Toxicity of nano and bulk forms of Cerium oxide in different cell lines
Fatemeh Soltani¹, Kamal Yavari², Mahdi Sadeghi³*, Ali Bahrami Samani², Shirvani Arani Simindokht²

¹ Department of Medical Radiation Engineering, Science & Research Branch, Islamic Azad University, Tehran, Iran
² Radiopharmaceutical Research and Development Lab (RRDL), Nuclear Science and Technology Research Institute (NSTRI), Tehran, Iran
³ Medical Physics Department, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

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ABSTRACT
In recent years, nanotechnology has gained serious attention for diagnosis, prevention and treatment roles. In this study we synthesized nanoceria or CeO2NPs (cerium oxide nanoparticles) and compared toxicity of cerium oxide powder in nano and bulk forms in two cancerous and one normal cell lines. The cell lines were cultured in a standard humidified incubator, at 37 °C in a 5% CO2 atmosphere, in RPMI 1640 medium. The cells were incubated with different concentrations of cerium oxide (from 2 μg/mL to 64 μg/mL) in bulk and nano forms. To determine the effect of cerium oxide on cell viability after 24 h, 48 h, and 72 h incubation, a MTT assay was performed using SKBR3 (human breast cancer cell line), A431 (Human epidermoid carcinoma cell line) and C2C12 (ATCC mouse skeletal muscle cell line) cells. Analysis of variance followed by Sidak post-hoc test, shows the toxicity of nanoceria is significantly deferent from bulk form on three cell lines in this study and is more on cancerous cells in compared to normal cells especially in higher level of concentrations after 24, 48 and 72 hours (All P<0.05). Additionally, the effect of cell lines, cerium oxide forms and concentrations cerium oxide leads in significantly the lowest amount of viability after 72 hours compared with 24 hours and 48 hours.

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INTRODUCTION
The recent rapid progress in nanotechnology has led to a great deal of concern due to the needs and applications of NMs (nanomaterials) in many areas such as industry, agriculture, business, medicine and public health [1]. With the increasing production volumes and number of commercially available, environmental exposure to NMs seems inevitable, and as a result, further testing and research on nanotoxicity are needed [2]. CeO2NPs have shown promising biomaterial for biomedical applications, and it is foreseen that their importance will increase in future technological developments [3-12]. In addition to great benefit of nanotechnology, it is important to consider assessing toxicological properties of NMs [13]. As current findings about the toxicology of bulk materials may not be adequate for predicting toxic forms of nanoparticles, further investigations on nanotoxicity will be necessary [14]. The physical and chemical characteristics of nanoparticles can differ substantially from their bulk counterparts [15]. In contrast to conventional chemicals, the possible risks of using NMs for human health and the environment have not been yet fully evaluated [3, 16, 17]. Evaluating strategies for risk assessment of nanotoxicity,
extensive research efforts were directed toward developing toxicity assays such as the MTT. The objectives of these assays are the quantitative determination of the viability of living cells that were incubated with NMs [3, 16]. In recent years, many studies have investigated the proliferation of a wide range of cell lines with respect to a wide variety of engineered nanoparticles [1-2]. It is expected that the importance of nanoceria as rare-earth metal oxide nanoparticles with multiple industrial and biomedical uses will increase in future research efforts [18]. Only few studies have been performed describing the effects of nano cerium oxide toxicity [19-20].

In the present paper, we synthesized nanoceria, CeO₂ using precipitation method and investigated the in vitro toxicity of nanoceria and its bulk counterpart in SKBR3 (human breast cancer cell line), A431 (Human epidermoid carcinoma cell line) and C2C12 (ATCC mouse skeletal muscle cell line) cells.

MATERIALS AND METHODS

Material
In this study MTT dye (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma Aldrich), phosphate-buffered saline (PBS), trypsin-EDTA solution, dimethyl sulfoxide (DMSO, Merck), trypan blue (Sigma Aldrich), cerium oxide powder in bulk form (CeO₂; Merck), Cerium (III) nitratehexahydrate (Ce(NO₃)₃·6H₂O, Merck), Hexamethylenetetramine ((CH₂)₆N₄, Fluka), RPMI1640 medium (Gibco) were used. C2C12, A431 and SKBR3 cell lines were purchased from pasture institute.

Synthesis of nanoceria
CeO₂NPs have been produced using many different preparation methods such as sol-gel [21-22], thermal decomposition [23], solvothermal oxidation [24], microemulsion methods [25], flame spray pyrolysis [26], microwave-assisted solvothermal process [27] and precipitation [28].

In this study CeO₂NPs were synthesized using precipitation technique [28]. A 0.007 kg (7 g) of hexamethylenetetramine (HMT) and a 0.0016 kg (1.6 g) of Ce(NO₃)₃·6H₂O were dissolved in 100 mL distilled water separately and each of them stirred for 30 min. The two solutions were then combined and stirred for 23 h. The obtained solution was then centrifuged for 10 min at 4000 revolutions per minute (rpm). Precipitates were dried at 70°C for 15 h in a hot air oven.

Crystal structures were identified with a powder X-ray diffractometer (Stoe, Stidy-MP) employing the Cu Kα radiation (k= 154.18 pm) line. Actual X-ray diffraction (XRD) nano-particles patterns were verified comparing with JCPDS (Joint Committee on Powder Diffraction Standards) data.

The morphology of the synthesized CeO₂NPs were imaged by transmission electron microscopy (TEM). CeO₂NPs were analyzed for their size distribution by TEM image (Fig. 1) and XRD spectrum (Fig. 2). The purity of bulk and nano powder was analyzed with X-ray fluorescence analysis (XRF). The synthesis has been previously described, and we refer to this work for more...
Cerium oxide nanoparticles were synthesized by precipitation method. The XRF results showed 97.70% purity for purchased cerium oxide powder in bulk form and 97.48% for synthesized nano powder of ceria. From the XRD data shown in Fig. 2, it is clear that the precipitate powder was already CeO$_2$. The Transmission electron microscopy (TEM) results confirm that grains are nanometer in size and show good agreement with the XRD results.

According to the XRD pattern (Fig. 2), the average particle size was obtained using the Debye–Scherer equation [21]. Comparison of the XRD patterns with the JCPDS data (File No. 34-0394) confirms the samples are cerium oxide with cubic structure. Using XRD (X-ray diffraction) and TEM, the average crystallite size obtained 7 nm and 6.6 nm respectively.

Cytotoxicity effect of Nano and Bulk ceria

The effect of intervention after 24 hours: Assessing the effect of CeO$_2$ nano and bulk materials on viability of three cell lines of SKBR3, A431 and C2Cl2 in various concentrations after 24 h incubation, the results of three way ANOVA showed a three way interactions of cell line*cerium oxide*concentration ($F_{(12,42)}=3.97, P<0.001$), all two ways interactions of cell line*cerium oxide ($F_{(2,42)}=39.48, P<0.001$), cell Line*concentration ($F_{(2,42)}=6.04, P<0.001$), and cerium oxide*concentration ($F_{(6,42)}=17.62, P<0.001$) and all main effects of cell line ($F_{(2,42)}=133.19, P<0.001$), cerium oxide ($F_{(4,42)}=565.23, P<0.001$) and concentration ($F_{(6,42)}=104.55, P<0.001$). This means that the effect of nano and bulk ceria on viability varies within the levels of three cell lines for each concentration. Additionally considering the significance of the three ways interaction, the results of Sidak simultaneous post hoc tests showed the significant lowest amount of viability in SKBR3*nano*concentration8 (C8), C2Cl2* nano*64, SKBR3*nano*C16, C2Cl2* nano*C16, SKBR3*nano*C32, SKBR3*nano*C64 levels, and the other levels of three factors were significantly in higher amount of viability. In the other words, after 24 hours, nano cerium oxide of SKBR3 cell line leads in lowest amount of viability especially in higher level of concentration (Fig. 3).

The effect of intervention after 48 hours: To investigate how CeO$_2$ nano and bulk materials affect the viability of three cell lines of SKBR3, A431 and C2Cl2 in various concentrations after 48 hours incubation, the results of three way ANOVA showed significant three way interactions of cell line*cerium oxide*concentration ($F_{(12,42)}=9.03, P<0.001$), all two ways interactions of cell line*cerium oxide ($F_{(2,42)}=101.62, P<0.001$), cell line*concentration ($F_{(2,42)}=30.30, P<0.001$), and cerium oxide*concentration ($F_{(6,42)}=44.37, P<0.001$) and all main
effects of Cell line (F\((2,42)\)=1017.53, P<0.001), cerium oxide (F\((1,42)\)=1361.97, P<0.001) and concentration (F\((6,42)\)=853.77, P<0.001). In the other words, the effect of CeO\(_2\) nano and bulk materials on viability varies within the levels of three cell lines for each concentration. Furthermore taking into account the significance of the three ways interaction, the results of Sidak post hoc tests showed the significant lowest value of viability in A431\(^{-}\)nano\(^{-}\)C32, SKBR3\(^{-}\)nano\(^{-}\)C64, A431\(^{-}\)nano\(^{-}\)C64 levels, and the other levels of three factors were significantly in higher amount of viability. Hence, after 48 hours, nano cerium oxide of SKBR3 and A431 cell lines lead in the lowest amount of viability in higher level of concentration (Fig. 4).

The effect of intervention after 72 hours: After 72 hours incubation, the results of three way ANOVA to assess the effect of CeO\(_2\) nano and bulk materials on viability of three cell lines of SKBR3, A431 and C2Cl2 in various concentrations showed significant three way interactions of cell line\(^{-}\)cerium oxide\(^{-}\)concentration (F\((12,42)\)=6.87, P<0.001), all two ways interactions of cell line\(^{-}\)cerium oxide (F\((2,42)\)=91.37, P<0.001), cell line\(^{-}\)concentration (F\((2,42)\)=55.44, P<0.001), and cerium oxide\(^{-}\)concentration (F\((6,42)\)=27.10, P<0.001) and all main effects of Cell line (F\((2,42)\)=1193.08, P<0.001), cerium oxide (F\((1,42)\)=696.47, P<0.001) and concentration (F\((6,42)\)=2472.66, P<0.001). In the other words, the effect of cerium oxide on the viability amount varies within the levels of three cell Lines for each concentration. Furthermore pertaining to the significance of three ways interaction, based on the results of Sidak post hoc tests the significant lowest value of viability was observed in SKBR3\(^{-}\)nano\(^{-}\)C32, A431\(^{-}\)nano\(^{-}\)C4, A431\(^{-}\)nano\(^{-}\)C8, A431\(^{-}\)nano\(^{-}\)C16, A431\(^{-}\)nano\(^{-}\)C32, SKBR3\(^{-}\)nano\(^{-}\)C64 and SKBR3 levels, and other levels of three factors had significantly higher amount of viability. Therefore, after 72 hours, nano cerium oxide of SKBR3 and A431 cell lines result in the lowest amount of viability in higher level of concentration (Fig. 5).

**DISCUSSION**

The use of nanotechnology in drug delivery has been increased rapidly. Since many people such as researchers, manufacturers of NMs, patients and ordinary people who may use products containing nanostructures can get exposed to nanostructures; there is a great need for investigating on toxicity of NMs. Because of deficiency of knowledge in human health risks associated with toxicity of NMs, we designed a novel in vitro system to examine the interactions of manufactured CeO\(_2\) NMs and their bulk
Toxicity of nano and bulk forms of Cerium oxide in different cell lines

There are few investigations that compare toxicity of nano and bulk forms of cerium oxide nanoparticles especially in higher level of concentration. Darroudi et al showed CeO₂ NPs via the sol–gel method and performed an in vitro cytotoxicity study using neuro2A cell line via MTT assay and showed a concentration-dependent toxicity of cerium oxide nanoparticles with non-toxic effect of concentration below 10 mg/mL after 24 h incubation. Their research does not include any investigation on bulk form of CeO₂ [22].

Arnold et al and Rosenkranz et al showed CeO₂ NPs are more toxic than equimolar bulk cerium oxide [30-31]. Similarly Grover et al indicated bulk compound of cerium oxide is less cytotoxic than its counterpart NMs with the four cell lines tested. The cell lines exposed to CeO₂-NM for 24 hours. The statistical significant change in MTT assay between treated and control groups were analyzed by one-way [33].

In present study, we considered four variables or effects which are: incubation time point (24h, 48h and 72h), cell line (cancer cell lines, SKBR3 and A431 and normal cell line, C2Cl2), form of cerium oxide (nano and bulk) and concentration (C2 to C64); so that 72*SKBR3*nano*C32, 72*A431*nano*C64, 72*SKBR3*bulk*C8, 72*A431*bulk*C16, 72*SKBR3*bulk*C32, 72*A431*bulk*C64, 72*SKBR3*nano*C64, 72*SKBR3*nano*C32, 72*SKBR3*bulk*C8, 72*A431*bulk*C16, 72*SKBR3*bulk*C32, 72*A431*bulk*C64, 72*SKBR3*nano*C64, 72*SKBR3*nano*C32, 72*SKBR3*bulk*C8, 72*A431*bulk*C16, 72*SKBR3*bulk*C32, 72*SKBR3*bulk*C64, 72*A431*nano*C16, 72*A431*nano*C32, 72*SKBR3*nano*C64, 72*A431*nano*C64 combination levels had the lowest amount of viability. In the other words, as can be seen the effect of cell lines, cerium oxide and concentration leads in significantly the lowest amount of viability after 72 hours based on the results obtained from the Sidak post hoc tests. CeO₂ powders have been prepared using a high-yield homogeneous precipitation method using hexamethylenetetramine and trivalent cerium salt. This study demonstrate that nano Cerium Oxide of SKBR3 and A431 cell lines leads in lowest amount of viability especially in higher level of concentration after 24, 48 and 72 hours. Additionally, the effect of cell lines, cerium oxide and concentration leads in significantly the lowest amount of viability after 72 hours.

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CONFLICT OF INTEREST

The authors declare that this research does not have any conflict of interest with anyone or any institute.

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Figure 5. Comparative effects of CeO₂ nano and bulk materials on viability of three cell lines after 72 h incubation