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In vitro evaluation of the genotoxicity of CeO_2 nanoparticles in human peripheral blood lymphocytes using cytokinesis-block micronucleus test, comet assay, and gamma H2AX

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Abstract

Engineered nanoparticles (ENPs) are used in a wide range of applications because of their unique properties. Cerium dioxide nanoparticles (CeO_2 NPs) are one of the important ENPs, and they can cause negative health effects, such as genotoxicity, in humans and other living organisms. The aim of this work was to analyze the genotoxic effects of short-term (3–24 h) CeO_2 NPs exposure to cultured human blood lymphocytes. Three genotoxicity systems "cytokinesis-block micronucleus test, comet assay, and gamma H2AX test" were used to show the genotoxic potential of CeO_2 NPs (particle size <25 nm, concentrations: 6, 12, and 18 μg/mL). Hydrogen peroxide was selected as the positive-control genotoxic agent. Our results indicate that CeO_2 NPs have genotoxic potential on human peripheral blood lymphocytes cells even at 3–24 h exposure under *in vitro* conditions.

Keywords

CeO_2 NPs, comet assay, micronucleus test, gamma H2AX, blood lymphocytes, *in vitro*

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Introduction

The use of nanomaterials (size between 1 nm and 100 nm) in the world has become an important component of consumer products in recent years. This fact has gradually led to an increase in concerns and debates about the impact of nanomaterials on different biological systems (Courbiere et al., 2013; Dhawan and Sharma, 2010). Nanoparticles (NPs) can be classified into two groups such as natural (soil colloids, fullerenes, carbon nanotubes, biogenic magnetite, and airborne nanocrystals of sea salts) and anthropogenic NPs (carbon black, carbon nanotubes, platinum, and engineered/manufactured NPs) (Dhawan and Sharma, 2010). According to assessments in recent years, engineered NPs, which have small size, large specific surface area and surface reactivity, are extensively used in many industries such as electronic, textile, cosmetic, pharmacology, and medicine (Courbiere et al., 2013). However, their effects on health and potential biological impacts need to be determined for risk assessment.

Nanomaterials have frequently been used in nanotechnology products. Lanthanide cerium dioxide nanoparticles (CeO_2 NPs) have a wide application in a variety of fields, such as catalysts, solid fuel cells, ultraviolet absorbents, gas sensors, glass and ceramic applications (Srinivas et al., 2011). Therefore, the risk of both occupational and environmental exposure to CeO_2 NPs has risen for humans. According to an OECD report, CeO_2 NPs are one of the most important NPs for which human and environmental impacts need to be assessed (Srinivas et al., 2011). Although some researchers reported that CeO_2 NPs exhibit antioxidant properties (Colon et al., 2010; Rubio et al.,

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2016; Schubert et al., 2006), some recent reports have shown that CeO_2 NPs' exposure can give rise to negative effects on human health such as genotoxicity, toxicity, and apoptosis (Aalapati et al., 2014; Mittal and Pandey, 2014; Marzi et al., 2013).

Some tests, including the micronucleus test, comet assay, and gamma H2AX, are commonly used to assess the genotoxicity of many different chemicals (drug, pesticide, NPs, and phthalates) *in vivo* as well as *in vitro* studies (Balasubramanyam et al., 2009; Jugan et al., 2012; Tsaousi et al., 2010).

The *in vitro* micronucleus test is a standard cytogenetic test for genotoxicity. It is simple, useful, and applicable in different cell types (Kirklanda et al., 2011; Lindberga et al., 2009; Meschini et al., 2015). The type performed using cytochalasin B is the most useful micronucleus assay which produces binucleated cells (BNCs) (Battal et al., 2015). The comet assay, also called single-cell gel electrophoresis (SCGE) assay, is extensively used as a marker for the assessing of DNA break in single cell (Tice et al., 2000). This technique can even be applied to a limited number of cells. The alkaline type of SCGE has also been used to evaluate single-stranded DNA breaks and DNA repair of individual cells (Collins, 2004). Additionally, phosphorylated histone H2AX, also known as ϒH2AX, is susceptible and fast in revealing DNA double-strand breaks in the cells. This test evaluates genotoxic damage, which is DNA damage caused by various agents in interphase cells (Cavaş et al., 2012).

There is limited knowledge related to genotoxicity of cerium oxide NPs (Li et al., 2016). In this study, we aimed to evaluate *in vitro* genotoxic potential of CeO_2 NPs in human blood lymphocytes exposed to different concentrations (concentrations: 6, 12, and 18 μg/mL; particle size <25 nm). During our studies, the micronucleus test, the SCGE assay, and gamma H2AX foci formation assays were used as genotoxicity endpoints.

Materials and methods

Chemicals

Pure cerium dioxide NPs, phosphate buffered saline (PBS), Ethylenediaminetetraacetic acid (EDTA), Triton X-100, normal melting point agarose, and low melting point agarose were purchased from Sigma-Aldrich (St. Louis, USA). Anti-gamma H2AX (phospho S139) antibody (9f3) primary and Alexa Fluor® 488 Goat anti-Mouse IgG secondary antibodies were supplied by Abcam (Cambridge, MA, USA) and BioLegend (San Diego, CA, USA). All other chemicals used in the experiments were also purchased from Bio Ind (Cromwell, CT, USA). Human blood lymphocytes were treated with 100 μM hydrogen peroxide (H_2O_2) as positive control. CeO_2 NPs dissolved in sterile distilled water and were used at concentration of 0.5% (v/v).

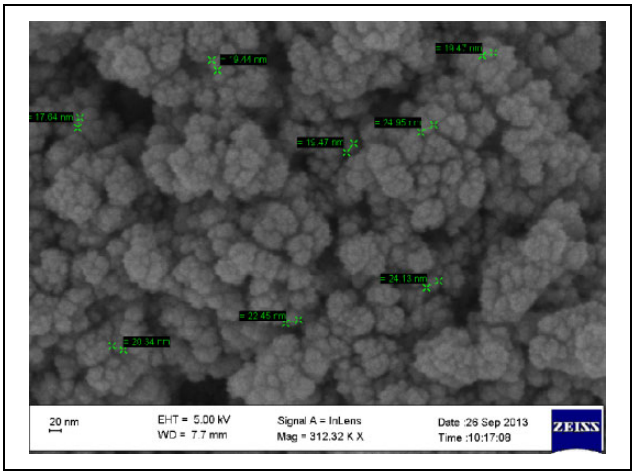

Figure 1. SEM images of CeO_2 NPs.

Characterization of NPs

CeO_2 NPs in stock solution were dispersed by ultrasonication for 20 min, before each treatment dose was prepared. Once the particle stock solution was prepared in the cell culture medium, the addition of NPs to the cell culture medium caused discoloration at high concentration. Therefore, distilled water was used as solvent of NPs. Moreover, others researchers used distilled water for NP stock solution (Estevez et al., 2011; Molina et al., 2014; Selvaraj et al., 2015). The morphology and the size of powdered NP were determined using scanning electron microscopy (SEM) (Figure 1) and X-ray diffraction analysis. The average particle size was confirmed by BET (≤25 nm).

Blood sampling

Human blood lymphocytes were attained from healthy nonsmoking donors (four men and four women, mean age 24 years). The donors were interviewed about the possible influence of this study. Each person read the questionnaire and signed it. The study protocol was designed according to ethical standards and was approved by the Ethical Committee of the Medical Faculty at Mersin University (document

number: 2012/81). The genotoxicity tests were evaluated using three culture tubes for each individual. Each test was applied three times in order to avoid inaccuracy (three repeated, total individual: 8).

Cell culture

Blood sample (3 mL) and Histopaque-1077 (3 mL) was centrifuged at 2000 rpm for 30 min. The visible opaque layer was used as lymphocyte suspension after centrifugation. Subsequently, the lymphocyte suspension (0.5 mL) was added to RPMI1640 cell culture medium (4.5 mL) supplemented with 20% fetal calf serum, 2 mM L-glutamine, 10 mg/mL phytohemagglutinin, 100 U/mL penicillin, and 100 mg/mL streptomycin. The cell cultures were incubated at 37°C with 5% CO_2 for 24 h.

Using doses

CeO_2 NPs doses were determined and selected due to the available data of NPs dose ranges humans are exposed to both occupationally and environmentally (Gehlhaus et al., 2009; Hussain et al., 2012). Three doses were used, a low dose (6 μg/mL), a middle dose (12 μg/mL), and a high dose (18 μg/mL).

Cytokinesis-block micronucleus test

The cytokinesis-block micronucleus test was utilized to evaluate chromosomal damages. Lymphocytes, which were transferred in sterile cell culture dishes (15 mL), were exposed to different concentrations of CeO_2 NPs and H_2O_2(100 μM) for 24 h. Following this exposure, cells were resuspended in 0.075 M KCl, and incubated for 3 min. The cells were fixed three times in Carnoy fixative (methanol: glacial acetic acid, 3:1) and then dropped onto clean slides. Finally, the number of micronuclei in BNCs was evaluated under light microscope in 2000 exposed to cells after the nucleus was stained by 5 % giemsa for 10 min. (Figure 2).

Alkaline comet assay

The comet assay was performed to detect the DNA strand breaks induced by CeO_2 NPs. After exposure to different concentrations of CeO_2NPs for 3 h, 100 μL of lymphocyte, the suspension was mixed with 2% low melting temperature agarose and embedded on slides precoated with 5% normal melting point agarose. Subsequently, the slides were placed in a bottle containing the lysis solution (pH 10), including 2.5 M NaCl, 0.1 M EDTA, 10 mM Tris Base, and 1% Triton X for 1 h at 4°C. The slides were denatured in alkaline buffer (0.3 M NaCl, 1 mM EDTA) for 30 min and performed at 25 V and 300 mA for 20 min, and then washed with neutralization buffer (0.5 M Tris-HCl, pH 7.5) for 10 min. After neutralization, the slides were dehydrated by 70% ethanol, air dried, stained with ethidium bromide, and analyzed with a fluorescence microscope (Figure 3). All processes were performed in dark to prevent DNA damage.

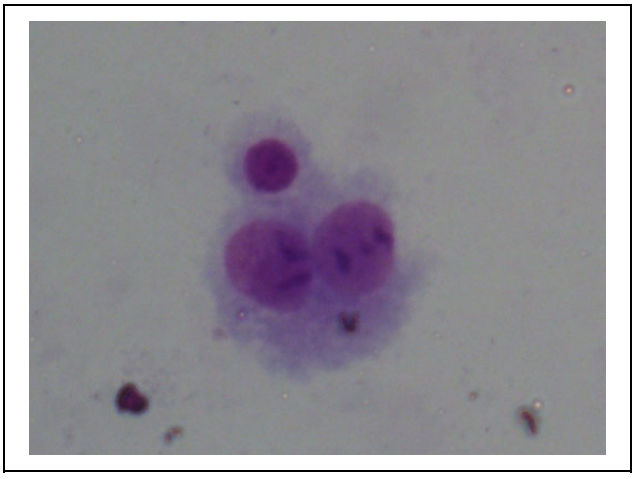

Figure 2. Binucleated cell including micronucleus in lymphocyte cultures exposed to CeO_2 NPs.

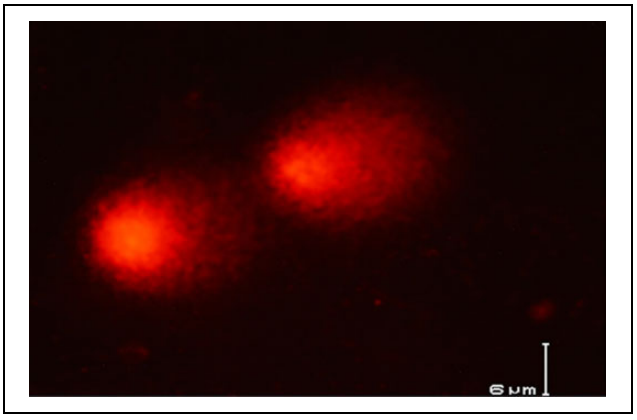

Figure 3. Comet assay views in lymphocyte cultures exposed to CeO_2 NPs.

Immunofluorescence for ϒ-H2AX foci formation

ϒH2AX foci were used to show that CeO_2 NPs lead to double-strand DNA breaks. All experiments were designed according to Scarpato et al. (2013). Briefly, cells that were exposed to CeO_2 NPs were centrifuged and resuspended in 0.075 M KCl, and incubated for 3 min. Then the cells were centrifuged again and

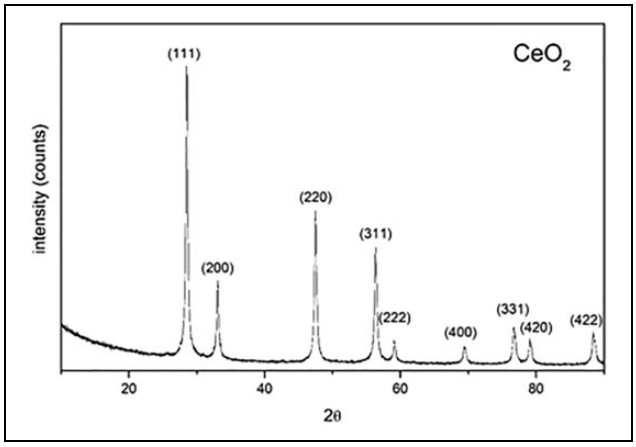

Figure 4. XRD patterns of CeO_2 NPs.

gradually fixed in methanol: glacial acetic acid (5:1), methanol and methanol: glacial acetic acid (3:1). Following this fixation, the cell solution was transplanted onto pre-cleaned slides. After the slides were washed twice in 1X PBS, the cells were blocked in 1X PBS/0.3% Triton X-100 (PBS/Triton; Sigma-Aldrich) and washed again three times in 1X PBS. The slides were steeped overnight at 4°C with primary antibody diluted 1:100 PBS/Triton. The following day, the cells were washed three times in 1X PBS and then incubated at room temperature for 2 h with secondary antibody diluted 1:100 in PBS/Triton. After three washes in 1X PBS, the slides were counterstained with 3–4 drops of 4′,6-Diamidino-2-phenylindole. Then the slides were investigated using a fluorescence microscope.

Statistical analysis

The effect of the NP treatment on genetic material (nuclei) was analyzed using the SPSS 11.5 program.

Results

Characterization of NPs

We evaluated the size of individual NPs by SEM, which was approximately 20.7 ± 2.7 nm. X-ray diffraction analysis confirmed the crystalline size of CeO_2 NPs from diffraction peak (Figure 4). According to XRD analysis, the crystalline phase was determined as cubic. The BET result of CeO_2 NPs was determined by Sigma-Aldrich and it was indicated as ≤25 nm at the product information sheet. So the BET of NPs was not evaluated.

Table 1. Effects of CeO_2 NPs on the frequencies of MNBN‰ and CBPI values in human lymphocyte cells.

Dose	Micronucleus (‰), mean ± standard deviation	CBPI (%), mean ± standard deviation
Negative control	1.00 ± 0.76	2.08 ± 0.05
Positive control	11.25 ± 1.03[a]	0.89 ± 0.01[a]
6 μg/mL CeO_2 NPs	10.00 ± 1.31[a]	1.19 ± 0.01[a]
12 μg/mL CeO_2 NPs	15.00 ± 1.30[a]	1.16 ± 0.01[a]
18 μg/mL CeO_2 NPs	18.87 ± 1.12[a]	1.13 ± 0.02[a]

MNBN: micronucleated binucleated cells; CBPI: cytokinesis-block proliferation index; CeO_2 NPs: cerium dioxide nanoparticles.
[a]Statistical difference from negative control $p < 0.0001$.

Cytokines-blocked micronucleus test

The frequency of micronuclei was determined in human lymphocytes treated with various doses of CeO_2 NPs. The frequency of micronuclei was 1‰ in the negative control group. The micronucleated cell frequency enhanced to 11.25‰ in the H_2O_2 (100 μM) positive control group. The lowest concentration of CeO_2 NPs (6 μg/mL) caused micronucleus frequency (10‰) as well as positive control (11.25‰). The frequency of micronuclei increased to 15‰ and 18.87‰ when CeO_2 NP doses increased to 12 and 18 μg/mL, respectively (Table 1).

Comet assay

Table 2 shows the genetic damage index (GDI) values in human peripheral lymphocytes treated with CeO_2 NPs. Two doses used of CeO_2 NPs (12 and 18 μg/mL) significantly increased the control GDI value of 0.5 to 1.01 and 1.19, respectively ($p < 0.05$). The lowest CeO_2 NP dose did not significantly increase the GDI value in human peripheral lymphocytes. However, treatment with positive control (H_2O_2) significantly increased the GDI value in human lymphocytes ($p < 0.001$), while the solvent control group did not significantly increase the GDI value. Our results showed that the GDI value induced by positive control was similar to that of the highest concentration of CeO_2 NPs (Table 2).

Gamma H2AX foci formation

Treatment with CeO_2 NPs was evaluated with gamma H2AX foci formation that shows DNA double-strand breaks in human lymphocytes. Any significant

Table 2. Comet assay results in human lymphocyte cells exposed to CeO_2 NPs.

Exposed dose	Exposed duration	Proportion of damaged nuclei (%)					Percentage of damaged cells	GDI
		T0	T1	T2	T3	T4		
NC	3 h	71.62 ± 2.28	15.25 ± 1.27	3.00 ± 0.53	2.12 ± 0.54	8.00 ± 1.52	13.12 ± 1.93	0.59 ± 0.07
PC	3 h	25.87 ± 2.15	20.62 ± 2.07	10.62 ± 1.19	12.25 ± 1.23	30.37 ± 0.80	53.25 ± 1.76[b]	2.00 ± 0.04[b]
6 μg/mL CeO_2 NPs	3 h	60.25 ± 3.28	20.00 ± 2.25	6.50 ± 1.25	2.62 ± 0.41	10.62 ± 1.79	19.75 ± 2.78	0.83 ± 0.08
12 μg/mL CeO_2 NPs	3 h	51.12 ± 3.66	23.00 ± 2.85	10.75 ± 1.87	3.37 ± 0.62	11.75 ± 1.90	25.87 ± 2.00	1.01 ± 0.07[a]
18 μg/mL CeO_2 NPs	3 h	45.87 ± 2.80	23.50 ± 2.55	10.62 ± 1.53	4.87 ± 0.98	15.12 ± 2.86	30.62 ± 3.40[a]	1.19 ± 0.11[a]

NC: negative control; PC: positive control (hydrogen peroxide); CeO_2 NPs: cerium dioxide nanoparticles; GDI: genetic damage index.
[a]Statistical difference from negative control $p < 0.05$.
[b]Statistical difference from negative control $p < 0.001$.

Table 3. Gamma H2AX results in human lymphocyte cells exposed to CeO_2 NPs.

Group	Concentration	Gamma H2AX ± SE
Negative control	—	2.12 ± 0.35
Positive control	100 μM	20.37 ± 0.80[a]
Cerium dioxide	6 μg/mL	7.75 ± 0.36[a]
Cerium dioxide	12 μg/mL	10.25 ± 0.52[a]
Cerium dioxide	18 μg/mL	33.50 ± 0.82[a]

CeO_2 NPs: cerium dioxide nanoparticles; SE: Standard Error.
[a]Statistical difference from negative control $p < 0.0001$.

increase in gamma H2AX foci formation was not detected for the solvent control group, while the positive control H_2O_2 also significantly induced double-strand DNA breaks ($p < 0.0001$; Table 3). Furthermore, the negative control group did not induce the gamma H2AX formation. Treatment with three doses of CeO_2 NPs significantly increased DNA double-strand breaks in lymphocytes. Moreover, the highest dose of CeO_2 NPs induced a higher DNA damage than in positive control.

Discussion

Because of their unique properties, NPs may be useful in a diverse range of applications, such as the biomedical field, the use of nano vaccines and nano drugs. (Saptarshi et al., 2013). Hence, NPs' increased use in nanotechnology enhances the risk of its exposure to humans and other living organisms. These risks may cause cytotoxic, genotoxic, and biochemical adverse effects on the biological systems. Therefore, there is an urgent need to demonstrate these adverse effects of NPs on biological systems (Kumari et al., 2014a).

Because of the increasing evidence of NPs adverse effect on biological systems, scientists became concerned. As a consequence, studies on the possible adverse effects of NPs, especially genotoxicity, have increased (Gonzalez et al., 2008). Genotoxic effects of different types of NPs have been evaluated by several researchers (Asha Rani et al., 2009; Ghosh et al., 2010; Kumari et al., 2009; Trouiller et al., 2009; Wise et al., 2010). Shukla et al. (2011) investigated titanium dioxide NPs (TiO_2 NPs) in human epidermal cells which cause reactive oxygen species (ROS)–mediated genotoxicity. Their data demonstrated that TiO_2 NPs have a mild cytotoxic potential. Moreover, both *in vitro* and *in vivo* genotoxicity research of oxide NPs has increased recently. Singh et al. (2016) made a

research on genotoxic effects of chromium oxide NPs and microparticles in *Wistar* rats after 28 days of repeated oral exposure. Their finding provided initial data on the probable genotoxicity.

The genotoxic effects of CeO_2 NPs on different organism have been reported by some researchers (Ali et al., 2015; Gaiser et al., 2012; Kawai et al., 2015). However, these reports are conflicting. For example, several researchers showed (Aalapati et al., 2014; Marzi et al., 2013; Mittal and Pandey, 2014) that CeO_2 has genotoxic effects, whereas some others have claimed (Colon et al., 2010; Rubio et al., 2016; Schubert et al., 2006) that CeO_2 has antigenotoxic effects. Auffan et al. (2009) measured the potential *in vitro* cyto- and genotoxicity of nano-sized CeO_2 (7 nm) on human dermal fibroblasts. Nano-sized CeO_2 at these dosages caused strong DNA lesions and these genotoxic effects occurred at very low doses. Kumari et al. (2014b) evaluated genotoxicity of cerium oxide NPs and cerium oxide micro in Wistar rats after 28 days of repeated oral administration. They used concentrations of 30, 300, and 600 mg/kg body weight (bw)/day of cerium oxide NPs. Their results showed that DNA damage has significantly increased in peripheral blood leukocytes and liver. Also, MN and CA have increased in peripheral blood and bone marrow. Benameur et al. (2015) evaluated the genotoxic effects of cerium oxide NPs' environmentally relevant doses on primary human dermal fibroblasts. They used a cytokinesis-blocked micronucleus assay for the genotoxicity study. The results showed that nano-CeO_2 increased the frequency of micronuclei in dermal fibroblasts. Moreover, the frequency of centromere negative micronuclei increased in cells, which were exposed to nano-CeO_2 for 48 h. In addition, Kaki et al. (2015) studied the effects of cerium NPs (CeNP) and CeO_2 on oxidative toxic stress in human lymphocytes. Although cerium NPs have an interesting potential in drug delivery, gene therapy, and molecular medicine, they found that CeO_2 is able to induce oxidative toxicity in human lymphocytes (24, 48, and 72 h exposure; 15, 30, 60, 120 µmol/mL; 30 nm). Similar results were also observed in different studies (Aalapati et al., 2014; Mittal and Pandey, 2014; Marzi et al., 2013). Different from these studies, Pierscionek et al. (2009) claimed that nanoceria did not cause any DNA damage and significant increases in the number of sister chromatid exchanges. Additionally, Pedram et al. (2015) used different concentrations of CeO_2 (0, 0.5, 1, and 2 ng/mL) in human lymphocytes. Their results showed that CeO_2NPs have protective effects in very low concentrations against CP.

The difference of this study is that it used different doses and genotoxicity tests in human lymphocytes. The earlier studies showed that CeO_2 NPs have a cytotoxic effect, oxidative damage, and decrease of cell viability in human blood lymphocytes. In our study, a gamma H2AX foci formation test has been used for the first time to investigate the genotoxic effects of CeO_2 NPs in human lymphocytes *in vitro*. We used three doses of CeO_2 NPs (6, 12, and 18 µg/mL) in our study. Being exposed to NPs for 72 h significantly decreased the viability of human lymphocytes. Therefore, the lymphocytes for comet and gamma H2AX foci formation tests have been exposed to NPs for 3 h. However, the lymphocytes for micronucleus test have been exposed to NPs for 24 h. Exposure to three doses of NPs significantly increased the potential genotoxic effect. All the test systems showed that CeO_2 NPs have a potential genotoxic effect.

Although several researchers have demonstrated the protective and antioxidant properties of CeO_2 NPs in their work, we reported a potential genotoxic effect of CeO_2 NPs. Prior to our study, Auffan et al. (2009) investigated the genotoxic effects of these NPs on human dermal fibroblasts *in vitro*. Moreover, Srinivas et al. (2011) evaluated a 24-h, 48-h, and 14-day postexposure period on cytotoxicity, oxidative stress, and inflammation in rats with CeO_2 NPs. They found a significant reduction in cell viability and their results suggested that acute exposure toCeO_2 NPs through an inhalation route may cause cytotoxicity via oxidative stress.

Consequently, our results showed that the genotoxic effect of CeO_2 NPs is strictly dose dependent, and human lymphocytes are the sensitive cells. Additionally, the cells are useful tools to show DNA damage produced by nanomaterial samples. The genotoxic effect of CeO_2 NPs is thought to be due to the presence of ROS and oxidative DNA damage (Benameur et al., 2015; Kaki et al., 2015; Kawai et al., 2015; Mittal and Pandey, 2014). However, the genotoxic effect of CeO_2 NPs depends on cell types and from time to time these NPs have antigenotoxic effects when another genotoxic compound is also present (Marzi et al., 2013). Therefore, more studies are necessary to evaluate the genotoxic effect of CeO_2 NPs in specific cells.

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Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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