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Research Article

Effects of the Absorption Behaviour of ZnO Nanoparticles on Cytotoxicity Measurements

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ZnO absorbs certain wavelengths of light and this behavior is more pronounced for nanoparticles of ZnO. As many toxicity measurements rely on measuring light transmission in cell lines, it is essential to determine how far this light absorption influences experimental toxicity measurements. The main objective was to study the ZnO absorption and how this influenced the cytotoxicity measurements. The cytotoxicity of differently sized ZnO nanoparticles in normal and cancer cell lines derived from lung tissue (Hs888Lu), neuron-phenotypic cells (SH-SY5Y), neuroblastoma (SH-SY5Y), human histiocytic lymphoma (U937), and lung cancer (A549) was investigated. Our results demonstrate that the presence of ZnO affected the cytotoxicity measurements due to the absorption characteristic of ZnO nanoparticles. The data revealed that the ZnO nanoparticles with an average particle size of around 85.7 nm and 190 nm showed cytotoxicity towards U937, SH-SY5Y, differentiated SH-SY5Y, and Hs888Lu cell lines. No effect on the A549 cells was observed. It was also found that the cytotoxicity of ZnO was particle size, concentration, and time dependent. These studies are the first to quantify the influence of ZnO nanoparticles on cytotoxicity assays. Corrections for absorption effects were carried out which gave an accurate estimation of the concentrations that produce the cytotoxic effects.

1. Introduction

The industrial use of nanoparticles in a wide variety of applications has been rapidly expanding in the last decade [1]. Such applications include the use of zinc, titanium, magnesium, and other metallic oxide nanoparticles, thereby increasing the occupational and other environmental exposure of these nanoparticles to humans and other species [2].

Zinc oxide (ZnO) has properties such as wide band gap (3.37 eV), high exciton binding energy (60 meV), and a variety of morphologies [3]. ZnO nanoparticles have unique properties including small size and correspondingly large-specific surface area to volume ratio. ZnO has been increasingly employed in a variety of industrial applications including production of wave filters, UV detectors, catalysts, paint, transparent conductive film, cosmetics, gas sensor and catalytic processes, solar cells and microelectronics [4–12], personal care products (toothpaste, beauty products, and sunscreens [13]), and textiles [14]. Increased applications in industry will increase chances of exposure of the nanoparticles to humans. Thus, zinc oxide nanoparticles are the subject of much research because of their high probability exposure to human and environment [1].

Nanoparticles exhibit unique physiochemical properties and can have many unknown biological effects. Effects of ZnO nanoparticles on bacteria [12, 15], algae [16, 17], crustaceans [15], nematodes [18], and yeast *Saccharomyces cerevisiae* [19] have been studied. Many research groups

studied the effects of ZnO nanoparticles on eukaryotic cell lines such as human neuron cells [2, 20], epidermal [21], lung cancer [22, 23], intestinal [24], lymphocytes [25], and normal cells [22, 25, 26]. Lockman et al. (2004) reported that nanoparticles can alter blood-brain barrier integrity and permeability [27]. Later Tiwari and Amiji (2006) demonstrated nanocarrier-based central nervous system (CNS) delivery system for enhancing drug transport into the CNS [28]. Another group investigated zinc oxide nanoparticle penetration in human skin *in vitro* and *in vivo* [29]. However, none of these studies took into account the light absorption effects of the nanopowders themselves and the consequences on toxicology measurements.

In this study, we evaluated the possible contribution of ZnO nanoparticles absorption in the readings of cytotoxicity measurements. We also demonstrated cytotoxicity of two different sizes of spherically shaped ZnO nanoparticles in normal and cancer cell lines derived from different histological origin. Finally, we evaluated that the cytotoxicity of ZnO was particle size, concentration, and time dependent.

2. Materials and Methods

2.1. ZnO Nanoparticles. The ZnO nanoparticles were synthesized using a method detailed elsewhere [30]. Two groups of ZnO nanoparticles with the average particle size of around 85.7 nm and 190 nm named Z1 and Z2 were used, respectively. The ZnO nanoparticle size of one sample is twice that of the other sample. The absorption of the ZnO nanoparticles was measured using Perkin Elmer Lambda 750 UV-Vis-NIR spectrophotometer with a range of about 330 to 700 nm. The reflectance mode was used for the measurements.

The micrographs from the field emission transmission electron microscope (FETEM) are obtained from the JEOL JEM2100F. The microstructures can clearly be seen from the phase contrast image.

The stock solutions of ZnO nanoparticles (Z1 and Z2) were prepared in phosphate buffer saline (PBS, 0.01 M, Sigma, USA), with 100 mM stock concentration, sonicated for 30 minutes, and stored at 4°C. The nanoparticles stock concentrations were vigorously vortexed and then diluted with complete medium prior each experiment, resulting in a series of final concentrations ranging from 50 μM to 10 mM. Dose response effects of ZnO nanoparticles to cancer and normal cell lines were evaluated at two different exposure times, 24 and 96 h.

2.2. Cell Lines and Culture Conditions. Human medulloblastoma SH-SY5Y cell line was a gift from Dr. Carol Sanfeliu (Department of Pharmacology and Toxicology, Institute of Biological Research, Barcelona, Spain). Lymphoma U937 and lung cancer A549 cell lines were a gift from Dr. Mohamed Saifulaman (Faculty of Applied Sciences, UiTM, Malaysia). Normal lung Hs888Lu cell lines were purchased from American Type Culture Collection (ATCC, The Global Bioresource Centre, Manassas, USA).

The SH-SY5Y, U937, and A549 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma, USA) with high glucose content, 1% nonessential amino acids (100×) (PAA Laboratory GmbH, Austria), 1% L-glutamine (200 mM) (Sigma, USA), and 1% gentamicin (10 mg/mL) (PAA Laboratory GmbH, Austria) and supplemented with 10% fetal bovine serum (FBS, PAA Laboratory GmbH, Austria). For treatment experiments neuroblastoma (SH-SY5Y) cells were adapted to grow in 1 : 1 of Minimum Essential Medium Eagle: nutrient mixture F12-Ham (EMEM: Hams-F12, Sigma, USA) with 1% nonessential amino acids, 1% L-glutamine, and 1% gentamicin and supplemented with 10% FBS. Hs888Lu was adapted to grow in DMEM with high glucose content, 1% nonessential amino acids, 2% L-glutamine (200 mM), 1% penicillin/streptomycin (100×) (PAA Laboratories GmbH, Austria), and 1% sodium pyruvate (1 mM) (Sigma-Aldrich, USA) and supplemented with 10% FBS. All cell lines were maintained in an incubator (Contherm Scientific Ltd, New Zealand) at 37°C in a 5% CO_2 atmosphere with 95% humidity. Culture conditions were optimized for each cell line.

2.3. Assay for Cytotoxic Activity. Cells (1×10^5 cells/mL) were seeded in 96-well plates and left to grow overnight in humidified atmosphere containing 5% CO_2 at 37°C. On the following day, cells were treated with serial dilution of ZnO concentrations ranging from 50 μM to 10 mM. After 24 h and 96 h cell viability was measured by CellTiter 96 AQ_{ueous} Assay which uses the novel tetrazolium compound (3-(4,5-dimethylthiazol2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) (MTS, Promega, USA) using Glomax multidetection system (Promaga, USA) and read at 490 nm [31]. Results were representative of at least three independent experiments and were expressed as percentage of the value observed with a control which contained no ZnO.

2.4. Assay for Neurotoxicity. Retinoic acid (RA, Sigma, USA) will induce the differentiation of the neuroblastoma cells (SH-SY5Y) to behave like neuron-phenotypic cells [32, 33]. Approximately, 1×10^4 cells/mL were seeded in 96-well plates. After 24 h, RA was added at a final concentration of 10 μM in complete EMEM-F12 media. The medium in the plate was changed at day 3 with fresh RA and cultures were ready to be tested on day 6. ZnO nanoparticles (both groups) were tested for their neurotoxicity effect. The nanoparticles serial dilutions in EMEM-F12 were made fresh prior to each test. For neurotoxicity, the differentiated SH-SY5Y cells in each well were tested with final concentrations of ZnO nanoparticles ranging from 50 μM to 10 mM. The wells were agitated lightly and incubated for 24 h or 96 h. Following treatments, cell viability was assessed with Cell Titer 96* Aqueous Non-Radioactive Cell Proliferation Assay (MTS, Promega, USA). The MTS assay is a colorimetric assay based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3 carboxymethoxyphenyl)2-(4 sulfophenyl)-2H-tetrazolium (MTS) by dehydrogenase enzymes found in metabolically active cells. The amount of colour formazan product is proportional to the number of viable cells. Briefly, 20 μL MTS solution was added to each well and incubated in humidified incubator at 37°C in 5%

CO_2 for 2–4 h in the dark. The quantity of formazan product present was determined by measuring the absorbance at 490 nm with a microtiter plate reader (GloMax Integrated Systems by Promega, USA). Values were expressed as the percentage of optical density of control cells (nontreated).

2.5. Interference of ZnO Nanoparticles with the Cell Viability Measurements. ZnO absorption and its influence on the reading of the cytotoxicity measurements were studied. A nanoparticles-free assay was developed to investigate the direct interference of ZnO nanoparticles (Z1 or Z2) with cell viability assay. Cells (1×10^5 cells/mL) were seeded in 96-well plates and left to grow overnight. The following day, cells were treated with serial dilution of ZnO concentrations ranging from 50 μM to 10 mM. After 24 or 96 h the supernatant containing excess of ZnO nanoparticles that did not attach to the cells or culture plates was disposed and gently the cells were washed with PBS twice. 100 μL of fresh media was then added to each well followed by 20 μL MTS reagent. Thereafter, the absorbance for cell with ZnO and cell-free ZnO system was determined by reading at 490 nm wavelength of the MTS assay. Nontreated cultures were used as controls.

2.6. Interference of MTS Reagents with the Cell Viability Measurements. Cells (1×10^5 cells/mL) were seeded in 96-well plates and left to grow overnight. On the following day, cells were treated with serial dilution of ZnO concentrations ranging from 50 μM to 10 mM. After 24 or 96 h the interference of MTS reagent with the cell viability assay was assessed by measuring the absorbance of treated culture in the absence of MTS reagent. The absorbance with and without MTS was measured at 490 nm.

2.7. Cell Morphology. Interference of ZnO nanoparticles was also evident by morphological changes that appeared in cell lines. Prior to strong changes in metabolism or proliferation, cells often change their shape in response to toxic reagents. The changes in the morphology of cell lines treated with various ZnO nanoparticles concentrations for 24 and 96 hours were compared to that of corresponding cell-free ZnO and untreated cells, by confocal microscopy. Bright field images of cells were acquired with 40× objective using a Leica confocal microscope (Leica DMI 4000B, Wetzlar, Germany) equipped with a digital camera.

2.8. Statistical Analysis. GraphPad PRISM version 5.0 program was used to create the graphs. All determinations were performed at least in triplicate. Means and standard deviations were determined. All comparisons were made using two-tailed Student's t-test, ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$) using GraphPad PRISM version 5.0.

3. Results and Discussion

3.1. ZnO Characterization. The ZnO nanoparticles used are phase pure as shown in the indexed X-Ray diffraction (XRD) pattern in Figure 1. The ZnO belongs to the hexagonal crystal structure with no impurity peaks present. The morphology

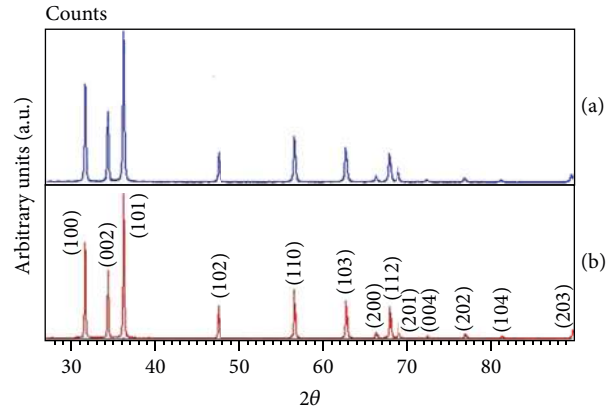


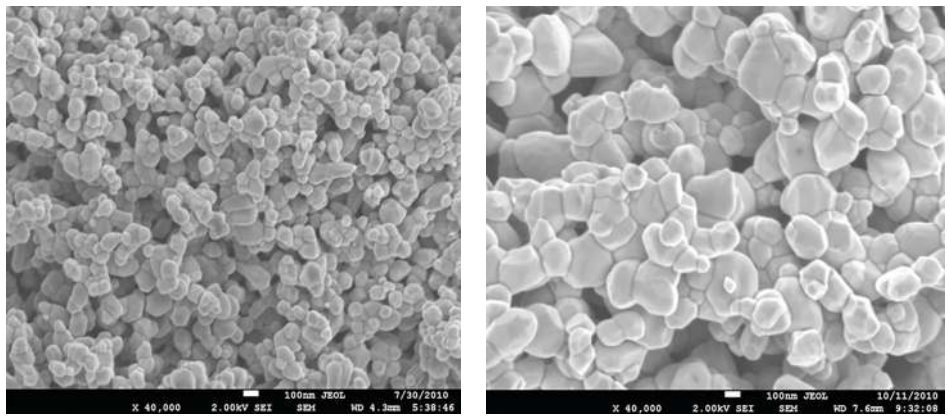
Figure 1: X-Ray diffraction patterns of ZnO samples. (a) ZnO annealed at 700°C for 30 min and (b) ZnO annealed at 700°C for 3 h.

of the ZnO samples is spherical in shape as can be seen in the scanning electron microscopy (SEM) results in Figure 2. From the micrographs, it is obvious that the particle size of the ZnO has an average size of 85.7 nm (sample Z1) and 190 nm (sample Z2).

The microstructure of the nano-ZnO can be clearly seen from the TEM images showing spherical geometries in Figure 3. The size is as estimated earlier using SEM. This is because SEM can give a wider field of view enabling measurements of size on more number of particles giving a statistically better accuracy for average size.

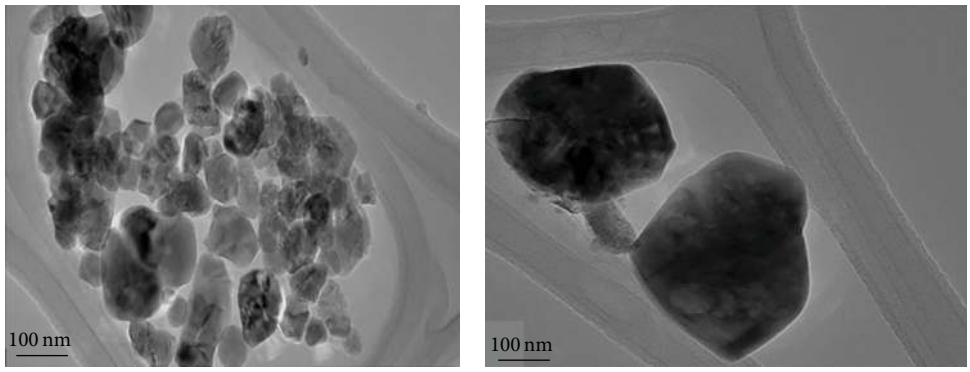
3.2. UV-Vis of ZnO. The reflectance measurement of ZnO is shown in Figure 4. It can be seen that the absorption edge of the materials is about 390 nm. The measurement for the toxicity studies is at 490 nm. Even though the absorption edge is at 390 nm, there is still about 1.3% of absorption of light at 490 nm. This absorption at 490 nm will affect the toxicity measurements made in the experiments, especially when the density of ZnO in the samples is high.

3.3. Studies of the Effects and Interference of Nanoparticles with Cytotoxicity Data. Absorbance reading at 490 nm shows an increase in cell viability compared to the control (nontreated) after treatment with ZnO nanoparticles above 1 mM concentration (Figures 8–10), which is not logical. Thus, questions arise as to the effect of the ZnO nanoparticles themselves and/or MTS reagent on the measurements for the absorbance at 490 nm. Therefore, a nanoparticles-free assay was developed to investigate the direct interference of ZnO nanoparticles with absorbance measurements and then with cell viability assay. The results show a significant decrease (P's < 0.01) in the relative percentage absorbance after removing ZnO nanoparticles from the culture system compared with the system in the presence of ZnO nanoparticles at the concentrations of more than 1 mM (Figure 5). The cells treated with ZnO nanoparticles concentrations equal or less than 1 mM do not show significant change under the ZnO



(a) (b)

Figure 2: SEM micrographs (a) of ZnO annealed at 700°C for 30 min and (b) ZnO annealed at 700°C for 3 h.



(a) (b)

Figure 3: TEM images of ZnO annealed at 700°C for (a) 30 minutes and for (b) 3 h.

and ZnO-free conditions. This implies that absorbance of the 490 nm wavelength of light is only significant for higher concentrations of ZnO.

Previous researches lead us to explore the applicability and limits of MTS assay for the evaluation of cytotoxicity data. Previously, several studies highlighted the interference of carbon nanotubes and other nanomaterials with cytotoxicity dyes, including 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide (MTT), neutral red, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1), MTS, and coomassie blue and alamar blue [34–40]. Only one publication by Ostrovesky et al. (2009) addressed the cytotoxicity effects of high concentration ranges of ZnO nanoparticles between 1.5–10 mM in glioma cell lines and normal human astrocytes [41], but the interference of ZnO nanoparticles with the absorbance measurement at 490 nm was not investigated or reported; it should be noted that they examined cytotoxicity of ZnO nanoparticles using lactate dehydrogenase (LDH) release. Our findings showed an interference of ZnO nanoparticles with absorbance readings at 490 nm at concentrations above 1 mM (Figure 5) and these results are novel.

3.4. Studies of the Effects and Interference of MTS Reagent with Cytotoxicity Data. The interference of MTS reagent with the cell viability assay was also assessed by measuring the absorbance of treated cell lines in the absence of MTS reagent. Our novel findings with regard to MTS interference demonstrated the dramatic increase (P's < 0.01) in absorbance reading at 490 nm in the absence of MTS reagents at concentrations above 1 mM compared to the control cells (no ZnO). No increase in absorbance measurements was observed below 1 mM ZnO nanoparticles (Figure 6).

This observation is suggesting that MTS reagent is not involved and the nanoparticles themselves may be interfering in the increase in absorbance reading. The results demonstrated that after the elimination of ZnO nanoparticles from the culture condition, the percentages of relative absorbance significantly decrease at the concentrations above 1 mM. This has not been reported previously.

3.5. Cell Morphology. Interference of ZnO nanoparticles was also evident by morphological changes that appeared in cell lines. The current study therefore routinely included bright field microscopic analysis of cell cultures. After 96 h

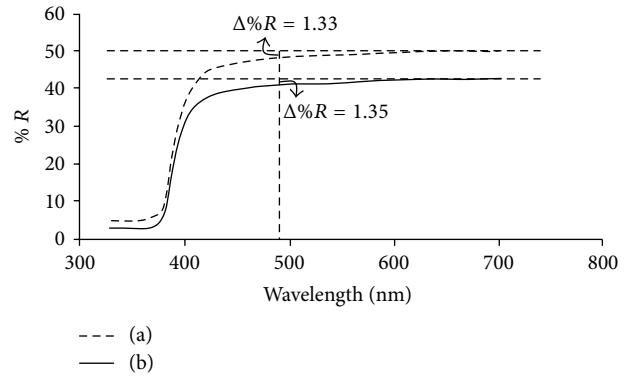


FIGURE 4: UV-Vis results of (a) ZnO annealed at 700°C for 3 h and (b) ZnO annealed at 700°C for 30 min.

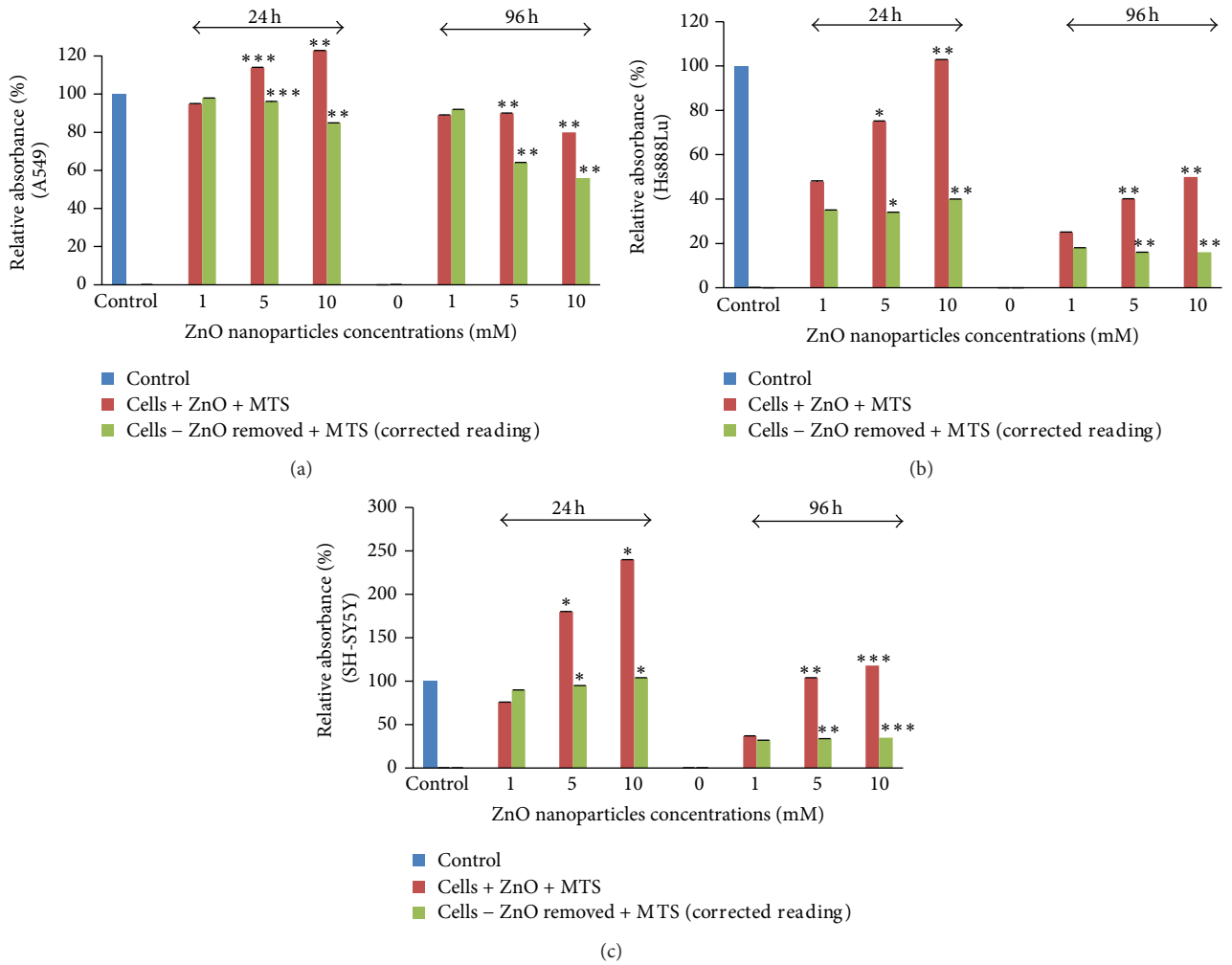


FIGURE 5: The interference of ZnO nanoparticles (85.7 nm) in absorbance reading in (a) lung cancer, (b) normal lung, and (c) neuroblastoma cells. Control represents untreated cell lines. Black bars represent cell lines treated with Z1 followed by MTS addition. Gray bars represent cell lines treated with Z1 followed by disposing ZnO and addition of MTS (corrected reading). Values are means ± SD ($n = 3$) and all comparisons were made using two-tailed Student's t-test ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

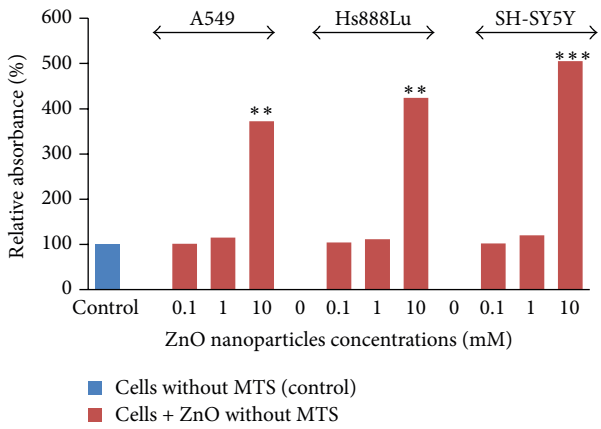


Figure 6: The absorbance measurements without MTS reagents in different cell lines. Control represents untreated cell lines. Cells were treated with Z1 for 24 h. Black bars represent measurements in the absence of MTS. Values are means ± SD ($n = 3$) and all comparisons were made using two-tailed Student's t-test ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

incubation with Z1 nanoparticles at 1 mM concentration, SH-SY5Y cells have undergone morphological changes into spherical shape, gained in volume, and formed clusters in media after detachment from cell culture plate (Figure 7(c)). Cellular shrinkage and detachment from the surface of the plate as well as increase in cell death at doses 1 mM and 5 mM were also observed in normal lung cells and neuron-like cell (not shown). In contrast, lung cancer (A549) cell lines treated with 1 mM of ZnO nanoparticles (Z1) and incubated for 96 h exhibited no visible morphological changes (Figure 7(d)).

The numbers of dead cells increase with increasing nanoparticles concentration to 5 mM in both SH-SY5Y and A549 cells (Figures 7(e) and 7(f)). While a significant increase in absorbance reading at wavelength of 490 nm was observed at selected concentration (Figures 8(a) and 9(a)).

Figures 7(g) and 7(h) show the dramatic decrease in the cell number after removing ZnO nanoparticles and dead cells from the culture system compared with the system in the presence of ZnO particles at the concentration of 1 mM (Figures 7(c) and 7(d)). The absorbance readings dropped dramatically in the sample where ZnO nanoparticles have been removed (Figure 5).

In this study we found out that no effects are observed for lung cancer (A549) cell lines exposed to 1 mM of ZnO nanoparticles, Z1 and Z2 (85.7 nm group and 190 nm group, resp.), and incubated for 24 or 96 h. In contrast, other studies have described that the cell viability in A549 cell lines was reduced by 75–80% between 18 and 25 μg/mL (0.2 and 0.3 mM) with ZnO particles (70 and 420 nm) between 6 and 12 h [23]. The more pronounced toxicity was observed at 24 h [23]. Recently, Kim and coworkers (2010) reported that ZnO nanoparticles exhibited the cytotoxicity in terms of cell proliferation, cell viability, and membrane integrity in A549 cells [22].

We have shown that Z1 nanoparticles did induce approximately 50–70% decrease in cell survival in normal lung cells Hs888Lu at 1 mM after 24 and 96 h, respectively. ZnO nanoparticles activity has not been reported previously in such cells by any research group. While, Kim and coworkers (2010) demonstrated the cytotoxic effect of ZnO nanoparticles in another normal lung cell types (L-132).

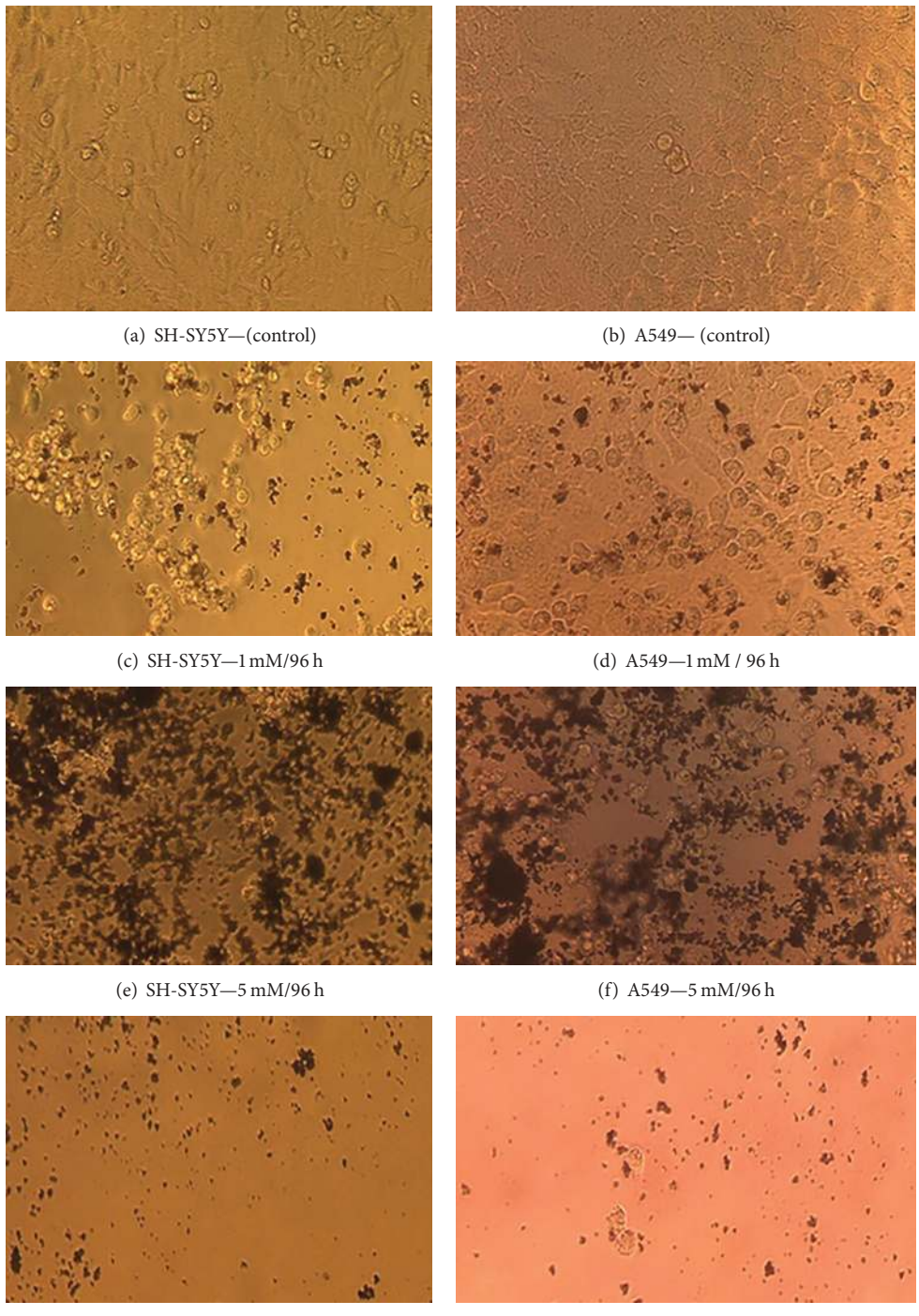
3.6. ZnO Cytotoxicity and Neurotoxicity. It is known that different cell lines might exhibit different sensitivities towards zinc oxide nanoparticles [2, 20–28], so the use of more than one cell line is therefore considered necessary in the evaluation of antiproliferative nanoparticles.

Cell lines are treated with ZnO nanoparticles concentrations ranging from 50 μM to 10 mM of average particle sizes 85.7 nm (Z1) and 190 nm (Z2) and incubated for 24 or 96 hours. Our data indicated that exposure to either size of ZnO nanoparticles samples Z1 and Z2 induced different toxic effects in the human cell lines tested in this study (Figures 8–10). ZnO nanoparticles do not show toxicity in lung cancer cells A549, and they almost maintain 90–100% cell viability up to 1 mM concentration after exposure to either size of ZnO nanoparticles for 24 or 96 h (Figure 8(a)).

We further examined the cytotoxic effect of the ZnO nanoparticles in normal lung cells Hs888Lu. Cells were exposed to both particle sizes Z1 and Z2 for 24 and 96 h (Figure 8(b)). It seems that the difference in particle sizes behaves differently in normal lung tissue. Z1 particles induced almost 50% decrease in cell viability after 24 h incubation at 0.8 mM and 1 mM concentrations (P's < 0.01). Some cytotoxic effect was already observed after 96 h treatment with the same particles at 0.2 mM (data not shown), but the most pronounced effect was observed between 0.8 mM to 1 mM concentrations with 70% (P's < 0.01) viability loss (Figure 8(b)). Z2 particles were less potent in Hs888Lu cell lines, reducing viability by 20% after 96 h treatment at 0.8 mM and 1 mM concentrations (P's < 0.05), while no effects were observed after 24 h incubation.

The cytotoxic responses of different cell lines to ZnO nanoparticles were found to be different. To assess if ZnO nanoparticles may exert cytotoxicity on human neuron cells similar to those of cancer and normal lung cells, we also studied the cytotoxicity and neurotoxicity effects of ZnO nanoparticles on neuroblastoma (SH-SY5Y) and neuron-like cells (differentiated SH-SY5Y). Exposure of SH-SY5Y and differentiated SH-SY5Y cells to ZnO nanoparticles Z2 for 24 h at 1 mM concentration induces a decrease in cell survival to 60% and 80% (P's < 0.01), respectively (Figure 9). The longer incubation for 96 h significantly decreases the cell survival to less than 50% in both cell lines at 0.8–1 mM concentrations (P's < 0.01).

It seems that smaller particles average size of 85.7 nm (Z1) has exhibited more cytotoxic activity in both cell lines, where potent cytotoxic effect had been observed at a low concentration of 0.4 mM with Z1 nanoparticles. Some effects of Z2 were observed at 0.8 mM in both SH-SY5Y (P's < 0.01) and differentiated SH-SY5Y (P's < 0.05) cell lines after 96 h. Exposure of these cells to Z1 particles for 96 h at concentrations ranging between 0.4 mM and 1 mM induced a decrease (P's < 0.01) in cell survival to almost 30–40% (Figure 9).



(a) SH-SY5Y—(control)

(b) A549— (control)

(c) SH-SY5Y—1 mM/96 h

(d) A549—1 mM / 96 h

(e) SH-SY5Y—5 mM/96 h

(f) A549—5 mM/96 h

(g) SH-SY5Y—5 mM/96 h (ZnO disposed)

(h) A549—5 mM/96 h (ZnO disposed)

Figure 7: Morphological changes of A549 and SH-SY5Y cell lines after 96 h incubation in Z1 (85.7 nm) nanoparticles: (a) and (b), control cells in regular cell culture (no nanoparticles); (c) and (d), cell lines treated with 1 mM of Z1 nanoparticles; (e) and (f), cell lines treated with 5 mM of Z1; (g) and (h), cell lines treated with 5 mM of Z1 followed by disposing ZnO.

Chen and colleagues reported that ZnO and iron oxide (Fe_2O_3) nanoparticles do not induce significant decrease of cell viability in SH-SY5Y cell lines at a concentration range of 0.01–100 μM for 48 h [20]. This is in agreement with our finding for SH-SY5Y and differentiated SH-SY5Y, where no effects were observed after exposure to ZnO nanoparticles at concentration 100 μM for 24 and 96 h (data not shown). From our literature review no studies were reported on nanoparticles cytotoxicity in neuron-like (differentiated SH-SY5Y) cell lines.

Another human cell line from different histological origin, lymphoma (U937), was tested for its sensitivity to

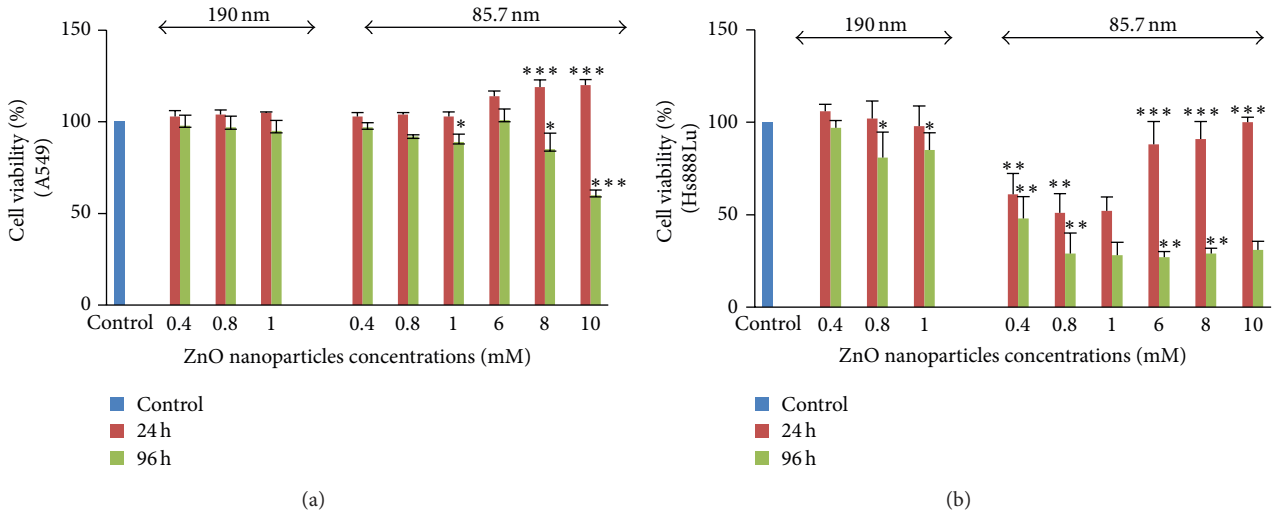


FIGURE 8: Cytotoxicity effects of ZnO nanoparticles, Z1 (85.7 nm) and Z2 (190 nm), after 24 or 96 h incubation in (a) lung cancer cell lines (A549) and (b) normal lung (Hs888Lu) cells. Control represents untreated cell lines. Values are means ± SD ($n = 3$) and all comparisons were made using two-tailed Student's t-test ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

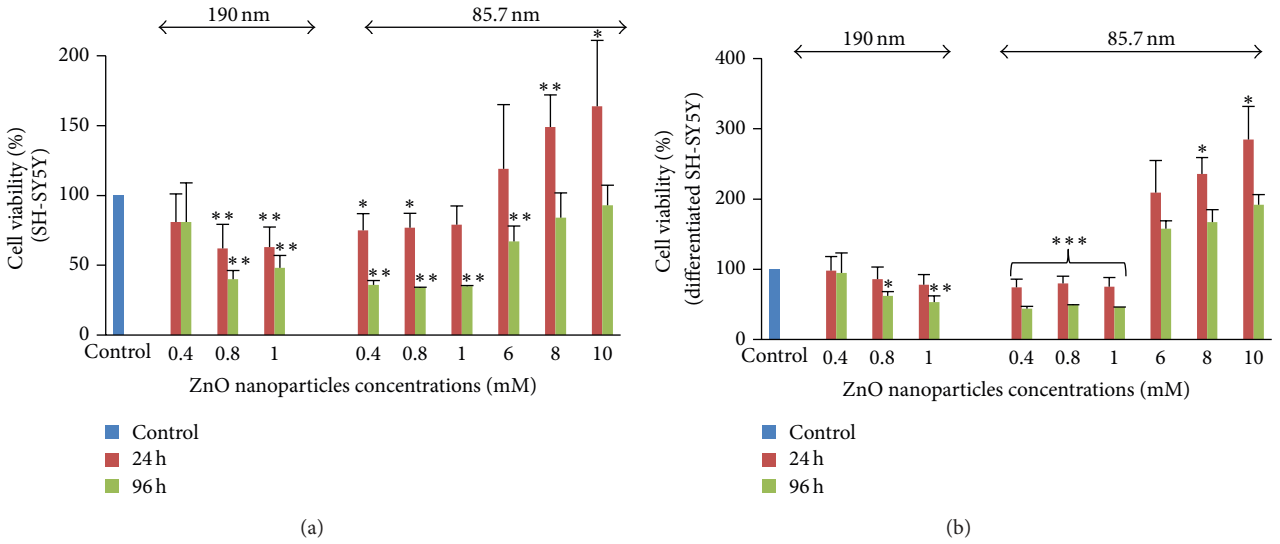


FIGURE 9: (a) Cytotoxicity effects of ZnO nanoparticles Z1 (85.7 nm) and Z2 (190 nm) in SH-SY5Y cells; (b) neurotoxicity effects of Z1 and Z2 on cell viability in differentiated SH-SY5Y cell lines after 24 or 96 h incubation. Control represents untreated cell lines. Values are means ± SD ($n = 3$) and all comparisons were made using two-tailed Student's t-test ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$)

ZnO nanoparticles. The lymphoma cell lines treated with ZnO nanoparticles were under the same conditions as described above for the other cell lines. U937 cell lines almost maintain 95–100% cell viability up to 1 mM concentration after exposure to ZnO nanoparticles Z1 and Z2 for 24 h (Figure 10). While the longer incubation for 96 h significantly decreases the cell survival to 40–50% with either size of ZnO nanoparticles at concentrations ranging between 0.8 and 1 mM (P's < 0.01). Cytotoxicity of ZnO nanoparticles of either size did not show significant differences from each other in the U937 cell lines at concentrations ranging between 0.8 and 1 mM. From our literature review no studies were reported on nanoparticles cytotoxicity in lymphoma (U937) cell lines.

3.7. Particles Size and Duration of Exposure. It has been demonstrated that different particle size of the ZnO may have distinctly different physical properties and behave differently in cytotoxic studies. Our results show that smaller particles (85.7 nm) have more cytotoxic activity in cell lines tested (Hs888Lu, SH-SY5Y, and differentiated SH-SY5Y). While ZnO nanoparticles do not show toxicity in lung cancer (A549) and lymphoma (U937) cell lines after exposure to either size of ZnO nanoparticles. This is in agreement with the study demonstrated by Lin and coworkers (2009) stating that cell viability was not particle size dependent at all particle diameters (70 and 420 nm) in A549 cell lines at 24 h incubation. The size dependent results of our work can be explained by the fact that for small particle size ZnO, diffusion

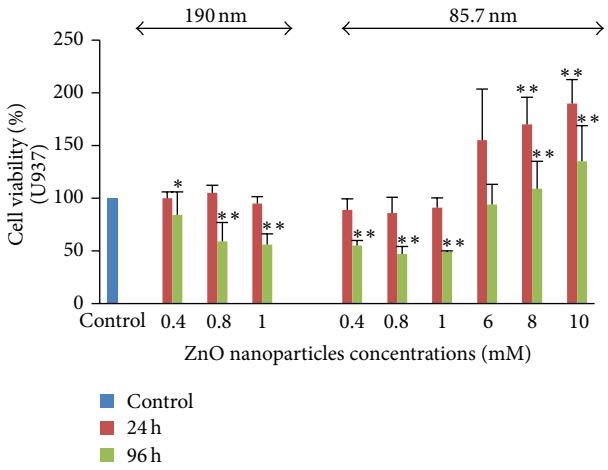


FIGURE 10: The response of lymphoma cells (U937) exposed to ZnO nanoparticles Z1 (85.7 nm) and Z2 (190 nm) for 24 or 96 h. Control represents untreated cell lines. Values are means ± SD ($n = 3$) and all comparisons were made using two-tailed Student's t-test ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

into the cells will be facilitated and thus the detrimental effect on them more pronounced.

The duration of exposure is one of the important parameters in any *in vitro* cytotoxicity assay. Thus, longer exposure periods may be necessary for screening the effects of metal oxide nanoparticles. The results in Figures 8–10 prove that the longer the incubation time with ZnO nanoparticles is (either group) the more harmful the effect to the cell lines is. This is in agreement with previous studies that revealed a time dependent cytotoxicity of A549 [23], human epidermal (A431) [21], human mesothelioma MSTO-211H, and rodent 3T3 fibroblast cells [42] on exposure to ZnO nanoparticles. Similar cytotoxic effects were obtained in the glioma cell lines (LN18, LN229) and normal human astrocytes, treated with 10 mM ZnO nanoparticles for 24 and 72 h [41].

4. Conclusion

The data from the present study demonstrate that the presence of ZnO at high concentrations affected the cytotoxicity measurements due to the absorption characteristic of ZnO nanoparticles. The data revealed that the ZnO nanoparticles with an average particle size of around 85.7 nm and 190 nm induced cytotoxicity towards U937, SH-SY5Y, differentiated SH-SY5Y, and Hs888Lu cell lines. The ZnO nanoparticles were found inactive in lung cancer cell line (A549). Moreover, our data have also indicated that the cytotoxicity of ZnO was particle size, concentrations, and time dependent. Hence, the results of this study demonstrated that a ZnO nanoparticle at concentrations above 1 mM has a profound influence on the cytotoxic effects of nanoparticles in different cell lines. Further studies will attempt to investigate the underlying mechanisms of this phenomenon.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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