Synthesis and biocompatibility study of ceria-mildronate nanocomposite in vitro

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ABSTRACT Nanoscale cerium dioxide (CeO$_2$, nanoceria) possesses notable redox activity, which is actively used in advanced biomedical applications. The low toxicity, high biocompatibility and antioxidant activity of nanoceria make it a new generation nanzyme with a unique activity. Combination of nanoceria with various biologically active substances results in organic-inorganic nanocomposites possessing enhanced activity. Here, we synthesized a novel organic-inorganic hybrid material (Mil-CeO$_2$) based on 2-(2-carboxylatoethyl)-1,1,1-trimethylhydrazinium and nanoceria, which has an ultra-small particle size, high antioxidant activity and pronounced biological activity. The analysis of cytotoxicity of the composite did not reveal any negative effects on the NCTC L929 mouse fibroblasts at concentrations below 10 mM. It was shown that the nanocomposite did not cause morphological changes in cells, or lead to cell death and mitochondrial membrane potential disruption, while maintaining viability in mouse fibroblasts in vitro. Additionally, we showed that Mil-CeO$_2$ is capable of protecting cells from hydrogen peroxide (H$_2$O$_2$)-induced or radiation-induced oxidative stress.

KEYWORDS cerium oxide nanoparticles, nanoceria, 2-(2-carboxylatoethyl)-1,1,1-trimethylhydrazinium, toxicity, nanocomposite

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1. Introduction

Cerium oxide nanoparticles (CNPs) are considered as the most versatile inorganic antioxidant [1–3]. CNPs redox activity is probably due to a high degree of oxygen nonstoichiometry, which is due to the presence of defects in the crystal lattice of CeO$_2$ nanoparticles [4–7]. Cerium oxide nanoparticles are capable of inactivating a wide range of reactive oxygen species (ROS) and free radicals [8–11], preventing the development of intracellular oxidative stress in vitro [12,13] and in vivo [14–16]. It is also well known that cerium oxide nanoparticles mimic the activity of endogenous enzymes such as superoxide dismutase (SOD) [17–19] and catalase [20]. Cerium oxide nanoparticles are considered as a promising anticancer [21–23], antibacterial [24–26] and antiviral [27–29] agent as well as a promising inorganic antioxidant for diverse biomedical applications [30–32].

We have previously shown that cerium dioxide nanoparticles demonstrate a synergistic effect with well-known anticancer drugs or another therapeutic substances, enhancing their effect [33–36]. In particular, it has been shown that the panthenol-cerium dioxide complex protects testicular epithelial cells (ST cells) from oxidative stress caused by hydrogen peroxide or UV radiation [37]. Similarly, conjugation with cerium dioxide affects the cytotoxicity and photocytotoxicity of curcumin, depending on the cell type, being more toxic for cancer cells. The nanoceria-curcumin conjugate demonstrated highly selective cytotoxicity under oxidative stress conditions induced by UVA/UVC irradiation or H$_2$O$_2$, causing a
dramatic inhibition of metabolic activity of cancer cells and protecting normal cells from these damaging factors [38]. The combination of recombinant TNF-alpha with cerium dioxide nanoparticles has been shown to provide a stronger and more stable cytotoxic effect in Hep-2 and A-549 tumor cell lines [39]. Thus, the design and study of hybrid cerium-containing nanocomposites could be a promising tool for creating advanced functional materials with a therapeutic activity exceeding that of the individual components (synergism).

There are a large number of promising drugs that could act as a synergistic agent for cerium dioxide nanoparticles. One possible candidate is meldonium, which is a small, highly hydrophilic molecule that has no affinity for plasma proteins. Upon administration, the maximum plasma concentration of meldonium is reached after about 1 hour, and its half-life depends on the dosage, treatment time and pharmaceutical form. Meldonium is metabolized mainly in the liver by gamma-butyrobetaine hydroxylase (BBOX) to form dimethylamine, 2-hydroxymethyl-2-(hydroxymethylamino) propane-1,3-diol, 3-amino-4-(hydroxymethylmethylamino). The latter metabolite can be converted to succinic acid, the main metabolite found in plasma. Mildronate is regarded as a mitochondria-targeted drug as it easily penetrates into mitochondria and enters the metabolic cycle [40, 41]. Mitochondrial damage can activate free radical processes and lead to the initiation of apoptosis, as well as disruption of mitochondrial oxidative phosphorylation, damage to the endoplasmic reticulum and changes in gene activity [42–44]. Mitochondria are the main site of ROS formation in the cell. There are several factors that determine ROS generation in mitochondria: the presence of different metabolic intermediates, hypo/hyperoxia, an increase in the concentration of NADH or membrane mitochondrial potential (MMP). Thus, the development and study of a new mitochondria-targeted biologically active nanocomposites is an urgent task. To summarize, the ceria-mildronate composite can be considered as a promising substance for inhibiting the oxidative stress and increasing the cell metabolic status.

2. Materials and methods

2.1. Synthesis and characterization of nanocomposite

The ceria-mildronate composite (CeO$_2$-Mil) was synthesized in two stages. Initially, cerium (III) trimeldonate solution was obtained by mixing 500 mg sodium mildronate (3.48 mM) with cerium chloride (CeCl$_3$·7H$_2$O) in a molar ratio of 3:1 with thorough stirring. Next, we synthesized nanoscale cerium dioxide composite with mildonium (CeO$_2$-Mil) by precipitation using NaOH (1 M). The composite was separated by decantation and then centrifuged at 10,000 rpm and washed three times with MQ water. CeO$_2$-Mil composite sol was sonicated for 1 hour before using. The hydrodynamic diameter and the $\zeta$-potential values were measured using a Zetasizer Nano ZS analyzer (Malvern Instruments Ltd., UK). High-resolution transmission electron microscopy (HR-TEM) analysis was performed using a Libra 200 MC microscope (Zeiss, Germany). TEM images were recorded by a CCD camera (Gatan, USA) with a matrix size of 4096×4096 pixels.

2.2. Cell culture

The analysis of cytotoxicity and bioactivity was carried out using NCTC clone L929 cell culture from the Institute of Cell Biophysics collection; NCTC clone L929 fibroblasts were obtained from the subcutaneous connective tissue of C3H/An mice. The cells were seeded in 96-well plates at a density of $2\times10^4$ per cm$^2$ in DMEM/F12 medium (1:1) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin solution. Cells were cultivated under 5% CO$_2$ at 37°C.

2.3. MTT assay

Cell viability was assessed using the MTT test. The cells were seeded in 96-well plates and cultivated for 24 hours. 6 h after cell seeding, the culture medium was replaced with a medium containing CeO$_2$-Mil nanocomposite at various concentrations (0.05-11 mM). Cells with the medium, but without CeO$_2$-Mil nanocomposite, were used as a control. 24 h after the introduction of the nanocomposite, the medium in wells was replaced with a medium containing 3,4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazole (5 mg/ml). The optical density of the formed formazan was determined at a wavelength of $\lambda=540$ nm using a BIO-RAD model 680 photometer.

2.4. Live/Dead assay

To assess the ratio of living/dead cells in culture, we used the L-7007 LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen) containing SYTO 9 (stains all cells, $\lambda=485/498$ nm) and propidium iodide (stains the nuclei of dead cells, $\lambda=535/617$ nm) dyes. Cell staining was performed by replacing the culture medium with a dye mixture (5 $\mu$M). Observation of morphology and fluorescent staining was carried out on an Axiosvert 200 inverted microscope (Carl Zeiss). Microphotography was performed using a Power Shot A620 digital camera (Canon). Stained cells were counted using the Image J software.
2.5. Mitochondrial membrane potential (MMP level) analysis

The MMP level was assessed using tetramethyl rhodamine, TMRE (Thermo Fisher Scientific, USA). TMRE is a positively charged red-orange dye that penetrates cells and accumulates readily in active mitochondria due to their negative membrane potential. Depolarized or inactive mitochondria have a reduced membrane potential and are unable to accumulate TMRE. The TMRE excitation peak is 488 nm, the emission peak is 575 nm. The preparation of the working solution with the dye was carried out in Hanks solution, upon the addition of the prepared solution to the cells, cultivation was carried out for 15 minutes. Before the experiment, using microscopic methods, the solution with the dye was replaced with the Hanks solution. The work was carried out under sterile conditions in a laminar flow hood. For the study, we used an Axiovert 200 Zeiss inverted fluorescence microscope. Then, the level of fluorescence intensity was calculated using ImageJ.

2.6. Fluorescent staining of cell nuclear with Hoechst 33342 dye

Cells were cultured on 96-well plates, as described above. After 24 hours of culturing with nanocomposite, the cells were washed with HBSS, prior to 20 min staining with Hoechst 33342 (5 µM). Images of stained cells were captured by a fluorescence microscope Zeiss Axiovert 200.

2.7. Oxidative stress model in vitro

The protective action of CeO$_2$-Mil nanocomposite was analyzed using two experimental models of oxidative stress: single dose X-ray irradiation (15 Gy) or hydrogen peroxide treatment (1 mM for 30 minutes). Cell viability analysis was performed using MTT test after 24 hours for hydrogen peroxide treatment and after 72 hours for X-ray irradiation, respectively.

2.8. Statistical analysis

Data are presented as standard deviation from the mean value. The significance of differences between experimental groups was assessed by the Mann-Whitney U-test.

3. Results and discussion

The synthesis of CeO$_2$-Mil nanocomposite was carried out according to the scheme shown in Fig. 1a. The hydrodynamic radius of the nanocomposite upon dilution in water was approximately 15–20 nm (Fig. 1b). The zeta potential of the particles when diluted in distilled water was $+23.3.2$ mV. According to TEM data, the particle size of the nanocomposite was 4–5 nm (Fig. 1c). The synthesized CeO$_2$-Mil nanocomposite demonstrated good colloidal stability and can be stored for at least 7 days without any signs of sedimentation.

It is well known that cerium oxide nanoparticles coated by biocompatible ligands are not toxic to mammalian cells in vitro and in vivo, even at high concentrations [45–47]. For example, citrate-stabilized cerium oxide nanoparticles do not cause toxic effects in the culture of mouse embryonic fibroblasts, while maintaining a high level of their migratory, proliferative and metabolic activity [48]. In turn, dextran-stabilized gadolinium-doped cerium oxide nanoparticles have demonstrated selective toxicity against cancer cells [49]. Nevertheless, each new synthesis scheme for CeO$_2$ nanoparticles preparation and stabilization requires a comprehensive analysis of cytotoxicity to analyze the prospects for biomedical use.

The cytotoxicity of the CeO$_2$-Mil nanocomposite was analyzed using mouse fibroblasts cell line NCTC L929 (Fig. 2). The CeO$_2$-Mil nanocomposite was shown to have no toxic effect and provided a high level of metabolic activity at the concentrations below 1 mM. It should be noted that cerium (III) chloride in high concentrations (above 1 mM) was toxic for mouse fibroblasts, while this was not the case for CeO$_2$-Mil nanocomposite. Additionally, an assessment of the toxicity of milderenate and cerium chloride at the same concentrations was carried out. Analysis of the ratio of live and dead cells using the differential staining method has shown that the CeO$_2$-Mil nanocomposite does not cause cell death at all studied concentrations (0.05–11 mM), retaining the native morphology and phenotypic features characteristic of fibroblasts (Fig. 3b).

Mitochondria are redox-sensitive organelles [50]. The mitochondrial disruption is accompanied by the changes in the membrane potential and alterations to the oxidation–reduction potential of the mitochondria. Previously, it was shown that nanoceria could depolarize mitochondrial membrane of human colon cancer cells [51]. In the current study, it was revealed that preliminary incubation of NCTC L929 with CeO$_2$-Mil nanocomposite did not lead to any changes in the MMP in any of the concentrations thus confirming the high biocompatibility of the nanocomposite (Fig. 3c).

The results of Ce-Mil nanocomposite protective effects study are presented in Fig. 4. The radioprotective action of Ce-Mil nanocomposite was analyzed after X-ray exposure. Earlier, we have demonstrated radioprotective action of cerium oxide nanoparticles using mouse fibroblasts upon X-ray irradiation [52]. The optimal dose for NCTC L929 cell culture irradiation was selected at 15 Gy. The dose of radiation and cultivation time after irradiation were chosen on the basis of preliminary experiments. Live/Dead and MTT assays were performed 72 hours after X-ray irradiation. It was shown that the CeO$_2$-Mil nanocomposite after X-ray radiation exposure (15 Gy) showed protective action only at the highest concentration (0.5 mM), which ensured the preservation of cell viability by about 25% compared to the irradiated control.
FIG. 1. Synthesis scheme and the structure of the CeO$_2$-Mil nanocomposite (a), dynamic light scattering in MQ water (b) and transmission electron microscopy (c).

FIG. 2. Cytotoxicity analysis by the MTT test of CeO$_2$-Mil nanocomposite using NCTC L929 cell line (24 hours after incubation)
Fig. 3. Microphotographs of NCTC L929 mouse fibroblasts 24 hours after incubation with CeO$_2$-Mil nanocomposite (0.05–11 mM) dyed with SYTO9/PI (top line), Hoechst 33342 (middle line) and TMRE (bottom line) (a). Quantitative analysis of live/dead assay (b) and MMP level (c).

Fig. 4. Protective effect of CeO$_2$-Mil nanocomposite (0.05–0.5 mM) on NCTC L929 cell line under oxidative stress conditions induced by H$_2$O$_2$ treatment (left) and X-ray irradiation (right) as assessed using MTT assay. The cells were pretreated with a CeO$_2$-Mil nanocomposite in different concentrations (0.05–0.5 mM) and then exposed to X-ray irradiation (15 Gy) or treated with hydrogen peroxide (1 mM for 30 min). Data are presented at mean ±SD, * p≤0.05%, ** p≤0.001%
Lower concentrations of CeO$_2$-Mil nanocomposite (0.125–0.05 mM) did not provide a radioprotective effect. Meanwhile, the protective effect of CeO$_2$-Mil nanocomposite upon H$_2$O$_2$ treatment was observed in all the studied nanocomposite concentrations (0.05–0.5 mM) maintaining a high level of NCTC L929 cell viability close to the control values.

The protective action of the CeO$_2$-Mil nanocomposite under H$_2$O$_2$ induced oxidative stress can be explained by the pronounced catalase-like activity of the cerium oxide nanoparticles. We assume that the CeO$_2$-Mil nanocomposite, after being introduced into the cell culture, is localized not only in the cytoplasm of the cell, but is also partially adsorbed on the outer surface of cell membranes, which makes it possible to effectively decompose hydrogen peroxide in the selected concentration range (0.05–0.5 mM). At the same time, upon X-ray exposure, oxidative stress and the corresponding damaging factors develop through the water radiolysis inside and outside the cell. When exposed to X-rays, hydroxyl and superoxide radicals are first formed, which can dismutate to hydrogen peroxide. At the same time, it is well known that under acidic conditions, cerium oxide nanoparticles lose their catalase-mimetic activity while maintaining their SOD-mimetic ability, which leads to the accumulation of H$_2$O$_2$, being more toxic than superoxides, resulting in the selective radiation-induced cytotoxicity in relation to transformed cells [53]. Cerium oxide nanoparticles possess pronounced antioxidant properties and thus they can inactivate almost all types of ROS and free radicals formed as a result of radiation-induced water radiolysis, including the superoxide anion [54, 55] and hydroxyl radicals [56, 57]. Radiation-induced damage also develops in mitochondria, which is expressed in damage to mitochondrial DNA, uncoupling of the respiratory chain due to the development of oxidative stress, and a drop in the mitochondrial membrane potential [58–60]. Thus, the use of mitochondria-targeted radioprotectors based on cerium oxide can be considered as a promising strategy for protecting healthy surrounding tissue during radiation therapy.

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