with different exposure concentrations determined by the WST assay. Cells were respectively treated with 5, 10, 20, 50 and 100 μg ml^{-1} of CB, CNT, SiO_2 and ZnO for 24 h. The viability was measured with the WST assay and results are given in percent related to untreated to controls. Results are the mean ± SEM (vertical bars) of three independent experiments each carried out in triplicate. *$P < 0.05$; **$P < 0.01$ in comparison to untreated controls.

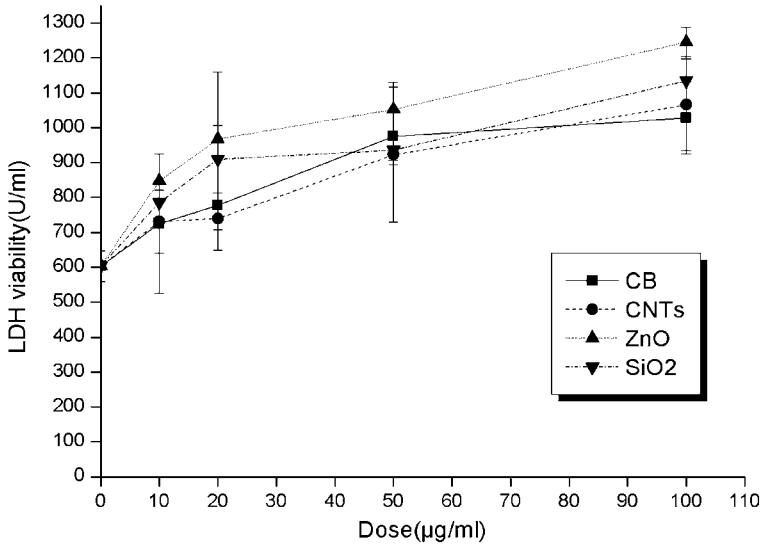

Figure 4. LDH leakage from PMEF after 24 h treatment with nanoparticles. Cells were respectively treated with 10, 20, 50 and 100 μg ml^{-1} of CB, CNT, SiO_2 and ZnO for 24 h. The LDH viability in supermarket was measured by the LDH assay and results are given in activity unit per milliliter. Results are the mean ± SEM (vertical bars) of three independent experiments each carried out in triplicate.

so we adopted the result obtained from the WST assay as the indication of cytotoxicity of nanoparticles. Compared with CNTs, CB and SiO_2 induced less inhibition of viabilities, according to the WST assay. ZnO produced more significant cytotoxicity ($P < 0.01$) than the other three types of particles, especially at higher dosage levels.

LDH Leakage

All four types of nanomaterials induced apparent LDH leakage from PMEF cells treated for 24 h, which revealed the impact of nanoparticles on cell membrane integrity (Fig. 4). Compared with the controls, LDH levels in cell medium were gradually elevated as particle concentrations increased. Following exposure to CB, CNTs, SiO_2 and ZnO at the highest dosage levels, LDH releases were increased by 70.4, 88.0, 76.6 and 106.4%, respectively, significantly higher than the untreated control ($P < 0.01$). However, the effect was not as significant as that on cellular viability inhibition. In addition, it was noted that no statistically significant difference was found when comparing the effects among different types of nanoparticles at the same dosage level. This was not in accordance with the results obtained from the WST assays which showed obvious diversity in cytotoxicity of four nanomaterials. Therefore, it probably suggested that the acute cytotoxicity

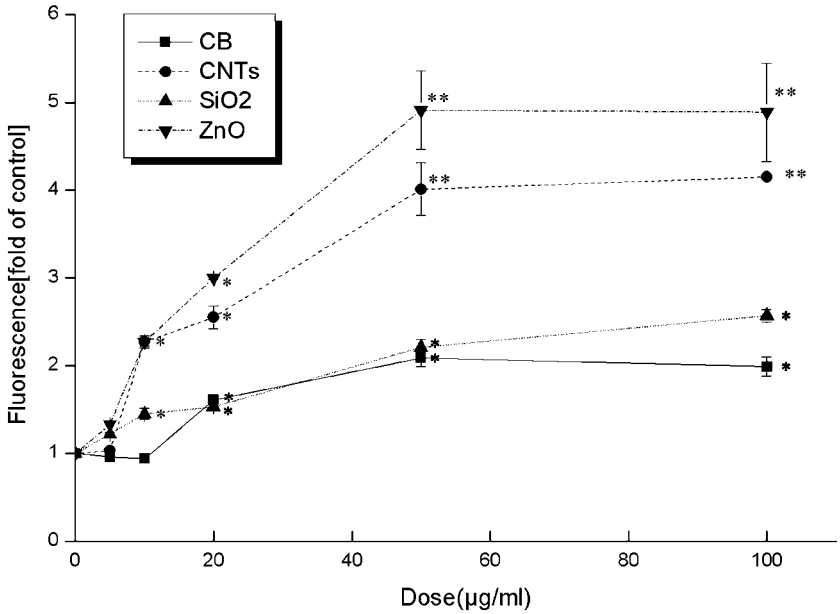

Figure 5. Effect of nanoparticles on ROS generation in PMEF cells. Cells were treated with 5, 10, 20, 50 and 100 μg ml^{-1} of CB, CNT, SiO_2 and ZnO for 24 h. ROS was detected by fluorescence measurement of the reported DCF and results are given in fold of untreated controls. Results are the mean ± SEM (vertical bars) of three independent experiments each carried out in triplicate. *$P < 0.05$; **$P < 0.01$ in comparison to untreated controls.

primarily originated from the cellular internalization of nanoparticles rather than physical damage on the cellular membrane. Moreover, it has been proved by previous studies that nanoparticles can cross the cell membrane and enter the cytoplasm through several different routes (Geiser *et al*., 2005; Bianco *et al*., 2005; Kam *et al*., 2006).

Reactive Oxygen Species Generation

The ability of nanoparticles to induce intracellular oxidant production in PMEF cells was assessed using DCF fluorescence as a reporter of ROS generation. DCF fluorescence intensity statistically increased after 24 h exposure to all examined nanoparticles within the dose range of 10–50 μg ml^{-1}, but no significant increase in ROS was observed at 100 μg ml^{-1} (Fig. 5). The disappearance of the increase in ROS concentration at 100 μg ml^{-1} may be due to the leakage of fluorescent product from the cell, since significant membrane damage (LDH leakage) was apparent at the highest dosage level (Hussain *et al*., 2005). The effects of the four types of nanoparticles were obviously different: ZnO appeared more effective, CNTs was moderate while SiO_2 and CB induced relatively less ROS generation. For example, 50 μg ml^{-1} of CB, CNTs, SiO_2 and ZnO respectively elevated the ROS levels 2-, 4-, 2- and 5-fold, compared with the controls.

Intracellular Oxidative Stress Levels

Intracellular GSH levels in PMEF cells exhibited a dose-dependent decrease after 24 h exposure to nanoparticles [Fig. 6(A)]. In particular, ZnO induced the most significant reduction of GSH in the four types of nanoparticles ($P < 0.01$). After exposure to CB, CNTs, SiO_2 and ZnO nanoparticles at 100 μg ml^{-1}, the GSH levels were reduced by 61, 50, 36 and 91%, respectively, compared with the control groups.

As another indication of intracellular oxidative stress, the activity of SOD in treated cells was determined by the xanthine oxidase method. Compared with the GSH measurement, parallel results were observed in this experiment [Fig. 6(B)]. For example, the greatest decrease of SOD activity also occurred in ZnO exposed cells. Additionally, there was a significant linear correlation between GSH levels and SOD activities for each particle group ($r^2 > 0.8$, $P < 0.05$).

In order to elucidate the lipid peroxidation induced by nanoparticles, the MDA concentration was measured. Each type of nanoparticle elevated the intracellular MDA concentration in a dose-dependent manner [Fig. 6(C)]. ZnO treatment at 50 and 100 μg ml^{-1} resulted in the greatest MDA generation, approximately twice the CB, CNT and SiO_2 group levels or 5-fold the control level. We also found that MDA levels were highly correlated with the SOD activities in all the four particle groups ($r^2 > 0.8$, $P < 0.01$).

Overall, four types of nanoparticles resulted in dose-dependent GSH depletion, SOD activities reduction and MDA generation, which reflected the oxidative stress in the PMEF cells. However, the comparative analysis of these oxidative effects demonstrated that there are significant differences between the four nanomaterials. Concretely, ZnO nanoparticles induced the most significant oxidative stress while SiO_2 induced the least. In addition, CB nanoparticles appeared slightly more effective than CNTs.

DNA Damage

Comet assay, also called single cell gel electrophoresis (SCGE), determines a combination of single-strand breaks, double-strand breaks and alkaline labile sites (Olive and Durand, 1992). In this assay, there were significant increases in tail length, percentage of DNA in tail, tail moment and Olive tail moment after PMEF cells were treated with four types of nanoparticles at both examined concentrations (Fig. 7). CNTs and ZnO caused more DNA damage than CB and SiO_2 nanoparticles; however, there was no significant difference between them at 5 μg ml^{-1} dosage level. As for 10 μg

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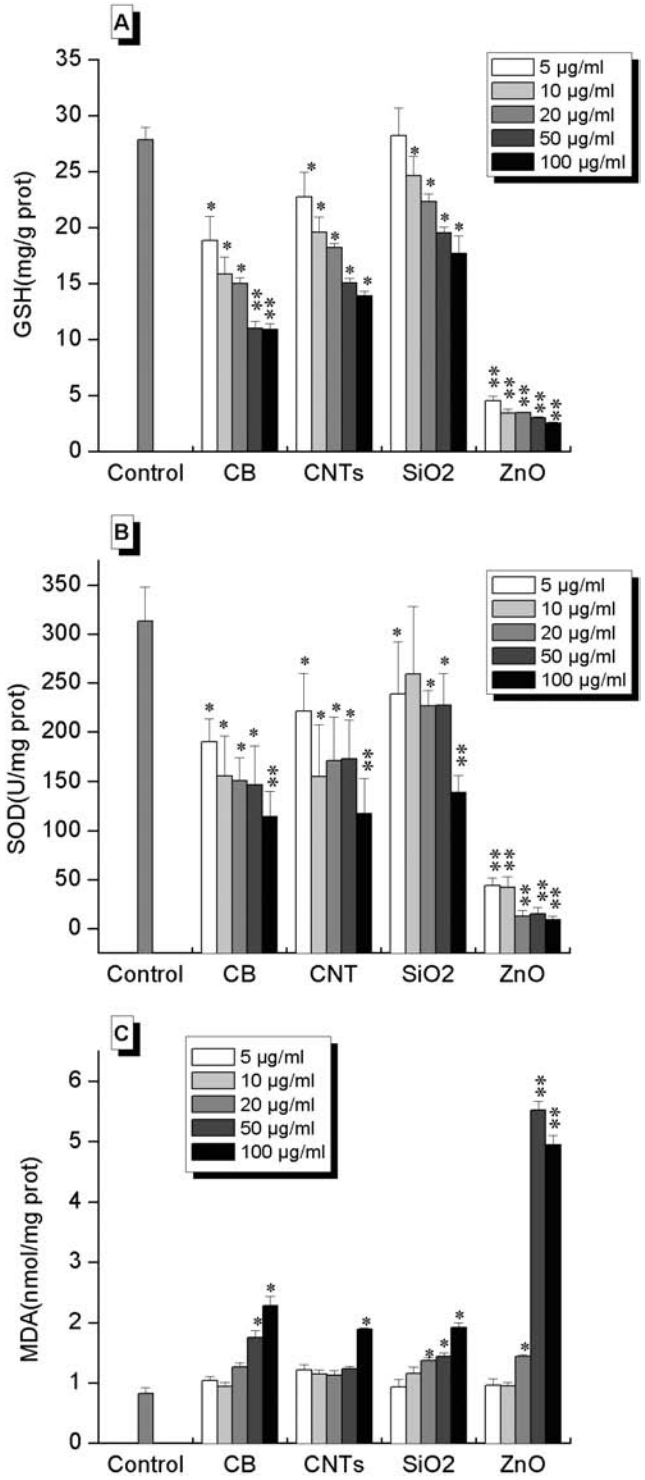

Figure 6. Intracellular oxidative stress levels in the PMEF cells exposed to nanoparticles for 24 h. Cells were treated with 5, 10, 20, 50 and 100 μg ml^{-1} of CB, CNT, SiO_2 and ZnO for 24 h. (A) GSH levels; (B) SOD activities; (C) MDA concentrations. Results are the mean ± SEM (vertical bars) of three independent experiments carried out in triplicate. *$P < 0.05$; **$P < 0.01$ in comparison to untreated controls.

ml^{-1}, their genotoxicity can be classified from most to least toxic as follows: CNTs > ZnO > CB > SiO_2. Take tail DNA% as example, 38.2, 18.8, 12.8 and 6.8%, respectively, for the group of CNTs, ZnO, CB and SiO_2, vs 3.26% for the untreated control.

Discussion

Cytotoxicity and the underlying mechanism

Oxidative stress as a common mechanism for cell damage induced by nano- and ultrafine particles is well documented. A wide range of nanomaterial species have been shown to create ROS both *in vivo* and *in vitro* (Donaldson *et al*., 2001; Zhou *et al*., 2003; Oberdorster, 2004; Nel *et al*., 2006). Similarly, in our research, four types of nanoparticles induced significant GSH depletion and ROS generation in a dose-dependent manner. The accumulation of ROS, e.g. superoxide radicals ($O_2^{\cdot -}$) and hydroxyl free radicals ($^{\cdot}OH$) depleted cellular GSH and resisted the defensive effects of cellular antioxidant enzymes, e.g. SOD. Consequently, redundant free radicals would interact with biomolecules including proteins, enzymes, membrane lipids and even DNA which could be oxidized, modified, destructured and ultimately dysfunctioned. As a marker of lipid peroxidation elicited by ROS, dose-dependent increased MDA production was also observed in our study. In addition, the inverse correlation between cell viabilities decline vs ROS level elevation further proved that oxidative stress was probably a key route by which nanoparticles induce cytotoxicity.

A key finding of our research was that ZnO nanoparticles, as the only particle type of metal oxide tested in our study, induced significantly more viability reduction and oxidative damage than the other three types of nanomaterials. Since SiO_2 and ZnO had similar crystal shape and particle size, the toxicity diversity between them may be attributed to their chemical compositions. Additionally, although having the smallest particle size, CB exhibited much lower cytotoxic and oxidative effects than ZnO nanoparticles. Therefore, it seems like the impact of metal content in nanomaterials may be more significant than particle size, which was regarded as the essential parameter in the toxicological response caused by nanoparticles. On the whole, it is reasonable to believe that chemical composition possibly played a primary role in the toxicological effects of different nanomaterials.

The Interrelationship between Particle Composition and Oxidative Effect

Then, how did particle composition play the primary role in the underlying oxidative mechanism through which cytotoxicity was induced by nanoparticles? An investigation demonstrated that cobalt chrome alloy nanoparticles appeared to be rapidly dissolved or corroded and might consequently spread more evenly around the cell cytoplasm with a high concentration of metal within 24 h after exposure (Papageorgiou *et al*., 2007). The increased solubility of metal nanoparticles might be expected *a priori* from their hugely increased surface area compared with the usual particles at equivalent mass. Metal ions such as Fe^{2+}, Cu^{+}, Mn^{2+}, Cr^{5+} and Ni^{2+} released from nanoparticles may contribute a lot to yield free radicals via the Fenton-type reaction. Transition metal composition of ultrafine particles in ambient air pollutant has been shown to induce oxidant radical formation via the Fenton-type reaction and to elicit intracellular oxidative stress (Prahalad *et al*., 1999). According to this model, single-component materials as well the presence of transition metals on the surface can participate in the formation of such active sites. For instance, ultrafine particles contain transition metals (e.g. Fe and vanadium) and are also coated with redox-cycling organic chemicals (e.g. quinones), whereas carbon nanotubes contain metal impurities that can amplify chemical changes in

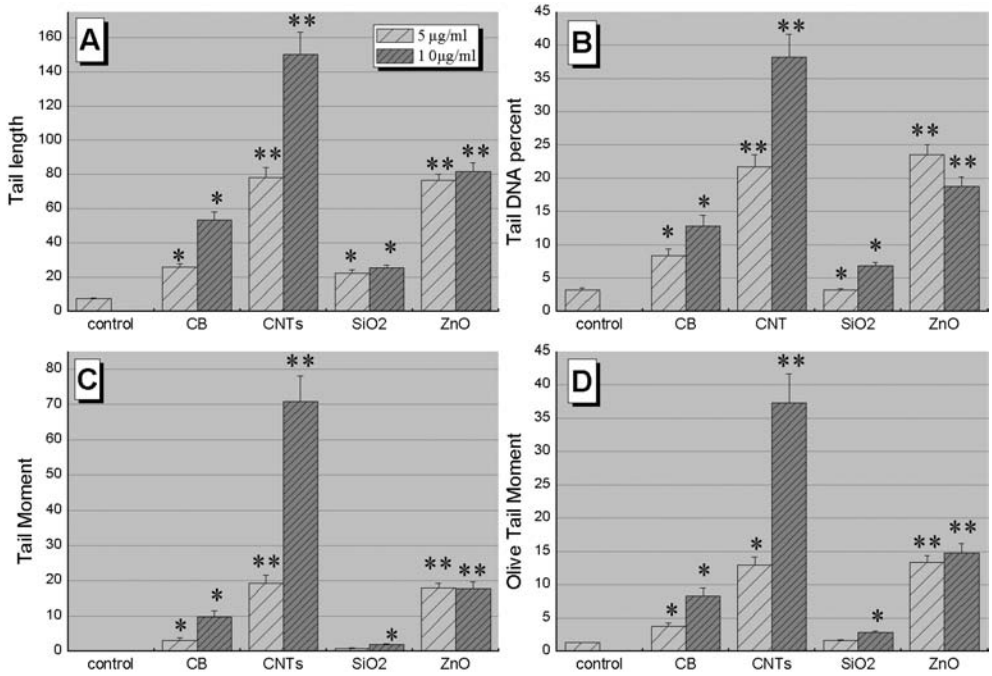

Figure 7. DNA damage in PMEF cells exposed to nanoparticles determined by comet assay. Cells were respectively treated with 5 μg ml^{-1} of CB, CNT, SiO_2 and ZnO for 24 h. DNA damage was evaluated by (A) tail length, (B) tail DNA percent, (C) tail moment, (D) Olive tail moment. Values shown are mean from 50 randomly selected comet images of each sample. $^*P < 0.05$; $^{**}P < 0.01$ in comparison to untreated controls.

the nanomaterials environment. Several studies have demonstrated that the cytotoxicity of CNTs may stem from its metal content. In an animal study, CNTs containing 26% nickel and 5% yttrium killed about 50% of the animals within 7 days after treatment. The deaths were attributed to nickel that was released during ultrasonication (Lam *et al*., 2004). Other studies also implied that the oxidative changes induced by raw CNTs in cell cultures were dependent on metal contaminants (Shvedova *et al*., 2003; Pulskamp *et al*., 2007).

In contrast, there were studies suggesting that SiO_2 nanoparticles and pure quartz dusts can generate HO in the absence of trace metal (Fenoglio *et al*., 2006; Lin *et al*., 2006). Similarly, as indicated by our study, pure CNTs also induced obvious ROS formation, GSH depletion, SOD inhibition and MDA generation in PMEF cells. Therefore, the metal contaminants cannot account for the oxidative effects exerted by various nanomaterials. On the other hand, zinc was not usually recognized as a transition metal because of its invariable quantivalency (+2) in biosystem, and it could not catalyse the Fenton reaction to generate ·OH in the form of zinc ion. Accordingly, the most significant toxicological response induced by ZnO nanoparticles in our investigation cannot be ascribed to particle dissolution and zinc ions release. In addition, a recent study demonstrating that the possible release of reactive metal species from several metal oxide nanoparticles including ZnO does not contribute significantly to the toxicological response in HAECs (Gojova *et al*., 2007) further corroborates the notion that the impact of free metal ions released from ZnO nanoparticles on oxidative stress is minimal.

The toxicological effects of different nanoparticles may be attributed to their surface properties that originate from the specific 'nano' size but are ultimately determined by chemical compositions. Shrinkage in particle size may create discontinuous crystal planes that increase the number of structural defects as well as disrupting the well-structured electronic configuration of the material, so as to give rise to altered electronic properties on the particle surface (Oberdorster *et al*., 2005b; Donaldson *et al*., 2002). This could establish specific 'surface groups' that could function as reactive sites. 'Surface groups' can make nanoparticles hydrophilic or hydrophobic, lipophilic or lipophobic, or catalytically active or passive. The extent of these changes and their importance strongly depends on the chemical composition of the material (Nel *et al*., 2006). In our research, different surface properties between SiO_2 and ZnO nanoparticles which have identical particle size and shape could be responsible for the disparity in their toxicological effects. An explanation of how their surface properties can lead to diverse cytotoxicity is the interaction of electron donor or acceptor active sites with molecular dioxygen (O_2). For instance, since ZnO was more chemically active in nature than SiO_2, electron capture on the surface of ZnO nanoparticles can lead to more formation of the superoxide radical ($O_2^{\cdot-}$) which culminates in ROS accumulation and oxidative stress.

Potential Genotoxicity and Particle Shape

Besides chemical composition, other particle properties such as size and shape may also affect the addressed specific physicochemical and transport properties, with the possibility of negating or amplifying the surface effects. In our study, two reasons might account for the DNA damage caused by nanoparticles. Firstly, ROS generation and oxidative stress in the cell may cause oxidative damage to DNA through free radical attack. Previous work demonstrated that sunscreen TiO_2 and ZnO can catalyze oxidative DNA damage in cultured human fibroblasts measured by comet assay (Dunford *et al*., 1997). Moreover, this has been proved by the determination of 8-hydroxy-deoxyguanosine (8OHdG), a good marker of oxidative DNA lesion (Schins *et al*., 2002; Shi *et al*., 2004; Papageorgiou *et al*., 2007). However, in this study,

CNTs exhibited greater genotoxicity than ZnO nanoparticles which elicited more oxidative stress. Therefore, it is educible that the DNA damage caused by CNTs may come from mechanical injury and not oxidative effect. It is likely that CNTs might penetrate into cell nucleus through nucleopores, and then destruct the DNA double helix (Pantarotto *et al*., 2004). Secondly, although several studies have shown that some spherical nanoparticles such as titanium dioxide or silica nanoparticles can also enter the nucleus (Geiser *et al*., 2005; Stearns *et al*., 2001; Chen and von Mikecz, 2005) and it has been demonstrated that C_{60} nanoparticles can bind to and deform nucleotides (Zhao *et al*., 2005), CNTs induced significantly more DNA damage than other nanoparticles with the sphere shape or crystal structure in our research. To combine the above two points, the genotoxicity of different nanoparticles may primarily be due to particle shape rather than chemical composition. However, since the available techniques are really scarce at present, it is rather difficult to inspect intracellular translocation of nanoparticles. Unfortunately, we cannot directly confirm the actual process from our data.

Practical Importance and Limitations

In contrast to the growing literature on application of nanomaterials, the information about biological effects of nanoparticles is insufficient and the publications available on this topic are often controversial. We focused on CB, SiO_2, CNTs and ZnO nanoparticles as examples of typical manufactured nanomaterials that are associated with environmental and occupational exposures. These nanoparticles are produced on an industrial scale serving as raw materials of printer toners, semiconductors, catalyst and cosmetics. Recently, some specific metal nanomaterials have even been directly applied in human body as varnishes, contrast agents, drug carriers as well as surgical implants. Previous studies have demonstrated that exposure to some types of nanoparticles induces toxicological effects in different cell lines and key organs in general. However, on account of lacking standard strategies and methods for toxicological evaluation on nanomaterials, it is rather difficult for us to decide which kind of nanoparticle may be the greater health hazard. Additionally, comparative studies which could provide useful references on this question are very sparse. In the present study, we examined the effect of a wide range of nanoparticle concentrations on PMEF cells. It is reasonable to suggest that, according to our results, more attention should be paid to the bio-safety evaluation on the reactive metal oxide nanomaterials. In addition, the potential genotoxicity of CNTs at lower exposure concentrations should also be given sufficient caution. On the other hand, the PMEF cells utilized in our study were freshly derived from mouse embryos and they may be genetically identical with normal cells *in vivo* while more sensitive to extraneous stimulating factors than cell lines. Though these qualities can help us achieve more practical results, the conclusions should be further tested *in vivo*. Finally, we have to point out that this investigation, similar to most previous researches, did not elucidate the particle state during reaction with cells, e.g. agglomeration, distribution and metabolism, because of the difficulties in relevant techniques.

Conclusions

Notwithstanding its limitation, this study can clearly indicate that engineered nanoparticles of CB, CNTs, SiO_2 and ZnO induced statistically significant cytotoxicity through oxidative stress mechanism, but the effects of various nanomaterials were different; ZnO nanoparticles induced more cytotoxicity and oxidative impairment, whereas CNTs caused more DNA damage. In conclusion, our results suggested that particle surface properties determined by chemical composition possibly played a critical role in the ROS generation which is currently the best-developed paradigm for nanoparticle toxicity. However, the genotoxicity of nanoparticles at lower exposure doses may be primarily due to particle shape.

This study, using freshly derived primary cells, adds to the growing literature on the biological effects of nanomaterials. Although interaction of nanoparticles with fibroblasts *in vivo* was not tested, we can infer from this *in vitro* study that manufactured nanoparticles with reactive metal oxide composition are more effective than carbonaceous and silica nanoparticles. This differential toxicity according to particle composition could be an important concept to take note of in the manufacture of future nanomaterials, especially for biomedical applications in humans. Finally, it will be important to establish whether the present results obtained from PMEF cells in culture also apply to the *in vivo* environment. Future investigations may provide more understanding of the relationship between surface properties and cellular uptake, translocation, metabolism, oxidative effects and other biological effects of different nanoparticles *in vivo* and *in vitro*.

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