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Research Article

Induction of Cytotoxicity by Selected Nanoparticles in Chinese Hamster Ovary-K1 Cells

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Abstract

The aim of the present study is to analyze the cytotoxicity of selected nanoparticles on Chinese Hamster Ovary-K1 (CHO-K1) cells using methyl tetrazolium (MTT) assay and lactate dehydrogenase (LDH) release assay. Four different metal oxide nanoparticles namely silicon dioxide (SiO_2-NPs, 1 nm), aluminium oxide (Al_2O_3-NPs, 16.7 nm), titanium dioxide (TiO_2-NPs, 11.4 nm) and iron oxide (Fe_3O_4-NPs, 15.65 nm) were exposed to CHO-K1 cells at 25, 50, 75 and 100 µg/ml concentrations for 24 h maintaining the control group. The percentage of cell viability using methyl tetrazolium (MTT) assay showed significant reduction in cell viability from 63.82 to 31.19% in SiO_2-NPs, 96.68 to 34.14% in Al_2O_3-NPs, 65.69 to 14.32% in TiO_2-NPs and 120.69 to 59.86% in Fe_3O_4-NPs when compared with the untreated cells. Assessment of cytotoxicity by using lactate dehydrogenase (LDH) release assay revealed that Al_2O_3–NPs showed more cytotoxicity followed by Fe_3O_4-NPs, TiO_2-NPs and SiO_2-NPs in concentration-dependent manner. Therefore, it is reasonable to conclude that size and the composition of the nanoparticles could contribute to the relative cytotoxicity in CHO cells.

Keywords: nanoparticles; cytotoxicity; CHO-K1 cells; MTT; LDH.

Introduction

Nanotechnology is the fastest growing branch of science with a wide range of utility and applications. The use of nanoparticles ranges from industrial, pharmaceutical and electrical to biomedical and personal care products. Nanoparticles were present in the nature as natural nanoparticles and man began to synthesize nanoparticles from the pre-civilisation period itself. However, during the last two decades, the production and application increased to large extend and a new branch called nanotoxicology has emerged to meet the adverse effects caused by the engineered nanoparticles. The toxicity of nanoparticles mainly depends upon the physicochemical properties, dose, route and duration of exposure (Oberdorster *et al*., 2005, Klien *et al*., 2012). The toxic effects and the mechanism of toxicity of nanoparticles are highly complicated and incomparable in many aspects.

Organisms are in continuous contact with the nanoparticles and the entry of nanoparticles inside the cell follows three routes namely simple diffusion, through ion channel and by endocytosis (Rappoport *et al*., 2011). Nanoparticles get accumulated into the body of exposed organisms and induce various adverse effects. Both *in vitro* and *in vivo* studies are widely used to evaluate the cytotoxicity and genotoxicity of nanoparticles. The cytotoxicity of nanoparticles can be generalized as impairment of cellular metabolism, membrane damage and nuclear anomalies like DNA damage or cell death (Singh *et al*., 2010). Impairment of cell cycle and altered gene expression are shown by iron oxide nanoparticles *in vitro* (Singh *et al*., 2009, Lie *et al*., 2013). Copper oxide nanoparticles has been shown to cause decreased cell viability, and dose-dependent increase in the DNA damage and oxidative damage in Murine macrophages as well as in peripheral blood lymphocyte culture (Bucchianico *et al*., 2013). Aluminium oxide and titanium oxide nanoparticles have been reported to cause cytotoxicity and genotoxicity in dose-dependent manner in CHO-K1 cell lines (DiVirgilio *et al*., 2010). The internalization of nanoparticles and its persistence inside a cell varies with cell types; therefore, the toxic potential of nanoparticles also greatly varies among the cell type (Bahadar *et al*., 2016).

In the present study, the cytotoxicity of metal oxide nanoparticles such as aluminium oxide (Al_2O_3-NPs), silicon dioxide (SiO_2-NPs), titanium dioxide (TiO_2-NPs) and iron oxide (Fe_3O_4-NPs) are compared in Chinese Hamster Ovary

(CHO-K1) cell lines. Al_2O_3-NPs have wide application in biological systems as biosensors, biofiltration, drug delivery and antigen delivery for immunization purposes (Prakash *et al*., 2011). SiO_2-NPs have been shown to enter the nucleus and result in aggregation of intra nucleus proteins, inhibiting DNA replication, transcription and translation (Chen and Von, 2005). TiO_2-NPs coated with a corona of adsorbed serum proteins has been shown to induce oxidative stress response and cytotoxicity in different cell lines (Runa *et al*., 2014). Based on the surface coating and particle size Fe_3O_4-NPs have been shown to possess cytotoxicity, genotoxicity, neurotoxicity and developmental toxicity (Valdiglesias *et al*., 2015). Nanoparticles possess diverse applications in human, despite its target specific health risk continues to grow as a huge concern to human health. *In vitro* cytotoxicity of nanoparticles is evaluated using different cell lines, incubation period and several assays. The present study focused to test the cytotoxicity of four metal oxide nanoparticles in Chinese Hamster Ovary (CHO-K1) cell lines using methyl tetrazolium (MTT) and lactate dehydrogenase (LDH) release assay, as it is recognised as the standard measures to determine the cytotoxicity of nanoparticles. To date, there is lack of data regarding the comparative cytotoxicity of the selected nanoparticles using CHO-K1 cell lines. Therefore, the present study is targeted to evaluate the nanotoxicity as well as to compare the nanotoxic potential of selected nanoparticles based on the size and composition by using simple, rapid and the most sensitive methods.

Materials and Methods

Nanoparticles

TiO_2-NPs (Cat. No: 634662, 11.4 nm, Titanium (IV) oxide, mix of anatase and rutile) and Fe_3O_4-NPs (Cat. No. 637106, 15.65 nm, iron oxide) were obtained from Sigma Aldrich, Germany. Al_2O_3-NPs (Cat. No: 0140408, 16.7 nm, aluminium oxide) and SiO_2-NPs (Cat. No: 1940323, 1 nm, silicon dioxide) were obtained from SISCO Research Laboratory (SRL), India. The purity and size of the nanoparticles are further confirmed by X-ray diffraction and Transmission Electron Microscopy. The nanodispersions were prepared just before exposure by ultra-sonication at 100 kHz for 30 min (except SiO_2 for 10 min) using double distilled water and maintained as stock.

Cell Culture

Chinese Hamster Ovary (CHO-K1) cell line was purchased from National Centre for Cell Sciences (NCCS), Pune, India and maintained in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, Invitrogen). The cell line was cultured in T-25 culture flask with DMEM supplemented with 10% foetal bovine serum, L-glutamine, sodium bicarbonate and antibiotic solution containing penicillin (100U/ml), streptomycin (100µg/ml), and amphoteracin B (2.5µg/ml). Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, then 100µl cell suspension ($5x10^4$ cells/well) was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany).

Preparation of Nanoparticles for Exposure

From the prepared stock of nanodispersions, the particles were dispersed in cell culture medium to a concentration of 1mg/ml. CHO-K1 cells were then plated into a 96-well plate at a density of 5×10^4 cells/well. The concentrations of nanoparticles were selected as 25, 50, 75 and 100 µg/ml based on the previous report on cytotoxicity (Awasthi *et al*., 2015) and maintained for 24 h. Cells free of nanoparticles were used as control cells throughout each assay.

MTT Assay

CHO-K1 cells were treated with 25, 50, 75 and 100 µg/ml concentrations of selected nanoparticles for 24 h. After the treatment period, the medium was changed and cells were incubated with 30 µl of reconstituted MTT (Sigma–Aldrich, St. Louis, MO, USA) under normal culture conditions for 4 h. Cell viability was marked by the conversion of the tetrazolium salt, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide) to dark-blue coloured formazan by mitochondrial dehydrogenases using the method as described by Mosmann (1983). Colour development was measured photometrically in a microplate reader at 570 nm after cell lyses in DMSO (100 µl/well) against the blank.

Lactate Dehydrogenase (LDH) Leakage Assay

LDH assay was performed according to the method as described by Decker and Lohmann-Matthes (1988). Briefly, to the supernatant of the treated and control cultures, phosphate buffer (100mM; pH 7.4) sodium pyruvate (30mM) and NADH (6.6mM) were added. Then the absorbance was read in microplate reader immediately and after 5 min at 490 nm to determine LDH activity. The amount of LDH released is proportional to the number of cells damaged or lysed.

Statistical Analysis

All experiments were performed in triplicates for the accuracy of the results. Statistical analysis of the data was performed using the statistical package SPSS 17.0. Students t-test was used to determine the statistical significance where $P<0.05$ is set significant against the control sample.

Results and Discussion

The increased use of metal oxide nanoparticles in the industrial and medical field expanded great concern on their potential impact on the environment and human health. Nanoparticles are synthesised and released continuously into the environment and owing to its small size it possess the ability to readily diffuse through the protective cellular barriers which results in possible toxic impacts on the exposed organisms. Recently, *in vitro* studies are gaining more attention in cytotoxic evaluation of nanoparticles

because cell line studies are cost effective, time saving, easy to handle, comparable, reproducible and highly acceptable method. In the present study evaluation of cytotoxicity of selected metal oxide nanoparticles at four different concentrations were tested in Chinese Hamster Ovary (CHO-K1) cell lines. The present observations demonstrated that when CHO cell lines incubated with different concentrations of metal oxide nanoparticles showed less cell viability and high cytotoxicity at increasing concentrations.

MTT tetrazolium assay is the most prominent method adopted in laboratory for evaluation of cell viability. MTT assay is based on the mitochondrial conversion of tetrazolium salt into formazan and this conversion occurs only in living cells. MTT is a positive compound that can readily enter into the viable cells which require the incubation of a reagent with cell culture (Mosmann 1983). Therefore, it is the direct measure of cell viability in toxicological studies. Viable cells convert the reagent into a colour or a fluorescent product, which is detected photometrically. The percentage of cell viability assessed using methyl tetrazolium assay (MTT) showed significant concentration-dependent decrease from 63.82 to 31.19% in SiO_2-NPs, 96.68 to 34.14% in Al_2O_3-NPs, 65.69 to 14.32% in TiO_2-NPs and 120.69 to 59.86% in Fe_3O_4-NPs at the concentrations from 25 to 100µg/ml (Fig. 1). In the present study, the percentage of cell viability decreased in the order of TiO_2, SiO_2, Al_2O_3 and Fe_3O_4 and the result revealed that the decrease in cell viability of metal oxide nanoparticles could be due to the increased internalization of nanoparticles. Nanoparticles with almost similar properties may give differential cellular uptake and cellular dysfunction. Nanoparticles have been shown to cross the plasma membrane and get accumulated inside various mammalian cell lines (Prakash *et al*., 2011, Saquib *et al*., 2012, Willman *et al*., 2012).

LDH assay is another method that determines the leakage of the cellular enzyme lactate dehydrogenase, through the damaged plasma membrane. Assessment of cytotoxicity by using lactate dehydrogenase (LDH) release assay revealed 4.5 fold increase in the cytotoxicity in Al_2O_3–NPs, 3 fold increase in Fe_3O_4-NPs, 1.9 fold increase in TiO_2-NPs and 1.8 fold increase in SiO_2-NPs, respectively in concentration-dependent manner (Fig. 2). The present result showed the dose-dependent increase in cytotoxicity in the order of Al_2O_3, Fe_3O_4, TiO_2 and SiO_2 from 25 to 100 µg/ml concentrations and the percentage of cytotoxicity is based on the particle size. Al_2O_3 and TiO_2 nanoparticles have reported with internalization and cytotoxicity in CHO cells where it has been shown to induce vesicle formation and got trapped inside the vesicles along with free nanoparticles observed inside the cell (DiVirgilio *et al*., 2010). It is believed that nanoparticles with smaller size can easily enter into the biological system and the resulting cytotoxicity or genotoxicity is size-dependent (Balasubramanyam *et al*., 2009). However, titanium dioxide nanoparticles even in range of 150 nm can also internalized and elicit toxic responses as DNA damage and genetic instability (Trouiller *et al*., 2009). It has been reported that TiO_2–NPs of higher dose of 100-250 µg/ml elicit more toxicity than at lower doses of 10-50 µg/ml in BRL3A rat liver cells (Hussain., 2005). Therefore, apart from the particle size certain properties of nanoparticles like composition, stability, solubility, surface area etc also account for cytotoxicity.

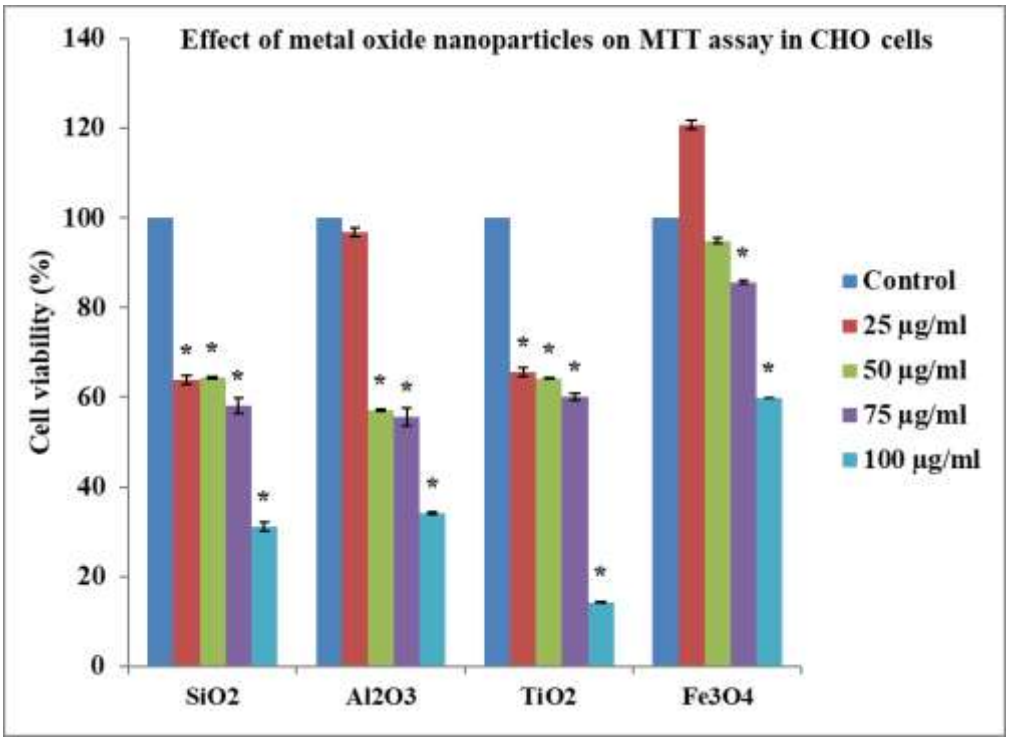

Fig. 1: Effect of metal oxide nanoparticles on MTT assay in CHO cells.

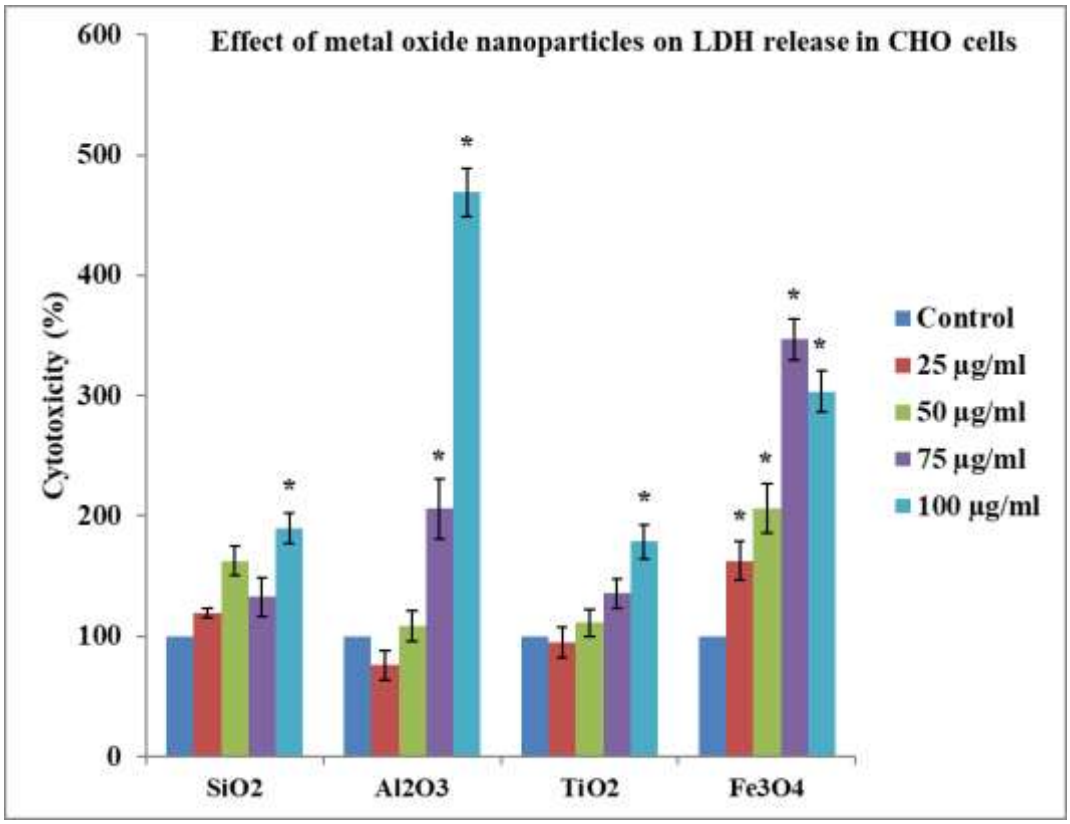

Fig. 2: Effect of metal oxide nanoparticles on LDH release in CHO cells

Toxicity of metal oxide nanoparticles has been mainly due to the disruption the antioxidant system and increase in oxidative stress (Manke *et al*., 2013; Afifi *et al*., 2015; Vidya and Chitra, 2015). Increased cellular level oxidative stress results in free radical mediated membrane damage including mitochondrial and plasma membrane which results in the damage of cellular protein, lipids and DNA and finally leads to cell death and dysfunction of electronic chain (Huang *et al*., 2010; Ramkumar *et al*., 2012). The cytotoxicity observed in the present study could be due to oxidative stress-mediated cellular damages. Likewise, the increased cytotoxicity shown by all nanoparticles in a dose-dependent manner may be due to the leakage of cellular enzymes including lactate dehydrogenase through the damaged plasma membrane. Different factors like size, shape and composition of nanoparticles, internalization, metabolism and excretion by the tissue or cell type may account for the differential toxicity of nanoparticles (Yoo *et al*., 2012; Manke *et al*., 2013; Sadiq *et al*., 2015). Further research on particokinetics and cytokinetics is needed to understand the exact mechanism behind nanotoxicity at cellular and genetic level. There is a need for establishment of standard test protocols and comprehensive toxicity data regarding the nanoparticles to ascertain realistic toxicity level and risk assessment. Nanoparticles are proved as cytotoxic, therefore, there is major concern regarding the risk assessment and biologically and ecologically safe utilization of nanoparticles.

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