Arch Toxicol (2011) 85:695–704
DOI 10.1007/s00204-010-0608-7

SHORT COMMUNICATION

Cytotoxicity of zinc oxide (ZnO) nanoparticles is influenced by cell density and culture format

Boon Chin Heng · Xinxin Zhao · Sijing Xiong · Kee Woei Ng · Freddy Yin-Chiang Boey · Joachim Say-Chye Loo

Received: 23 June 2010 / Accepted: 20 September 2010 / Published online: 12 October 2010

Abstract A parameter that has often been overlooked in cytotoxicity assays is the density and confluency of mammalian cell monolayers utilized for toxicology screening. Hence, this study investigated how different cell seeding densities influenced their response to cytotoxic challenge with ZnO nanoparticles. Utilizing the same volume (1 ml per well) and concentration range (5–40 μg/ml) of ZnO nanoparticles, contradictory results were observed with higher-density cell monolayers (BEAS-2B cells) obtained either by increasing the number of seeded cells per well (50,000 vs. 200,000 cells per well of 12-well plate) or by seeding the same numbers of cells (50,000) within a smaller surface area (12-well vs. 48-well plate, 4.8 vs. 1.2 cm^2, respectively). Further experiments demonstrated that the data may be skewed by inconsistency in the mass/number of nanoparticles per unit area of culture surface, as well as by inconsistent nanoparticle to cell ratio. To keep these parameters constant, the same number of cells (50,000 per well) were seeded on 12-well plates, but with the cells being seeded at the edge of the well for the experimental group (by tilting the plate) to form a dense confluent monolayer, as opposed to a sparse monolayer for the control group seeded in the conventional manner. Utilizing such an experimental set-up for the comparative evaluation of four different cell lines (BEAS-2B, L-929, CRL-2922 and C2C12), it was observed that the high cell density monolayer was consistently more resistant to the cytotoxic effects of ZnO nanoparticles compared to the sparse monolayer for all four different cell types, with the greatest differences being observed above a ZnO concentration of 10 μg/ml. Hence, the results of this study demonstrate the need for the standardization of cell culture protocols utilized for toxicology screening of nanoparticles, with respect to cell density and mass/number of nanoparticles per unit area of culture surface.

Keywords Cell density · Confluent · Nanoparticle · Toxicology · Viability · Zinc Oxide

B. C. Heng (✉) · X. Zhao · S. Xiong · K. W. Ng · F. Y.-C. Boey · J. S.-C. Loo
Division of Materials Science,
School of Materials Science and Engineering,
Nanyang Technological University, N3-B4-W310,
Nanyang Avenue, 639798 Singapore, Singapore
e-mail: Alexishengbc@ntu.edu.sg

J. S.-C. Loo (✉)
Division of Materials Science,
School of Materials Science and Engineering,
Nanyang Technological University, Block N4.1,
#01-04a, 50 Nanyang Avenue,
639798 Singapore, Singapore
e-mail: JoachimLoo@ntu.edu.sg

Introduction

In recent years, the rapidly advancing field of nanotechnology has spawned a veritable menagerie of newly formulated nanomaterials (Staggers et al. 2008) that have found diverse applications in various aspects of human life, from cosmetics and medical products to water purification and solar energy capture. Due to increasing widespread contact and interaction of the human body with these various newly formulated nanomaterials, there has been growing concern over their potential hazard to human health and safety, which in turn has spurred intensive efforts in evaluating the toxicity of these new materials (Klaine et al. 2008; Schulte et al. 2008).

Mammalian cell lines are commonly utilized in the in vitro toxicity screening (International standard ISO-10993–5 1999; Andersen and Krewski 2009) of nanomaterials, as well as various soluble chemical products, despite their obvious limitations in recapitulating the intricate complexity of tissues and organs within whole organisms. Currently, there is a lack of standardization of cell culture protocols utilized for toxicity assays. This in turn leads to varying results in different studies investigating the same potentially toxic nanomaterials with the same cell lines. Variation in cell culture parameters such as oxygen tension (Boatman et al. 2004; Siim et al. 1994), as well as presence of antioxidants and free radical scavengers (Zhu et al. 2009; Erkekoglu and Baydar 2010), has been reported to influence the outcome of cytotoxicity assays. Nevertheless, a parameter that has often been overlooked when mammalian cell lines are utilized for in vitro toxicology screening is the density and confluency of the cell monolayer. It is speculated that the density and confluency of the cell monolayer would in turn have a profound influence on cellular response to toxic challenge. This is because the mitotic activity, surface area, intercellular contacts, cytoplasmic coupling (i.e., gap junctions), paracrine signaling and metabolism of cells would obviously be affected by the density and confluency of the monolayer (Wang et al. 2009; Grayson et al. 2008). Hence, this study investigated the cytotoxic effects of ZnO nanoparticles on mammalian cell lines (BEAS-2B, L-929, CRL-2922 and C2C12) seeded at different densities. Additionally, the possibility of a colloidal suspension of nanoparticles to undergo agglomeration and sedimentation within aqueous solution would make it imperative to investigate whether alteration of other cell culture parameters such as exposure volume and cell plating surface area could lead to variation in the observed experimental results. If nanoparticle sedimentation does occur, then alteration of these parameters would be expected to change the mass/number of sedimented nanoparticles per unit area and hence the degree of cellular exposure to nanoparticles.

The choice of ZnO nanoparticles for this study is based on its diverse and wide-ranging applications in various industrial and consumer products, from ceramic and cement manufacture to skin and haircare products (Schilling et al. 2010; Osmond et al. 2010). This would make it imperative to rigorously characterize the toxicological properties of nanoparticulate ZnO with regard to human health and environmental safety. Indeed, several previous studies have already thoroughly evaluated the toxicology of ZnO nanoparticles on various different mammalian cell lines (Xia et al. 2008; Yang et al. 2009; Heng et al. 2010), making it a well-established model system for toxicological studies in vitro.

Materials and methods

Cell line, culture media, reagents, chemicals and labware consumables

BEAS-2B (human bronchial epithelial cell line), L-929 (murine fibroblast cell line), CRL-2922 (human endothelial hybridoma cell line) and C2C12 (murine myoblast cell line) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All four cell lines were subjected to at least three serial passages after freeze-thawing, prior to being utilized for experiments. Unless otherwise stated, all reagents and chemicals were purchased from Sigma–Aldrich Inc (St. Louis, MO, USA); all culture media, serum and phosphate-buffered saline (PBS) were purchased from Gibco-BRL Inc. (Gaithersburg, MD, USA), while all labware consumables were purchased from Corning Inc. (Corning, NY, USA). The ZnO nanoparticles were purchased from Meliorum Technologies Inc. (Rochester, NY, USA). The transmission electron micrograph (TEM) of ZnO nanoparticles is shown in Fig. 1.

Nanoparticle characterization with transmission electron microscopy and dynamic light scattering

To evaluate particle morphology, the dried ZnO powder was observed under a transmission electron microscope (2010 TEM, JOEL, Japan) at an accelerating voltage of

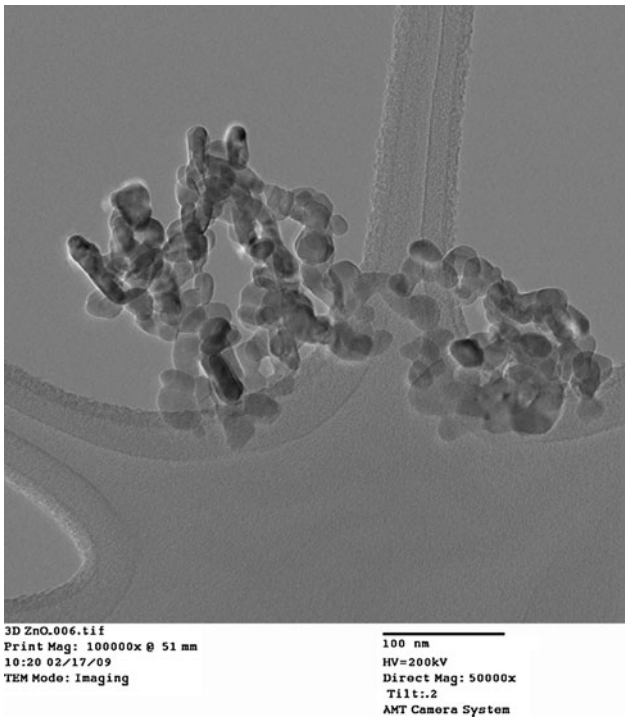

Fig. 1 Transmission electron micrograph image of ZnO nanoparticles

200 kV with a LaB6 cathode. Samples were prepared by mixing a small quantity of ZnO in ethanol followed by 10 min of sonication treatment within a water bath and collected on carbon-coated copper grids. The hydrodynamic sizes of the ZnO nanoparticles within culture media (Dulbecco's minimum essential medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 40 μg/ml sodium hexamonophosphate) were characterized at three concentrations (5, 20 and 40 μg/ml) using dynamic light scattering (DLS) (Malvern Co., UK). All samples were sonicated in culture media to form colloids prior to testing. Each sample was tested in duplicates and the mean values are recorded.

Varying cell density by seeding different cell numbers and by varying the surface area of the culture well

BEAS-2B cells were seeded on 12-well culture plates (≈4.8 cm^2 per well) at two different densities of 5.0×10^4 cells per well and 2.0×10^5 cells per well. At the same time, BEAS-2B cells were also seeded on 48-well culture plates at a density of 5.0×10^4 cells per well (≈1.2 cm^2). The culture media were composed of Dulbecco's minimum essential medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotic–antimycotic solution (Cat No. A5955, Sigma–Aldrich Inc.). The seeded cells were cultured for 24 h prior to being utilized for experiments.

Comparison of the cytotoxic effects of zinc oxide nanoparticles on BEAS-2B cells seeded at different densities

The following day after seeding, BEAS-2B cells in the control and experimental groups were exposed to varying concentrations (5, 10, 15, 20, 25, 30 and 40 μg/ml) of ZnO nanoparticles constituted in culture media (1 ml) supplemented with 40 μg/ml of sodium hexametaphosphate (SHMP), for 24 h at 37°C within a 5% CO_2 incubator. For consistency, the zero concentration reference control was also exposed to 40 μg/ml SHMP. The SHMP acted as a surfactant to facilitate dispersion and minimize the aggregation of the ZnO nanoparticles. Prior to incubation with the BEAS-2B cells, the ZnO nanoparticle colloids were sonicated for 15 min within an ultrasonic cleaner (MRC laboratory instruments Inc., Holon, Israel). Altogether, there were three replicates for each ZnO nanoparticle concentration in both the control and experimental groups. Subsequently, the cells were subjected to the WST-8 assay (Ishiyama et al. 1997) after 24 h of culture, so as to quantify the proportion of cells that remained viable after exposure to varying concentrations of ZnO nanoparticles within the control and experimental group.

WST-8 assay for assessing cell survival after exposure to zinc oxide nanoparticles

The WST-8 assay for cell viability (Ishiyama et al. 1997) was carried out with the cell counting kit solution (CCK-8) from Dojindo Molecular Laboratories Inc. (Kumamoto, Japan). The CCK-8 kit utilizes the water-soluble tetrazolium salt WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) in measuring NADH production resulting from the dehydrogenase activity of viable cells. The subsequent reduction in WST-8 by viable cells produces an orange-colored formazan product with an absorbance at 450 nm. The cells were washed three times in PBS (phosphate-buffered saline), prior to the addition of 25 μl of CCK-8 solution and 225 μl of culture media within each well of the 12-well culture plate. After incubation for 2 h at 37°C within a 5% CO_2 incubator, 100 μl aliquots of the reaction mixture were transferred onto a fresh 96-well plate, and absorbance readings were measured spectrophotometrically at 450 nm using an Infinite200® microplate reader (Tecan Inc., Maennedorf, Switzerland). The cell survival upon exposure to varying concentrations of ZnO nanoparticles in the control and experimental groups was calculated as the ratio of absorbance readings (450 nm) yielded by the treated and untreated (negative control) wells, after correction for blank absorbance reading of the reaction mixture incubated without cells for the same duration (2 h) at 37°C. As a control, the WST-8 reagent solution was incubated with increasing concentrations (10 to 40 μg/ml) of ZnO up to 3 h, and there was marginal change in absorbance values (450 nm) in the absence of cells (data not shown).

Exposure of BEAS-2B cells to increasing volumes of ZnO nanoparticle suspension

BEAS-2B cells were also seeded at a density of 5.0×10^4 cells per well of 12-well culture dishes (4.8 cm^2 per well) and subsequently exposed to increasing volumes (0.5, 1.0, 2.0 and 4.0 ml) of ZnO nanoparticle suspension at a fixed concentration of either 20 or 25 μg/ml. This was done to evaluate whether possible sedimentation of a colloidal suspension of ZnO nanoparticles could lead to variation in cytotoxicity data. If sedimentation does occur, then increasing the exposure volume would inevitably result in increased mass/number of sedimented nanoparticles per unit area, which mean that the cells would be exposed to more nanoparticles. On the other hand, if no sedimentation occurs and all ZnO nanoparticles remain in free suspension within aqueous solution, then altering the exposure volume would theoretically not lead to any change in the mass/number of nanoparticles that the cells are exposed to. After incubation for 24 h at 37°C within a 5% CO_2 incubator,

the WST-8 assay (Ishiyama et al. 1997) was utilized to assess the proportion of cells that remained viable. For further comparison, BEAS-2B cells were seeded at the same density on both 12-well and 48-well cultures dishes (5.0×10^4 and 1.25×10^4 cells per well of 12-well (4.8-cm^2) and 48-well (1.2-cm^2) culture dishes, respectively, approximately 10,000 cells/cm^2) and subsequently exposed to the same mass of ZnO nanoparticles per unit area, with either 20 or 25 μg/ml ZnO (0.25 ml per well of 48-well plate and 1.0 ml per well of 12-well plate). Because the surface area of a single well of a 48-well plate is four times less than that of a 12-well plate (1.2 vs. 4.8 cm^2, respectively), we can achieve the same cell seeding density, simply by reducing the total number of seeded cells by a factor of four. Similarly, we can achieve the same mass of ZnO nanoparticles per unit area of culture surface, simply by reducing the volume of ZnO nanoparticle suspension by a factor of four (i.e., 0.25 ml per well of 48-well plate and 1.0 ml per well of 12-well plate). After incubation for 24 h at 37°C within a 5% CO_2 incubator, the WST-8 assay (Ishiyama et al. 1997) was utilized to assess the proportion of cells that remained viable.

For comparison of ZnO with a soluble zinc salt, an additional control experiment was performed using $ZnCl_2$. BEAS-2B cells were seeded at a density of 5.0×10^4 cells per well of 12-well culture dishes (4.8 cm^2 per well) and subsequently exposed to increasing concentrations (0, 10, 25, 30, 35, 40, 45 and 50 μg/ml) of a soluble zinc salt $ZnCl_2$ within culture media. This corresponds to a Zn^{2+} molarity range of 73.4 to 366.8 μM. Subsequently, another control experiment was performed in which BEAS-2B cells (5.0×10^4 cells per well) were exposed to increasing volumes of culture medium, with and without supplementation of a fixed concentration of $ZnCl_2$ at 30 μg/ml (220.1 μM).

Varying cell density by localized seeding at edge of culture well

BEAS-2B, L-929, CRL-2922 and C2C12 cells were seeded at a density of 5.0×10^4 cells per well (4.8 cm^2) of 12-well culture plates. The culture media were composed of Dulbecco's minimum essential medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotic–antimycotic solution (Cat No. A5955, Sigma–Aldrich Inc.). Different methods of seeding were utilized for the experimental and control groups. In the experimental group, the 5.0×10^4 cells were initially concentrated in 0.1 ml of culture media and seeded at the edge of the well (Fig. 2), with the culture plate slanted at an angle of 30 degrees. After 4 h of incubation at 37°C to allow cell adhesion into a dense confluent monolayer at the edge of the well (Fig. 3a), 0.4 ml of culture media was added to each well and the plate was kept level within the incubator. For the control group, the same number of cells constituted in 0.5 ml of culture media was placed within each well, with the culture plate being kept level within the incubator. This resulted in a sparse non-confluent cell monolayer for the control (Fig. 3e). The seeded cells were cultured for 24 h prior to being exposed to varying concentrations (0, 5, 10, 15, 20, 25, 30 and 40 μg/ml) of colloidal ZnO nanoparticles, for 24 h at 37°C within a 5% CO_2 incubator. Subsequently, the cells were subjected to the WST-8 assay (Ishiyama et al. 1997) after 24 h of culture, so as to quantify the proportion of cells that remained viable after exposure to varying concentrations of ZnO nanoparticles within the control and experimental group.

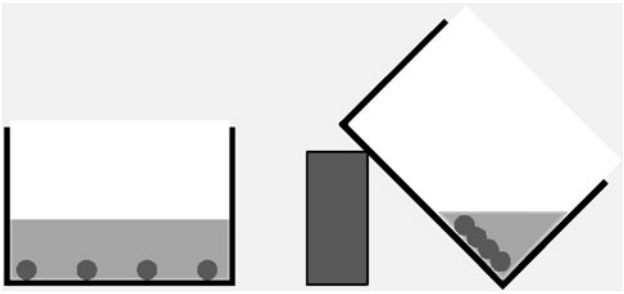

Fig. 2 A high-density confluent cell monolayer can be achieved by concentrating the cells within a small volume and seeding only at the edge of the wells within multi-well culture plates tilted at an angle; while a sparse non-confluent monolayer is achieved by seeding the same number of cells with a larger volume of culture media in culture plates placed level with the ground

Statistical analysis of data

The results from each data set were expressed as mean ± standard deviation ($n = 3$ for all data sets). Statistical differences between data sets were assessed by the Student's *t*-test, with a *P*-value less than 0.05 being considered significantly different.

Results

Nanoparticle characterization with transmission electron microscopy and dynamic light scattering

The morphology of the ZnO nanoparticles as shown by TEM (Fig. 1) displayed much heterogeneity in shape and size, ranging from approximately 10 to 40 nm. Subsequently, DLS analysis of colloidal ZnO nanoparticles within culture media demonstrated increasing hydrodynamic size with higher concentration. At 5 μg/ml, the average hydrodynamic size was 27.3 ± 4.2 nm, when compared to readings of 50.3 ± 4.5 and 196.9 ± 4.9 nm at concentrations

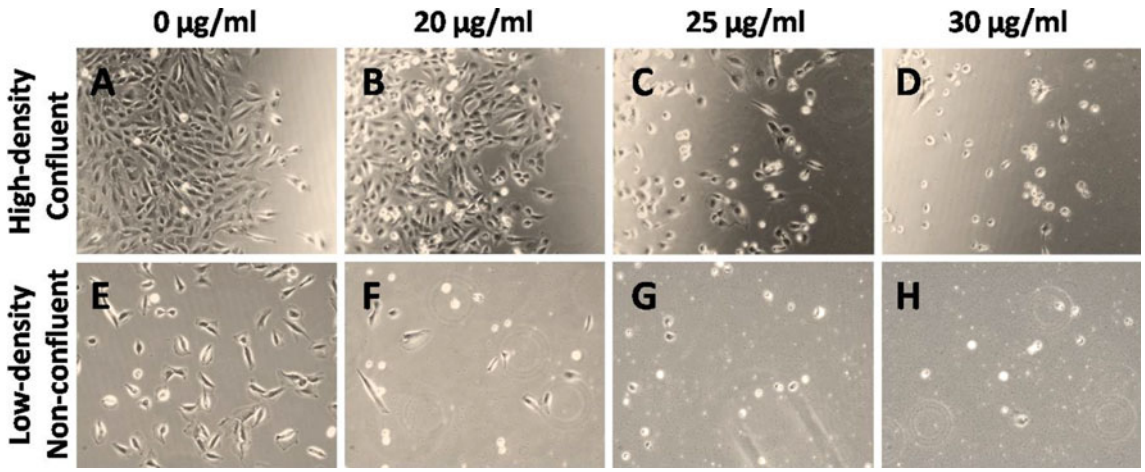

Fig. 3 Bright-field microscopy images of BEAS-2B cells exposed to varying concentration of ZnO nanoparticles. (**a**–**d**) High-density confluent cell monolayer, (**e**–**h**) Low-density non-confluent cell monolayer. In the experimental group, 5.0×10^4 cells were concentrated in 0.1 ml of culture media and seeded at the edge of the well (12-well plate), so as to achieve a high-density confluent monolayer. In the control group, the same number of cells was evenly seeded throughout the entire surface of the culture well, giving rise to a low-density non-confluent monolayer

of 20 and 40 µg/ml, respectively. This could be indicative of some degree of nanoparticle agglomeration at higher concentrations.

Cytotoxic effects of zinc oxide nanoparticles on different densities of BEAS-2B cells, achieved through seeding different cell numbers and by varying the surface area of the culture well

As seen in Fig. 4, the cell viability was consistently higher for the higher-density BEAS-2B cell monolayer seeded on 12-well plates (2.0×10^5 cells per well), when compared to the lower-density control group (5.0×10^4 cells per well), over the entire range of ZnO nanoparticle concentrations examined (from 5 to 40 µg/ml). The greatest differences were observed over the sub-lethal concentration range of ZnO nanoparticles (20, 25 and 30 µg/ml), where there was a steep decrease in cell viability for both the control (5.0×10^4 cells in 12-well) and experimental group. The higher-density monolayer of BEAS-2B cells (2.0×10^5 cells per well) displayed $87.5 \pm 1.9\%$, $73.1 \pm 2.4\%$ and $46.5 \pm 1.2\%$ cell viability at ZnO nanoparticle concentrations of 20, 25 and 30 µg/ml, respectively. By contrast, the corresponding values for the control (5.0×10^4 cells per well) were significantly lower ($P < 0.01$) at 69.5 ± 2.8, 31.9 ± 2.5 and $19.6 \pm 1.4\%$, respectively.

However, contrary results were obtained upon comparing 5.0×10^4 cells seeded (per well) on 12-well (4.8-cm^2) versus 48-well (1.2-cm^2) culture plates and exposed to the same volume (1 ml) and concentration of ZnO nanoparticles; with the higher cell density group now yielding lower viability readings at ZnO concentrations of 15 and 20 µg/ml. The higher-density monolayer of BEAS-2B cells (5.0×10^4 cells per well of 48-well plate) displayed $66.4 \pm 3.3\%$ and $51.3 \pm 4.4\%$ cell viability at ZnO nanoparticle concentrations of 15 and 20 µg/ml, respectively. By contrast, the corresponding values for the lower-density control (5.0×10^4 cells per well of 12-well plate) were significantly higher ($P < 0.01$) at $92.0 \pm 3.6\%$ and $69.5 \pm 2.8\%$, respectively.

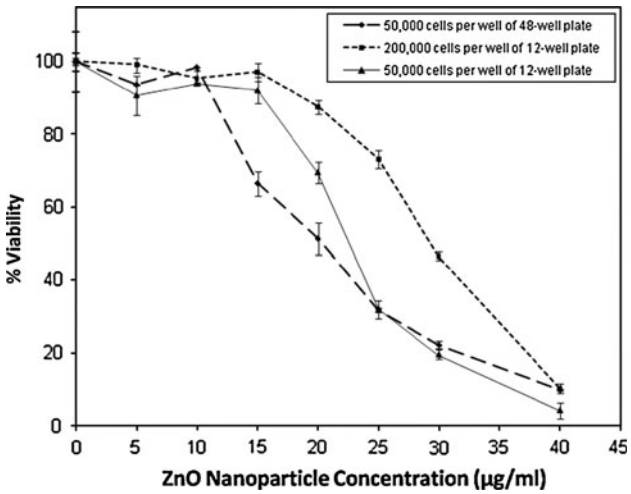

Fig. 4 Effects of increasing ZnO nanoparticle concentration on the viability of BEAS-2B cells seeded at different densities (WST-8 assay). Cell density was varied either by seeding a larger number of cells within the same surface area (i.e., 2.0×10^5 vs. 5.0×10^4 cells per well of 12-well plate) or by seeding the same number of cells on different surface areas (i.e., 5.0×10^4 cells per well on 12-well and 48-well plates)

Effect of increasing volume of ZnO nanoparticle suspension (of fixed concentration) on cell viability

Due to the contradictory cytotoxicity data observed in Fig. 4, it was necessary to investigate whether the toxic dose of the ZnO nanoparticles correlated with the concentration or net amount of the ZnO nanoparticles in suspension. A pertinent question was whether increasing the total volume of ZnO nanoparticle suspension, while keeping the concentration constant, could increase the toxicity on the exposed cells. As seen in Fig. 5a, there was observed to be a reduction in cell viability upon exposure to increasing volumes of ZnO nanoparticle suspension at either 20 or 25 μg/ml. When cells were seeded at the same density on 12- and 48-well plates (≈10,000 cells/cm^2) and exposed to the same mass of nanoparticles per unit area of culture surface (0.25 ml per well of 48-well plate and 1.0 ml per well of 12-well plate), with the nanoparticle to cell ratio also being kept constant, there was observed to be no significant differences in cell viability (Fig. 5c).

Control experiments with zinc chloride

As seen in Fig. 6, soluble $ZnCl_2$ is marginally more toxic than the nanoparticulate ZnO, upon comparison in terms of molarity. Upon exposure of BEAS-2B cells to increasing volumes (0.5 to 4.0 ml) of culture media with and without supplementation of 30 μg/ml (220.1 μM) $ZnCl_2$, the cell viability remained relatively constant (Fig. 5b).

Cytotoxic effects of zinc oxide nanoparticles on different densities of BEAS-2B, L-929, CRL-2922 and C2C12 cells, achieved by localized seeding at the edge of culture wells

To achieve different confluency of the cell monolayer, while keeping the total cell number, culture surface area and the volume/concentration of nanoparticle suspension constant within the experimental system, localized seeding at the edge of culture wells (Fig. 2) was carried out to obtain a confluent monolayer (Fig. 3a). This was compared with a sparse monolayer obtained by seeding the same cell number in the conventional manner (Fig. 3b). As seen in Fig. 7, the cell viability was consistently higher for the high-density confluent monolayer of all four cell lines within the experimental group compared to the control (sparse cell monolayer), over the entire range of ZnO nanoparticle concentrations examined (from 5 to 40 μg/ml). Differences between the experimental and control groups were most obvious and significant above a ZnO concentration of 10 μg/ml. Hence, a high-density confluent monolayer was more resistant to the cytotoxic effects of ZnO nanoparticles compared to a sparse non-confluent cell monolayer. The data were corroborated by the bright-field

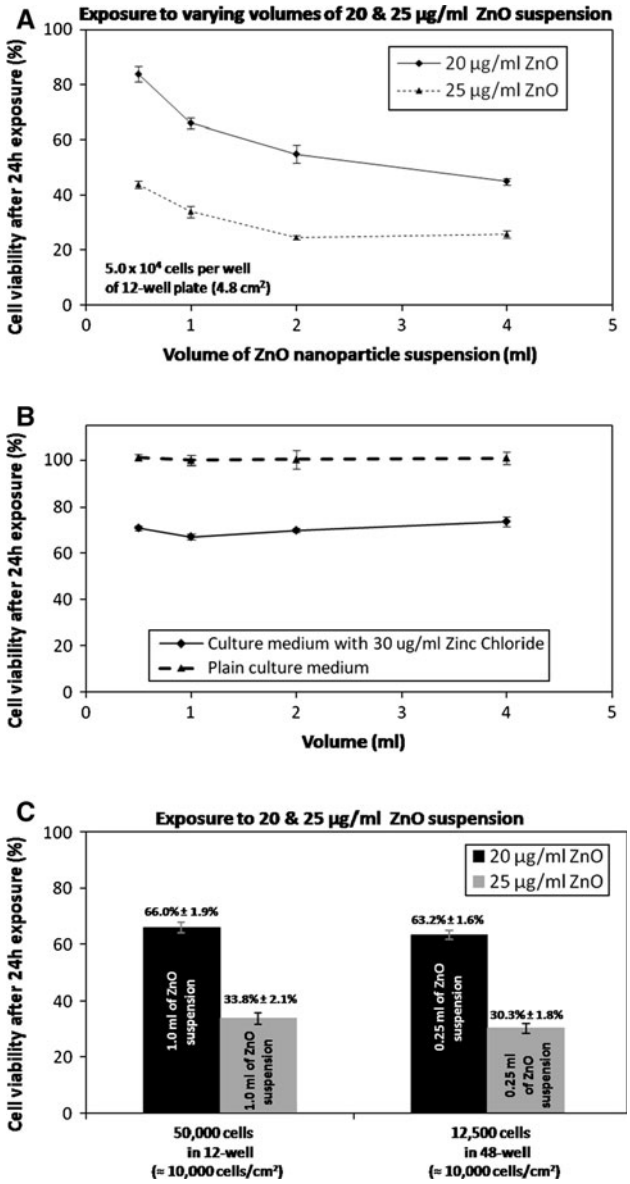

Fig. 5 **a** There was a reduction in BEAS-2B cell viability upon exposure to increasing volumes of ZnO nanoparticle suspension at either 20 μg/ml or 25 μg/ml within 12-well plates. **b** By contrast, cell viability remained relatively constant upon exposure to increasing volumes of culture medium with and without zinc chloride (30 μg/ml). **c** When cells were seeded at the same density on 12- and 48-well plates (≈10,000 cells/cm^2) and exposed to the same mass of nanoparticles per unit area, there was observed to be no significant differences in cell viability

images of the high-density and sparse cell monolayers, after 24-h exposure to varying concentration of ZnO nanoparticles (Fig. 3).

Discussion

It is challenging to characterize how the cytotoxic effects of nanomaterials are influenced by the confluency of cell

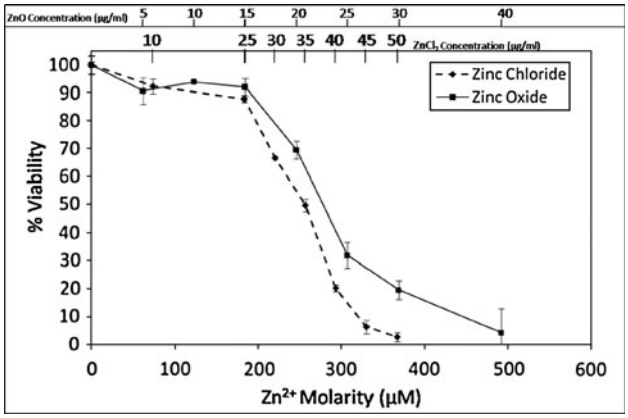

Fig. 6 Comparison of the cytotoxic response of BEAS-2B cells (1.0×10^4 cells/cm^2) to increasing molarities of soluble zinc chloride and nanoparticulate zinc oxide

monolayers, within the context of an experimental investigation. This is because varying cell densities by varying the total cell number within different formats of multi-well culture plates would invariably alter (1) nanoparticle to cell ratio and (2) nanoparticle to culture surface area ratio, for a given fixed volume and concentration of nanoparticle suspension in aqueous solution, thereby confounding the results observed. As our results showed, when the culture surface area was kept constant (12-well format), a higher cell density of 2.0×10^5 cells per well gave consistently higher viability readings compared to 5.0×10^4 cells per well (Fig. 4). However, in this setup, the nanoparticle to cell ratio was not constant. On the contrary, when 5.0×10^4 cells were cultured on different culture surface areas (12-well vs. 48-well) with the volume of nanoparticle suspension being kept constant (1 ml), it was the culture with lower cell density that registered higher viability readings (Fig. 4). However, in this setup, the nanoparticle to culture surface area ratio varied.

If the ZnO nanoparticles remained in suspension during the experiment, changing the nanoparticle to cell ratio should logically not affect cellular response to the particles because the local environment surrounding the cells would not change regardless of how much more nanoparticles were added, as long as its concentration remained the same. On the other hand, the observation that the same number of cells cultured in different well-format elicited different cytotoxic response implied that sedimentation of ZnO nanoparticles could be an important contributing factor to cytotoxicity, since this would change the local environment of the cells. Placing 1 ml of nanoparticle suspension within a single well of 48-well plate (1.2 cm^2) will result in four times the total number of nanoparticles sedimented per unit area of culture surface, when compared to placing 1 ml of the same nanoparticle suspension within 1 well of a 12-well plate (4.8 cm^2). Indeed, it was subsequently shown that there was a reduction in cell viability upon exposure to increasing volumes of ZnO nanoparticle suspension at a fixed concentration of either 20 or 25 μg/ml (Fig. 5a).

The pertinent question that now arises is whether the cytotoxic dosage of nanoparticles correlates with the concentration of the nanoparticle suspension to which the cells are exposed to. The data presented in Fig. 5 would suggest

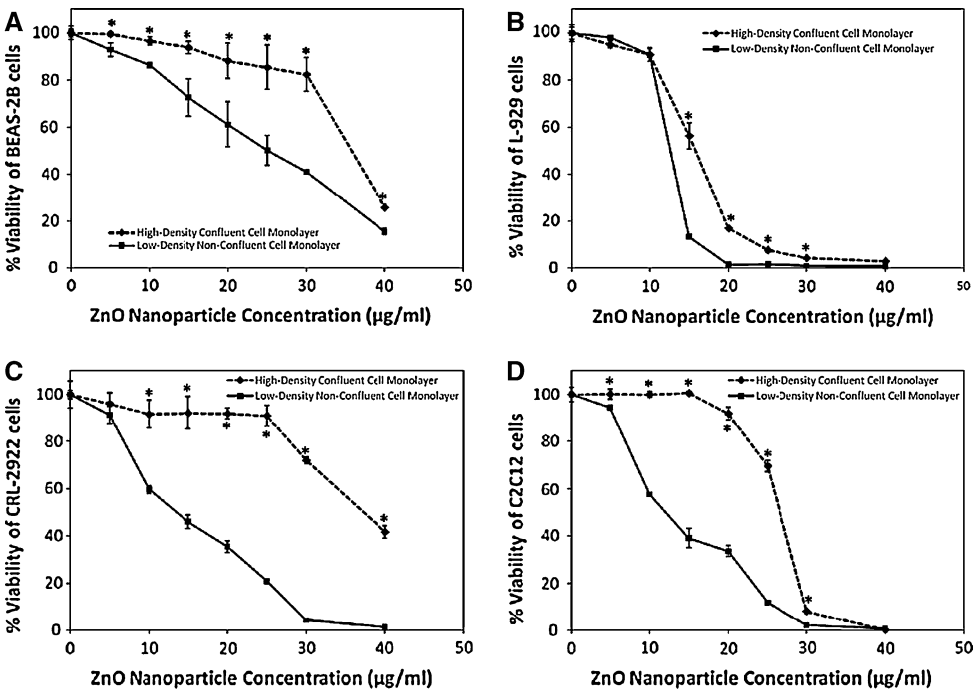

Fig. 7 Effects of increasing ZnO nanoparticle concentration on the viability of BEAS-2B (**a**), L929 (**b**), CRL-2922 (**c**) and C2C12 (**d**) cells seeded at different densities (WST-8 assay). In the experimental group, 5.0×10^4 cells were concentrated in 0.1 ml of culture media and seeded at the edge of the well (12-well plate), so as to achieve a high-density confluent monolayer. In the control group, the same number of cells was evenly seeded throughout the entire surface of the culture well, giving rise to a low-density non-confluent monolayer. Concentration points that display statistically significant differences ($P < 0.05$) between high- and low-density group (Student's *t*-test) are denoted by '*'

otherwise. When cells were seeded at the same density on 12- and 48-well plates (≈10,000 cells/cm^2) and exposed to the same mass of nanoparticles per unit culture area, there was observed to be no significant differences in cell viability (Fig. 5c). This would thus imply that the mass or number of nanoparticles per unit area of culture surface could in fact be a better measure of cytotoxic dosage. Nevertheless, the overwhelming majority of nanotoxicity studies invariably equate toxic dose with concentration of the nanoparticle suspension. One possibility is that no matter how well-dispersed a nanoparticle suspension is; there will always be some degree of gravity sedimentation over prolonged periods of in vitro culture. Hence, the cytotoxic effects of nanoparticles could in fact be a combination of freely suspended nanoparticles and sedimented nanoparticles in direct contact with the cell membrane. The data would thus cause us to rethink whether it is still appropriate to equate the toxic dosage of nanoparticles with only the concentration of the nanoparticle suspension.

There is evidence that ZnO cytotoxicity could be partially affected by the release of Zn^{2+} ions through the dissolution of nanoparticulate ZnO within aqueous culture media (Xia et al. 2008; George et al. 2010). Indeed, it was recently demonstrated that the cytotoxicity of nanoparticulate ZnO is reduced if its dissolution within culture media is inhibited by iron doping (George et al. 2010). Hence, a control experiment was performed with the salt $ZnCl_2$, because of its high solubility and also because chloride ions are naturally present within the culture media (i.e., sodium chloride). The results show that $ZnCl_2$ is marginally more toxic than ZnO, upon comparison in terms of molarity (Fig. 6). The pertinent question that arises is to what extent the observed cytotoxicity of the nanoparticulate ZnO is being effected by solubilized Zn^{2+} ion. Previously, the solubility of nanoparticulate ZnO in culture media was characterized by the study of Xia et al. (2008), which demonstrated that the maximal solubility of ZnO in complete DMEM (supplemented with 10% v/v FBS) at physiological temperature is 225 μM. Based on this molarity of Zn^{2+}, only about 25 to 30% of BEAS-2B cells lost their viability, according to the $ZnCl_2$ data (Fig. 6), assuming that $ZnCl_2$ is 100% soluble in aqueous solution. Hence, the observed further decrease in cell viability at higher concentrations of nanoparticulate ZnO is not likely to be effected by solubilized Zn^{2+} ions within the extracellular aqueous culture milieu, but is instead attributed to the insolvent ZnO nanoparticles per se. Certainly, it is likely that upon cellular uptake of the insolvent ZnO nanoparticles, the acidic conditions within the lysosomal and calveolar compartments of the cell might cause further dissolution of ZnO and subsequent release of additional Zn^{2+} ions (Xia et al. 2008). Nevertheless, this is a separate mechanism from the cytotoxic effect of solubilized Zn^{2+} ions within the extracellular environment.

As a further control, BEAS-2B cells (50,000 cells per cm^2) were exposed to increasing volumes (0.5 to 4.0 ml) of $ZnCl_2$ at a fixed concentration (30 μg/ml, 220.1 μM). This particular concentration was chosen because it corresponds to a sub-lethal dose, whereby the BEAS-2B cells retained 65 to 70% viability after 24-h exposure (Fig. 6). The results (Fig. 5b) showed that there was marginal change in the viability of BEAS-2B cells with increasing volumes of 30 μg/ml $ZnCl_2$, in sharp contrast to what was observed with increasing volumes of nanoparticulate ZnO at 20 and 25 μg/ml (Fig. 5a). Hence, this would confirm that for a soluble salt like $ZnCl_2$ or other completely dissolving nanoparticle, cytotoxic dosage is more appropriately expressed as a concentration or molarity. For completely insolvent nanoparticles, the cytotoxic dosage could possibly be more appropriately expressed as absolute mass or number of nanoparticles per unit area of cell culture surface (Rushton et al. 2010). Because ZnO is partially dissolving, the cytotoxic dosage would probably need to be expressed as a complex mathematical formula that combines solubility constant, concentration and mass/number of nanoparticles per unit area. Such a complex mathematical formula needs to be elucidated in a future study.

The challenge is thus to achieve different confluency of the cell monolayer, while keeping the concentration of nanoparticle suspension and amount of sedimented nanoparticles (if any) constant within the experimental system. One way of achieving this is by varying the cell seeding procedure (Fig. 2). It is demonstrated in this study that a high-density confluent cell monolayer can be achieved by concentrating the cells within a small volume (0.1 ml) and seeding only at the edge of the wells within multi-well culture plates tilted at an angle (Fig. 2); while a sparse non-confluent monolayer is achieved by seeding the same number of cells (5.0×10^4) with a larger volume (0.5 ml) of culture media in culture plates placed level with the ground. Hence, both the high-density experimental group and the low-density control group would have exactly the same nanoparticle to cell ratio, as well as the same number of nanoparticles per unit area of culture surface, for any given fixed volume and concentration of nanoparticle suspension.

Utilizing such an experimental set-up (Fig. 2), this study investigated the cytotoxic effects of ZnO nanoparticles on four different cell lines (BEAS-2B, L-929, CRL-2922 and C2C12) seeded at different densities. The results demonstrated that a dense and confluent monolayer of cells is more resistant to the cytotoxic effects of ZnO nanoparticles, when compared to a sparse non-confluent monolayer, with the greatest differences being observed above 10 μg/ml ZnO concentration. The contradictory results observed in Fig. 4, whereby the higher-density monolayer within 48-well plates (50,000 cells in 1.2 cm^2) was more sensitive to increasing ZnO concentrations compared to the lower-density

monolayer (50,000 cells in 4.8 cm^2), are due to the same volume of ZnO suspension (1 ml) being utilized for both experimental groups. Hence, the higher-density monolayer within the 48-well plate (1.2 cm^2) is effectively exposed to four times the absolute mass/number of nanoparticles per unit area, compared to the low-density monolayer within the 12-well plate (4.8 cm^2).

The observed differences in the cytotoxic effects of ZnO nanoparticles on cells seeded at different densities could be because the density and confluency of the cell monolayer would in turn affect their mitotic activity, metabolism, intercellular contacts, paracrine signaling (i.e., secreted cytokines and growth factors), cytoplasmic coupling (i.e., gap junctions) and even surface area occupied by individual cells (Wang et al. 2009; Grayson et al. 2008). For example, cell division involves extensive re-organization of the cytoskeleton and plasma membrane (Schweitzer and D'Souza-Schorey 2004), and this might have had a profound influence on the uptake of ZnO nanoparticles. It is possible that mitotic activity is reduced within dense confluent cells monolayers, due to more intensive competition for oxygen and nutrients among neighboring cells within a confined area. A slower rate of cell division may result in a lower rate of cellular metabolism within the dense confluent monolayer, as opposed to the sparse non-confluent monolayer. Although the raw absorbance values measured by the WST-8 assay will undoubtedly be affected by a reduction in metabolism within dense confluent cell cultures, this in itself is unlikely to contribute to the observed differences in cytotoxic response of the high and low seeding density groups to increasing concentrations of ZnO nanoparticles. This is because in the computation of percentage viability values, the raw data of both experimental groups were compared to their corresponding reference control (zero nanoparticle concentration) seeded at exactly the same cell density. One possibility is that a slowdown of metabolic pathways within dense confluent monolayers can somehow slow the cellular uptake and processing of ZnO nanoparticles. This in turn might confer greater resistance to the cytotoxic effects of ZnO nanoparticles, which have been shown to elicit its detrimental effect on cell viability partially through cellular uptake and processing of nanoparticles within the lysosomal and calveolar compartments of the cell (Xia et al. 2008). Additionally, the phase-contrast microscopy images (Fig. 3a and e) suggested that individual cells within a dense confluent monolayer occupied lesser surface area on the culture dish surface, when compared to cells within a sparse non-confluent monolayer. This could mean that individual cells within a dense confluent monolayer were exposed to lesser numbers of ZnO nanoparticles, either in suspension or in sedimented, when compared to cells within a sparse non-confluent monolayer. Another hypothesis was that cells seeded at high densities have a greater degree of intercellular adhesion contacts, cytoplasmic coupling through gap junctions, as well as paracrine signaling through secreted growth factors and cytokines, which in turn made them more resistant to the cytotoxic effects of ZnO nanoparticles, when compared to cells seeded at lower densities.

It is possible that these results on the effect of seeding density on the cytotoxic response of adherent cells to ZnO nanoparticles may also be extrapolated to non-adherent cells within static culture, whereby cells are allowed to sediment to the bottom of the culture container. In that case, a higher seeding density would also mean more sedimented non-adherent cells per unit area. Again there would expected to be reduced cellular metabolism due to increased competition for oxygen and nutrient by a greater number of sedimented non-adherent cells per unit area, which in turn could lead to similar results as observed in this study. On the other hand, if non-adherent cells are cultured within a dynamic culture system like a stirred-tank bioreactor with constant recirculation and replenishment of oxygen and nutrients, then the seeding density may have much less effect on metabolism and cell proliferation, which in turn could lead to similar cytotoxic response of high and low seeding density groups to ZnO nanoparticles. However, this is required to be confirmed through a separate study on non-adherent cells.

Hence, the results of this study demonstrated that cell seeding density has a profound influence on the cytotoxic effects of nanoparticles. Specifically, cells seeded at higher densities were more tolerant of the cytotoxic effects of ZnO nanoparticles. Future studies would attempt to investigate the underlying mechanisms. In addition, this data demonstrated clearly the need for standardization of laboratory protocols utilized for the toxicology screening of nanoparticles with mammalian cell lines. Besides the field of nanotoxicology, the results of this study could also have important implications for pharmacokinetic analysis of nanoparticle-mediated drug delivery systems i.e., drug encapsulation within nano-sized micelles and liposomes. The observation that variation in cell culture parameters such as seeding density, exposure volume and culture surface area can drastically alter the cytotoxic response of cells to potentially toxic nanoparticles may also be applicable to the pharmacokinetic response of cells to biologics and small-molecule drugs delivered by nanoparticles. This needs to be confirmed in a further study.

References

Andersen ME, Krewski D (2009) Toxicity testing in the 21st century: bringing the vision to life. Toxicol Sci 107(2):324–330

Boatman RJ, English JC, Guerin TS, Cummings LM (2004) The effect of oxygen tension on the cytotoxicity of hydroquinone and

selected hydroquinone metabolites to isolated rat renal proximal tubular cells. Arch Toxicol 78(8):443–452

Erkekoglu P, Baydar T (2010) Evaluation of the protective effect of ascorbic acid on nitrite- and nitrosamine-induced cytotoxicity and genotoxicity in human hepatoma line. Toxicol Mech Methods 20(2):45–52

George S, Pokhrel S, Xia T, Gilbert B, Ji Z, Schowalter M, Rosenauer A, Damoiseaux R, Bradley KA, Mädler L, Nel AE (2010) Use of a rapid cytotoxicity screening approach to engineer a safer zinc oxide nanoparticle through iron doping. ACS Nano 4(1):15–29

Grayson WL, Bhumiratana S, Cannizzaro C, Chao PH, Lennon DP, Caplan AI, Vunjak-Novakovic G (2008) Effects of initial seeding density and fluid perfusion rate on formation of tissue-engineered bone. Tissue Eng Part A 14(11):1809–1820

Heng BC, Zhao X, Xiong S, Ng KW, Boey FY, Loo JS (2010) Toxicity of zinc oxide (ZnO) nanoparticles on human bronchial epithelial cells (BEAS-2B) is accentuated by oxidative stress. Food Chem Toxicol 48(6):1762–1766

International standard ISO-10993–5 (1999) Biological evaluation of medical devices—part 5: tests for in vitro cytotoxicity. International Organization for Standardization, Geneva

Ishiyama M, Miyazono Y, Sasamoto K, Ohkura Y, Ueno K (1997) A highly water-soluble disulfonated tetrazolium salt as a chromogenic indicator for NADH as well as cell viability. Talanta 44(7):1299–1305

Klaine SJ, Alvarez PJ, Batley GE, Fernandes TF, Handy RD, Lyon DY, Mahendra S, McLaughlin MJ, Lead JR (2008) Nanomaterials in the environment: behavior, fate, bioavailability, and effects. Environ Toxicol Chem 27(9):1825–1851

Osmond MJ, McCall MJ, Newman MD, Stotland M, Ellis JI (2010) Zinc oxide nanoparticles in modern sunscreens: an analysis of potential exposure and hazard. Nanotoxicology 4(1):15–41

Rushton EK, Jiang J, Leonard SS, Eberly S, Castranova V, Biswas P, Elder A, Han X, Gelein R, Finkelstein J, Oberdörster G (2010) Concept of assessing nanoparticle hazards considering nanoparticle dosemetric and chemical/biological response metrics. J Toxicol Environ Health A 73(5):445–461

Schilling K, Bradford B, Castelli D, Dufour E, Nash JF, Pape W, Schulte S, Tooley I, van den Bosch J, Schellauf F (2010) Human safety review of "nano" titanium dioxide and zinc oxide. Photochem Photobiol Sci 9(4):495–509

Schulte OPA, Trout D, Zumwalde RD, Kuempel E, Geraci CL, Castranova V, Mundt DJ, Mundt KA, Halperin WE (2008) Options for occupational health surveillance of workers potentially exposed to engineered nanoparticles: state of the science. J Occup Environ Med 50(5):517–526

Schweitzer JK, D'Souza-Schorey C (2004) Finishing the job: cytoskeletal and membrane events bring cytokinesis to an end. Exp Cell Res 295(1):1–8

Siim BG, Atwell GJ, Wilson WR (1994) Oxygen dependence of the cytotoxicity and metabolic activation of 4-alkylamino-5-nitroquinoline bioreductive drugs. Br J Cancer 70(4):596–603

Staggers N, McCasky T, Brazelton N, Kennedy R (2008) Nanotechnology: the coming revolution and its implications for consumers, clinicians, and informatics. Nurs Outlook 56(5):268–274

Wang L, Seshareddy K, Weiss ML, Detamore MS (2009) Effect of initial seeding density on human umbilical cord mesenchymal stromal cells for fibrocartilage tissue engineering. Tissue Eng Part A 15(5):1009–1017

Xia T, Kovochich M, Liong M, Mädler L, Gilbert B, Shi H, Yeh JI, Zink JI, Nel AE (2008) Comparison of the mechanism of toxicity of zinc oxide and cerium oxide nanoparticles based on dissolution and oxidative stress properties. ACS Nano 10(2):2121–2134

Yang H, Liu C, Yang D, Zhang H, Xi Z (2009) Comparative study of cytotoxicity, oxidative stress and genotoxicity induced by four typical nanomaterials: the role of particle size, shape and composition. J Appl Toxicol 29:69–78

Zhu Y, Kalen AL, Li L, Lehmler HJ, Robertson LW, Goswami PC, Spitz DR, Aykin-Burns N (2009) Polychlorinated-biphenyl-induced oxidative stress and cytotoxicity can be mitigated by antioxidants after exposure. Free Radic Biol Med 47(12):1762–1771