

***In Vitro* Cytotoxicity and Molecular Effects Related to Silicon Nanoparticles Exposures**

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Geliş tarihi : 01.08.2016 Kabul tarihi : 02.03.2017

Abstract

Silicon nanoparticles are widely used for various applications including environmental, biological, chemical and physical. And, to translate these nanomaterials to the clinic and industrial domains, their safety needs to be verified, particularly in terms of genotoxicity and cytotoxicity. Therefore, in this study, we aimed to investigate of cytotoxicity and changes in gene expression profiles influenced by commonly silicon (as silicon carbide, silicon dioxide, silicon nitride) nanoparticles in human alveolar epithelial (HPAEpiC) and pharynx (HPPC) cell lines in vitro since inhalation is an important pathway for exposure to these nanoparticles. HPAEpiC and HPPC cells were treated with silicon (0-100 µg/mL), nanoparticles for 72 h, and then cytotoxicity was detected by, [3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyltetrazolium bromide] (MTT) and lactate dehydrogenase (LDH) release assays, while genotoxicity was also analyzed by cDNA array - RT-PCR assay. According to the results of MTT and LDH assays, all tested nanoparticles induced cytotoxicity on both HPAEpiC and HPPC cells in dose-dependent manner. Determining and analyzing the gene expression profiles of HPAEpiC and HPPC cells, silicon nanoparticles showed changes in genes related to apoptosis, DNA damage or repair and oxidative stress. This study of gene expression profiles affected by nanotoxicity provides critical information for the clinical and environmental applications of silicon nanoparticles.

Keywords

Airway epithelial cell;
Genotoxicity; *In vitro*
gene expression;
Nanotoxicity; Silicon
nanoparticles

Silikon Nanopartikül Maruziyetine Bağlı Olarak Oluşan *In Vitro* Sitotoksik ve Moleküler Etkiler

Özet

Silikon nanopartikülleri çevresel, biyolojik, kimyasal ve fiziksel amaçlarla çeşitli alanlarda yaygın olarak kullanılmaktadır. Bu nanopartiküllerin klinik ve endüstriyel alanlarda kullanılabilmesi için güvenilirlikleri özellikle genotoksisite ve sitotoksisite açısından doğrulanmalıdır. Bu yüzden mevcut çalışmada, yaygın olarak kullanılan silikon nanopartiküllerinin (silikon karbid, silikon dioksit, silikon nitrit) insan alveolar epitel (HPAEpiC) ve farinks (HPPC) hücrelerindeki sitotoksisitesi ve gen ekspresyon profillerindeki değişimlerin araştırılması amaçlanmıştır. HPAEpiC ve HPPC hücreleri 72 saat boyunca silikon nanopartikülleriyle (0-100 µg/mL) muamele edildi. Nanopartiküllerin sitotoksisite değerlendirmeleri için 3-(4,5 dimetylthiazol -2-yl) - 2,5 diphenltetrazolium bromide (MTT) ve laktat dehidrogenaz salınım (LDH) yöntemleri kullanılırken; genotoksisite analizi için cDNA array - RT-PCR yöntemi kullanıldı. MTT ve LDH yöntemi sonuçlarına göre, uygulanan bütün test nanopartikülleri hem HPAEpiC hem de HPPC hücre hatlarında doza bağlı olarak sitotoksisiteyi indüklemiştir. HPAEpiC ve HPPC hücrelerinin ilgili genler açısından (apoptozis, DNA fasarı ve tamiri, oksidatif stres) gen ekspresyon profilleri incelendiğinde silikon nanopartiküllerinin ekspresyonu değiştirdiği gözlenmiştir. Bu çalışmadan elde edilen nanotoksisiteye bağlı olarak oluşan gen ekspresyon profilleri, silikon nanopartiküllerin klinik ve çevresel uygulamalarda kullanılabilmesi için önemli bir kaynak oluşturmaktadır.

Anahtar kelimeler

Alveolar epitel
hücreleri;
Genotoksisite; *In vitro*
gen ekspresyonu;
Nanotoksisite; Silikon
nanopartikülleri

1. Introduction

Nano-scale materials have at least one size in the range of 100 nm or less (The Royal Society and The Royal Academy of Engineering 2004). Nano-scale natural products can be produced by inherently consisting of processes such as volcanic events, fire, and erosion; as such, living things have been exposed to these particles. On the other hand, non-natural nanoparticles can enter the natural environment indeliberately via atmospheric diffusion, household waste, and farming and inadvertently release in the case of production/transportation (Zhang and Elliott 2006; Stampoulis *et al.* 2009). However, owing to singular their physical, mechanical and chemical properties, nanomaterials are commonly used in commercially available in personal care products, pharmaceuticals and foods (Yolanda 2016, Ong *et al.* 2016). The overwhelming growing production and use of nanomaterials result in an increasing number of workers and consumers exposed to nanomaterials via inhalation, dermal contact, or gastrointestinal tracts and other routes (Huang *et al.* 2009). Generally, nanomaterials are thought to have more serious adverse effects on organisms than micromaterials. Because, nanomaterials have smaller sizes and corresponding larger specific surface area than micromaterials (Kipin and Laskin 2005, Oberdorster *et al.* 2005, Nel *et al.* 2006). Many researchers and we have studied the different potential effects of nanomaterials with various compositions and properties on human and environmental health *in vitro* and *in vivo* settings (Sonmez *et al.* 2015, Leite-Silva *et al.* 2016, Turkez *et al.* 2016).

Nanostructures of silicon, including particles, dots, wires, rods and ribbons, have sparked much interest due to their unique electronic/mechanical properties and the improved performances, and make their potential application in many areas such as optoelectronic devices, solar cells and biological markers (Chantrenne and Lysenko 2005, Cavarroc *et al.* 2006, Zschech *et al.* 2007, Baca *et al.* 2007, Schmidt *et al.* 2009, Heintz *et al.* 2010). Among the different routes of nanoparticles exposure include inhalation, dermal, oral, and in the case

of biomedical applications. Inhalation is the most important from these possible exposure routes (Amoabediny *et al.* 2009). The exposure of silicon dioxide (SiO₂) caused pulmonary fibrosis in rats (Chen *et al.* 2004). In previous study, exposure of human bronchoalveolar carcinoma-derived cells to SiO₂ nanoparticles revealed dose- and time-dependent cytotoxicity (Lin *et al.* 2006). Mechanistic studies suggest that the cytotoxicity is due to elevated oxidative stress, oxidative DNA damage and lipid peroxidation (Gurr *et al.* 2005, Gerloff *et al.* 2009, Asadpour *et al.* 2016).

In this study, we investigated silicon nanoparticles (as silicon carbide, silicon dioxide, silicon nitride)-mediated changes in cellular pathway-specific gene expression associated with DNA damage or repair (ATM, Rad23 and Rad50), apoptosis (Anxa5 and FasI) and oxidative stress (Gpx2, Gs, Mt2, Cyp4a10) in human alveolar epithelial (HPAEpiC) and pharynx (HPPC) cell lines. Also, the relationships between cytotoxicity and gene expression of selected genes were determined.

The objectives of the present study were to understand the relationships between the following responses of HPAEpiC and HPPC to silicon nanoparticles: (1) cytotoxicity, and (2) genotoxicity (expression of selected genes).

2. Material and Methods

Cell cultures and treatment with silicon nanoparticles

HPAEpiC and HPPC cell lines (Science Cell, USA) was obtained from Sciencell Research Laboratories, Carlsbad, USA. Prior to the experiments, the cells were thawed and grown in tissue culture flasks as a monolayer in Dulbecco-modified Eagles-F12 medium (Sigma-Aldrich, USA) supplemented with 1% glutamine, 0.5% penicillin/streptomycin (PAN Biotech), and 10% fetal bovine serum at 37 °C in a humidified (95%) incubator with CO₂ (5%). The cultured cells were trypsinized with trypsin/EDTA for a maximum of 5 min and seeded with a subcultivation ratio of 1:3-1:8. HPAEpiC and HPPC cells were incubated with different concentrations (0, 5, 10, 20, 40, 80 and 100 µg/mL) of silicon NPs [silicon carbide (SiC; CAS No. 409-21-2), silicon dioxide (SiO₂; CAS No.

7631-86-9), silicon nitride (Si₃N₄; CAS No. 12033-89-5)](Sigma-Aldrich, USA) and incubated in a 96-well microtitre plates in triplicate at 37 °C, 5% CO₂ for 72 h.

2.1. Cytotoxicity testing

MTT assay

Cytotoxicity was assessed by measuring the formation of a formazan from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) spectrophotometrically test. HPAEpiC and HPPC cells were incubated with 0.7 mg/ml MTT for 30 min at 37 °C for 72 h at the end of the experiment. After washing with PBS the blue formazan was extracted from cells with isopropanol/formic acid (95:5). Cytotoxicity was photometrically determined at 560 nm using a Microquant reader.

Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) activity was measured in the culture medium after 72 h as an index of cytotoxicity, employing an LDH kit (Bayer Diagnostics®, France) adapted to the auto analyzer (ADVIA 1650, USA). Enzyme activity was expressed as the extra-cellular LDH activity percentage of the total activity in the wells.

2.2. Gene expression alteration by silicon nanoparticles

cDNA array-real-time RT-PCR assay was performed and scored with slight modifications according to Melo *et al.* (2010). Gene expression was determined by cDNA array-RT-PCR assay. HPAEpiC and HPPC were lysed using 500 µL Trizol reagent. After 10 min incubation at room temperature, 200 µL of chloroform, which is chilled in - 20°C, was added to the tubes and incubated 5 min at room temperature. At the end of the incubation period, tubes was centrifuged at 12,000g for 15 min in + 4°C. Including RNA the top aqueous phase was removed and transferred to a fresh tube. The RNA was pelleted by centrifugation at 12,000g (+ 4°C) for 15 min and precipitated with ice-cold ethanol. The RNA pellet

was air-dried and resuspended in 200 µL RNase-free water, treated with 200 µL DNase I, then immediately was maintained at -70 °C. RNA amount was measured using UV spectrophotometry at 260 nm. The purity and structural integrity of the RNA samples was evaluated by UV absorbance of the 260/280 nm ratio and visually checked on a 1% agarose gel stained with ethidium bromide (5 mg/mL). cDNA probes were synthesized using the High-Capacity cDNA Reverse Transcription Kit including the Moloney murine leukemia virus reverse transcriptase. 10 µL of 2x Real Time master mix was added into each well of a 96-well reaction plate. Then, into the same wells was added 10 µL of RNA sample, and was pipetted up and down two times to mix. Plates was centrifuged to spin down the contents and to eliminate any air bubbles for a very short time. Plates were placed to the thermal cycler. Thermal cycling conditions were 10 min 25 °C, followed at 37 °C for 120 min, and finally 85 °C for 5.

RT-PCR Assay

A simple RT-PCR method of using the specific primers was applied to detect the differential mRNA expression of HPAEpiC and HPPC cells. The primer sequences were retrieved benefiting data from the public database. While PCR analysis was performed, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene sequence was used as housekeeping control. PCR amplification products were analyzed on 1.5% agarose gels in the presence of ethidium bromide (0.5 µg/mL). Then, it was electrophoresed for 1 h at 100 V, and was photographed. Finally, the densities of the bands on the agarose gels were measured using the DNA imaging system. Data were measured as densitometry units according to the density of the GAPDH bands.

Statistical analysis

Statistical analysis was performed using SPSS Software (version 18.0, SPSS, Chicago, Illinois, USA). For statistical analysis of the obtained data,

Duncan's test was used. Statistical decisions were made with a significance level of 0.05.

3. Results

Similar results were observed in both cell lines treated with all kinds of silicon nanoparticles. According to determination of cell viability using MTT assay, applied all silicon nanoparticles

showed a dose- and time-dependent cytotoxicity in HPAEpiC and HPPC cells (Table 1.). Silicon nanoparticles exposure exhibited a significant cytotoxicity at all tested concentrations in both cell lines in 72 h. The IC20 values of MTT, which show the limiting value of toxicity, calculated to be closely 10 µg/ml for all silicon nanoparticles in both cell lines.

Table 1. Cell viability in HPAEpiC and HPPC cell cultures maintained 72 h in the presence of silicon NPs. (Control: HPAEpiC and HPPC cells without silicon NPs. Values inside the rectangle are statistically different from the corresponding control,* symbol presents significant differences at the p<0.05 level from the control group.)

Concentrations	SiC		SiO ₂		Si ₃ N ₄	
	HPAEpiC	HPPC	HPAEpiC	HPPC	HPAEpiC	HPPC
0 µg/mL	57,95 ± 1,09	60,36 ± 1,06	57,95 ± 1,09	60,36 ± 1,06	57,95 ± 1,09	60,36 ± 1,06
5 µg/mL	48,76 ± 1,1*	53,14 ± 1,06*	50,64 ± 1,08*	55,39 ± 1,08*	52,65 ± 1,1*	55,29 ± 1,08*
10 µg/mL	45,39 ± 1,08*	48,36 ± 1,07*	46,95 ± 1,09*	50,72 ± 1,09*	47,59 ± 1,1*	51,36 ± 1,07*
20 µg/mL	43,17 ± 1,07*	44,17 ± 1,1*	42,37 ± 1,08*	46,81 ± 1,08*	42,59 ± 1,07*	46,11 ± 1,09*
40 µg/mL	38,67 ± 1,07*	39,41 ± 1,1*	38,59 ± 1,08*	41,29 ± 1,07*	38,41 ± 1,07*	41,38 ± 1,08*
80 µg/mL	31,13 ± 1,1*	34,07 ± 1,08*	33,39 ± 1,07*	37,45 ± 1,07*	35,43 ± 1,09*	37,48 ± 1,08*
100 µg/mL	25,19 ± 1,09*	30,25 ± 1,08*	30,43 ± 1,09*	33,48 ± 1,1*	31,49 ± 1,08*	32,34 ± 1,09*

As shown in Table 2, LDH leakage in HPAEpiC and HPPC cell lines increased in a time- and doses-dependent manner. A statistically significant increases in LDH concentration was observed at

all tested concentrations of silicon nanoparticles in both cell lines in 72 h. Achieved results from LDH assay was in accordance with the cell viability results.

Table 2. LDH activities on HPAEpiC and HPPC cells treated with different concentrations of silicon NPs for 72 h. (Control: HPAEpiC and HPPC cells without silicon NPs. Values inside the rectangle are statistically different from the corresponding control,* symbol presents significant differences at the p<0.05 level from the control group.)

Concentrations	SiC		SiO ₂		Si ₃ N ₄	
	HPAEpiC	HPPC	HPAEpiC	HPPC	HPAEpiC	HPPC
0 µg/mL	67,23 ± 0,8	71,62 ± 0,9	67,23 ± 0,8	71,62 ± 0,9	67,23 ± 0,8	71,62 ± 0,9
5 µg/mL	75,49 ± 0,7*	83,49 ± 0,6*	78,39 ± 0,9*	83,49 ± 0,8*	74,59 ± 0,9*	89,34 ± 0,8*
10 µg/mL	97,92 ± 0,8*	101,27 ± 0,9*	94,95 ± 0,9*	104,59 ± 0,8*	87,30 ± 0,6*	109,45 ± 0,8*
20 µg/mL	117,85 ± 0,9*	126,62 ± 0,8*	107,31 ± 0,8*	129,48 ± 0,9*	98,43 ± 0,7*	127,38 ± 0,8*
40 µg/mL	139,97 ± 0,9*	150,36 ± 0,7*	121,34 ± 0,6*	153,44 ± 0,9*	123,54 ± 0,6*	156,48 ± 0,7*
80 µg/mL	179,12 ± 0,8*	192,97 ± 0,7*	149,47 ± 0,6*	172,39 ± 0,7*	155,43 ± 0,7*	182,39 ± 0,9*
100 µg/mL	195,62 ± 0,7*	210,74 ± 0,6*	173,26 ± 0,7*	189,26 ± 0,8*	183,49 ± 0,9*	195,33 ± 0,7*

We analyzed, using RT-PCR, the expression changes of 9 selected genes associated with different metabolic pathways in HPAEpiC and HPPC cell lines. The selected molecular markers were annexin A5 (Anxa 5) and Fas ligand (FasL), apoptosis markers; ataxia-telangiectasia mutated (ATM), RAD 23 and RAD50, DNA damage markers; cytochrome P450, family 4, subfamily a, polypeptide 10 (Cyp4a10), glutathione peroxidase (GPX2), glutathione reductase (GSR) and melatonin receptor 1b (MT2), oxidative stress markers.

Statistical analysis of silicon NPs were shown in Figures 1-3. In 10 µg/ml SiC, SiO₂ and Si₃N₄ nanoparticles-exposed both cells, Anxa, FasL, ATM, RAD23, RAD50, Cyp4a10, GPX2, GSR and MT2 genes were clearly induced by silicon nanoparticles exposure. In 10 µg/ml SiC, SiO₂ and Si₃N₄ nanoparticles increased the expression levels of all selected genes in both cell lines.

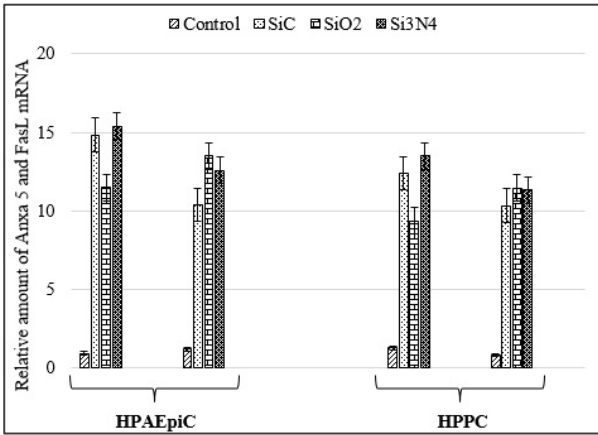


Figure 1. mRNA expression of apoptosis markers in silicon nanoparticles-exposed HPAEpiC and HPPC cells.

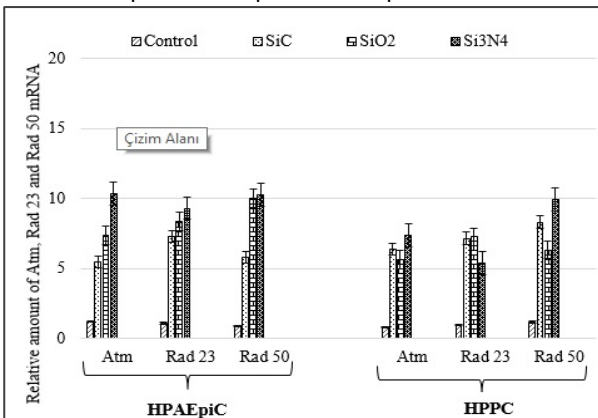


Figure 2. mRNA expression of DNA damage markers in silicon nanoparticles-exposed HPAEpiC and HPPC cells.

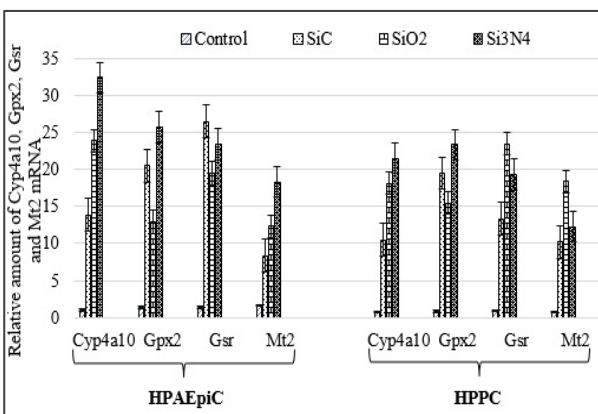


Figure 3. mRNA expression of oxidative stress markers in silicon nanoparticles-exposed HPAEpiC and HPPC cells.

4. Discussion and Conclusion

So far, although nanotechnological products and nanoparticles were used in many areas has been

mostly ignored the potential effects in environment and human health. Especially nanoparticles, therefore smaller than 100 nm and specific features of their size can be easily integrated into biological systems. Thanks to these features, nanoparticles was used in many fields such as biomedical and medical field, intelligent drug delivery, imaging, biosensors, nanomachines (biorobot), the nucleic acid analysis, nanofabrication of DNA chip for bioinformatics and genomic applications, stem cell-based organ engineering applications, implant materials, artificial tissue and nanosurgical as well as they can cause irreversible damage in cells and tissues (Gök 2007, Kocaeffe 2007, Tomalia *et al.* 2007, Portakal 2008, Syed *et al.* 2013, Atlı-Şekeroğlu 2013). Recently, various studies were identified the biological effects on tissues and organs of nanoparticles. But, it is necessary to investigate the potential toxicological effects in vivo and *in vitro* model systems (Ziady *et al.* 2003, Chen *et al.* 2006, Fischer and Chan 2007, Hwang *et al.* 2011). Thus, in the present study was focused on the nanotoxicity in order to understand of potential toxicity of nanoparticles in the genetic and cellular levels.

In this study, while MTT assay were used to determine the viability after exposure silicon nanoparticles, LDH assay was used to determine cytotoxicity in HPAEpiC and HPPC cells. The MTT assay was commonly used to define as quantitation of living cells still mitochondrially active in cultures (Brown *et al.* 1994). Also, LDH released into the medium provides an index of cell death and membrane permeability to LDH and an increase in LDH activity in the medium occurs as a result of cell membrane disintegration and enzyme leakage (Yokogawa *et al.* 2004). Applied all methods are often preferred in cytotoxicity studies. We demonstrated that all tested silicon nanoparticles have cytotoxic effect depending on dose- and time in HPAEpiC and HPPC cells. In previous study was investigated the cytotoxic effect of silicon dioxide (SiO₂) NPs in human epidermal keratinocyte (HaCaT) cells using Cell Counting Kit-8 (CCK-8) assay. According to study results, HaCaT cells lines exposed to

SiO₂ NPs showed cytotoxicity in a concentration-dependent and time-dependent manner (Gong *et al.* 2012). In a research conducted on murine macrophages (RAW 264.7), Choi *et al.* (2009) found that silicon NPs increased the cytotoxicity. Again, the results also support the observations of Yang *et al.* (2010), who have showed that exposure of HaCaT cells to 15-nm and 30-nm SiO₂ particles resulted in significantly decreased cell viability in a dose-dependent manner.

The RT-PCR was used for the studies of gene expression. The working with high sensitivity RT-PCR assay is a measurement method that reliable, fast and requiring a small amount of sample (Ünlü and Sağlar 2012). The expression of genes associated with the processes DNA damage or repair, apoptosis and oxidative stress increased in different rates compared to control all concentrations of SiC, SiO₂ and Si₃N₄ NPs in both cells. To my knowledge, there are no studies in the literature. Therefore, we discussed on different nanoparticles to this section. In a previous study Park *et al.* (2008) reported that exposure of BEAS-2B cells to titanium dioxide NPs (5, 10, 20 and 40 µg/ml) induced and increased

the expression of the oxidative stress-related genes (including heme oxygenase-1, thioredoxin reductase, glutathione-S-transferase, catalase and hypoxia inducible gene). Okuda-Shimazaki *et al.* (2010) found, using quantitative RT-PCR, that titanium dioxide nanoparticle induce inflammation-related genes, including heat shock protein (HSP) and IL-6 in human acute monocytic leukemia cells (THP-1) and human bronchial epithelial cells (NCI-H292).

In the present study, the cytotoxicity and genotoxicity potentials of 3 diverse silicon nanoparticles in HPAEpiC and HPPC cells was evaluated by using MTT, LDH and RT-PCR methods, respectively. The obtained findings exhibited that all silicon NPs have cytotoxic effect as dose- and time-dependent and each nanoparticle increased the expressions of different genes which involved in metabolic processes in cells. As a result, if silicon nanoparticles use in the diagnosis and treatment of diseases such as cancer, they can have toxic effects on patients. Therefore, the safety standards at genetic and cellular levels of NPs must have been well identified.

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