Comparison of the Cytotoxic Effects of Bulk and Nano-sized CeO$_2$ on Lymphocyte Cells

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Abstract: Cerium(IV) oxide (CeO$_2$) is widely used in industrial fields such as biomedical, glass, electronics, automotive, and pharmacology. In this study, the cytotoxic effects on human peripheral blood cultures of two forms of cerium(IV) oxide with different particle sizes (Bulk-sized Cerium(IV) oxide: BC and Nano-sized Cerium(IV) oxide: NC) in concentrations range of 0.001-200 ppm were investigated. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay were used to determine the cytotoxicity of these forms. According to the test results, it was determined that both forms caused severe cytotoxicity at all concentrations studied. It was observed that cytotoxicity increased with increasing concentration. NCs are more toxic at all concentrations except 100 and 200 ppm concentrations.

Keywords: Cerium(IV) oxide, biomaterial, cytotoxicity, MTT assay, lymphocyte, nanoparticle.

Submitted: July 26, 2021. Accepted: October 08, 2021.


DOI: https://doi.org/10.18596/jotcsa.974814.

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INTRODUCTION

Cerium(IV) oxide is one of the significant rare earth oxides used in many applications such as catalyst, hydrogen generation, optical displays and UV absorber due to its unique properties (1). The reason why CeO$_2$ is used in many industrial and biomedical applications is its redox capability (2). Due to the increasing use of cerium compounds, it is important to determine their possible toxic effects on living things and the environment. Because organic compounds can be converted into non-toxic compounds in nature, inorganic compounds often decompose into components that can cause toxicity (3).

Nanotechnology is a research area that brings innovation to technological processes with a different perspective. Today, nanomaterials are used in almost every application. In this early adopted and rapidly advancing technology, the effects of exposure to the nanoparticles used are not yet fully known. The amount, transition, degradation, change and accumulation in nature of nanomaterials released into the environment are not known exactly. Nanoparticles that enter the body through the skin, mouth, or lungs can cause direct or indirect harm to genetic material (4,5). It is known that nano-sized cerium(IV) oxide is used in different applications such as catalyst, conductor, electrode, ultraviolet absorber, luminescence devices and glass polisher (6). In addition to these physical applications, CeO$_2$ nanoparticles are also used in many biological applications due to their antibacterial, antioxidant and anti-inflammatory properties (2,7–10). Without a doubt, the application of nanoceria as a biomaterial will remain a highlight of biology, biomedical and materials research in the next years. However, the Organization for Economic Cooperation and Development (OECD) has classified this nanoparticle as a hazardous chemical. The OECD advises that the toxicity profile of CeO$_2$ nanoparticles be studied further through in vitro and in vivo research (11).
Particle-related parameters such as size, shape, surface charge, crystal structure, concentration, pH, and exposure time are factors that affect the toxicity of the nanoparticle. Among these parameters, the most intensively investigated parameters are usually particle size and concentration. In toxicity studies on many cell lines using different methods, it has been determined that CeO$_2$ in nanoparticle size is generally toxic (1,12–14). There are many studies showing that CeO$_2$ with nanoparticle size is more toxic than that in the bulk size (15–18). Lymphocytes are critical components of the immune system as they are able to elicit a response to bacteria, viruses and existing cells that enter the human body that develop into a cancerous cell type. They are widely used in in vitro drug development studies because they play an active role in the synthesis of lymphocyte cells, immunoglobulins, and a wide variety of other proteins in peripheral blood. In addition, researchers and clinicians use lymphocytes in fields related to immunology, infectious disease, hematological malignancies, vaccine development, transplant therapy, personalized medicine, and toxicology. In general, in vitro lymphocyte studies contribute to research on cell function, biomarker identification and disease modeling (19,20). In this context, it is necessary to know the toxic properties of cerium(IV) oxide, which is widely used in many industrial areas, in terms of the sustainability of its use as a biomaterial. For this purpose, in this study, we determined and compared that cytotoxic properties of BC and NC on lymphocyte cells using MTT test.

**EXPERIMENTAL SECTION**

**Chemicals and Instruments**

Primary Peripheral Blood Mononuclear cells (ATCC PCS-800-011™) (containing a minimum of 25x10$^6$ viable cells), Phosphate Buffered Saline (PBS), Fetal Bovine Serum (FBS), Penicillin-Streptomycin Solution, Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma), Dimethylsulfoxide (DMSO), Cerium(IV) oxide nanoparticles and Cerium(IV) oxide bulk sized particles (Sigma-Aldrich) and 3-(4,5-dimethylthiazol-2-yi)-2,5-diphenyltetrazolium bromide (Acros) were purchased commercially. In this study, NICHIRYO Nichipet Single Channel (10-100-1000 µL) automatic pipettes, Nüve BM 101 Water bath, ISOLAB vortex mixer, Panasonic MCO 170AICUVH-PE CO$_2$ Incubator, Hed Lab X BIO MSC CLASS II biosafety cabine, Thermoscientific-Countess II cell counter and Thermoscientific Multisakan Sky Microplate Spectrophotometer were used. Powder X-Ray Analysis were performed Philips X’Pert Pro diffractometer with Cu, Kα radiations, 40 kV of voltage and a current of 35 mA. The patterns of the samples were recorded from 5 ° to 70 ° (2 °) with 0.2 °/ min and a step size of 0.02 °.

**Determination of particle sizes**

The particle sizes of BC and NC were calculated with Scherrer’s equation (1) from obtained data by powder X-ray diffraction method.

$$d = \frac{K \lambda}{\beta \cos \theta}$$

where d is the mean particle size, K is the grain shape–dependent constant 0.89, λ is the wavelength of the incident beam in nanometer, θ is the Bragg reflection angle and β is the line broadening at half the maximum intensity in radians (21,22).

**Preparation of Cell culture and MTT assay**

MTT method, which is a colorimetric method, was used to determine the cytotoxicity of bulk and nanosized CeO$_2$ (23,24). Human peripheral blood mononuclear cell (PBMC) stock (1 mL) maintained at -80 °C were thawed by using a water bath. 1 mL of cell stock was diluted by adding 9 mL of culture medium that contains 89 % DMEM, 10 % FBS and 1 % penicillin-streptomycin. The number of cells in the cell suspension was calculated using a cell counter. 100 µL aliquots of the prepared cell suspension were added to the 96-well plate at approximately 25000 cells per well. After cells were seeded, 100 µL of culture medium was added to each well and incubated for 24 h at 37 °C in a 5% CO$_2$ and 95% humidity in a CO$_2$ incubator. The cells were removed and the supernatant was discarded. For each concentration, 100 µL of MTT solution prepared in PBS with a concentration of 5 mg/mL was added to each well. Incubation was continued for 4 h and then 200 µL of DMSO used as formazan crystal solvent was added to each well. Incubation was continued for another 18 h. After the end of the incubation, absorbance values at a wavelength of 570 nm were recorded with the help of a spectrophotometer. Experiments were carried out in triplicate.

**Statistical analysis**

Two-way ANOVA (Tukey) test included in the IBM SPSS statistics for Windows (version 22.0, IBM Corp., Armonk, NY, USA) package program was used for the statistical calculations of the absorbance values obtained. Statistically significance level is accepted at 95% (p<0.05).

**RESULTS AND DISCUSSION**

**Characterization of particles**

The average particle size of BC and NC was calculated as 231.61 nm and 27.15 nm,
respectively. Powder X-ray patterns of BC and NC were given in Figure 1.

![Powder X-ray patterns of BC and NC.](image)

**Figure 1**: Powder X-ray patterns of BC and NC.

**MTT assay**

When Figures 2 and 3 are examined, it is seen that all application concentrations of BC and NC cause statistically significant cytotoxicity compared to the cell control group. Especially at 100 and 200 ppm concentrations, the highest cytotoxic effect was determined and cell viability decreased below 25%. Remarkably, it is observed that BC and NC reduce cell viability to more than 50% at all concentrations.

![Effect of BC on cell viability at different concentrations.](image)

**Figure 2**: Effect of BC on cell viability at different concentrations.
In this study, the effects of BC and NC on human lymphocyte cell viability were also compared. It was determined that NC was more toxic than BC at all concentrations except at 100 and 200 ppm. BC was found to have a stronger toxic effect than NC at only 100 and 200 ppm concentrations. According to the International Standard 10993-5 (25), chemicals that reduce cell viability by more than 50% are considered moderately cytotoxic. The results of this study reveal that both forms of cerium(IV) oxide are moderately cytotoxic (Figure 4).

There are many studies in the literature investigating the cytotoxicity of CeO\textsubscript{2}. When the literature is examined, the toxic effects of the cerium(IV) oxide compound on cancer cell lines have been studied more. However, this compound is widely used especially in dental treatment. Therefore, it is important to know the effects of this compound on normal cells. In many of these studies, the cytotoxicity of the nanoparticle sized form of the cerium(IV) oxide compound was investigated. There are very few studies investigating the cytotoxicity of cerium(IV) oxide in bulk form. There are two different studies investigating the cytotoxicity of cerium(IV) oxide nanoparticles with a particle size of 25-30 nm. In these studies, the effects of this compound on lymphocyte cells were investigated using the MTT method. In one of these two studies, it was stated that the nanoparticle did not cause any toxicity in the concentration range of 2.5-20 ppm (11), while in the other study, it was shown that the nanoparticle caused cytotoxicity at high concentrations (50, 100 and 200 ppm). In the same study, it was claimed that nanoparticles were not cytotoxic at 1 and 10 ppm concentrations (12). Although the method and cell type used are the same, the data obtained of our study and these two studies partially overlap. The reason for this inconsistency is thought to be due to the difference in particle size and experimental conditions. The cytotoxicity of cerium(IV) oxide on different cancer cells other than lymphocyte cells has been extensively investigated. For example, Abid et al. determined that nano CeO\textsubscript{2} caused cytotoxicity in the concentration range of 0.93-120 ppm on RD rhabdomyosarcoma and L20B cell lines by using
MTT method (1). Using the same method, CeO$_2$ with a particle size of 2-6 nm has been reported to be cytotoxic on bone marrow mesenchymal stem cells (13). Cerium(IV) oxide with a particle size of 15-20 nm was determined by the WST-1 method to be cytotoxic on the MCF-7 cell line in the concentration range of 1-1000 ppm (14). Rasouli et al. determined that CeO$_2$ with a particle size of 11-17 nm was more cytotoxic on HFFF2 cancer cells than on HT29 normal cells and cytotoxicity increased with increasing concentration and exposure time (26). According to the results of a similar study, 100-nm size CeO$_2$ caused cytotoxicity on PC-3 cancer cells, but not on L929 normal cells (27). Similarly, Kargar et al. found that nano-CeO$_2$ at 8x10$^3$ ppm concentration did not cause toxicity on L929 cells (28). Contrary to these results, 11-33 nm-sized cerium(IV) oxide in the 125-1000 ppm concentration range (29) and 21-32 nm cerium(IV) oxide in the 1-500 ppm concentration range (30) was found to be non-cytotoxic on PC12 and A549 cancer cells, respectively. De Marzi et al. (2013) investigated the cytotoxic properties of 40 nm CeO$_2$ on A549, CaCo2 and HepG2 cell lines in the concentration range of 5x10$^{-2}$-5x10$^5$ ppm. While it did not cause cytotoxic effects on cell lines exposed to nanoparticles for 24 hours, they determined that the nanoparticle caused cytotoxicity after ten days of exposure (31). In two different studies comparing the toxicity of nano and bulk sized cerium(IV) oxide, it was determined that CeO$_2$ with nano size was more toxic than bulk size (17,18). This result is consistent with the data obtained in our study. In a study using the MTT method, it was determined that cerium(IV) oxide with a particle size of 13.04 nm did not cause any cytotoxicity on A549, Calu-3 and 3T3 cells at 10, 100 and 500 ppm concentrations (32). Cerium(IV) oxide with a size of 4-13 nm exhibited a cytotoxic effect as the concentration increased on A549 cells in the concentration range of 1.95-500 ppm. However, it has little effect on the viability of these cells (33). It was found that cerium(IV) oxide nanoparticles (14 nm) obtained by green synthesis at a concentration of 0.01 ppm on A549 cells were mildly cytotoxic according to the WST-1 method and not cytotoxic according to the MTT method (34).

CONCLUSION

In this study, the cytotoxic properties of bulk and nano-sized cerium(IV) oxide on human peripheral blood cultures in the concentration range of 0.001-200 ppm were investigated. The particle sizes of bulk and nano-sized cerium(IV) oxide are 231.61 nm and 27.15 nm, respectively. Both forms of the compound caused cytotoxicity on lymphocyte cells at all applied concentrations. The nano form is more cytotoxic at 0.001-50 ppm concentrations and the bulk form is more cytotoxic at 100 and 200 ppm concentrations. It was determined that cell viability decreased with increasing concentration, that is, cytotoxicity increased. It has been reported in many studies, including our study, that this compound, which is widely used as a biomaterial, causes cytotoxicity on both normal and cancer cells. In this context, it is recommended to limit the use of the compound as a biomaterial and to examine the toxicity profile in more detail with in vivo studies.

CONFLICT OF INTEREST

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

ACKNOWLEDGMENTS

The author thanks Füreya Elif Öztürkkan and Mustafa Sertçelik for their technical supports.

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