Romanian Biotechnological Letters *Vol. 15, No.1, 2010*

ORIGINAL PAPER

Antioxidative response induced by SiO_2 nanoparticles in MRC5 cell line

Received for publication, September 20, 2009
Accepted, January 4, 2010

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Abstract

Amorphous SiO_2 nanoparticles may be harmful to humans and also ecotoxic. In our study, the effect of these nanoparticles on some antioxidant scavenging enzymes belonging to MRC-5 cells was investigated. The cells were exposed to 6.3 x 10^5 SiO_2 nanoparticles (3-14 nm) per individual cell for 24, 48 and 72 hours, respectively. The treatment resulted in the appearance of a significant number of cells with modified shape. In more detail, the variation of SOD specific activity showed an increase of 88% only after 72 hours, whereas CAT activity significantly increased with 15% and 30% after 48 and 72 hours respectively. The enhancement of the GPX and GST specific activity with 56% and 43%, respectively, after 72 hours suggested that important lipid peroxidation occurred.

In conclusion, it appeared that up to 72 hours of exposure, MRC-5 cells can counteract the SiO_2 nanoparticles induced oxidative stress.

Key words: MRC-5 cells, SiO_2 nanoparticles, antioxidant scavenging enzymes

Introduction

Silica (SiO_2) is most commonly found in nature as sand or quartz, as well as in the cell walls of diatoms. It is a chemical substance used as filler, additive or a rheological modifier in the formulas of many products such as paints, coatings, plastics, synthetic rubber, insulation materials, etc. Amorphous SiO_2 is added to concrete for the improvement of its strength and durability as well as for the decrease of its porosity. It is also used in eutectic mixtures with appropriate additives as boron oxide or phosphorous oxide in order to lower the necessary sintering temperature and increase the chemical stability of the coatings of magnesium alloys [1]. Silica nanoparticles can be released in a time-dependent manner from all these materials. Tribological studies on SiO_2/acrylate nanocomposites showed that friction led to the gradual loss of SiO_2 nanoparticles [2]. At the same time, SiO_2 nanoparticles applied in tires are released by the interaction between tires and road [3].

Silica is potentially an ideal nanomaterial for biomedical applications because it can be easily surface functionalized for bioconjugation, and it is quite biocompatible and resistant to biodegradation in cellular environments. Silica nano-sized particles are known to bear a negative charge due to the dissociation of surface silanol groups, which can be easily modified by various functional groups. Taking into account the fact that modified silica

nanoparticles can effectively penetrate the cell membrane, great effort has been put forth into research in order to use them as carriers for drug or gene delivery [4, 5, 6, 7, 8].

Human risks are a major concern due to nanoparticles being present in the workplace and outer atmosphere, as well as being used in medical applications [9, 10]. Several *in vitro* studies revealed some key factors, according to which nanoparticles introduced in cell cultures could influence biological functions or induce cytotoxicity [11, 12, 13,14,15,16]. Their toxicity greatly depends on various parameters, such as the chemical composition, size, their dosage forms, etc.

Exposure to silica nanoparticles could generate potential risks to the cardiorespiratory systems of old subjects [17]. Pulmonary retention and extrapulmonary redistribution of inhaled silica nanoparticles have been considered to be important contributing factors of cardiorespiratory diseases.

Previous studies showed that amorphous SiO_2 nanoparticles may be dangerous to humans [18] and may be ecotoxic [19].

Many studies have indicated that the toxicity of insoluble materials increases with decreasing particle size. The precise mechanisms by which these materials exhibit higher levels of toxicity, at smaller particle sizes, have yet to be elucidated. Inflammation, autonomic nervous system activity, procoagulant effects, stimulation of capsaicin/irritant receptors and ROS production were taken into consideration, although it is now understood that oxidative stress plays an important role in initiating the chain of events, at the molecular and cellular level, leading to the observed health effects [20]

The reactive oxygen species (ROS)-mediated toxicity has been taken into consideration as a possible mechanism responsible for silica nanoparticles-induced cell injury in mammals**.**

ROS are molecules or ions formed by the incomplete one-electron reduction of oxygen. These reactive oxygen intermediates include singlet oxygen, superoxides, peroxides and hydroxyl radicals. ROS are formed continuously in cells as a consequence of both oxidative biochemical reactions and external factors, such as certain environmental pollutants. Organisms have developed numerous cellular defense mechanisms, which under normal metabolic conditions, regulate the level of ROS and protect against the ill-effects of free radicals. The defense system includes both low-molecular-weight free radical scavengers, such as the tripeptide glutathione (GSH),as well as antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX)[21].

When the production of ROS in the cells exceeds the ability of the antioxidant system to eliminate them, oxidative stress results [22]. Due to the high reactivity of ROS, most cellular components are likely to be targets of oxidative damage: lipid peroxidation, protein oxidation, GSH depletion, DNA single strand breaks, are all initiated by ROS excess. All of these events ultimately lead to cellular dysfunction and injury [23].

The aim of this study was to investigate the antioxidative response in MRC-5 cell line exposed to silica nanoparticles.

Materials and methods

Chemicals

GIBCO® Modified Eagle's Medium (MEM), fetal bovine serum, gentamycin (10 mg/ml), L-glutamine and vitamins solution (100X) were purchased from Invitrogen (Carlsbad, California, USA). Nicotinamide adenine dinucleotide phosphate disodium salt ($NADP^+$) and nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt

(NADPH) were supplied by Merck (Darmstadt, Germany). All the others chemicals used were of analytical grade and were supplied by Sigma (St. Louis, USA).

Cell lines and treatment

MRC-5 cells were maintained in Modified Eagle's Medium (MEM) containing non-essential amino acids, Earle's salts, L-glutamine and 10% fetal bovine serum. Cells were grown as monolayers in a humidified 5% CO_2 air atmosphere at 37°C in 75cm^2 culture flask. The cells were seeded at a density of 2.5x10^5 cells/ml. The stock suspension of SiO_2 nanoparticles was previously sterilized. In each study, the stock suspensions were sonicated and freshly diluted to appropriate concentrations in the cell medium. The primary amorphous nanoparticle size distribution was a lognormal function, in the range 3-14 nm, most of them being of 5-8 nm. The cells were incubated with silica nanoparticles at concentrations of 1.26 x 10^5 , 3.15 x 10^5 , respectively 6.3 x 10^5 SiO_2 particles per individual cell for 24, 48 and 72 hours. Controls without treatment were performed for each analysis.

Cell viability assay

Cell viability was determined by the MTT test [24]. The medium from each well was removed by aspiration, the cells were washed with 200 µl phosphate buffer solution (PBS)/well and then 50µl (1mg/ml) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to each well. After 2 hours of incubation the MTT solution from each well was removed by aspiration. A volume of 50 µl isopropanol was added and the plate was shaken to dissolve formazan crystals. The optical density at 595nm, for each well, was then determined using a Tecan multiplate reader (Tecan GENios, Grödic, Germany).

Preparation of Cell lysat

MRC-5 cells were harvested from culture flasks, washed with phosphate buffer solution (PBS) and centrifuged at 1,500xg for 10 min at 4^{0}C. Cell pellets were re-suspended in 0.5 ml of phosphate buffer solution (PBS) and then, ultrasonicated on ice three times, for 30 seconds. The total extract was centrifuged at 3,000xg for 15min at 4^{0}C. Aliquots of the supernatant were used for enzyme assays.

Antioxidant enzymes assay

The total SOD (EC 1.15.1.1) activity was measured according the spectrophotometric method of Paoletti [25], based on NADPH oxidation. The method consists of a purely chemical reaction sequence which generates superoxide anion from molecular oxygen in the presence of EDTA, manganese (II) chloride and mercaptoethanol. The decrease in absorbance at 340 nm was followed for 10 min to allow NADPH oxidation. A control was run with each set of three duplicate samples and the percentage inhibition was calculated as (sample rate)/ (control rate) ×100. One unit of activity was defined as the amount of enzyme required to inhibit the rate of NADPH oxidation of the control by 50%.

The CAT (EC 1.11.1.6) activity was assayed by monitoring the disappearance of H_2O_2 at 240 nm, according to the Aebi method [26]. The CAT activity was calculated in terms of U/mg protein, where one unit is the amount of enzyme that catalyzed the conversion of one µmole H_2O_2 in a minute.

The total GPX peroxidase (EC 1.11.1.9) was assayed by the Beutler [27] method, using H_2O_2 and NADPH as substrates. The conversion of NADPH to $NADP^+$ was followed by recording the changes in absorption intensity at 340 nm, and one unit was expressed as one µmole of NADPH consumed per minute, using a molar extinction coefficient of 6.22x10^3 M^{-1} cm^{-1}.

The glutathione *S*-transferase (GST) (EC 2.5.1.18) activity was assayed spectrophotometrically at 340 nm by measuring the rate of 1-chloro-2,4-dinitrobenzene (CDNB) conjugation with GSH, according to the Habig et al. method [28]. One unit of GST

activity was defined as the formation of one μmole of conjugated product per minute. The extinction coefficient 9.6 $mM^{-1}cm^{-1}$ of CDNB was used for the calculation.

Protein concentration

The protein concentration, expressed as mg/mL, was determined by the Bradford method [29] using bovine serum albumine as standard.

Statistical analysis

All values were expressed as means of triplicate ± SD. The differences between control and SiO_2 nanoparticles-treated cells were compared by means of the Student's test using standard statistical packages. The results were considered significant only if the p value was less than 0.05.

Results and discussion

Previous studies revealed that a strong interaction of uptake of silica nanomaterials with intracellular biomolecules might trigger inflammation and generation of ROS and oxidative stress (30, 31, 32, 33, 34).

Cytotoxicity induced by SiO_2 nanoparticles

The viability of MRC-5 cells treated with silica nanoparticles at concentrations of 1.26 x 10^5, 3.15 x 10^5, respectively 6.3 x 10^5 SiO_2 particles per individual cell for different periods of time are presented in Figure 1. The results suggested that the cytotoxic effect of these nanoparticles appeared to be time-and concentration- dependent. Treatment of the cells with the three different concentrations for 24 hours each resulted in no change of viability for the concentration of 1.26x10^5 and to an increase with about 14% for 3.15 x 10^5 and 6.30 x 10^5 nanoparticles per individual cell (Figure 1). After 48 and 72 hours, a decrease of cell viability with 10% and 30%,respectively, compared to control, was noticed only at the highest concentration.

Taking into account that MRC-5 cells presented a sensitivity to exposure to SiO_2 nanoparticles at a concentration of 6.30 x 10^5 particles per individual cell, all the following experiments were done using this concentration at 24, 48 and 72 hours of exposure.

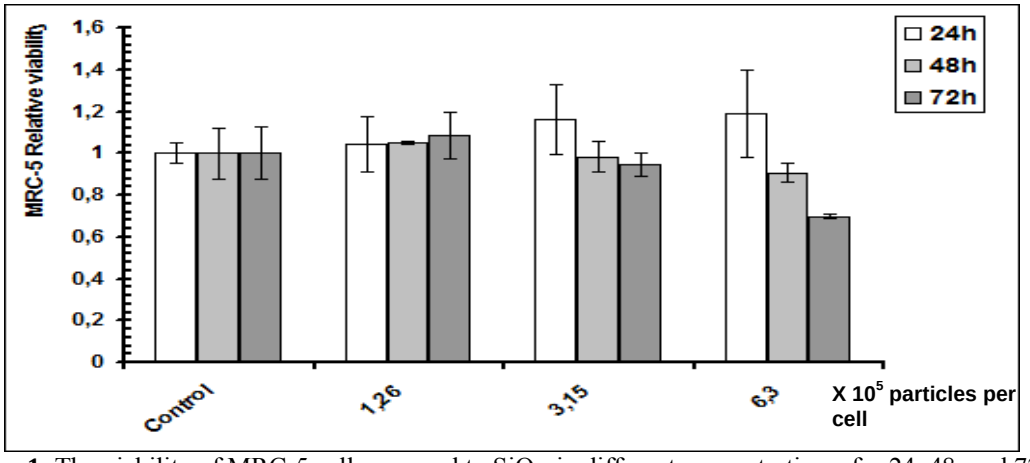

Figure 1: The viability of MRC-5 cells exposed to SiO_2, in different concentrations, for 24, 48, and 72 hours.

The phenotype of the MRC-5 cells treated with 6.3x10^5 SiO_2 particles per individual cell is shown in **Figure 2**. As observed, the control fibroblast (**Figure 2 – A, B, C**) presented a uniform morphology being uninucleated and adherent to support. After the treatment with SiO_2 (**Figure 2 – I, II and III**) the cells suffered several morphological changes, losing their

shape and remaining connected through extensions similar to dendrites. The exposure to $6.3x10^5$ SiO_2 particles per individual cell for 48 and 72 hours (**Figure 2 – II, III)** determined the appearance of an important number of cells with modified shapes.

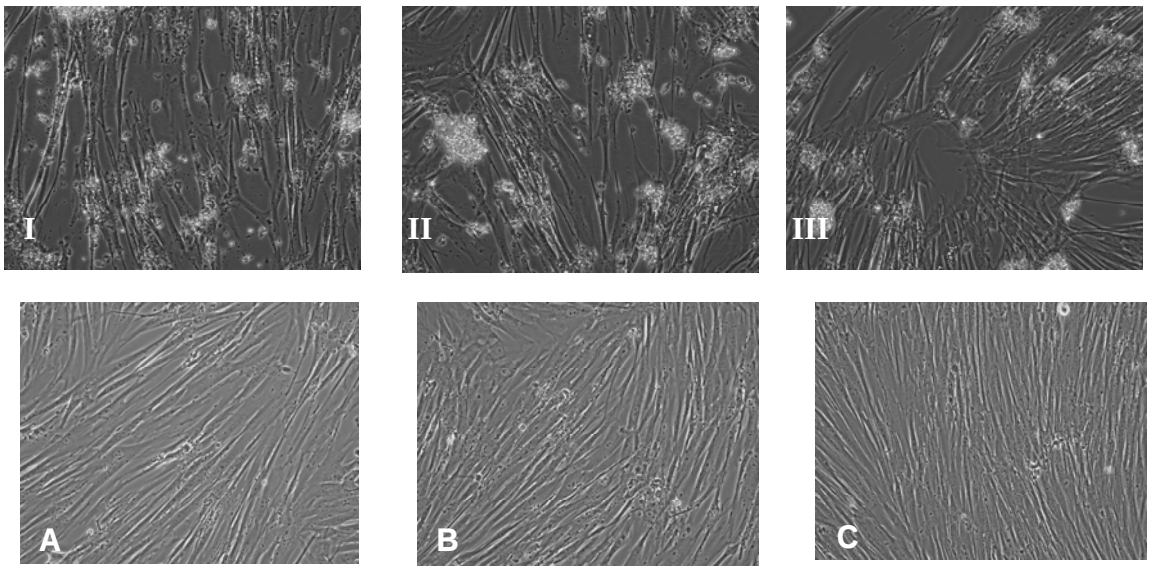

Figure 2. The effects of SiO_2 on MRC-5 cells morphology: control cells at 24 h (A), 48 h (B) and 72 h (C); cells treated 24 hours (I), 48h (II) and 72 h (III) with $6.3x10^5$ SiO_2 particles per individual cell

The antioxidant scavenging enzymes

The variation of SOD specific activity in MRC-5 cells treated with $6.3x10^5$ SiO_2 nanoparticles per cell remained unmodified after 24 and 48 hours and showed an increase with 88% only after 72 hours (Figure 3). In the case of CAT, after 24 hours of exposure, an unsignificant decrease of the specific activity was noticed, followed by a significant increase with 15% and 30% after 48 and 72 hours respectively.

An enhancement in specific activities of the enzymes involved in GSH metabolism was recorded after MRC-5 treatment with $6.3x10^5$ SiO_2 nanoparticles per cell (Figure 4).

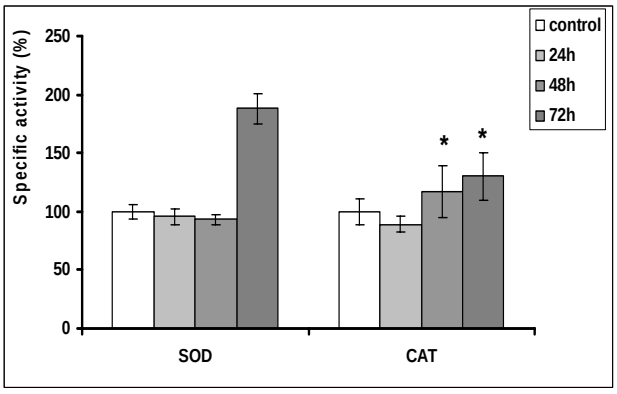

Figure 3. SOD and CAT specific activity of MRC-5 cell line exposed to $6.3x10^5$ SiO_2 particles per individual cell for 24, 48 and 72 hours. Data are expressed as mean±S.E. Significant changes vs. control * $P<0.05$

Our data showed that GPX specific activity increased after 48 and 72 hours of exposure with 39% and 56%, respectively, compared to control. For GST, the specific activity has enhanced with 38% and 43% after 48 and 72 hours of treatment, respectively.

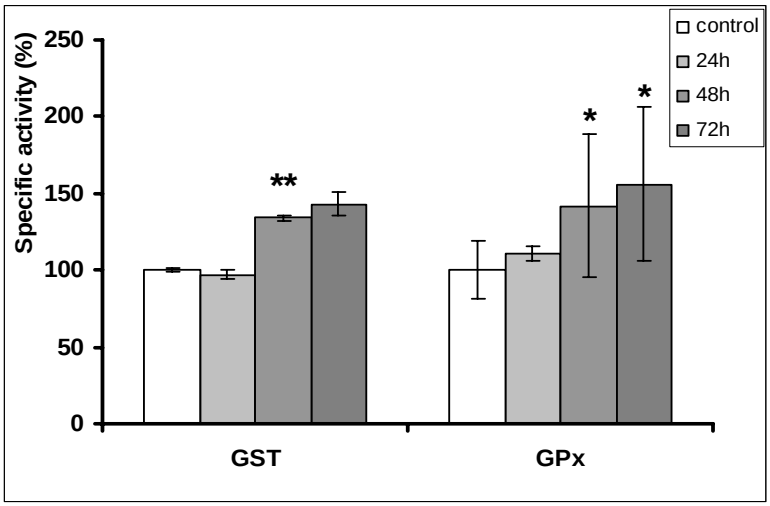

Figure 4. GST and GPX specific activity in MRC-5 cell line exposed to $6.3x10^5$ SiO_2 particles per individual cell for 24, 48 and 72 hours. Data are expressed as mean±S.E. Significant changes vs. control * $P<0.05$ and ** $P<0.01$

Discussions

The rapid development of nanotechnologies and anthropic activities created the possibility of nanoparticles release in the ambient atmosphere. Nowadays it is accepted that inhaled nano-sized particles may evade phagocytosis, cross cell membranes and redistribute to other sites of the body [35]. Our study was carried out in order to investigate the effects of silica nanoparticles on some biochemical parameters of the MRC-5 cell line, derived from human fetal lung fibroblasts and expressing α-smooth muscle actin, considered to be a myofibroblast [36].

The modified shapes of the cells after the treatment with the highest concentration of nanoparticles (Figure 2) for 48 and 72 hours suggest a stress-generating impact between the cells and silica particles.

Considering Figures 3 and 4, it is obvious that after 24 hours of exposure, there was no decreasing effect on the four enzymatic-specific activities. In the case of SOD activity, an increase compared to control was registered only after 72 hours, which could be due to a late appearance of oxidative stress. The time dependent variation of CAT activity had almost the same pattern as the SOD one.

The increase of the GPX specific activity with 56% and of the CAT activity only with 30% after 72 hours suggests that after the exposure of MRC-5 cells to SiO_2 nanoparticles, the generation of hydrogen peroxide is less significant than that of lipid peroxidation. GPX reduces the lipid hydroperoxides to hydroxylated lipid derivates. The GSTs family includes enzymes capable to catalyze multiple reactions in order to detoxify peroxidised lipids, as well as the metabolism of xenobiotics [37]. At the same time, the upregulation of GPX and GST activity can be correlated with an increase of GSH level, formed in order to counteract nanoparticles-induced oxidative stress. Our results differ from those obtained by other scientists, who observed a lower level of GSH in human kidney embrionic cells treated with silica nanoparticles [38] compared to control as well as those of Yu et al.[39]. in keratinocytes. The different results could be due to the difference between the size of particles used by us (3-14 nm) and those used by Wang et al. [38] (50 nm) and Yu et al.[39] (30 nm).

Our results suggest that the SiO_2 nanoparticles induced oxidative stress but the antioxidant enzymes maintained the cellular redox homeostasis in MRC-5 cells during the time of exposure. Nevertheless surviving cells showed a modified phenotype.

This preliminary data suggest that the treatment of the MRC-5 cell line with SiO_2 nanoparticles generated cellular morphological modifications and oxidative stress as indicated by the elevation of specific activities of SOD, CAT, GPX and GST. It seems that until 72 hours of exposure, MRC-5 cells can counteract the nanoparticle-induced oxidative stress. The excessive environmental exposure of lung fibroblasts to SiO_2 nanoparticles might activate molecular mechanisms of some human lung diseases based on oxidative damage.

Further evaluation of the relationship between toxicity and variety of sizes, shapes, and chemical modifications on the surface of particles is needed, and future studies based on these data will provide very useful information on the effects of environmental silica nanoparticles on lung cells.

Acknowledgments

This study was financially supported by the National Research Council of Higher Education, Romania, Grant number: 340/2007 and COST B35 Action/2006.

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