



Original Article

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Toxicity of nano and bulk forms of Cerium oxide in different cell lines

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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.

Conflicts of Interest: Declared None

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Keywords

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Nanoceria,
MTT assay,
C2C12 cells,
A431 cells,
SKBR3 cells

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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 nanocerium 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 nanocerium, CeO₂ using precipitation method and investigated the in vitro toxicity of nanocerium 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) nitrate hexahydrate (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 nanocerium

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

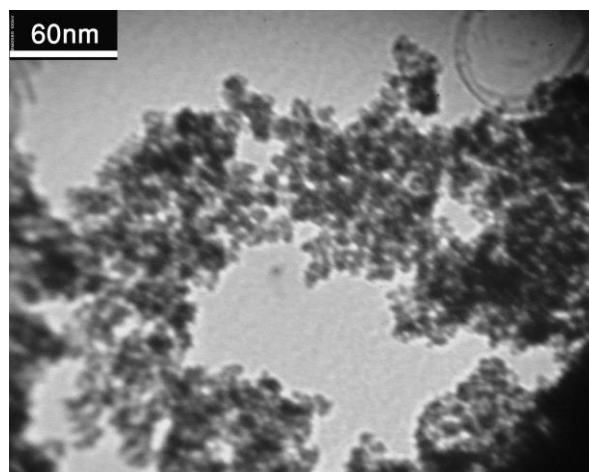


Figure 1. Transmission electron microscopic image of CeO₂ nanoparticles

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, Stady-MP) employing the Cu K α radiation ($\lambda = 154.18$ 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

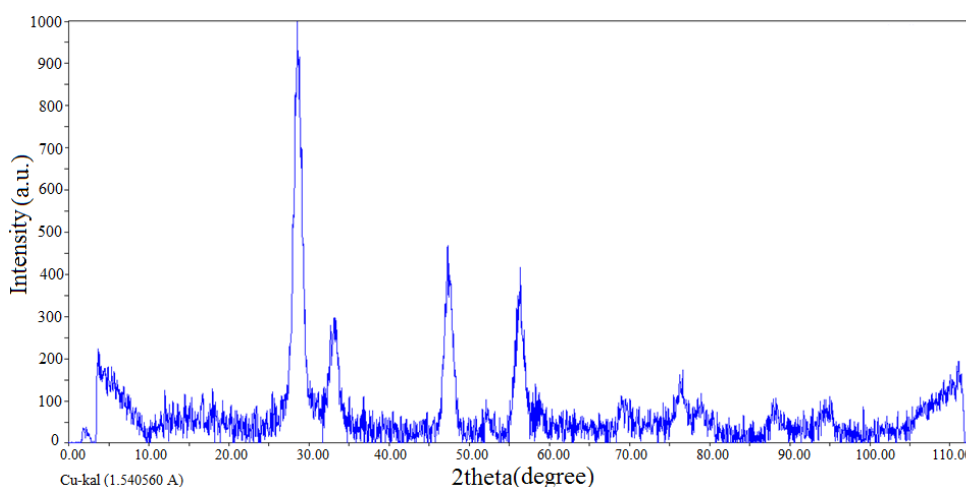


Figure 2. XRD pattern of CeO₂ nanoparticles

details [28].

Incubation of cell lines and MTT assay

The human C2C12, A431 and SKBR3 cells (from ATCC) were cultured in RPMI medium (Gibco BRL, Paisley, UK) supplemented with 10% of fetal bovine serum (FBS), 2 mM L-Glutamine and 1ml/L Gentamicin 40 mg/ml at 37 °C with 5% CO₂. Briefly, the day before MTT addition, confluent layer of the cells was trypsinated, counted and resuspended. Suspension of 4×10^3 of cells were plated in RPMI medium into each well of the 96 well plates, so that they could become about 60–80% confluence next day at the time of MTT examination. CeO₂NPs and their bulk counterpart were dispersed in RPMI medium. Homogenous dispersions were produced after 5 minutes of sonication. The suspensions at the concentrations of 2 µg/ml (C2), 4 µg/ml (C4), 8 µg/ml (C8), 16 µg/ml (C16), 32 µg/ml (C32), 64 µg/ml (C64) were added into three 96-well plates and incubated for three time points 24 h, 48 h, 72 h, respectively. MTT assay was used for evaluating cytotoxicity of all cell lines. The assay is dependent on the reduction of the tetrazolium salt MTT (3-(4, 5-dimethylthazol-2-yl)-2, 5-diphenyl tetrazolium bromide) by the mitochondrial dehydrogenase of viable cells to form a blue formazan product. 20 µl MTT (5 mg/ml in PBS) was added by a multi-channel pipette into all wells of three plates treated with cerium oxide in bulk and nano forms and cells were incubated for another 3–4 hours at 37°C. Supernatants were collected from each well by a multi-channel pipette and 150 µl of DMSO was added to each well and incubated at room temperature protected from light for 15 min prior to determining cell viability. All absorbance values were measured by ELISA reader at 570 nm. Cell viability was calculated from the absorbance ratios in each treated groups and the corresponding control group. It should be noted that, control groups for each cell line incubated into another plate in RPMI medium and cell viability was measured for them at all time points as well.

Statistical Analysis

Data were presented using mean (SD). To assess four effects of cell lines, Cerium Oxide (in two different forms of nano and bulk), concentrations and time points on the viability of the cells, a four way analysis of variance was used considering the interaction effect and main effects followed by Sidak post hoc test for combination levels of these factors.

To evaluate the research hypotheses separately for each time, investigating the effect of cell lines, Cerium Oxide, and concentrations on the viability of the cells, a four three analysis of variance was used considering the interaction effect and main effects followed by Sidak post hoc test for combination levels of these factors.

In each model the assumptions of normality, homogeneity of variances and independence of residuals were assessed graphically and confirmed. All analyses were performed using Minitab16 statistical software.

Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Preparation of ceria nanoparticles

Ceria 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 CeO₂. 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 CeO₂ nano and bulk materials on viability of three cell lines of SKBR3, A431 and C2C12 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 ($F_{(12,42)}=6.04$, $P < 0.001$), 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 ($F_{(1,42)}=565.23$, $P < 0.001$) 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), C2C12*nano*C64, SKBR3*nano*C16, C2C12*nano*C16, C2C12*nano*C32, 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 CeO₂ nano and bulk materials affect the viability of three cell lines of SKBR3, A431 and C2C12 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 ($F_{(12,42)}=30.30$, $P < 0.001$), and cerium oxide*concentration ($F_{(6,42)}=44.37$, $P < 0.001$) and all main

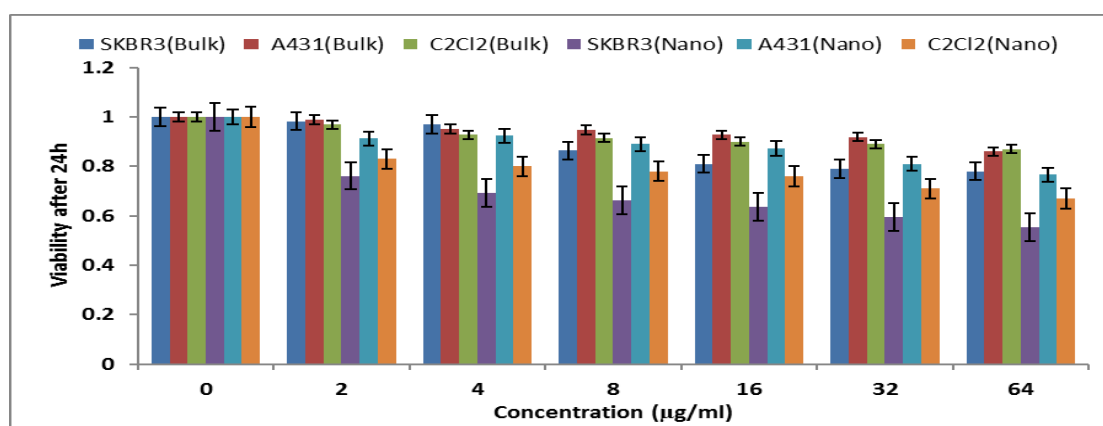


Figure 3. Comparative effects of CeO₂ nano and bulk materials on viability of three cell lines after 24 h incubation

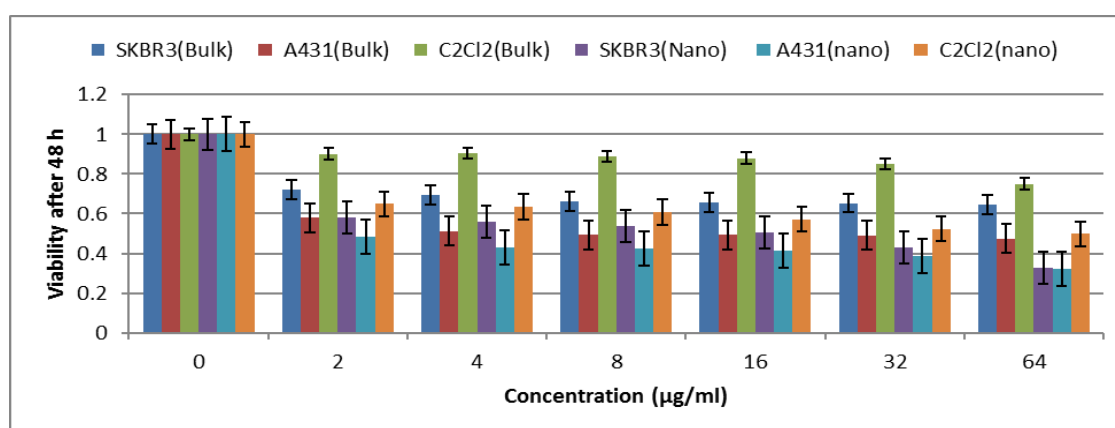


Figure 4. Comparative effects of CeO₂ nano and bulk materials on viability of three cell lines after 48 h incubation

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 CeO₂ 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 CeO₂ nano and bulk materials on viability of three cell lines of SKBR3, A431 and C2C12 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 ($F_{(12,42)}=55.44$, $P<0.001$), 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 CeO₂ NMs and their bulk

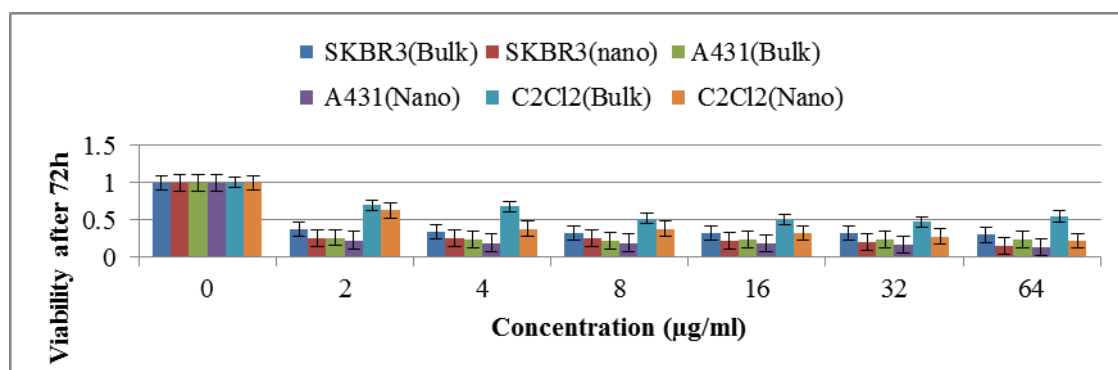


Figure 5. Comparative effects of CeO₂ nano and bulk materials on viability of three cell lines after 72 h incubation

counterpart materials within the cells. Bulk analogues were used to assess the effect of particle size on the toxicity. In the present study, we synthesized CeO₂ nanoparticles using an easy and low-cost precipitation method. Characteristics of the nanopowders was studied by X-ray fluorescence (XRF) analyzer, X-ray diffraction (XRD) analyzer and transmission electron microscopy (TEM). The comparison of XRD results with JCPDS files confirmed compound identity and cubic structure of the sample, and TEM studies confirmed the calculated value of particle size from Debye Scherrer's formula [29]. Comparing our synthesis results with previously published works, [28, 30] indicates that we achieved the same results found earlier by them.

There are few investigations that compare toxicity of nano and bulk forms of ceria within cells. Darroudi et al synthesized CeO₂NPs 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 CeO₂NPs 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, C2C12), form of cerium oxide (nano and bulk) and concentration (C2 to C64) and in a consistent statistical analysis evaluated the interaction of variables using four, three, two and one way ANOVA followed by Sidak post hoc.

To compare the changes in the viability during three time points of 24, 48 and 72 hours along with the effect of cell line, cerium oxide and concentration, the findings of four way analysis of variance revealed the significant four

way interaction effect of time*cell line*cerium oxide*concentration ($F_{(24,126)}=3.26$, $P<0.001$) which means that the changes in the viability during three time points of 24, 48 and 72 hours varies for cell lines, each cerium oxide (nano and bulk) and within the levels of concentrations (C2 to C64); so that 72*A431*bulk*C4, 72*A431*bulk*C8, 72*A431*bulk*C16, 72*A431*bulk*C32, 72*A431*bulk*C64, 72*A431*nano*C64, 72*SKBR3*nano*C16, 72*SKBR3*nano*C32, 72*A431*nano*C4, 72*A431*nano*C8, 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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