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RESEARCH ARTICLE

SiO_2 Nanoparticule-induced size-dependent genotoxicity – an *in vitro* study using sister chromatid exchange, micronucleus and comet assay

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Abstract

Fine particles with a characteristic size smaller than 100 nm (i.e. nanoparticlesspread out in nowadays life. Silicon or Si, is one of the most abundant chemical elements found on the Earth. Its oxide forms, such as silicate (SiO_4) and silicon dioxide, also known as silica (SiO_2), are the main constituents of sand and quartz contributing to 90% of the Earth's crust. In this work, three genotoxicity systems ''sister chromatid exchange, cytokinesis block micronucleus test and single cell gel electrophoresis (comet) assay'' were employed to provide further insight into the cytotoxic and mutagenic/genotoxic potential of SiO_2 nanoparticules (particle size 6 nm, 20 nm, 50 nm) in cultured peripheral blood lymphocytes as *in vitro*. It was observed that there is a significant decrease in Mitotic index (MI), Cytokinesis block proliferation index (CBPI), proliferation index (PRI) values expressed as Cell Kinetic parameters compared with negative control ($p < 0.05$). There is a statistically significant difference between negative control culture and culture exposed to SiO_2 (6 nm, 20 nm, 50 nm) ($p < 0.01$, $p < 0.01$, $p < 0.05$, respectively). It is found that SiO_2 nanoparticles at different size (6, 20, 50 nm) progressively increased the SCE frequency and DNA damage on the basis the AU values compared with negative control ($p < 0.05$). Results showed that the genotoxic/mutagenic and cytotoxic effects of SiO_2 nanoparticules is dependent to particule size.

Keywords

Blood lymphocytes, cytotoxicty, genotoxicity, micronucleus, nanomaterial, single cell gel electrophoresis, SiO_2, sister chromatid exchange

History

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Introduction

Nanotechnology industry is rapidly going on expanding in the many area such as medical healthcare, environmental remediation, nano-electronic and aerospace engineering. According to estimatives in recent years, there are over 800 consumer products already available containing nanomaterials (Singh et al., 2009). A nanomaterial is defined as substance with at least one dimension <100 nm in size and they can take many different forms such as tubes, rods, wires or spheres, with more elaborate structures devised, such as nano-onions and nano-peapods (Park et al., 2013). These nanomaterials contain particles of metal (gold, and silver nanoparticles), semiconductors (quantum dots), carbon (nanotube and buckball), and oxides (iron oxide, titanium dioxide, and silica). As one of the commonly used members of nanomaterials in nanotechnology-based consumer products, silica nanoparticles are widely used in a variety of fields, such as chemical industry, medicine, cosmetics, and agriculture (Hansen et al., 2008; Park et al., 2013; Passagne et al., 2012).

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Toxicity of nanoparticles is dependent on their physicochemical properties such as size and surface area, surface chemistry, charge, composition, solubility, crystal structure, aggregation, agglomeration. If nanoparticles are able to gain entry into the body via different route there are a number of direct and indirect mechanism that can subsequently promote DNA damage. Indeed, it has been shown that silica nanoparticles can enter the nucleus. Silica nanoparticles also have an effect on nuclear integrity by forming intranuclear protein aggregates leading to the inhibiton during the replication, transcription, and cell proliferation. (Geiser et al., 2005; Liu et al., 2007). Besides, these nanoparticles have been shown to reduce replication activity down to 67% and 60% after 6–24 hours, respectively (Chen & von Mikecz, 2005). Despite these abnormalities, there is limited evidence in relation to genotoxicity of silica nanoparticles and some recent studies utilising the comet assay have demonstrated that silica nanoparticles ranging in size from 20–400 nm do not exert significant genotoxicity (Barnes et al., 2008; Jin et al., 2007).

The comet assay, micronucleus test and sister chromatid exchange analysis have been used to access the toxicity and genotoxicity of many different chemicals, drugs and pesticides in *in vivo, in vitro* studies in different organisms (Çelik & Akbaş, 2005; Çelik et al., 2013).

Comet assay, also known single-cell gel electrophoresis (SCGE) assay is verdatil sensitive and rapid method for measuring DNA single- and double strand breaks at the level of individual cells and is capable of detecting DNA damage with great sensitivity and has been used widely both *in vitro* and *in vivo* protocols to identify potentially environmental genotoxins (Tsuda et al., 2000). The Comet assay has been widely accepted as a simple, sensitive, and rapid tool for assessing DNA damage and repair in individual eukaryotic as well as some prokaryotic cells, and has increasingly found application in diverse fields ranging from genetic toxicology to human epidemiology. Comet assay has been widely used in different models from bacteria to man, employing diverse cell types to assess the DNA-damaging potential of chemicals and/or environmental conditions by many research groups and researchers (Çavaş, 2011; Narendra, 2000; Sandal et al., 2008).

Countryman and Heddle proposed the human micronucleus test in 1976 as a faster and alternative method to metaphase analysis. The most popular type is the use of cytochalasin B to block cytokinesis, which causes the accumulation of binucleate cells at first mitosis (Fenech & Morley, 1985). There are, in fact, four recognized mechanisms by which micronuclei and microucleus like structures can arise; mitotic loss of acentric fragment, a variety of mechanical consequences of chromosomal breakage, an exchange, mitotic loss of whole chromosomes, and apoptosis. The latter is a form of nuclear destruction in which the nucleus disintegrates and nuclear fragments are formed (Koç-Başer et al., 1999; Tomanin 1991). The simplicity of screening, scoring, and the wide applicability of the *in vitro* micronucleus test in different cell types make it a useful tool to evaluate cytogenetic damage (Kirsh-Volders et al., 2003). Micronuclei (MN) have been used as an indicator of chromosome damage for more than 20 years in *in vitro* and *in vivo* studies. (Çelik, 2006; Çelik & Kanık, 2006; Chung et al., 2002).

Genotoxic agents have the potential to interact with DNA and may cause DNA damage. SCE occurs spontaneously in proliferating cells and is regarded as a manifestation of damage to the genome. It has been commonly used as a test of mutagenicity in order to evaluate cytogenetic responses to chemical exposure, and dose-response relationships for different chemicals have been reported in both *in vivo* and *in vitro* studies (Bal et al., 1998; Çelik & Akbaş, 2005; Çelik & Aras Ateş, 2006).

As previously demonstrated, the Cytokinesis block proliferation index (CBPI) is an accurate and biologically relevant index in detecting cellular toxicity or cell-cycle delay (Fenech & Morley, 1985). Proliferation index (PRI) and mitotic index (MI) were evaluated in order to determine the cytotoxicity of various compounds in human peripheral blood lymphocyte cultures (Bal et al., 1998; Çelik, 2006; Çelik & Akbaş, 2005; Çelik & Aras Ateş, 2006; Eke & Çelik, 2008)

This study was designed to evaluate the genotoxic and mutagenic potential of $S\dot{I}O_2$ nanoparticles in human peripheral blood lymphocytes exposed to different particle size (6 nm, 20 nm and 40 nm). The aim of present study was to demonstrate the damage caused by SiO_2 nanoparticle under in *vitro* conditions and to compare the potential genotoxic/toxic effects produced by different sizes of SiO_2 nanoparticles in human blood lymphocyte.

Materials and methods

Chemicals

APTES (Aminopropyl triethoxysilane) was purchased from ACROS Organics, TEOS (Tetraethyl orthosilicate) from Aldrich Chemistry, Ethanol absolut from Sigma Aldrich (St. Louis, MO), Ammonium hydroxide (Ammonia solution min. 25%) from Analar Normapur for silica nanoparticles. Culture medium RPMI 1640 (Gibco, Grand Island, NY), fetal calf serum (Gibco) phytohemagglutinin (Gibco), 5-Bromo-2-deoxyuridine (BrdU, Sigma), Mitomycin C (Kyowa).

The preparation of SiO_2 nanoparticles

Silica nanoparticles were prepared according to Stöber method (Costa et al., 2007) with slight modifications in Mersin University Advanced Technology, Education, Research and Aplication Center. One mix solution (1.2 mL TEOS, 0.1 mL APTES, 1.2 mL NH_4OH, 5 mL ethanol) prepared under inert atmosphere was blended during over night. In the end of reaction, Silica nanoparticles were washed with ethanol for four times to remove artifact reactives. Then, silica nanoparticle samples were prepared for phisicochemical characterisation by drying under nitrogen atmosphere.

Field emission-scanning electron microscopy (FE-SEM) images were obtained using a Zeiss/Supra 55 FE-SEM, and the samples were platinum coated prior to the measurements. The shape and size of particles were verified by FE-SEM images, as shown in Figure 1, illustrating that the particles were uniformly spherical. The high magnification SEM image (Figure 1d) also revealed the formation of small spherical nanoparticles approximately 6 nm in diameter. Nanoparticles were also analyzed with DLS (Dynamic Light Scattering, Zetasizer Nano ZS) to determine their sizes (Figure 2a–c). DLS is a technique frequently used for the determination of particle size distribution of the nanoparticles dispersed in a solution. For this measurement, the nanoparticles were initially dispersed in a saline solution in a concentration of 150 μg/ml by ultrasonication for 30 min. Filtration is one of the simple techniques for the size control of the nanoparticles. So, dispersed nanoparticles were filtered by an inorganic membrane filter (Whatman Anotop syringe filter, pore size 0.02 μm and 0.1 μm) to remove agglomerated particles. As shown in Figure 2(a–c), DLS results confirm the presence of nearly monodisperse nanoparticles with a diameter of 6 nm, 20 nm, 50 nm. Such small inorganic colloidal nanoparticles can easily come close to each other to form agglomerates until the attractive forces balanced (such as van der Waals forces or hydrogen bonds). Therefore, before starting biological studies, nanoparticle solutions were filtered using the appropriate filters.

Subjects

Two healthy male non-smoking donors (mean age, 29.32 ± 2.33 years) provided blood samples. Subjects had not been exposed to radiation or drug 6 months prior to the study. Each person was interviewed about the possible

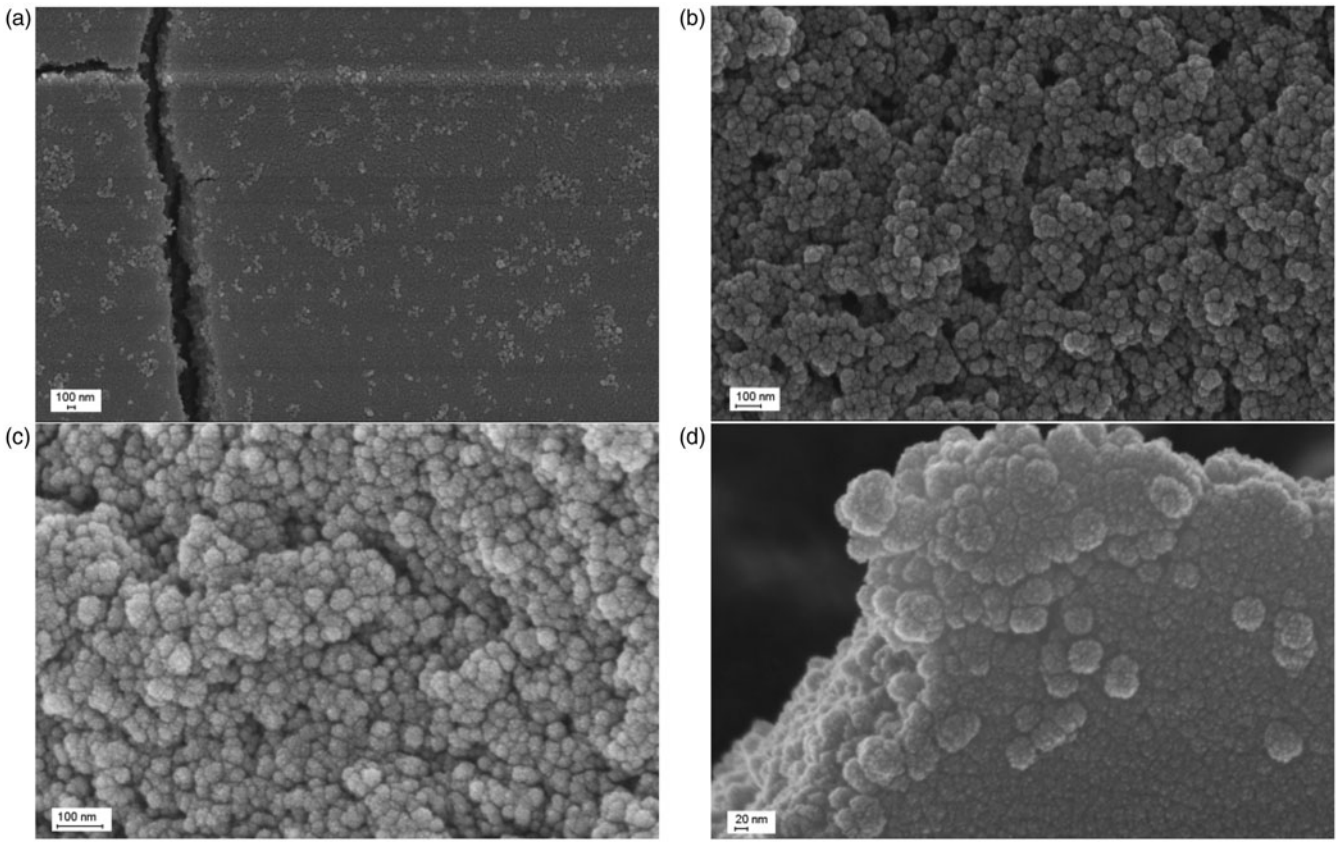

Figure 1. Representative FE-SEM images of SiO_2 nanoparticles obtained by Stöber method in different diameters. Particle sizes are (a) 50 nm, (b) 20 nm and (c, d) 6 nm. (d) High magnification image of (c).

influence of confounding factors. Questionnaires were given to each blood donor to evaluate exposure history; and informed consent forms were signed by each of them. For all the volunteers, hematological and biochemical parameters were analyzed and no disease was detected. Ethical Committee at Mersin University Medical Faculty approved the experiments described in this study.

Blood sampling, cell preparation and comet assay

The experiments were performed on peripheral blood lymphocytes obtained from two healthy donors, Peripheral blood mononuclear cells were isolated by Histopaque-1077 density gradient centrifugation, according to the manufacturer's instructions. Lymphocyte cultures were set up by adding 0.5 ml of lymphocyte suspension in 4.5 ml of RPMI 1640 medium supplemented with 20% fetal calf serum, 2 mM l-glutamine, 10 µg/ml phytohemagglutinin, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were incubated for 72 h at 37 °C.

Alkaline comet assay

Comet assay was performed with lymphocytes of two donors according to Singh et al. (1988). Firstly, lymphocytes from two donors were treated with SiO_2 nanoparticule at 150 µg/ml concentration at three sizes (6 nm, 20 nm, 50 nm). Briefly, 100 µl of cell suspension was mixed with 200 µl of 2% low melting temperature agarose at 37 °C and then placed on a slide pre-coated with thin layer of 0.5% normal melting agarose. The cell suspension was immediately covered with a coverglass and the slides were kept at 4 °C for 5 min to allow solidification of the agarose. After removing the coverglass, the cells were lysed in a lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, pH 10) for 1 h. After washing in re-distilled water the slides were placed in a horizontal gel electrophoresis chamber. The chamber was filled with cold electrophoretic buffer (1 mM EDTA, 300 mM NaOH, pH 13) and slides were kept at 4 °C for 40 min to allow the DNA to unwind. Electrophoresis was conducted at 20 °C using 25 V and 185 mA for 20 min. After electrophoresis, the slides were washed three times with neutralization buffer (0.4 M Tris, pH 7.5). All preparative steps were conducted in dark to prevent additional DNA damage. The slides were stained with with etidium bromide (0.1 mg/ml, 1:4) and analyzed with a fluorescence microscope (Olympus BX 51) equipped with a video camera CCD-4230.

Slide scoring

Comet images were analyzed according to Collins et al. (1995). One hundred comet images were scored for each treatment by two scorers (G.G and A.A.) visually under fluoresence microscopy (BX51 OLYMPUS). An intensity score from class 0 (undamaged) to class 4 (ultra high damage) (Sun et al., 2004) was assigned to each cell. The method of the observation was barred in a blind way during which the observer had no knowledge of the identity of the slide. Fifty cells per slide and two slide were examined per sample to evaluate DNA damage for each culture culture treated with

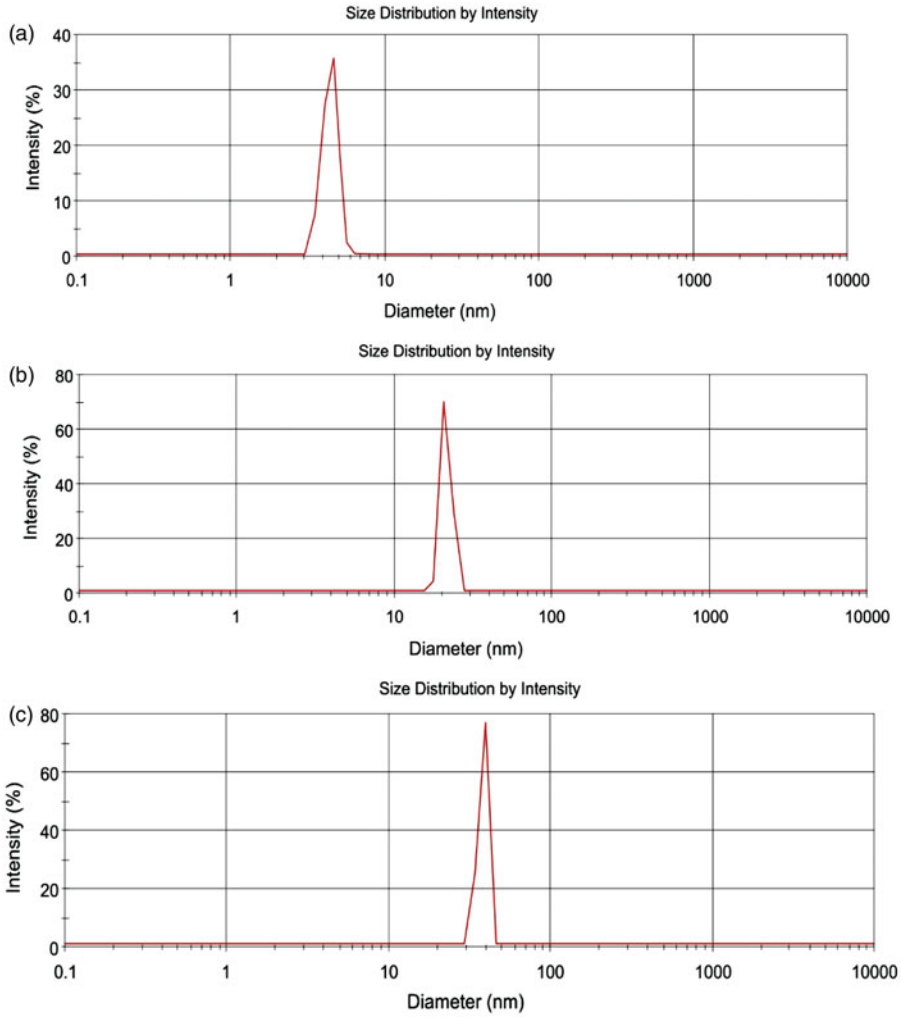

Figure 2. DLS histograms of the nanoparticles with sizes of 6 nm (a), 20 nm (b) and 50 nm (c), respectively. The nanopa were initially dispersed in a saline solution in a concentration of 150 μg/ml by ultrasonication for 30 min, and then were filtered by an appropriate filter.

different particle size. The cells were classified bt eye in the five categories on the basis of the extend of DNA migration, undamaged (class 0), very little damage (class 1), moderate damage (class 2), high damage (class 3) ultra high damage (class 4). The ''Arbitrary units (AU)'' was used to express the extent of DNA damage and calculated following formula.

$$AU = \sum_{i=0}^{4} i \times N_i$$

N_i = the number of scored cell in i level, i = the level of DNA damage (0, 1, 2, 3, 4).

Cytogenetic analysis

Cytokinesis block micronucleus test

Blood samples were taken by venipuncture using heparinized vacutainers. Lymphocytes cultures were set up in the laboratory at a sterilized place and prepared according to the technique described by Scarpato et al. (1996) with slight modifications. Heparinized whole blood (0.8 mL) was added to 5 mL of culture medium RPMI 1640 (Sigma), supplemented to 20% with fetal calf serum (Sigma), with 0.2 mL phytohemagglutinin (Sigma), and with antibiotics (10 000 IU/mL penicillin and 10 000 IU/mL streptomycin). Three nanomaterial solution of SiO_2 (6 nm, 20 nm, 50 nm) were added in lymphocyte cultures. A final concentration of 6 μg/mL of cytochalasin B was added to cultures 44 h later to arrest cytokinesis. At 72 h of incubation, the cultures were harvested by centrifugation at 2000 rpm for 10 min. Then, to eliminate red cell, and to keep the cytoplasm, the cell pellet was treated with a hypotonic solution (4–5 min 0.075 M KCl at 37°C). Cells were centrifuged, and Carnoy's fixative (methanol: acetic acid, 3:1, v/v) solution was freshly added. This fixation step was repeated five times. Next, cell pellets were resuspended in a small volume of fixative solution and dropped onto clean, cold slides. The slides were stained with 10% Giemsa dye solution.

Sister chromatid exchange

All SiO_2 nanoparticle samples were sterilized using autoclave. Three particule solution of SiO_2 were added to lymphocyte cultures under laboratory conditions. Lymphocyte cultures were prepared according to the technique of Moorhead et al. (1960) with slight modifications. Heparinized whole-blood (0.8 mL) was added to 4.5 mL of culture medium RPMI 1640 (Gibco), supplemented with 20% of fetal calf serum (Gibco), 0.1% mL phytohemagglutinin (Gibco), and antibiotics (10 000 IU/mL penicillin and 10 000 IU/mL streptomycin). 5-Bromo-2-deoxyuridine (9 μg/mL, BrdU, Sigma) was added to cultures at the beginning of the 72-h incubation period at 37°C for SCE analysis. Lymphocytes were cultured in the dark for 72 h, and

metaphases were blocked during the last 1.5 h with colcemid at final concentration of 0.2 μg/mL. Mitomycin C (2 μg/mL) was used as positive control. The cells were harvested by replacing the culture medium with KCl (0.075 M) in which cells were incubated for 20 min at 37°C. The cells were fixed in Carnoy's fixative (methanol:acetic acid, 3:1 v:v) five times, and slides were kept at room temperature overnight. Air-dried slides were stained according to fluorescence-plus Giemsa method by Perry & Wolff (1974) with slight modification. The number of SCEs was counted in 100 second- metaphase cells from each of the cultures on coded slides. Thus, 100 cells were scored in blind per culture for SCEs.

Cell proliferation kinetic (CPK) and mitotic index

The mitotic index (MI) was calculated as proportion of metaphases among the total cell population by counting a total of 1000 cells per culture. The cell proliferation kinetics was defined as the proportion of the relative frequency of first division metaphases (M1, identifiable by uniform staining of both the sister chromatids), second division metaphases (M2, identifiable by differential staining of the sister chromatids), and third and subsequent division metaphases (M3, identifiable by non-uniform pattern of staining). Replication index or proliferation index (RI) was calculated according to Ivett & Tice (1982). RI is the average number of replications completed by metaphase cells and is calculated as follows: RI = 1x (% first division metaphases + 2x (% second division metaphases) + 3x (% subsequent division metaphases)/100

Scoring criteria for micronuclei and cytokinesis-block proliferation index

In order to determine the frequency of binucleated cells with micronuclei (BNMN) and the total number of MN in lymphocytes (MNL), a total of 2000 binucleated cells with well-preserved cytoplasm were scored exactly for each subject and each dose on coded slides. MN were accepted only when (i) they were separated from the main nuclei, but included within the corresponding cytoplasm, (ii) they had a chromatin material similar to that of the main nuclei, (iii) they were coplanar to the main nuclei, (iv) it should be 1/16th to 1/3rd of the mean diameter of the main nuclei. In the MN study, toxicity was evaluated by classifying cells according to the number of nuclei. The well-known cytotoxicity index was used: an index for measuring the cell proliferation kinetics, called the cytokinesis-block proliferation index (CBPI), which was calculated following the expression: where MI–MIV represent the numbers of cells with one to four nuclei, respectively, and MIII and MIV are equally considered to be in their third cell cycle. As previously demonstrated, the CBPI is an accurate and biologically relevant index in detecting cellular toxicity or cell-cycle delay (Fenech & Morley, 1985).

Statistical analysis

Data were compared by one-way variance analysis. Statistical analysis was performed using the SPSS for Windows 16.0 package program (SPSS Inc., Chicago, IL). Post hoc analysis was performed by Student Newman Keuls (SNK) test. $p < 0.05$ was considered as level of significance.

Results

Peripheral human lymphocytes were exposed to SiO_2 nanoparticles at different particle sizes (6, 20, 50 nm). The induction of genotoxicty was evaluated using cytokinesis block micronucleus test and sister chromatid Exchange analysis and DNA damage in cellular DNA was analysed via single cell gel electrophoresis method (comet assay). Figure 3(a–c) show SCE points in metaphase, micronucleus in binucleated cells and comet assay views in periperal blood lymphocytes exposed to SiO_2 nanoparticles at three different size (6, 20, 50 nm), respectively.

Data related with Comet assay give to us an clue about oxidative DNA damage. Table 1 represents AU values indicating DNA damage at various level.

UD = 0 (undamaged) LD = 1 (low damaged), MD = 2 (moderate damaged), HD = 3 (High damaged) UHD = 4 (ultra high damaged,) comet figures in peripheral blood lymphocytes exposed to SiO_2 nanoparticle (6, 20, 50 nm) (mean ± S.E.). In lymphocyte cultures, exposed to all the particle sizes of SiO_2, DNA damage was induced in particle size-dependent manner at different levels, and a sitatistically significant difference was observed compared with control. AU value reached to 54.0 ± 6.0 in negative control group, to 134.5 ± 7.65 in group exposed to 6 nm SiO_2, to 123.0 ± 7.00 in group exposed to 20 nm SiO_2, to 83.5 ± 2.50 in group exposed to 50 nm SiO_2. It is also demonstrated that DNA damage increased with the shrinkage of particle size of SiO_2. In contrast to, AU value is 315.0 ± 15.0 in positive control group treated with H_2O_2. There is a statistically significant difference between negative control culture and culture exposed to SiO_2 (6 nm, 20 nm, 50 nm) ($p < 0.01$, $p < 0.01$, $p < 0.05$, respectively) for AU values. It is found that a significant difference between negative control and positive control ($p < 0.001$).

While MN frequency is 2.25 ± 0.25 in negative control group, in contrast to, 4.50 ± 0.50, 5.00 ± 1.00, 5.00 ± 00 in group exposed to 6 nm, 20 nm, and 50 nm SiO_2, respectively. There is a increase in MN frequency, but not statistically significant difference between negative control culture and cultures exposed all the particle size of SiO_2. MN frequency reached to 9.00 ± 1.00 in positive control group treated with Mitomycin C.

In peripheral lymphocyte cultures, a progressive increase in mutagenicity (increase in SCE frequency) was observed when SiO_2 nanoparticle was shrinked from 50 nm to 20 nm and 6 nm. There is a significant difference between control and exposed culture treated with 6 nm SiO_2 nanoparticle. The SCE frequency reached to 5.06 ± 0.06, to 5.00 ± 0.04, to 4.66 ± 0.02 in cultures exposed to, 6 nm, 20 nm, 50 SiO_2 nm nanoparticle, respectively. In contrast to, SCE frequency reached to 3.58 ± 0.06 in negative control cultures and to 6.52 ± 0.16 in positive control treated with mitomycin C (Table 2).

It was observed there is a significant decrease in MI, CBPI, PRI values expressed as Cell kinetic parameters compared with negative control ($p < 0.05$).

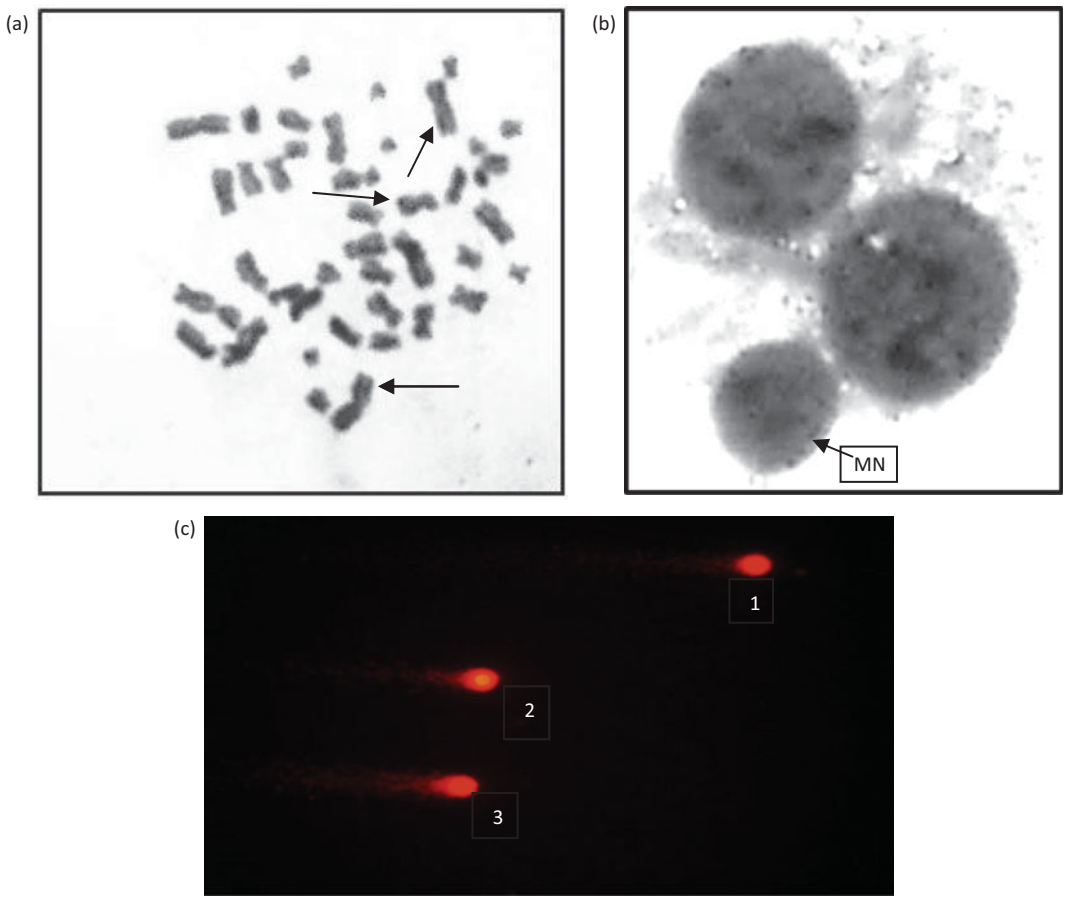

Figure 3. (a) SCE points in metaphase of lymphocyte cultures exposed to SiO_2 nanoparticles. (b) Binucleated cells including Micronucleus in lymphocyte cultures exposed to SiO_2 nanoparticles. (c) Comet assay views (class 1, 2, 3) in lymphocyte cultures exposed to SiO_2 nanoparticles (6 nm).

Table 1. Comet assay scores in peripheral blood lymphocytes exposed to SiO_2 nanoparticles.

	0 Mean ± SE	1 Mean ± SE	2 Mean ± SE	3 Mean ± SE	4 Mean ± SE	AU
6 nm	39.0 ± 1.80	21.0 ± 7.00	15.5 ± 2.50	14.5 ± 1.25	10.0 ± 1.00	134.5 ± 7.65**
20 nm	16.0 ± 5.00	55.0 ± 0.00	23.0 ± 6.00	4.00 ± 1.00	2.50 ± 0.50	123.0 ± 7.00**
50 nm	38.5 ± 1.50	46.0 ± 1.00	10.0 ± 0.00	4.50 ± 0.50	1.00 ± 0.00	83.5 ± 2.50*
NC	60.0 ± 0.00	28.0 ± 4.00	9.00 ± 3.00	3.00 ± 1.00	0.00 ± 0.00	54.0 ± 6.00
PC (H_2O_2-10 mM)	3.00 ± 1.00	9.00 ± 3.00	10.0 ± 2.00	26.0 ± 2.00	52.0 ± 4.00	315.0 ± 15.0***

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
AU: Arbitrary Units; NC: Negative Control; PC: Positive Control; SE: Standart Error.

Discussion

DNA damage may arise through indirect mechanisms where the nanomaterial does not physically interact with the DNA molecule, but with other cellular proteins such as those involved in the cell division process. Additionally, nanomaterials may induce other cellular responses that in turn lead to genotoxicity, such as causing oxidative stres, inflammation and aberrant signalling responses, all of which have been the primary focus of most studies to date and so are discussed in detail in the following section.

In this study, DNA damage levels/mutagenicity/genotoxicity were examined to define effects of silica nanoparticles at different particle size (6 nm, 20 nm, 50 nm) via three different assay. The first step was carried using *Sister Chromatid Exchange* analysis which measure genotoxicity and mutagenicity, second, *Comet assay* evaluated DNA damage levels such as strand breaks. In third, cytogenetic assay was performed to determine clastogenic/aneugenic effetcs using *Cytokinesis Block Micronucleus* assay in peripheral blood lymphocyte cultures. In addition, cytotoxicity and cell kinetic parameters were evaluated calculating PRI, CBPI and MI values. Our results indicate that the genotoxic/toxic effects and DNA damaging potential of SiO_2 nanoparticles were dependent on particle size.

Recently, some studies raported that nanoparticles tended to cause damage to living organism due to their higher activity and selectivity. In many researches performed as *in vitro*, nanoparticle concentration and nanoparticle size were considered for biological effects such as DNA damage

Table 2. SCE, MN frequency and CBPI, MI and PRI values in peripheral blood lymphocytes exposed to SiO_2 nanoparticles.

	NC	6 nm	20 nm	50 nm	PC (MMC-2 μg/ml)
Donor A					
SCE	3.64	5.00	4.96	4.64	6.36
MN	2.50	4	4	5	8
CBPI	2.02	1.71	1.48	1.82	1.70
MI	62	47	58	55	33
PRI	2.08	1.62	1.47	1.72	1.42
Donor B					
SCE	3.52	5.12	5.04	4.68	6.68
MN	2.00	5	6	5	10
CBPI	2.04	1.69	1.70	1.84	1.67
MI	64	46	55	52	30
PRI	2.06	1.64	1.46	1.78	1.50
Mean *S.E.*					
SCE	3.58 ± 0.06	5.06 ± 0.06*	5.00 ± 0.04*	4.66 ± 0.02*	6.52 ± 0.16**
MN	2.25 ± 0.25	4.50 ± 0.50	5.00 ± 1.00	5.00 ± 0.00	9.00 ± 1.00**
CBPI	2.03 ± 0.01	1.70 ± 0.01**	1.59 ± 0.11**	1.83 ± 0.01**	1.68 ± 0.01**
MI	63.0 ± 1.00	46.5 ± 0.50*	56.5 ± 1.50*	53.5 ± 1.50*	31.5 ± 1.50**
PRI	2.07 ± 0.01	1.63 ± 0.01*	1.46 ± 0.005*	1.75 ± 0.03*	1.46 ± 0.04*

*$p < 0.05$, **$p < 0.01$.

SCE: Sister chromatid Exchange; MN: Micronucleus; CBPI: cytokinesis Block Proliferation index; MI: Mitotic index; PRI: proliferation index; NC: Negative control; PC: Positive control; S.E.: Standard error.

and cell kinetic parameters. Our study underlines that the smaller a particle, the greater its genotoxicity because of larger specific surface area. Nel et al. (2006) explained that, in nano scale, smaller particle possessed greater surface area per unit mass. Therefore, lots of active sites were procreated on the particle which could capture oxygen molecules and produce various radical molecules through dismutation.

Comet assay, single cell gel electrophoresis, detect DNA breaks, crosslinks and transient DNA strand breaks. Nanoparticles may induce genotoxicity by interacting directly with DNA or through indirect means by virtue of a number of factors including surface stres through direct particle influences on DNA (Lin et al., 2006). In the present study, comet assay results indicated that all the nanoparticle size of SiO_2 a significant genotoxicity in human peripheral blood lympocytes.

In our study, SiO_2 nanoparticles (6, 20, 50 nm) lead to increase in SCE frequency. SCEs are often included as a genotoxic endpoint to reflect DNA damage, or a biological dosimeter or biomarker of exposure (Spronck & Kirkland, 2002). Several research raported that nanoparticles, for examle TiO_2, induced the SCE frequency in cultured human blood lymphocytes (Türkez, 2011; Turkez & Geyikoğlu, 2007). Wang et al. (2007) raported that membrane damage, DNA damage as well as apoptosis was based on oxidative stres. Peters et al. (2004) investigated the effects of the SiO_2 nanoparticles whose size spectrum were between 4- and 40 nm (mean particle size 14 nm). They indicated that SiO_2 nanoparticle exposure lead to inflammatory activation in human dermal microvascular endothelial cells (HDMEC).

In this study SiO_2 nanoparticles induced micronucleus frequency in human peripheral blood lymphocytes cultures in relation to particle size. In several researches, it is shown that nanoparticles such as SiO_2, TiO_2 induced the genotoxicty. Valko et al. (2006) raported that since silica nanoparticles can results in increased levels of reactive oxygen species and given that the hydroxyl radical is a highly reactive molecule the generation of OH close to the DNA could readily lead to the induction of DNA strand breaks and oxidised bases which could have important implications in the development of cancer. Rahman et al. (2002) demonstrated an increase in micronucleus frequency at all concentrations tested (0.5–5.0 mg/cm^2) in SHE fibroblasts. The study by Wang et al., also found an increased micronculeus frequency, at 65 mg/mL, using the *hprt* forward mutation assay and comet assay to demonstrate that TiO_2 nanoparticles are also capable of inducing point mutations and DNA strand breakages Karlsson et al. (2008) observed increased strand breakages by the comet assay following the exposure of TiO_2 nanoparticles to lung epithelial cells. Besides, some researchers indicated that TiO_2 did not induce genotoxicty. Theogaraj et al. (2007) did not find that TiO2 NPs were genotoxic; using the chromosomal aberration test in CHO cells, no increases in DNA damage frequency were observed in either the absence or presence of UV light.

Silica nanoparticles also have an impact on nuclear integrity by forming intranuclear protein aggregates that can lead to inhibition of replication, transcription, and cell proliferation. Many researcher has well documented size dependent cytotoxicity and explained that the biologic activity and biokinetics are dependent on many parameters: size, shape, chemistry, crystallinity, surface properties (area, porosity, charge, surface modifications, weathering of coating), agglomeration state, biopersistence, and dose. (Kipen & Laskin, 2005; Oberdorster et al., 2005).

Cell cycle arrest may reflect the degree of DNA damage because cells include the checkpoints at various stages of the cell cycle to maintain the accurate replicas of the genome. It is well documented that one of the most major cellular response observed both *in vitro* and *in vivo* after the exposure to chemicals, ionizing radiation, and/or other genotoxic agents is the inhibition or the delay of cell-cycle progression. MI and RI or CBPI are used as indicators of sufficient cell proliferation and cytotoxicity (Anderson et al., 1988; Scott

et al., 1991). The interaction between cytotoxicity and tumor promotion was indicated in several studies performed by many researchers (Albert & Magee, 2000). Also cytotoxicity causes the tumor promotion via inflammation and/or humoral immunity. SiO_2 nanoparticles exhibits a cytotoxic effect *in vitro* because of its inhibition of mitotic activity. The decrease in the mitotic activity is size-dependent. The frequency of MI decreased with smaller particle size of SiO_2, and statistically significant differences were observed compared with negative control. It is raported that other nanoparticle samples such as TiO_2, ZnO, may be occured cytotoxic effects (Geiser et al., 2005; Ohshima et al., 2005). RI and CBPI values reflect number of cells in second mitosis and third/forth mitosis. In the present study, SiO_2 nanoparticles lead to a decrease in RI ve CBPI values, cell kinetic parameters. Therefore, it may be said that SiO_2 has cytostatic effects on human peripheral blood lymphocyte cultures *in vitro* because it causes delay in the cell cycle. Li et al. (2011) investigated the effects of silica particles on cell phase proportion and found that the cell number in G0/G1 phase declined gradually with the dereasing size of treated silica particles; all the four particles blocked cell cycle at S stage and, what is more, S and /G2/M two-phase arrest of HepG2 cell even appeared in 19 nm Si particle treated group. The experimental results of study performed by Li et al. demonstrated that silica particle-induced cytotoxicity is size-dependent. These findings show paralellelism with our results on the base of cytotoxicity.

In conclusion, we have demonstrated that 6-nm, 20-nm and, 50-nm SiO_2 nanoparticles significantly reduced mitotic activity and increase DNA damage. It found that DNA damage effect was depending on particle size. Further study is needed to evaluate the potential toxicity of nanosized SiO_2 especially in different cell cultures and different organism including rodents.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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