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# Anti-cancer effects of cerium oxide nanoparticles and its intracellular redox activity

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ABSTRACT

Data on medical applications of cerium oxide nanoparticles $CeO_2$ (CONP) are promising, yet information regarding their action in cells is incomplete and there are conflicting reports about *in vitro* toxicity. Herein, we have studied cytotoxic effect of CONP in several cancer and normal cell lines and their potential to change intracellular redox status. The IC50 was achieved only in two of eight tested cell lines, melanoma 518A2 and colorectal adenocarcinoma HT-29. Self-propagating room temperature method was applied to produce CONP with an average crystalline size of 4 nm. The results confirmed presence of $Ce^{3+}$ and $O^{2-}$ vacancies. The induction of cell death by CONP and the production of reactive oxygen species (ROS) were analyzed by flow-cytometry. Free radicals related antioxidant capacity of the cells was studied by the reduction of stable free radical TEMPONE using electron spin resonance spectroscopy. CONP showed low or moderate cytotoxicity in cancer cell lines: adenocarcinoma DLD1 and multi-drug resistant DLD1-TxR, non-small cell lung carcinoma NCI-H460 and multi-drug resistant NCI-H460/R, while normal cell lines (keratinocytes HaCaT, lung fetal fibroblasts MRC-5) were insensitive. The most sensitive were 518A2 melanoma and HT-29 colorectal adenocarcinoma cell lines, with the IC50 values being between 100 and 200 μM. Decreased rate of TEMPONE reduction and increased production of certain ROS species (peroxynitrite and hydrogen peroxide anion) indicates that free radical metabolism, thus redox status was changed, and antioxidant capacity damaged in the CONP treated 518A2 and HT-29 cells. In conclusion, changes in intracellular redox status induced by CONP are partly attributed to the prooxidant activity of the nanoparticles. Further, ROS induced cell damages might eventually lead to the cell death. However, low inhibitory potential of CONP in the other human cell lines tested indicates that CONP may be safe for human usage in industry and medicine.



## 1. Introduction

Cerium oxide $CeO_2$ (CONP) crystal lattice, consists of a cerium core enveloped by an oxygen lattice. These particles have an interesting feature, coexistence of $Ce^{3+}$ and $Ce^{4+}$ ions and the ability of oxygen vacancies formation on their surface, which enables them to interact with and modulate free radicals. In such way these nanoparticles can pass many cycles of reactions with free radicals and thus regenerate themselves in every cycle [1,2]. This is a unique feature making CONP advantageous in comparison with the other nanoparticles. Besides, compared with other rare earth elements, CONPs have a high hydrogen-absorbing capacity, such that reactions with $H_2$, $O_2$, or $H_2O$ occur more readily, which may account for its regenerative capacity as a catalyst [3]. As such, they display antioxidant/antiradical activity [4,5] that encouraged studies of pharmacological potential of these nanoparticles, as well as their biomedical applications [6–8].

Some studies have shown protective effect of the CONP towards oxidant-mediated apoptosis [9,10]. However, in certain studies CONP induced oxidative stress either *in vitro* or *in vivo* [11,12]. It

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seems that environmental conditions, such as pH, direct opposite CONP activity regarding its oxidant or antioxidant properties [13]. In a neutral pH environment such as in normal cells, CONPs are cytoprotective and act as antioxidants. However, in an acidic pH environment characteristic for cancer cells CONPs may be cytotoxic and act as pro-oxidants.

Reactive oxygen species (ROS) accumulation in cells is generally associated with undesired effects. ROS generation, which also includes the generation of reactive nitrogen species (RNS) contributes to diabetes, neurodegenerative diseases, aging, and cancer. In cancer, ROS can trigger its development and progression through unique adaptation to oxidative stress and deregulation of antioxidant enzymes expression [14]. It is now widely accepted that ROS can interfere in intracellular processes, thus leading to the injuries. It has been demonstrated that several types of nanoparticles act as potent free radical scavengers and antioxidants. Thus nanoparticle antioxidants may counteract disorders/diseases which are related to oxygen levels and ROS production [15].

Rare studies of the CONP activity in cancer showed that CONP possess anti-invasive properties towards some cancer cells [16], induces radio-sensitisation [17], and simultaneously radio-protection of normal cells by regulating antioxidant enzymes and quantity of ROS [18,19].

Potential adverse health and ecological and environmental implications associated with the exposure of CONP are very limited, and the results vary among research groups, from absence of negative effects [11,20] to less or more deleterious effects on the organs and accumulation in some organs such are kidneys and lungs [21,22]. The final *in vivo* effects of CONP may depend on the way of their application and require further studies [6]. Because of wide potential for human usage in industry and medicine, we need more information about CONPs cytotoxicity in human cells.

In this study, we have evaluated the anti-cancer effects of CONP *in vitro* and its selectivity towards human cancer cells. To that end, the cytotoxic effect of CONP was investigated in different human normal and cancer cell lines. In order to see whether CONP induce changes in free radicals related antioxidant capacity of the cells, we studied total free radical production by the reduction of stable free radical TEMPONE in living cells using electron spin resonance (ESR) spectroscopy. Also, production of ROS/RNS and induction of cell death were analyzed by flow-cytometry. We chose pairs of the normal and cancer cell lines, to see if the effect of CONPs, regarding their interaction with free radical related antioxidant capacity of the cells differs in these cells.

## 2. Materials and methods

### 2.1. Drugs

The 10 mM CONP suspension was prepared in ethanol and dispersed for 20 min by using a sonicator (Bandelin Sonorex, Berlin, Germany). Before the experiment the suspension was diluted in 50% ethanol and vortexed. Cisplatin (CPt) was obtained from Pfizer (Perth) Pty. Ltd., Bentley, Australia. CPt was kept at room temperature. Before treatment, CONP and CPt were freshly diluted in sterile water.

### 2.2. Reagents

RPMI 1640 medium, Dulbecco's Modified Eagle's Medium (DMEM), penicillin–streptomycin solution, antibiotic–antimycotic solution, L-glutamine and trypsin/ethylenediaminetetraacetic acid (EDTA) were purchased from PAA, Vienna, Austria. 4-oxo-2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPONE), fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), sulforhodamine B (SRB) and MTT were obtained from Sigma–Aldrich Chemie GmbH, Germany. Annexin-V-FITC (AV)/Propidium Iodide (PI) kit was purchased from Abcam, Cambridge, UK. Dihydrorhodamine (DHR) was obtained from Molecular Probes®, Invitrogen, CA, USA. Nitrate of Ce was from Aldrich, USA, and NaOH from Zorka, Serbia.

### 2.3. Synthesis of the CONP

Nanometric size ceria powder particles with fluorite-type structure were obtained by applying self-propagating room temperature method (SPRT) method using as starting materials nitrate of Ce and NaOH, according to Matović et al. (2013) [23]. During SPRT synthesis hand mixing of nitrates with NaOH was performed in alumina mortar for ~10 min until the mixture got light brown. After being exposed to air for 4 h, the mixture was suspended in water. Rinsing out of $NaNO_3$ was performed in centrifuge – Centurion 1020D, at 3500 rpm for 10 min. This procedure was repeated three times with distilled water as well as with ethanol. Obtained powders are dried at 70 °C [23].

The compositions of reacting mixtures were calculated according to nominal composition of the final reaction product. Preparation of $CeO_2$ powder was performed according to reaction:

$$2[Ce(NO_3)_3 \cdot 6H_2O] + 6NaOH + (1/2 - \delta)O_2 \rightarrow 2CeO_{2-\delta} + 6NaNO_3 + 15H_2O \quad (1)$$

Obtained powder was characterized by X-ray diffraction and by Raman spectroscopy.

Crystal structure was identified by X-ray diffraction (XRD) using filtered Cu Kα radiation (Siemens D5000). XRD was also used to evaluate the crystallite size and lattice parameter. Before measurement the angular correction was done by high quality Si standard. Lattice parameters were refined from the data using the least square procedure. Standard deviation was about 1%.

The Raman spectra were obtained using a U-1000 (Jobin-Yvon) double monochromator in back scattering geometry. The Raman spectra were excited by the 514 nm line of an $Ar^+$ ion laser and taken at room temperature. In order to avoid sample heating we used a cylindrical focus and the laser power was kept lower than 10 mW.

The specific surface area was measured by nitrogen adsorption according to a Brunauer–Emmett–Teller (BET) method. The adsorption characteristics of synthesized and annealed samples were determined. Adsorption and desorption isotherms of $N_2$ were measured at −196 °C using the gravimetric McBain method. The specific surface area, $S_{BET}$, pore size distribution, mesopore including external surface area, $S_{meso}$, and micropore volume, $V_{mic}$, for the samples, were calculated from the isotherms. Pore size distribution was estimated by applying BJH method [24] to the desorption branch of isotherms and mesopore surface and micropore volume were estimated using the high resolution $\alpha_s$ plot method [24–26]. Micropore surface, $S_{mic}$, was calculated by subtracting $S_{meso}$ from $S_{BET}$. Also, the particle size, $D_{BET}$, was calculated from the specific surface area, $S$, using the following expression: $D_{BET} = 6/S \cdot \rho$, assuming isometric particles and using the bulk density, $\rho$, of $CeO_2$ (7.2 g/cm$^3$).

### 2.4. Cells and cell culture

Non-small cell lung carcinoma NCI-H460, human lung fetal fibroblast MRC-5 and colorectal adenocarcinoma cell lines DLD1 and HT-29 were purchased from American Type Culture Collection (Rockville, MD, USA). NCI-H460/R multi-drug resistant cancer cells were selected from NCI-H460 cells by continuous treatment with stepwise increasing concentrations of doxorubicin