

The Effect of Particle Size on the Cytotoxicity of Amorphous Silicon Dioxide: An in Vitro Toxicological Study

Athena Rafieepour¹, Mansour R. Azari^{2*}, Jalal Pourahmad Jaktaji³, Fariba Khodaghali⁴, Habibollah Peirovi⁵, Yadollah Mehrabi², Yousef Mohammadian⁶

Abstract

Introduction: Amorphous silicon dioxide (A-SiO₂) is abundant in the Earth's crust, the A-SiO₂ nano and microparticles are released into the air through industrial and manufacturing activities. Due to the limited available toxicological information, the objective of the present study was to evaluate the toxicity of different sizes of A-SiO₂ particles on the A549 cell-lines in an in vitro study. **Materials and Methods:** The A-SiO₂ particles in two categories of nano (10-100 nm) and micro (< 5µm) were used in this study. The human lung A549 cell-line was exposed to either nano- or micro-sized A-SiO₂ particles at 10, 50, 100, and 250 µg/ml, and the effects were investigated. **Results:** The cytotoxicity of A-SiO₂ nano and microparticles in both 24- and 72-hour exposure times resulted in decreased cell survival, mitochondrial membrane potential, and increased ROS generation which was concentration-time dependent (P < 0.05) but glutathione content was not affected in a time-dependent manner. Cytotoxicity of nanoparticles, contrary to the previous study, was not higher than microparticles in the comparable dose and exposure times. **Conclusion:** The rate of ROS generation in the A549 cell-line exposed to A-SiO₂ nanoparticles was higher than microparticles. And at the same time, cell survival for exposed cells to A-SiO₂ nano and microparticles were higher for nanoparticles in shorter exposure periods and was inversely concentration- and time-dependent. Further studies on exploring the effect of size and its possible toxic mechanism are recommended to achieve a more credible risk assessment.

Keywords: Amorphous silicon dioxide- nanoparticles- microparticles- A549 cell lines,- toxicity- in vitro

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Introduction

Airborne contaminants are one of the important causes of morbidity in the world, and according to a WHO (2014) report, there are 7 million premature deaths per year as a result of exposure to them. Airborne particle pollutants are divided into three groups of large particles (PM₁₀), fine particles (PM_{2.5}) and ultrafine particles (UFP, PM_{0.1}), and the most harmful effects can be observed in ultrafine particles with an aerodynamic diameter of less than 100 nm (Breitner et al., 2011; Franck et al., 2011; Luch, 2012). An increase in the surface-to-volume ratio, as well as an increase in the number of very small particles, is one of the reasons that can increase these toxic effects (Sioutas et al., 2005) and since respiratory tract and lungs with an entry rate of 200 billion particles per day are directly in contact with pollutants, impairing the function of the

respiratory system is very prevalent throughout the world (Donaldson et al., 2005; Cohen et al., 2017). Also, particles in fine and nanometer sizes can easily pass through the membrane and damage vital organs systemically (Hillyer and Albrecht, 2001; Donaldson et al., 2004; Fischer and Chan, 2007), although the mechanism of this effect is not clear, and some researchers believe that nanoparticles do not differ significantly in their toxic effects compared to microparticles (Karlsson et al., 2009). However, the production of nanoparticles and their widespread use in modern science, in comparison with microparticles, has increased the human exposure to these materials both intentionally and accidentally (Peixe et al., 2015).

The Organization for Economic Cooperation and Development (OECD) has reported that the use of common nanoparticles such as silicon dioxide nanoparticles are expected to increase in the future (Leung et al., 2012).

¹Student Research Committee, School of Public Health and Safety, Shahid Beheshti University of Medical Sciences, Tehran, Iran. ²School of Public Health and Safety, Shahid Beheshti University of Medical Sciences, Tehran, Iran. ³School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran. ⁴Neuroscience Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran. ⁵Nanomedicine and Tissue Engineering Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran. ⁶Department of Occupational Health Engineering, Tabriz University of Medical Sciences, Tabriz, Iran.
*For Correspondence: mrazari@sbmu.ac.ir

Amorphous silicon dioxide particles (A-SiO₂) are widely used in various processes, such as drug delivery and biomedicine (Sergent et al., 2012). These nanoparticles are unique in their physicochemical properties and are a byproduct in the manufacture of several types of commercial products. Also, harvesting agricultural products or burning waste in furnaces could expose the general population to A-SiO₂ (Merget et al., 2002; Barik et al., 2008; Napierska et al., 2010; Wang et al., 2015). Besides, respirable silicon dioxide particles are one of the most common occupational pollutants throughout the world (Dahmann et al., 2008) that can expose workers and people living around plants, mines, or dry areas (Greenberg et al., 2007; Santos et al., 2010). Generally, accurate toxicological evaluation has been recommended by many health experts (Karlsson et al., 2009).

The International Agency for Research on Cancer (IARC) has identified A-SiO₂ as non-carcinogenic to humans stating that it has less toxic effects compared to crystalline forms (Raju and Rom, 1998). In some studies, inhalation exposure to A-SiO₂ nanoparticles in animal subjects has been partially associated with reversible inflammation in the respiratory system and changes in blood parameters, but these studies do not provide evidence of the progression of fibrosis in the lungs (Shin et al., 2017). However, in an epidemiological study, the occurrence of four cases of silicosis was reported among people exposed to pure A-SiO₂ (Merget et al., 2002). In some cohort studies, A-SiO₂ has been known to increase mortality due to pulmonary diseases (Puntoni et al., 1988; Checkoway et al., 1993), and the results of some studies indicated that inhalation of A-SiO₂ nanoparticles could result in significant pulmonary toxicity in comparison to its crystalline form (Kaewamatawong et al., 2005; Yazdi et al., 2010). The importance of the study for comparison the toxicity of silicon dioxide micro and nanoparticles has been expressed by some researchers (Sahu et al., 2016). On the other hand, most of the particles' toxicity information was shown in animal models (Szalay, 2012a) that raises concerns about generalizing the toxicity effects in humans (May et al., 2009).

Laboratory work focused on investigating the effect of A-SiO₂ particles could play an important role in evaluating the adverse toxicity risk to humans; hence the objective of the present study was to investigate the toxicological effects of A-SiO₂ particle sizes of micron and nanometer in the A549 human lung cell line.

Materials and Methods

Materials and reagents

A-SiO₂ nanoparticles in the diameters of about 15-20 nanometers were purchased from the US Research Nanomaterials company. A-SiO₂ of micron size was not available in Iran. Therefore, with the consultation of the Faculty of Geology at the Shahid Beheshti University, Yellow Namibian opal stone was purchased and ground by a ball mill (Siebtechnik, Holland (380 V, 0.75 KW) for 40 hours. The powdered material was treated to the extraction of impurities such as metals from ball milling, according to the method recommended by Zong et al.,

(2015), using hydrochloric acid in 2M concentration and concentrated nitric acid.

Physicochemical properties of A-SiO₂ particles

The chemical structure and purity of the ground A-SiO₂ microparticles were confirmed using the XRD diffraction (STOE-STADV, Germany) and the ICP-OES (Agilent 7700, USA), respectively. The size of A-SiO₂ microparticles was measured by the phase-contrast microscopy (PCM, Dialux 22EB- Germany) method recommended by Stefaniak et al., (2007). The A-SiO₂ nanoparticles' sizes were also confirmed using a transmission electron microscope (TEM, Philips CM30, Netherlands).

The particle size distributions were measured in a 100 µg/ml concentration of A-SiO₂ nano and microparticles in culture media. The suspension was stored for 25 minutes at 25°C for 1 hour and then dispersed with a sonicator for 20 minutes at 240V and 60 Hz frequency. Particle size distribution was measured using a DLS (Nanophox 90-246V, Germany). The surface area of A-SiO₂ nano and microparticles were analyzed by Brunauer-Emmett-Teller (BET, Tristar II-3020, US).

Cell culture and exposure to A-SiO₂ particles

The A549 cell line was purchased from the Pasteur Institute in Iran and cultured in a Dulbecco's Modified Eagle Medium (DMEM, Bio Idea Co., Iran) + 10% Fetal Bovine Serum (FBS, GIBCO, USA) and 1% Penstrep (GIBCO, USA) in an incubator at 37°C and 5% CO₂. After reaching to an 80% confluency of cells, the supernatant of each plate was replaced by serum-free DMEM and DMEM containing A-SiO₂ particles for control and exposure groups, respectively. Toxicological tests were performed at four concentrations of 10, 50, 100, and 250 µg/ml of A-SiO₂ nano and microparticles with 24 and 72 hours exposure times according to the previous studies that showed that the entrance of SiO₂ particles into the perinuclear of A549 cells occurred in ≥ 24 h exposure time (Ahamed, 2013; Nowak et al., 2014). Serum-free medium was used to prepare different concentrations of A-SiO₂ nano and microparticles and homogenized for 20 minutes before use in a sonicator device.

Cell survival assay

1×10⁴ cells per well were seeded in a 96-well plate and were exposed overnight to nano and microparticles of A-SiO₂. After 24 and 72 hours exposure periods, MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) was added to each well and the color change resulting from the conversion of MTT salt to Formazan as a result of the mitochondrial activity of succinate dehydrogenase in survival cells (Mosmann, 1983) was measured by an ELISA plate reader (Biotek ELx 800, USA at 570 nm). Results were calculated by the ratio of the average cell survival in the exposed group to the average cell survival in the control group ×100.

Mitochondrial Membrane Potential assay

1×10⁴ cells per well of a 96 black well plate were

exposed to different concentrations of nano and microparticles of A-SiO₂ at 24 and 72 hours exposure times. 200 μ l of DMEM containing Rhodamine 123 at a concentration of 2 μ M was added to each well for 60 minutes before the test (Johnson et al., 1980). The fluorescence intensity of Rhodamine 123 was measured by a Multi-Mode Reader (Synergy-HTX, BioTEK, USA) at 485 and 528 nm of excitation and emission wavelengths, respectively. Finally, the mitochondrial membrane potential was calculated based on the difference in fluorescence intensity between the control and test groups (Villena et al., 2013; Sakamuru et al., 2016) and reported in comparison to the control group.

ROS assay

1 \times 10⁴ cells per well in a 96 black well plate were exposed to different concentrations of A-SiO₂ nano and microparticles at 24- and 72-hours exposure periods. The intensity of fluorescence induced by adding DCFH-DA reagent and converting to DCF was measured by a Multi-Mode Reader (Synergy-HTX, BioTEK, USA) at 485 and 525 nm of excitation and emission wavelengths, respectively and was considered intracellular ROS index (LeBel et al., 1992). Results were calculated by the ratio of the average ROS generation in the exposed group to the control group \times 100.

Intracellular glutathione content assay

The A549 cellular glutathione content in 24 and 72 hours exposure periods to A-SiO₂ micro and nanoparticles was measured according to the method recommended by Ellman (1959). In this test, the lysis cells were exposed to a DTNB reagent (5,5'-Dithiobis [2-nitrobenzoic acid]) at 2 mg/ml, and the yellow color resulting from the DTNB depletion by free protein thiols to TNB was measured by an ELISA reader (Biotek ELx 800, USA) at a wavelength of 405 nm (Requejo et al., 2010). The GSH content in exposed cells was reported in comparison to GSH content in control cells.

Statistical analysis

Toxicity tests were repeated three times and the results demonstrated as mean \pm standard deviation. SPSS software version 16 and t-test independent sample and one-way ANOVA tests were used to analyze the data obtained from toxicology tests and results showed that in a 95% confidence interval.

Results

Physicochemical properties of A-SiO₂ nano and microparticles

According to the information provided by US Research Nanomaterials corporation, the specific surface area and purity of A-SiO₂ nanoparticles were 170 - 200 m²/g and 99.5%, respectively (US-Research-Nanomaterials-Inc, 2020). The physical properties of A-SiO₂ microparticles were analyzed by BET and ICP-OES (Table 1). The results showed that the specific surface area and purity of A-SiO₂ microparticles were 34.71 \pm 0.5 m²/g and 99.75%, respectively. The size of A-SiO₂ nano and microparticles in their powdery state (Figure 1) and in suspension in DMEM are shown in Table 2.

After the purification process, the amorphous nature of SiO₂ microparticles was confirmed by X-Ray diffraction, and did not show a peak in the 2 θ = 27-26 $^\circ$ (Wang et al., 2012) (Figure 2).

Cell survival rate

A549 cell-line exposure to A-SiO₂ nano and microparticles in all concentrations and two time periods showed concentration and time-dependent reduction in cell survival. Reduction in cell survival during 24-hour exposure time with A-SiO₂ nano and microparticles expect 10 μ g/ml concentration of A-SiO₂ nanoparticles compared to the control group was statistically significant (P < 0.05). A549 cell-line exposed for 72 hours to A-SiO₂ nano and microparticles demonstrated significantly lower cell survival than the control group (P < 0.05).

The results of this study showed a significant

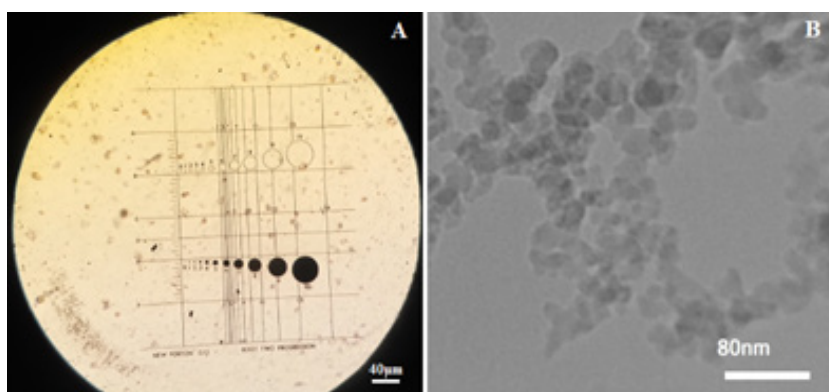


Figure 1. A-SiO₂ A) microparticles (PCM) and B) nanoparticles (TEM)

Table 1. Purification Efficiency of A-SiO₂ in Micron Sizes by ICP-OES

Metal impurity as (ppm)	Ag	Al	Be	Cd	Cr	Cu	Fe	Li	Mn	Ni	Pb
Before extraction	0.5>	1148.1	1.46	0.11	38.37	15.51	8077.24	20.78	43.31	14.26	11.16
After extraction	0.5>	451.15	0.39	0.1>	31.12	3.76	2055.41	20.45	5.9	4.08	4.34

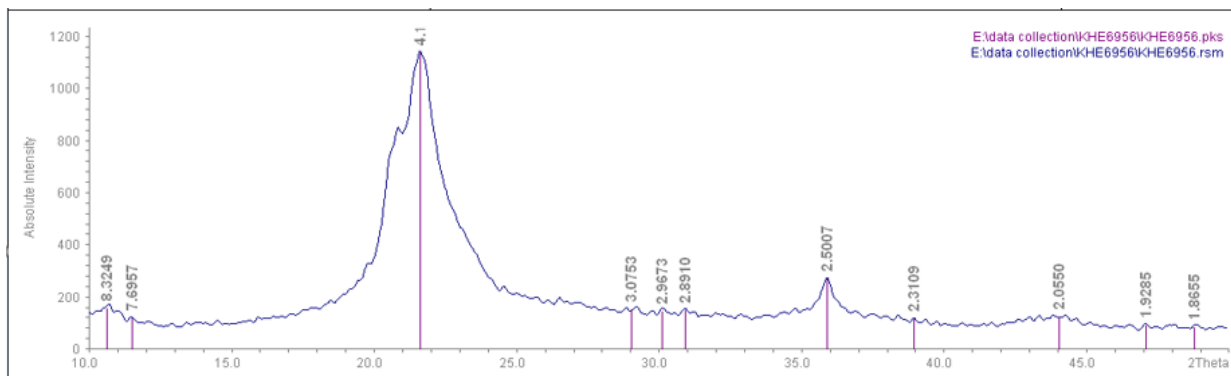


Figure 2. XRD Analysis for A-SiO₂ Microparticles

Table 2. Powdery Size, DLS (in DMEM, 50 µg/ml Concentration, 25°C) and Zeta Potential (in Analar Water, 25°C) Analysis Results for Nano and Micro A-SiO₂ Particles

	Average size in powdery state (nm)	Average size in suspension state (nm)	Zeta potential (mV)
A-SiO ₂ microparticles	3670	9927.08	-32.2
A-SiO ₂ nanoparticles	≤ 20	220. 58	-34.2

difference in A549 cell survival when exposed to A-SiO₂ microparticles compared to nanoparticles in all concentrations (P < 0.05). It must be added that cell survival at 24-hour exposure time with all concentrations of A-SiO₂ except for the 250 µg/ml microparticles was lower than nanoparticles, and at 72-hour exposure time at all concentrations of A-SiO₂ nanoparticles except 10 µg/ml was lower than A-SiO₂ microparticles.

A549 mitochondrial membrane potential rate

The decrease in the mitochondrial membrane potential of exposed cells in all concentrations of A-SiO₂ nanoparticles, except 10 µg/ml, in 72-hour exposure, was significantly higher than the control group and demonstrated a decrease proportional to exposure time and an increase in concentration (P < 0.05). Also, the decrease in the A549 mitochondrial membrane potential in cells exposed to all concentrations of A-SiO₂ microparticles was higher compared to exposure to A-SiO₂ nanoparticles

in both exposure periods (P < 0.05).

Intracellular ROS rate

The decrease in the mitochondrial membrane potential of exposed cells in all concentrations of A-SiO₂ nanoparticles, except 10 µg/ml, in 72-hour exposure, was significantly higher than the control group and demonstrated a decrease proportional to exposure time and an increase in concentration (P < 0.05). Also, the decrease in the A549 mitochondrial membrane potential in cells exposed to all concentrations of A-SiO₂ microparticles was higher compared to exposure to A-SiO₂ nanoparticles in both exposure periods (P < 0.05).

Intracellular glutathione content rate

In the 24-hour exposure to A-SiO₂ microparticles, the intracellular glutathione content was higher than the control group but in the 72-hour exposure to A-SiO₂ microparticles, the intracellular glutathione was

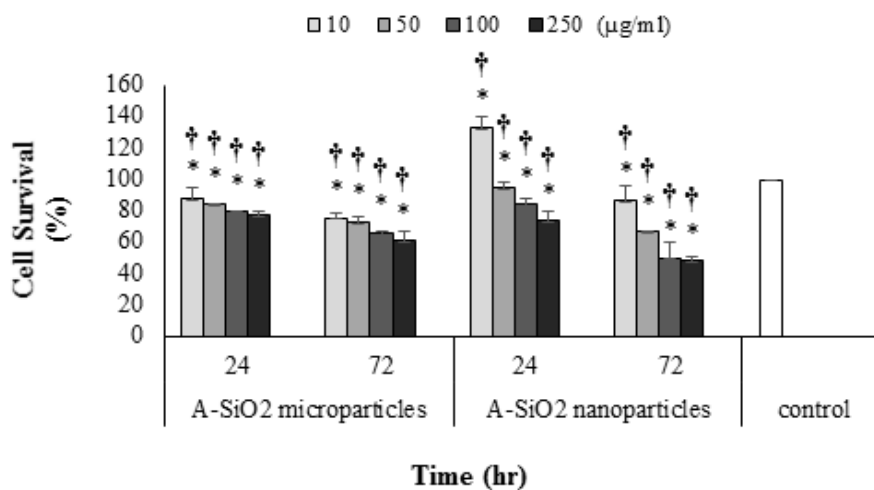


Figure 3. A549 Cell-Line Survival in 24- and 72-hour Exposure Periods and 10, 50, 100 and 250 µg/ml Concentrations of A-SiO₂ Nano and Microparticles. (Results of mean ± SD of cell survival percent from three experimental tests). * † Significant statistical differences; 1) between experimental groups compared to control and 2) between comparative experimental groups exposed to micro and nano-sized particles (p-value < 0.05)

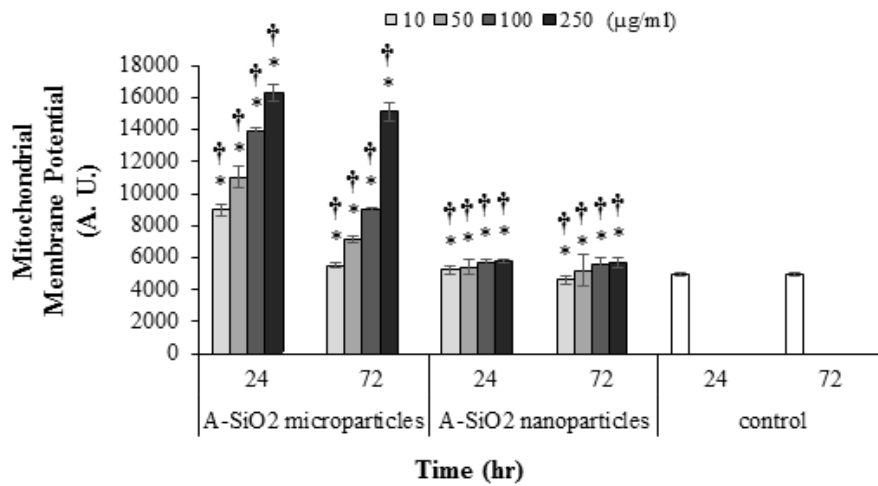


Figure 4. A549 Cell-Line Mitochondrial Membrane Potential Reduction in 24- and 72-hour Exposure Periods and 10, 50, 100 and 250 µg/ml Concentration of A-SiO₂ nano and Microparticles. (Results of mean ± SD of absorbance rate from three experimental tests). * † Significant statistical differences; 1) between experimental groups compared to control and 2) between comparative experimental groups exposed to micro- and nano-sized particles (p-value <0.05)

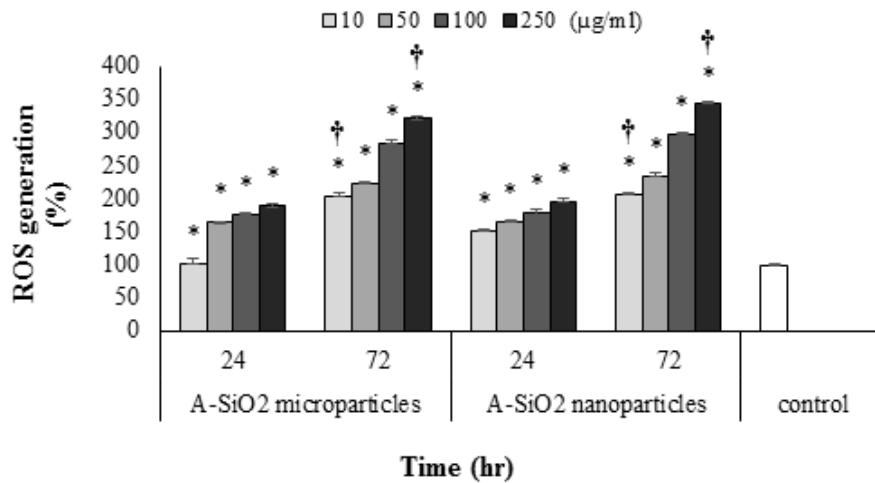


Figure 5. Intracellular ROS Generation in 24- and 72-Hour Exposure Periods to 10, 50, 100 and 250 µg/ml Concentrations of A-SiO₂ Nano- and Microparticles. (Results of mean ± SD of ROS generation percent from three experimental tests). * † Significant statistical differences; 1) between experimental groups compared to control and 2) between comparative experimental groups exposed to micro- and nano-sized particles (p-value <0.05)

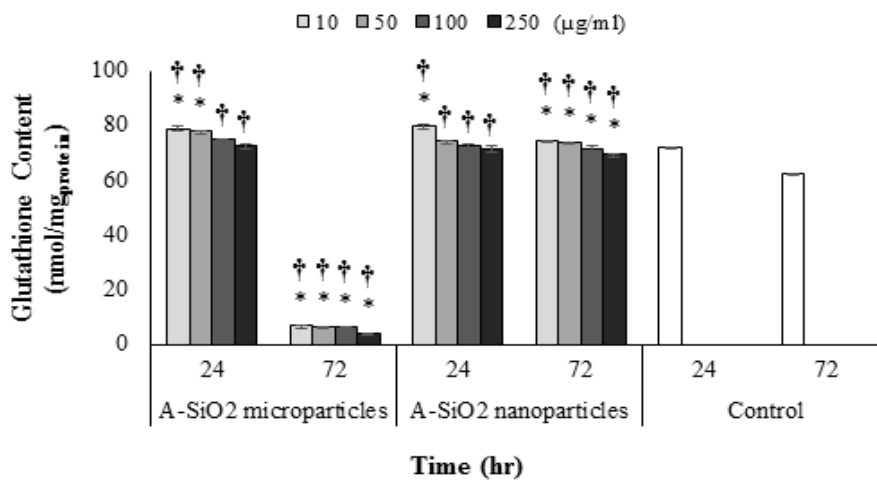


Figure 6. Intracellular Glutathione Content in 24- and 72-Hour Exposure time to 10, 50, 100 and 250 µg/ml Concentrations of A-SiO₂ Nano and Microparticles. (Results of mean ± SD of GSH concentration per mg of cell's protein from three experimental tests). * † Significant statistical differences; 1) between experimental groups compared to control and 2) between comparative experimental groups exposed to micro- and nano-sized particles (p-value <0.05)

significantly lower than the control group and showed concentration-dependent behavior.

The intracellular glutathione content in the 24-hour exposure to all concentrations of A-SiO₂ nanoparticles except 250 µg/ml, was lower than the control group. In the 72-hour exposure to A-SiO₂ nanoparticles, the amount of glutathione in all concentrations was higher than the control group and showed a negative proportionality to higher concentrations. Also, following the increase of exposure time to A-SiO₂ microparticles, the decrease in intracellular glutathione content was more than A-SiO₂ nanoparticles, and increasing the exposure time resulted in decreased intracellular glutathione content.

Discussion

Silicon dioxide particles are among the most commonly used in industries, constructions, and cities (Hurley et al., 2003; Karlsson et al., 2008; Faraji and Wipf, 2009; Rivas-Sánchez et al., 2009; Mishra, 2015). The size of silicon dioxide particles and the toxicity of nanoparticles has been a health concern in spite of their benefits in industries and pharmaceutical applications (Szalay, 2012b). Since most of the toxicological information of nanoparticles has been derived from experiments on the animal species (Szalay, 2012b), the present study was designed to investigate the effect of SiO₂ particles on the human pulmonary cell-line.

The findings of this study showed that the concentration of both nano and microparticles of A-SiO₂, and their exposure time affected the toxicity of the exposed A549 cell-line. Generally, this study demonstrated a lower cell survival, mitochondrial membrane potential, and higher ROS generation in cells exposed to A-SiO₂ microparticles compared to nanoparticles. Decrease of the intracellular glutathione content, A549 mitochondrial membrane potential, and cell survival and the increased ROS generation of exposed cells were concentration- and time-dependent. In a study by Lin et al., (2006) the A-SiO₂ particle toxicity in human bronchial cells was investigated in two sizes of 15 and 46 nm, and the results showed that the toxicities of the two nanoparticles were similar to each other. These results seemed fairly consistent with the present findings in terms of cell survival compared to the control group concluding that the toxic effect of A-SiO₂ was through damage to the mitochondrial membrane. However, in contrast to the present study, Napierska et al., (2009) stated that the cell viability of the EAHY926 cell-line exposed to A-SiO₂ nanoparticles was lower than A-SiO₂ microparticles.

Contrary to the results of the present study, He et al., (2009) stated that the toxicity of A-SiO₂ nanoparticles was higher than A-SiO₂ microparticles in the MDA-MB-468 and COS-7 cells. They reported that nanoparticles had a higher potential of penetration through the lysosomes compared to microparticles and hence have a more lethal effect on the cells through the rupturing of the lysosome and cell lysis. The disparity of the present findings with those of He and et al. could be explained through differences in test concentrations and the nature of the cell lines used in the two studies. However, Chen et al.,

(2004) also confirmed the results of the present study by examining the effect of A-SiO₂ nano and microparticles on the Wistar rat lung, and they reported a higher incident rate of lung fibrosis for microparticles, justifying the lower toxicity of A-SiO₂ nanoparticles through the easier movement in and out of the exposed cells, and pointed out that this could be a key factor in reduced toxicity. Guichard et al.,'s findings (2012) were in line with the results of the present study and stated that the toxicity of the cells exposed to particles is dependent on their concentration and exposure time as well as their size.

Despite findings of our study, which demonstrated lower toxicity for the A-SiO₂ nanoparticles compared with A-SiO₂ microparticles for a short exposure period of 24 hours, the cells exposed to nanoparticles had a higher ROS generation, as well as an increased the production rate of glutathione content which could nullify the potent ROS. The result of the present study defies other authors who have reported the amount of intracellular ROS as a reduction factor in the content of intracellular glutathione (Chen et al., 2018).

In conclusion, the results of the present study demonstrated the toxicity of A549 human cell lines exposed to A-SiO₂ nano and microparticles were concentration and time dependent. In shorter exposure periods, nanoparticles had lower toxicity than microparticles in similar exposure concentrations. Generally, it was demonstrated that particle size is not the only factor affecting the toxicity, and concentration and exposure time are also important factors. The authors of this study recommend further studies exploring the possible mechanisms of toxicity phenomena observed in this study.

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Conflict of interest

The authors declare no conflict of interest relating to the material presented in this Article. Its contents, including any opinions and conclusions expressed, are solely those of the authors.

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