AATEX **14**, Special Issue, 397-400
Proc. 6th World Congress on Alternatives & Animal Use in the Life Sciences
August 21-25, 2007, Tokyo, Japan

In vitro cytotoxicity assessment of selected nanoparticles using human skin fibroblasts

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Abstract
Zinc oxide (ZnO) and Titanium dioxide (TiO_2) are two chemical compounds with very wide industrial and commercial applications, particularly as pigments. Due to their physical properties, both compounds are also used as sunscreen ingredients for protect from UV radiation. At the nano-scale, ZnO and TiO_2 have proven to have a similar level of protection compared to normal-scale sunscreen particles. An advantage of the topical use of nano-scale ingredients in sunscreens is their transparency compared to the white residue left on skin with normal scale particles.

However, the potential toxicity of these nanoparticles is not well understood. The aim of this study was to assess the cytotoxicity of ZnO and TiO_2 nanopowders (particle size 50-70 nm and less than 150 nm, respectively). The cytotoxicity of these selected nanomaterials was investigated in human skin fibroblasts using the colourimetric MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2Htetrazolium) *in vitro* assay. Cytotoxicity endpoints including NOAEC (no observed adverse effect concentration), IC_{50} (50% inhibitory concentration) and TLC (total lethal concentration) of the test materials were determined after 4 and 24 h exposure. ZnO indicated higher toxicity at both 4 and 24 h. By increasing the exposure time the cytotoxicity of both nanoparticles increased substantially. At 24 h exposure the IC_{50} of ZnO was 49.56 ± 12.89 ppm, and for TiO_2 the IC_{50} was 2,696 ± 667 ppm.

Results of this study indicated that human skin fibroblasts were sensitive to both ZnO and TiO_2 nanoparticles through the MTS assay and they correlated well with the published *in vivo* and *in vitro* toxicity data. The data generated in this study can be used to assess human topical risk exposure to selected nanomaterials.

Keywords: nanotoxicity, titanium dioxide, zinc oxide, MTS assay, skin fibroblasts

Introduction

In recent years, the use of nanoparticles to replace normal-scale particles has been rapidly increasing. Zinc oxide (ZnO) and Titanium dioxide (TiO_2) are two chemical compounds with very wide industrial and commercial applications particularly as pigments. Due to their physical properties, both compounds are used as sunscreen ingredients for protection from UV radiation. ZnO and TiO_2 in nano-scale have proven to have a similar level of protection compared to normal-scale sunscreen particles (Popov et al., 2005). However, they give an outstanding advantage in providing a clear transparent appearance upon topical application compared to the white residue left on skin with normal-scale particles.

The smaller size and unique properties of the nanoparticles has substantially improved the application of these pigments however it has also generated greater concern in term of risks to human health and the environment (Colvin, 2003; Warheit, 2004; Adams et al., 2006). *In vivo* studies demonstrated that both nanoparticles of TiO_2 and ZnO were likely to cause greater adverse effects when compared to the normal-scale particles (Oberdörster, 2000; Wang et al., 2006) due to the increased in surface area (Oberdörster, 2000; Oberdörster et al., 2005). A limited number of *in vitro* studies have also been performed to assess the toxicities of the nanoparticles using different cellular systems and test methods (Cai et al., 1991; Dunford et al., 1997; Sayes et al., 2006). However, published toxicity data are still considered inadequate to earn a full understanding of the potential toxicity of these nanoparticles. Further studies are needed to clarify the risk of these materials as well as their application for human use.

Recently, *in vitro* methods have shown a significant potential for assessing the toxicity of environmental and occupational health risks (Bakand et al., 2006a, 2006b; Lestari et al., 2006). One such method is the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay which is based on the ability of viable cells to convert a soluble tetrazolium salt to a formazan product. This method has been proven to be user friendly, rapid and highly sensitive (Promega, 2005; Bakand et al., 2006a; Potera, 2007).

The aim of this study was to assess the cytotoxicity of ZnO and TiO_2 nanoparticles. The cytotoxicity of these nanoparticles was investigated in human skin fibroblasts using the colourimetric MTS *in vitro* assay.

Materials and methods

Chemical compounds

In vitro assay reagents were purchased from Gibco (USA), Promega (USA) and Sigma (USA). Titanium dioxide (TiO_2), CAS # 13463-67-7, was purchased from Sigma, Australia (product number 634662), Analytical reagent. Zinc oxide (ZnO), CAS # 1314-13-2, was purchased from Sigma, Australia (product number 544906), Analytical reagent.

Human skin fibroblasts and culture conditions

Human skin fibroblasts were obtained from fresh skin biopsies taken from the arm of healthy individuals (Cytogenetics Department, The New Children's Hospital, Westmead, Sydney, Australia). All cultures were maintained in a phenol red free culture medium DMEM/F12 (Dulbecco's modified essential medium/Ham's 12 nutrient mixture, Gibco), supplemented with 5% (v/v) fetal calf serum (JS Bioscience, Australia), and 1% (v/v) antibiotic (2 mM L-glutamine, 100 U/mL Penicillin and 0.1 mg/mL Streptomycin; Gibco). Cultured cells were kept at 37ºC in a humidified 5% CO_2 incubator.

Once the cells reached confluence, the culture medium was removed from the flask and the cells were rinsed three times with sterile HBSS (Hank's Balanced Salt Solution, Gibco). The confluent cell layers were enzymatically removed, using Trypsin/EDTA (Gibco, USA), and resuspended in culture medium. Cell viability was assessed by vital staining with trypan blue (0.4% (w/v); Sigma, USA), and cell number was determined using a light microscope (Leitz Wetzlar, Germany).

Preparation of nanoparticles

TiO_2 and ZnO were individually suspended in the culture medium at the concentration of 5000 ppm and dispersed by ultrasonic vibration for 15 min. In order to ensure the uniform suspension, they were stirred on vortex agitation (1 min) before every use.

Cytotoxicity testing

Cytotoxicity testing was performed using the Promega CellTiter 96 AQueous Non-Radioactive Cell Proliferation (MTS) assay to determine the number of viable cells in culture (Promega, 2005). The test protocol for cytotoxicity evaluation was adopted from previously published papers and manufacturers instructions (Malich et al., 1997; Hayes and Markovic, 1999; Bakand et al., 2005a; Bakand et al., 2005b; Lestari et al., 2006; Hayes et al., 2007).

Nanoparticles were suspended in culture media, serially diluted across 96-well microtiter plates (100 µL), and incubated at 37ºC with 5% CO_2. Two sets of exposure times were carried. These included 4 h and 24 h exposure periods. Four hours prior to the end of each exposure period a MTS mixture (20 µL/well) was added.

After the completion of exposure period, the plates were then placed on a microwell plate reader (Multiskan MS Labsystem, Finland), shaken for 10 s and the absorbance of the formazan product was read at 492 nm. Each experiment was repeated on three separate occasions.

Two internal controls were set up for each experiment: (1) an IC_0 consisting of cells only; and (2) IC_{100} consisting of medium only. Background absorbance due to the non-specific reaction between test compounds and the MTS reagent was deducted from exposed cell values (Hayes and Markovic, 2002).

Dose response curves of *in vitro* cytotoxicity data

Dose response curves were plotted for the test chemicals after correction by subtracting the background absorbance from the controls. NOAEC (No Observable Adverse Effect Concentration), IC_{50} (50% inhibitory concentration) and TLC (Total Lethal Concentration) values were extrapolated graphically from the plotted absorbance data.

Table 1. Toxicity values of zinc oxide and titanium dioxide

Toxicity Endpoints	Zinc Oxide (ZnO)		Titanium Dioxide (TiO_2)	
(ppm)	4 h	24 h	4 h	24 h
NOAEC	180.98 ± 110.2	1.13 ± 0.45	289.2 ± 73.5	80.38 ± 13.48
IC_{50}	n/a	49.56 ± 12.89	n/a	2,696 ± 667
TLC	n/a	298.18 ± 67.35	n/a	15,1333 ± 4,056

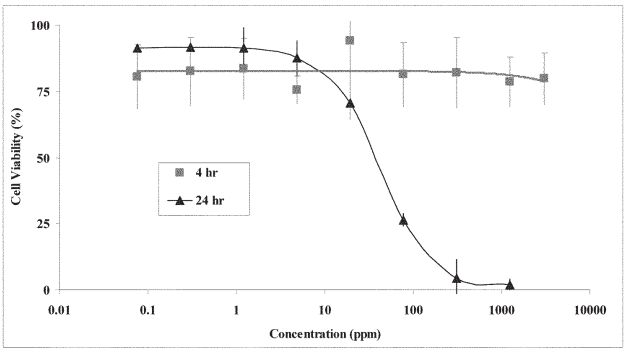

Fig. 1 Concentration-cell viability curves of zinc oxide nanoparticle following 4 and 24 hour exposure on human skin fibroblasts using the MTS assay.

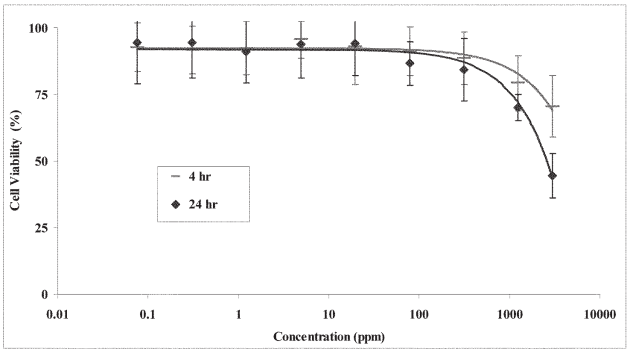

Fig. 2 Concentration–cell viability curves of titanium dioxide nanoparticle following 4 and 24 hour exposure on human skin fibroblasts using the MTS assay

Results

In vitro cytotoxicity data

Relationship between test chemical (ZnO and TiO_2) concentration (ppm) and cell viability (%) after 4 and 24 h exposures using the MTS assay are presented in Figs. 1 and 2, respectively. Each experimental curve represents the average of a series of three different experiments.

Cell viability was significantly reduced in a dose-dependent manner after exposure of human fibroblasts to nanoparticles using the MTS assay. The experimental data for NOAEC, IC_{50}, and TLC values on the cells are summarised in Table 1 with data presented as mean values ± standard deviations.

The experiment was conducted following 4 and 24 h exposure time. Slight cell viability reduction was observed after 4 h exposure time but, by increasing the exposure time to 24 h the cytotoxicity of both nanoparticles increased substantially. Following 24 h exposure time the IC_{50} of ZnO was 49.56 ± 12.89 ppm, and for TiO_2 the IC_{50} was 2,696 ± 667 ppm.

Discussion

Exposure to both ZnO and TiO_2 nanoparticles indicated a range of cytotoxicity responses to human skin fibroblast cells. NOAEC values at 4 h exposure using the MTS assay showed that ZnO showed greater toxicity compared to TiO_2. However, other toxic endpoints of IC_{50} and TLC could not be measured due to incomplete dose response curves. These results indicate that at 4 h exposure both nanoparticles present only a mild adverse effect to human cell fibroblasts. However, when the exposure time was increased to 24 h both nanoparticles had a substantial toxic impact to cells. This time dependent result is standard dose-response behaviour. These results are supported by previous published results that indicated that longer (3 day) exposure generated a greater toxicity to human bronchial epithelial cells (IC_{50} (TiO_2) 6.5 μg/mL) using the MTT assay (Gurr et al., 2005).

Results of this study demonstrated that the MTS assay could be implemented as an effective and sensitive tool to assess cytotoxicity of ZnO and TiO_2 nanoparticles on human skin fibroblasts. Recent published *in vivo* studies have shown significant adverse effects to mice using nano-scale Zn metal powder and TiO_2 at 5 g/kg body weight (Wang et al., 2006; Wang et al., 2007). Pulmonary toxicity has also been observed in rats after 24 h exposure to low concentration (5 mg/kg) of nano-scale TiO_2 particles (Warheit et al., 2007). *In vitro* toxicity assessment has become widely used for recent toxicity studies. Such assays provide rapid, cost effective and reliable results (Hayes and Markovic, 1999). Toxicity results of ZnO nanoparticles in Chinese hamster ovary cells indicated a NOAEC and IC_{50} at 54 and 340 μg/mL, respectively (Dufour et al., 2006).

Nanomaterial usage will continue to increase rapidly and widely in areas such as cosmetics, pharmaceuticals and other industrial applications. Accurately assessing the toxicity and safety of these nanomaterials to human health is of upmost importance. Toxicity data generated in this study can potentially be used to assess human topical risk exposure to nanomaterials. Future studies should be focused on investigating the potential risk of nanomaterials to human health at the microscopic cellular level by implementing appropriate microscopic techniques such as TEM (transmission electronic microscope) to reveal general mechanisms of toxicity and characterising exposure to nanomaterials.

Acknowledgements

The authors are grateful to the Consumer Coalition for Information on Cosmetics (CCIC) for the travel scholarship grant to FD to attend the Congress. This research was supported by a Postgraduate Safety Science Scholarship and Australian Postgraduate Award. The authors would also like to thank Dr. Preprame Pattanamahakul for valuable suggestions and Dr. Zhanhe Wu of Westmead Hospital, Sydney for providing human skin fibroblasts.

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