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Amorphous silica nanoparticles show concentration and time-dependent toxicity on Human HaCaT Cells

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Abstract

Background: The wide use of nanomaterials in medicine and biology and the unknown cytotoxicity of some nanoparticles heighten the demand for health and safety guidelines. The creation of these guidelines relies on experimental investigation to inform guidelines to eliminate the risk of exposure from newly synthesized nanoparticles. Skin contact of nanoparticles can cause skin cancer; therefore, the focus of this study was to identify the cytotoxicity of these particles to skin cells in an in vitro model.

Methods: The toxicity of 12-nm amorphous silica ($SiO_{2)}$ nanoparticles following 4, 24 and 48h exposure was investigated using a human keratinocyte cell line (HaCaT) with [3-(4,5-dimethylthiazol-2yl)-diphenyltetrazolium bromide] (MTT) and crystal violet assays. Eleven concentrationsof 12-nm SiO_2 ranging between 0.05-10 mg/mL were tested.

Results: At 4, 24 and 48h exposure, a dose dependent increase in cell killing with increasing concentration were observed when screened with the MTT assay. At 24h for concentrations ≥ 2 mg/mL, relative survival decreased when assayed by the MTT assay and relative cell number decreased when assayed with the crystal violet assay. After 48htreatment, cytotoxicity was observed at every treatment concentration assessed with the MTT and crystal violet assays. The level of cytotoxicity was also time dependent (4, 24, 48h)at every concentration. The level of cytotoxicity after 48h treatment was equal to or less than that of cell population streated for 24h.

Conclusion: Silica nanoparticles are toxic to cultured human skin cells at a concentration as low as 0.05 mg/mL for 48h treatment when screen by the crystal violet assay. When nanoparticles of consistent structure and surface area are used to treat human cells in culture, results varied between two well recognised colourimetric bioassays. However, with both assays, silica nanoparticles are toxic to human skin cells *in vitro* and toxicity is both concentration dependent and time dependent.

Keywords: Silica particles, nanoparticles, occupational health, HaCaT cells

Introduction

The fast development of nanotechnology has produced a myriad of engineered nanomaterials. Nanoscaled particles have been used in many areas, including chemical, medical research and related

industries. The potential hazard arising from nanomaterials has led to the study of their toxicological effects on human health and the environment [1-5]. Despite some nanomaterials such as quantum dots and carbon nanomaterials being intensively studied toxicologically, other engineered nanomaterials have not been [6-14].

This study focussed on amorphous silica nanoparticles. Exposure to Silica is linked to the development of lung cancer, and in 1997, the International Agency for Research on Cancer (IARC) classified inhalation of crystalline silica at occupational sources as group 1 human carcinogen [6-8]. The use of amorphous silica in nanotechnology has been important part in bioanalysis and imaging, diagnostics, drug delivery and gene transfer [9-13].This adds to the increasing industrial exposure to silica nanoparticles during production, transportation, storage, and consumer use by which human exposure and environmental burden were obviously increased. The National Institute of Occupation Safety and Health (NIOSH) recommended the limit of exposure to crystalline silica to 0.05 mg/m^3. However, miners are exposed at levels equal to or greater than is recommended[14]. Therefore, it is conceivable that amorphous silica could enter the human body through all possible routes, including inhalation, oral, intravenous injection and transdermal delivery. In this study, human keratinocyte cell line (HaCaT) is chosen as an ideal cell model for studying dermal toxicity [15]. To govern a safe use of silica in the future, it is important to gather toxicity information by testing the *in vitro* and *in vivo* properties of amorphous silica [16]. Recently, two studies revealed that amorphous silica may present toxicity concern at high doses in number of different organs[17, 18]. Human bronchoalveolar carcinoma cell line(A549) were exposed to 15-nm or 46-nm SiO_2 nanoparticles for 48h at dosage levels between 10 and 100 µg/mL and decreased cell viability was observed in a dose-dependent manner [26].The concentrations chosen for this study are based on previously published *in vitro* and *in vivo* studies[26, 27]. In this study, we investigated the response of a human keratinocyte cell line (HaCaT) to 11 range of concentrations of amorphous silica nanoparticles (0.05-10 mg/mL) using the Thiazolyl Blue Tetrazolium Bromide (MTT) and crystal violet assays.

Methods

Chemicals

Silica nanoparticles (12 nm (TEM), nanopowder, and 99.8% trace metals basis) were purchased from Sigma Aldrich[19]. The nanoparticles characteristics are shown in table 1. Culture media was Roswell Park Memorial Institute (RPMI) (Sigma Chemical Company (St. Louis, MO). with 10% heat-inactivated fetal bovine serum (FBS) (HYQ®, Hyclone, Utah, USA) Penicillin–streptomycin, [3-(4,5-dimethylthiazol-2yl)-diphenyltetrazolium bromide] (MTT), and Nicotinamide adenine dinucleotide (NADH). General reagent wasobtained from SigmaChemical Company (St. Louis, MO). HaCaT cell line originated from American Type Culture Collection (ATCC).

Cell culture

The cells were grown in RPMI medium, pH7.4, with 10% heat inactivated FBS, and 1% antibiotic mixture of penicillin (10,000units/mL) and streptomycin (10,000µg/mL). The treatment media was the same as growth media. The cells were seeded in a T-75 flask at $1x10^6$ cells/mL total volume of 25 mL fresh medium. When the cells reached 70-80% confluency, they were trypsinised and sub-cultured[20]. The cells were maintained in a humidified incubator with 5% CO_2at 37 °C[15].The number of viable cells after trypsinisation was determined by Trypan blue staining and counting using hemocytometer.

Table 1: The physical and chemical properties of silica nanoparticles are shown below, including boiling point (bp) and melting point (mp) (obtained from Sigma Aldrich).

Characteristic	Value
Assay	99.8% trace metal basis
Colour	colourless
Form	nanopowder
Primary Particle Size	12 nm (TEM)
SurfaceArea*	spec.surface area 175-225 m^2/g [35]
Formula	SiO_2
Molecular Weight	60.08 g/mol
boiling point (bp)	2230 ^{0}C(lit.)
melting point (mp)	> 1600 ^{0}C(lit.)
Relative Density	2.6 g.cm3

TEM: transmission electron microscopy.
*Ref [35]

Trypan Blue cell counting

For the cell counting method, an aliquot of 50 µl of cells suspension was mixed 50 µlof Trypan Blue , then the number of cells in flask was counted by a hemocytometer after trypsinization[21].

SiO_2treatment

For the Bioassays, HaCaT cells were seeded at 10,000 cells/well in 96 well-flat bottom microplates, then incubated overnight to allow adherence. Cells were exposed to SiO_2 at 11 range of concentrations (0.05, 0.1 , 0.5, 1.0, 1.5, 2.0, 4, 5, 7, 9, 10 mg/mL) for 4, 24 and 48h. Amorphous silica nanoparticles was diluted in a fresh medium (stock at 20mg/mL), then mixed by vortexing for 5 min, followed by dilution to the required concentration with media. After exposure of the cells for the appropriate time,the suspension was removed, and the cells were washed twice with phosphate buffered saline (PBS) to remove excess SiO_2.

Bioassays

Crystal Violet Assay (screening for cell adherence phenotype)

Cells were stained with crystal violet, and live cells remained adhered to the plate while dead cells were washed away. Plates for the crystal violet assayincluded6 technical replicate wells per treatment. After treatment (see above), the plates were washed and 50 µl of crystal violet stain was added and incubated at room temperature for 15 minutes. The Stain was washed off with demineralized water and the plates were left to dry overnight. A 33% (v/v) acetic acid solution was then added and the optical density (OD)at 570 nm was read within minutes using ELISA reader. Three biological replicates were carried out for each treatment experiment. The results were expressed as percentage viability compared to untreated control.

MTT Assay (screening for metabolic functioning)

The cytotoxicity of Silica nanoparticle was determined using the MTT assay as described [22, 23]. $1x10^4$ cells were seeded in volume of 100 µl into 96-well flat bottom plate. MTT was added to each well at 0.5 mg/mL, and then plates were incubated at 37 °C for 4h, then 80 µl of 20% SDS in 0.02 M HCl was added to each well. The plates were kept in the dark at room temperature for overnight. OD was read on ELISA reader at 570 nm, with 630 nm as reference wave length. In each experiment a standard curve was run to convert the OD values to cells/well.

Statistical analysis

The data were analysed as mean ± SEM of at least three independent experiments using one-way analysis of variance (ANOVA) and Tukey–Kramer multiple comparisons test

using SPSS software to compare exposure groups.

All comparisons were considered significant level $p< 0.05$.

Results

The silica particle size was approximately 12 nm when measured by TEM (Sigma Aldrich). Interference assays were carried out for the MTT and crystal violet assays. No interference was observed (data not shown). HaCaT skin cells were exposed to silica nanoparticles at increasing concentrations at three exposure time points. At each given time, there was no significant concentration-dependent cell killing. For example, no significant ($p>0.05$) difference in concentration-dependent was observed for 4 h treatment when assayed with the MTT assay or after crystal violet (Fig. 1 & 2). Decrease in relative viability and relative cell numbers were observed with increasing concentration after 24h and 48 h treatment, however they did not reach significance (Fig. 1 & 2). At a given exposure time there was a concentration dependent killing. At 4 h exposure a concentration dependent increase in cell killing only with MTT assays. At 24 h cell killing increased to 70% obvious with crystal violet assay. At 48 h more cell killing observed by up to 80% clearly in crystal violet assay.

There was a time-dependent increase in cell killing, observed when assayed with the crystal violet assay only (fig.1). This was significant when assay using the crystal violet assay cell number decreased up to 75%. At any given dose concentration, the change from 4 h to 24h to 48 h was examined.

The 4h treatment when assayed for metabolic activity with MTT assay showed very little change with dose (fig.1). However, for most concentrations following 4 h when assayed for adherence with the crystal violet assay there were increase in relative cell number (fig.2). This was also observed for low concentrations at 24 h (0.05-2 mg/ml).

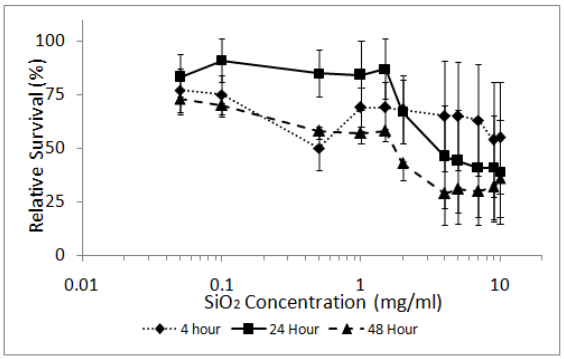

Fig. 1: The effect of treatment of HaCaT cell line with Silica particle assessed using the MTT assay. Data are shown as Relative survival (%) compared to the untreated control and are presented as mean ± SEM, n = 3, except for 2 mg/ml where n=6.

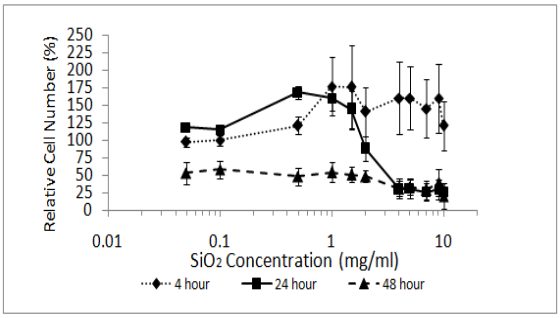

Fig. 2: The effect of treatment of HaCaT cell line with Silica particle assessed using the crystal violet assay. Data are shown as Relative cell number (%) compared to the untreated control and are presented as mean ± SEM of three separate experiments except for 2 mg/ml where n=6. All Treatments with crystal violet assay were significantly different from untreated control (100%) at $P< 0.05$ except in 1.5 mg/ml.

Discussion

The immortal human keratinocyte cell line (HaCaT) exhibits similar biological properties to those of normal human keratinocytes and is therefore a useful cell model for studying dermal toxicity[24]. Particle size and concentration influence cytotoxicity assessment, Also different cell lines respond differently to the same particle at the same concentration[25]. A recent

study on silver nanoparticles (20,80 and 113 nm) studied exposure to murine peritoneal macrophage cell line RAW 264.7, L929 mouse fibroblasts, D3 murine embryonic stem cell line and mouse embryonic fibroblasts (MEF-*LacZ*)[26]. The Silver nanoparticles at 20nm were more toxic than the larger nanoparticles. Furthermore, not all cell lines were affected by the 20 nm nanoparticles. Therefore, the ability of silver nanoparticles to induce cell killing is influenced by cell type and nanoparticle size. A range of characteristics of nanoparticles can influence the toxicity in *in vitro* biological systems, including investigation in cell culture systems[27-30]. The toxicity of nanoparticles is also dependent on dose and duration of exposure[31, 32].For silica nanoparticles depending on their shape and dimension, the silica particles are taken up by the cells via different mechanisms of endocytosis, and then undergo distinct intracellular processing until they are exocytosed[26].Smaller particles are understood to be easier to internalize in cells than larger particles[33].This suggests that the 12 nm particles in the current study would have been internalised due to their small size. This warrants further investigation, via such techniques as specialized microscopy.

Human bronchoalveolar carcinoma cell line (A549) treated with 15nm of silica nanoparticles at doses 10 and 100 ug/ml for 48hdecreased cell viability dose-dependently[17]. In figure 1, based on the MTT viability assay, a similar effect was observed for the 12 nm particle used in the current study. It was observed that cytotoxicity was a function of (1) particle concentration and (2) duration of exposure. A similar result was observed with the crystal violet assay at the higher doses and exposure times. At 4 h exposure there was a dose dependent increase in cell killing, which was also found at 24 h and at 48 h with an increased level of cell killing (fig 2).

According to Drescher et.al (2011), silica particle cytotoxicity in 3T3 fibroblast cell line was caused by the silica concentration and the content of fetal calf serum (FCS). Dynamic light scattering reveals the physical parameter of silica was affected by agglomeration[34]. Silica nanoparticles induced less toxicity by decreasing the relative viability parallel to dosage concentration. In the current study particle concentrations aregiven across a wide range of exposure time. Silica particles at concentrations of 0.05-2 mg/ml exhibit gradual increase toxicity which also increased with increasing time of exposure (fig 1). A concentration between 2-10 mg/ml exhibits a further decrease in cell viability which also increases with the duration of exposure. Chang et al (2007) exposed to WS1 and CCD- 966sk human skin cell lines to Silica for 48 h. The decrease of relative viability increased with the particle mass concentration in both cell lines they used. At a concentration of 667µM,cell viability decreased to approximately 80% of the control[20].

In figure 2, the relative cell number showed a gradual reduction over the time of exposure and concentration of treatment. A concentration between 0.05-2 mg/mL decreased relative cell number at 48 h. Dosage between 2-10 mg/mL similarly had a toxic effect at 24 and 48 h. The cytotoxicity of silica in both assays showed a variation of cell killing but both confirmed that the mass concentration of silica particles will affect the cell population. The effect was most marked using the crystal violet assay as an end-point.

Conclusion

The MTT and crystal violet assay reveal that silica nanoparticles become toxic to cultured human skin cells at the 11 concentrations tested and may also impede cell

proliferation. The outcome of this study suggests that even with nanoparticles of similar structure and dimensions, toxicity may vary even with the end-point measured in recognised colourimetric bioassays. We conclude that the toxicity is not just dependent upon particle size and surface area. Mass concentration and exposure time clearly influence the outcome. It is useful to carry out interference detection for colorimetric assays, as our work has suggested other parameters may influence OD readings (data not shown).

Thus the finding of this work demonstrates the importance of studying duration of exposure and nanoparticle concentration when studying additional cell lines for the toxicity assessment of nanoparticles.

Abbreviations

IARC: International Agency for Research on Cancer; NIOSH:National Institute of Occupation Safety and Health; HaCaT:human keratinocyte cell line; A549:Human bronchoalveolar carcinoma cell line; MTT:Thiazolyl Blue Tetrazolium Bromide; RPMI: Roswell Park Memorial Institute; FBS: fetal bovine serum; NADH:Nicotinamide adenine dinucleotide; ATCC:American Type Culture Collection; bp:boiling point; mp: melting point; PBS:phosphate buffered saline; OD: optical density; FCS; fetal calf serum; TEM: transmission electron microscopy.

Competing of interest

The authors declare that they have no competing interests.

Authors Contributions

AA carried out the experimental work, analysed the data and drafted the manuscript. BJSS participated in data analysis, design etc. as for AE. AE participated in the design of the study and reviewed the manuscript and gave her intellectual input. All authors read and approved the final manuscript.

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