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RESEARCH ARTICLE

Evaluation of *In Vitro* Cytotoxicity of Zinc Oxide (ZnO) Nanoparticles Using Human Cell Lines

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Abstract

The aim of the present study was to evaluate the *in vitro* toxicity of Zinc Oxide (ZnO) nanoparticles against human alveolar epithelial (A549) cells and human embryonic kidney cells (HEK cells). The toxic effects of nanoparticles were analyzed after 24 hours of incubation with different cell lines using trypan blue dye exclusion method. Incubation of Zinc Oxide (ZnO) nanoparticles with different cells produced a dose dependent inhibition of growth of the cells. The TC50 values (toxic concentration 50 i.e. concentration of particles inducing 50% cell mortality) of Zinc Oxide (ZnO) nanoparticles were found in the range of 33 - 37 µg/ml.

Keywords

Zinc Oxide (ZnO) nanoparticles, A459, HEK, Cytotoxicity, *In vitro*

Introduction

Nanoparticles (range of 1-100 nm) have possessing a much higher specific surface area (SSA) than their larger counterparts of the same materials Thus, Nanoparticles (NP) exhibit specific physicochemical properties and functions [1]. ZnO nanoparticles have received considerable attention in recent years because of their potential use in electronic and industrial applications. Nanoscale ZnO materials are already being used in the cosmetic and sunscreen industry due to their transparency and ability to reflect, scatter, and absorb UV radiation [2]. ZnO nanomaterials are also being considered for use in next-generation biological applications including antimicrobial agents, drug delivery, bioimaging probes, and cancer treatment [3,4]. ZnO nanoparticles are useful as antibacterial and antifungal agents when incorporated into materials, such as surface coatings (paints), textiles, and plastics. ZnO nanoparticles used as an additive into products including plastics, ceramics, glass, cement, rubber (e.g. car tyres), lubricants, paints, ointments, adhesives, sealants, pigments, foods (source of Zn nutrient), batteries, ferrites, fire retardants, etc. The increasing potential applications of zinc oxide nanoparticles provide strong impetus to investigate potential toxic effects of these materials. In occupational settings these nanoparticles get released as aerosol and may inhaled, ingested by the persons who are working there. We have previously reported the pulmonary and extrapulmonary toxicity of ZnO nanoparticles using rat intratracheal instillation model [5]. Kaviyarasu, et al. [6] reported the cytotoxic effects of cadmium selenide nanoparticles using A549 cell lines. Kaviyarasu, et al. [7] also reported the *In vitro* cytotoxicity effect and antibacterial performance of human lung epithelial cells A549 activity of Zinc oxide doped TiO_2 nanocrystals. Recently, Jia-Hui, et al. [8] reported the *in vivo* low toxicity of ZnO nanoparticles in mice. In support of *in vivo* studies, the present study was aimed to evaluate the *in vitro* cytotoxicity of zinc oxide nanoparticles using A549 and HEK cells.

Materials and Methods

Particle types-characterization

Carbonyl iron (4.5-5.2 ·m; > 98% purity) and Quartz fine powder (> 230 mesh; 58-68 µm; 99.94% purity) and were obtained from Sigma, USA and SD fine chemicals, Mumbai, India, respectively. ZnO nanoparticles obtained from Sigma Aldrich, St. Louis, USA. Size and crystallinity are determined by Dynamic Light Scattering

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Table 1: Characterization of ZnO Nanoparticles.

Nanoparticles	Size (nm)	Surface area (m^2/g)	Diameter (nm)	Length (nm)	Shape
ZnO	< 50 nm	> 10.8 m^2/g	< 50 nm	< 50 nm	crystalline

Table 2: Cytotoxicity of Zinc Oxide (ZnO) nanoparticles (Trypan blue assay).

Nanoparticles	Cell Type	TC_{50} (µg/ml)	TC_{25} (µg/ml)	TC_{75} (µg/ml)
ZnO	**A549 cells**	35.6	19.8	58.4
ZnO	HEK cells	33.5	16.8	49.22
Quartz	**A549 cells**	30.5	13.6	44.6

Spectroscopy (DLS), and X-Ray Diffraction (XRD), respectively (Table 1).

Experimental set-up

To evaluate the cytotoxicity of ZnO nanoparticles, two different human cell types, alveolar cells (A549 cells), human embryonic kidney cells (HEK cells) were employed using trypan blue assay. The toxic effects of nanoparticles were analyzed after 24 hours of incubation with these two cell lines.

Cell culture and treatment

Human alveolar epithelial (A549) cells and human embryonic kidney cells (HEK cells) were purchased from National centre for cell service (NCCS, Pune), India. These cell lines were grown and maintained using suitable media (DMEM/RPMI 1640, HiMedia, Mumbai, India). All the cell lines were grown in culture medium supplemented with 10% foetal bovine serum (FBS, HiMedia, Mumbai, India), 1% L-glutamine (HiMedia, Mumbai, India) and 1% penicillin-streptomycin-amphotericin B antibiotic solution (HiMedia, India). Cells were seeded in 25 cm^2 tissue culture flasks (tarsons, India), at 2,50,000 cells/flask in a total volume of 9 ml. When confluent, all the cells were trypsinized (using Trypsin-EDTA, HiMedia, Mumbai, India), and seeded in 6-well plates (tarsons, India) at a rate of 1,50,000/0.3 ml. Twenty-four hours after seeding, cells were washed 3 times with culture medium without any additive (FBS or antibiotics), and particle suspension (in phosphate buffer saline, (PBS) + 0.1% between 80) or medium alone was added to each well. For each nanomaterial, a stock solution of 1000 µg/ml particle in culture medium without any additive was prepared, vortex at maximum speed for 1 minute and bath-sonicated for 5 minutes. Different concentrations of nanoparticles in culture medium were prepared and used (100 - 1 µg/ml). Preliminary experiments demonstrated the necessity to add 0.1% Tween 80 to the culture medium to obtain a homogenous suspension for two nanoparticles. Cells were exposed for 24 h to medium alone or in presence of nanomaterials. At that time, trypan blue viability assay (see below) was performed to evaluate the toxicity of nanoparticles on different cell types.

Trypan blue dye exclusion method

Trypan blue dye exclusion method was used particularly to avoid the interference of nanoparticles during the assay procedure of absorbance [9,10]. For this assay, 6 well plates were used. At the end of the exposure (24 hours) of nanoparticles to cells, the medium was discarded and all the wells were washed with 200 µl Phosphate Buffer Solution. Now, all the cells were trypsinized and to this (100 µl) cell suspension, 100 µl of 0.4 w/v trypan blue (sigma, USA) was added and mixed well. After 5 min, an aliquot (10 µl) of dye-cell suspension mixture was loaded onto hemocytometer by carefully touching the edge of the cover slip with the pipette tip and each chamber is allowed to fill by capillary action. Viable cells were counted in all four 1 mm corner squares. A separate count was maintained for viable and non-viable cells. The % inhibition of growth was calculated by comparing the % viability in the well with test compound with that of the control.

Statistical analysis

When at least 2 viability values were below 50% of control condition, the TC50 (toxic concentration 50, concentration of particles inducing 50% cell mortality) was calculated using GraphPad Prism software (logarithmic transformation of X-values and non-linear regression - sigmoidal dose-response analysis with variable slope-with bottom and top constrains set at 0 and 100 respectively). If a TC_{50} could be calculated, TC_{25} and TC_{75} were calculated (respectively concentration corresponding to 75 and 25% viability), using the following equation:

$$TCf = [(f/100\text{-}f)^{**}1/H]^{*}TC_{50}$$

Where f: percentage that needs to be calculated; H: hillslope; *: multiply; **: to the power.

Results

The cytotoxicity data of ZnO nanoparticles using trypan blue dye exclusion assay on 2 different human cell lines are presented in Table 2.

Similar to the quartz (a known toxic agent), exposure of ZnO nanoparticles to the different cells produced a dose dependent inhibition of growth of cells, resulting in reduction in % viability of the cells in nanoparticles exposed wells. For all the nanoparticles TC_{50}, TC_{25} and TC_{75} values (respectively concentration corresponding to 50, 75 and 25% viability) were calculated on all the cell types and were shown in Table 2.

Discussion

In this study, the *in vitro* cytotoxicity of ZnO nanopar-

ticles against two different (A549, HEK cells) human cell lines using trypan blue dye exclusion assay method to assess the systemic toxicity of these nanoparticles after their exposure (by inhalation/aspiration, ingestion etc) to humans. The results of the present study showed the dose dependent cytotoxicity of nanoparticles against all cell types tested the TC_{50} values of ZnO nanoparticles were found in the range of 33 - 37 µg/ml, which were comparable to that of quartz (30.5 µg/ml), indicating the greater cytotoxicity.

The solubility of ZnO NPs is the pivotal factor in causing cytotoxicity *in vitro*. Zn^{2+} at a low concentration is essential for maintaining the cellular processes and metabolism; however, Zn^{2+} at a high concentration can cause toxicity [11]. ZnO NPs can induce a cell death via apoptosis through the intrinsic mitochondrial pathway [11,12]. ZnO NPs treatment *in vitro* has been shown to decrease mitochondrial membrane potential and conversely increase Bax/Bcl2 ratio. Moreover, excessive release of Zn^{2+} was shown to be sequestered by the mitochondrion [13]. Presence of cations aids to regulate ATP synthesis and ROS production in mitochondrion. Conversely, the rapid Zn^{2+} influx results in a rapid decline of mitochondrial membrane potential which subsequently activates the caspase-dependent apoptosis and release of LDH [11]. The present study supports the results of Cheng, et al. [14] which showed that ZnO NPs can cause toxicity by ER stress, cytotoxicity and genotoxicity, which are closely associated with ROS generation and inevitably lead to cell death *in vitro.*

Conclusion

The results of the study revealed the dose dependent cytotoxicity of zinc oxide nanoparticles using tested cell cultures which supports the results of *in vivo* toxicity studies of these nanoparticles.

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