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CeO_2-calcein nanoconjugate protective action against H_2O_2-induced oxidative stress *in vitro*

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ABSTRACT We studied cerium oxide-calcein nanoconjugate, which is capable of providing intracellular detection and simultaneous inactivation of reactive oxygen species (ROS). The synthesized nanoconjugate is easily uptaken by human mesenchymal stem cells (MSCs) and demonstrates antioxidant properties, protecting cells from H_2O_2-induced oxidative stress *in vitro*. Cerium oxide-calcein nanoconjugate neutralizes hydrogen peroxide, meanwhile releasing brightly fluorescent calcein from its surface, which is easily detected by fluorimeter or fluorescent microscope. This nanoconjugate is biocompatible and non-toxic to MSCs in concentrations below 2 mM. Such a theranostic agent can be considered as a promising tool for tracking the redox status of human MSCs *in vivo.*

KEYWORDS cerium oxide, calcein, bioimaging, cell uptake, oxidative stress, theranostics

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1. Introduction

Nanomedicine is a modern branch of medicine that uses a nanotechnological approach in creating new formulations of medical and diagnostic agents. Such agents are able to detect and to treat more effectively various neoplasms or pathological processes in the body. Cerium oxide (CeO_2) nanoparticles are among the most promising biomedical agents of modern nanotechnology [1–3]. CeO_2 nanoparticles are metal oxide-based nanoparticles that have an outstanding ability of reactive oxygen species (ROS) scavenging [4]. It was found that CeO_2 nanoparticles are able to effectively inhibit the development of a number of diseases caused by oxidative stress, including diabetes, cancer, atherosclerosis, etc. [5–7]. It has been shown that CeO_2 nanoparticles are able to mimic enzyme activity such as catalase [8], SOD [9], oxidase [10], peroxidase [11] phosphatase [12], which distinguish them from the other metal oxide nanoparticles. The biological activity of CeO_2 nanoparticles depends strongly on the scheme of their synthesis, the type of stabilizer and the microenvironment in which they perform their biological function. Dar et al. demonstrated size-dependent antibacterial activity of CeO_2 nanoparticles against the gram-negative strain of Escherichia coli (*E. coli*) HB101 K-12 [13]. It was found that the inhibition of bacterial growth depends on the average size and the concentration of cerium oxide nanoparticles. It has been shown that dextran-stabilized CeO_2 nanoparticles exhibit selective anticancer activity against MG-63 human osteosarcoma cells [14]. It was also demonstrated that CeO_2 nanoparticles are much more cytotoxic to human osteosarcoma cells in a slightly acidic environment (pH = 6) compared to physiological and basic pH values (pH = 7 and pH = 9, respectively). On the contrary, minimal toxicity was observed to non-transformed cells when cultured with CeO_2 nanoparticles at pH = 6 in the concentration range of 10 – 1000 μg/mL. Using various methods and approaches for the synthesis of bioactive CeO_2 nanoparticles, it is possible to control the mode of their activity. In addition, the catalytic activity of CeO_2 nanoparticles seems to depend on the chemical composition of their surface, which can be regulated using various ligands. For example, it has been shown that doping of the cerium oxide crystal lattice with gadolinium provides an increase in their catalytic activity and their suitability for MRI imaging [15]. Various functional agents are used to enhance the therapeutic effect of CeO_2 nanoparticles. Indocyanine green conjugated CeO_2 nanoparticles were used as anti-inflammatory agent in collagen-induced arthritis (CIA) mouse model [16]. It was found that functionalized

CeO_2 nanoparticles could be delivered systemically with accumulation in synovial tissues of joints through the SPARC-mediated mechanism, and they effectively inhibit inflammation *via* reducing hypoxia, scavenging excessive ROS, and restoring the disbalance of M1/M2 macrophages.

Thus, the design of new functional cerium oxide-based nanomaterials with specific therapeutic or diagnostic activity is an urgent task. Here we propose a new cerium oxide-based nanoconjugate with calcein that is capable of simultaneously inactivating and detecting ROS in the cell *in vitro*.

2. Materials and methods

2.1. Synthesis and characterization of CeO_2-calcein nanoconjugate

CeO_2-calcein nanoconjugate was synthesized by the method described earlier [17]. The hydrodynamic diameter and the ζ-potential values of CeO_2-calcein nanoconjugate were measured using a Zetasizer Nano ZS analyzer (Malvern Instruments Ltd., UK). Transmission electron microscopy (TEM) analysis was performed using a Libra 200 MC microscope (Zeiss, Germany).

2.2. Cell culture

The experiments were performed using a culture of primary human mesenchymal stem cells derived from the third molar bud, extracted in a healthy 16-year-old patient by orthodontic indications. Cells were cultured in DMEM/F12 (1:1) medium containing 10 % fetal calf serum and 200 units of penicillin/streptomycin at 37 °C in an atmosphere with 5 % CO_2. Cells were removed from culture flasks using 0.25 % trypsin-EDTA solution after washing them three times with Hanks' solution and then were seeded in 96-well plates at a density of $2.5 \cdot 10^4/\text{cm}^2$. The cytotoxicity of the CeO_2-calcein nanoconjugate was analyzed by replacing the initial cell culture medium with a medium, containing different concentrations of the conjugate.

2.3. MTT assay

Analysis of the cell viability after incubation with CeO_2-calcein nanoconjugate was performed after 24, 48 and 72 hours of incubation using the MTT assay. Cells were seeded in 96 well plates at a density of $2.5 \cdot 10^4/\text{cm}^2$ in a DMEM/F12 culture medium containing 10 % fetal calf serum. After 8 hours, CeO_2-calcein nanoconjugate (10 μM – 10 mM) were added by changing the culture medium. Then, after 24, 48 and 72 hours, the medium was replaced with a solution of the MTT reagent (0.5 mg/mL) and further analysis was carried out according to the standard method [18].

2.4. Fluorescent microscopy

Intracellular visualization of CeO_2-calcein nanoconjugate was performed using an inverted fluorescence microscope Zeiss Axiovert 200. Cells were seeded in 35 mm Petri dishes with a central hole (Ibidi, Germany) at a density of $2.5 \cdot 10^4/\text{cm}^2$ in a DMEM/F12 culture medium containing 10 % fetal calf serum. Afterwards, CeO_2-calcein nanoconjugate were added to the cells at a concentration of 1 – 10 mM. After 24 hours, microphotography of cell cultures was carried out after washing three times with a Hanks solution. The viability of MSCs after incubation with nanoconjugate was analyzed by Live/Dead assay using an inverted fluorescence microscope Zeiss Axiovert 200. LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen) was used containing SYTO 9 (stains all cells, $\lambda = 485/498$ nm) and propidium iodide (stains the nuclei of dead cells, $\lambda = 535/617$ nm) dyes. Microphotography was performed using a Power Shot A620 digital camera (Canon).

2.5. Oxidative stress model

The biological action of CeO_2-calcein nanoconjugate was analyzed using an experimental model of oxidative stress: treatment by 1 mM hydrogen peroxide for 30 minutes. Cell viability analysis was performed using MTT assay after 24 hours.

2.6. ROS and MMP monitoring *in vitro*

Detection of the free calcein fluorescence in the cells was carried out at excitation/emission wavelengths 501/521 nm using a Synergy H1 Hybrid multi-mode microplate reader (Biotek, USA) for 3 hours after 1 mM hydrogen peroxide addition. Membrane mitochondrial potential (MMP) was monitored using the fluorescent dye tetramethylrhodamine ethyl ester perchlorate (TMRE) at excitation/emission wavelengths 553/557 nm.

2.7. Statistical analysis

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

3. Results

Zeta potential of the nanoparticles diluted in distilled water was -26.7 ± 6.6 mV. According to the TEM data, nanoconjugate particle size was about 4 – 5 nm (Fig. 1a). The hydrodynamic diameter of the nanoconjugate particles dispersed in water was about 7 – 10 nm (Fig. 1b). The synthesized CeO_2-calcein nanoconjugate demonstrating good colloidal stability and can be stored for at least 1 year without sedimentation.

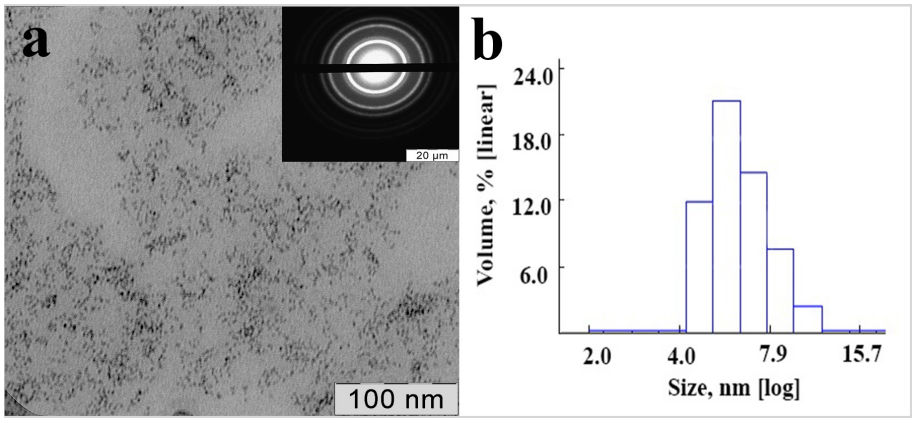

FIG. 1. Transmission electron microscopy of CeO_2-calcein nanoconjugate (a) and dynamic light scattering in MQ water (b)

At the first stage, biocompatibility of the CeO_2-calcein nanoconjugate was analyzed using the MTT assay. This assay determines the enzymatic activity of intracellular mitochondrial NADPH-dependent oxidoreductases, and reflects cells viability after their interaction with the test substance. The results of the MTT assay on human MSCs incubated with various concentrations of CeO_2-calcein nanoconjugate for 24, 48 and 72 hours are shown in Fig. 2. CeO_2-calcein nanoconjugate at a concentration of 200 μM and 1 mM ($p = 0.004$) significantly reduces the level of metabolic activity after 24 hours of incubation. There is also a significant decrease in cell viability in comparison with the untreated control (without the addition of a nanoconjugate) after 48 and 72 hours of incubation at concentrations of 1 – 10 mM ($p = 0.016$). A promoting effect of the CeO_2-calcein nanoconjugate is observed after 72 hours of incubation with 20 μM of nanoconjugate ($p = 0.016$). Such stimulating activity may be associated with the promoting effect of CeO_2 nanoparticles on the proliferative and migratory activity of human MSCs [19, 20]. Thus, according to the MTT assay, a high degree of biocompatibility of CeO_2-calcein nanoconjugate is observed in sufficiently low concentrations (below 1 mM) to human MSCs.

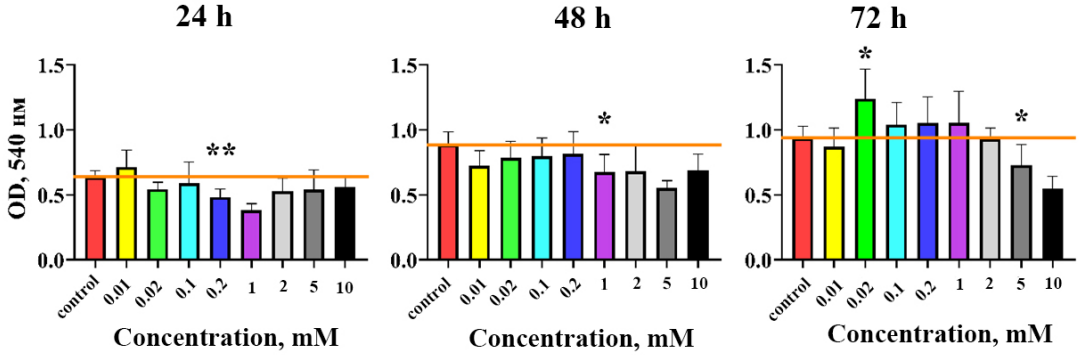

FIG. 2. MTT assay data for human MSCs after 24, 48 and 72 hours of incubation with CeO_2-calcein nanoconjugate in various concentrations (0.01 – 10 mM)

The effect of the CeO_2-calcein nanoconjugate on the morphofunctional state of human MSCs and the viability was analyzed by fluorescence microscopy. Fig. 3 shows micrographs of human MSCs stained with SYTO9/propidium iodide fluorescent dyes after their incubation with nanoconjugate in various concentrations (1 – 10 mM) for 24, 48 and 72 hours. 100 % absence of dead cells is confirmed in all the cases. This may simultaneously indicate both the absence of a pronounced toxic effect of the nanocomposite on mesenchymal stem cells, and the possible removal of dead cells at the

sample preparation stage. Also, excessive fluorescence is observed for nanoconjugate concentrations of 5 and 10 mM, that may be due to the luminescence of calcein, which has not entered the cells but has localized in the culture medium and on the cell surface [21].

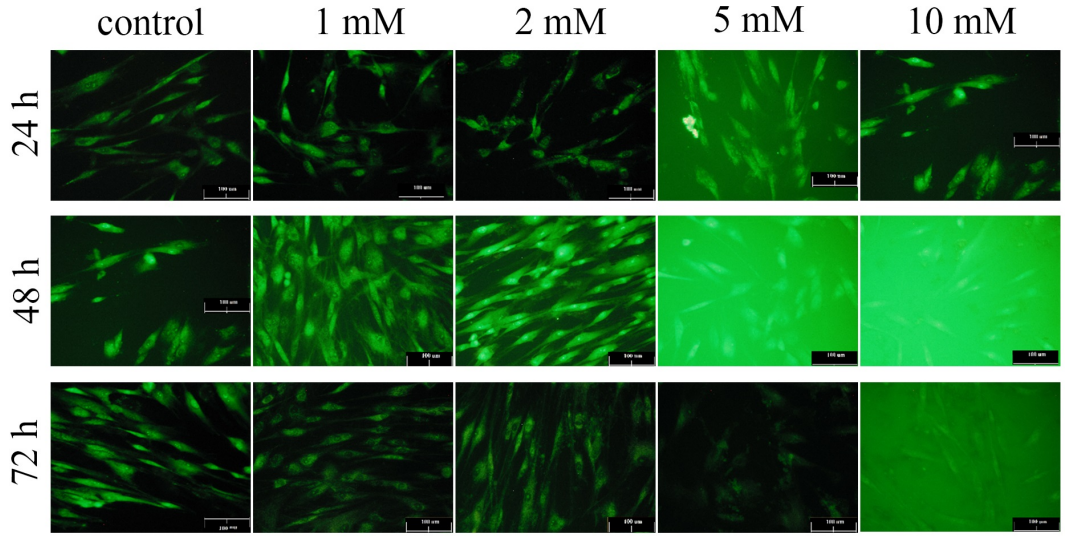

FIG. 3. Micrographs of human MSCs stained with SYTO9/propidium iodide fluorescent dyes after their incubation for 24, 48 and 72 hours with CeO_2-calcein nanoconjugate in concentrations from 1 to 10 mM. All cells are coloured green, and only dead cells are coloured both green and red. The scale is 100 μm

The micrographs of human MSCs incubated with hydrogen peroxide at concentration of 0.125 – 1 mM are presented on the top panel of Fig. 4. The microphotographs show that the morphology of cells changes significantly under the action of H_2O_2 at concentrations of 500 μM and 1 mM. Human MSCs lose focal contacts and reduce the degree of spreading after incubation with hydrogen peroxide. Moreover, in both cases, there is a statistically significant decrease in the metabolic activity of cells (Fig. 4, bottom panel). This indicates the cytotoxicity of hydrogen peroxide in both concentrations [22]. Thus, we selected a hydrogen peroxide concentration of 1 mM for further modeling of oxidative stress *in vitro*.

The antioxidant activity of the CeO_2-calcein nanoconjugate was analyzed in the model of H_2O_2-induced oxidative stress (Fig. 5). The dynamics of the calcein fluorescence intensity change (Fig. 5a) shows that the nanoconjugate at concentrations of 1 mM, 200 μM and 100 μM neutralizes readily exogenous ROS, that is confirmed by an increase in the fluorescence intensity. Therefore, CeO_2-calcein nanoconjugate can be used to detect the intracellular level of ROS and also to inactivate them locally. Next, the level of the membrane mitochondrial potential (MMP) of human MSCs after incubation with nanoconjugate was analyzed. MMP is an indirect indicator of intracellular oxidative stress [23]. An increase in the MMP compared to the control value was revealed in the cells which were pre-incubated with nanoconjugate (Fig. 5b). This confirms the antioxidant activity of the CeO_2-calcein nanoconjugate, which provides a high level of MMP, while maintaining the antioxidant status of human MSCs.

4. Conclusions

Here, we synthesized, characterized and investigated the CeO_2-calcein nanoconjugate, which can be considered as a promising theranostic agent for *in situ* monitoring of oxidative stress in the cells. It has been shown that the CeO_2-calcein nanoconjugate is biocompatible even in high concentrations (up to 1 mM). The CeO_2-calcein nanoconjugate does not cause any changes in morphofunctional characteristics of human MSCs. Also, CeO_2-calcein nanoconjugate is able to detect the level of ROS in cells in vitro at concentration of 0.1 – 1 mM. It has been shown that the nanoconjugate is capable of efficient inactivation of hydrogen peroxide, maintaining a high level of viability and mitochondrial potential of human MSCs under oxidative stress conditions. Thus, cerium-containing nanocomposites can be considered as a basis for the creation of new multifunctional theranostic agents.

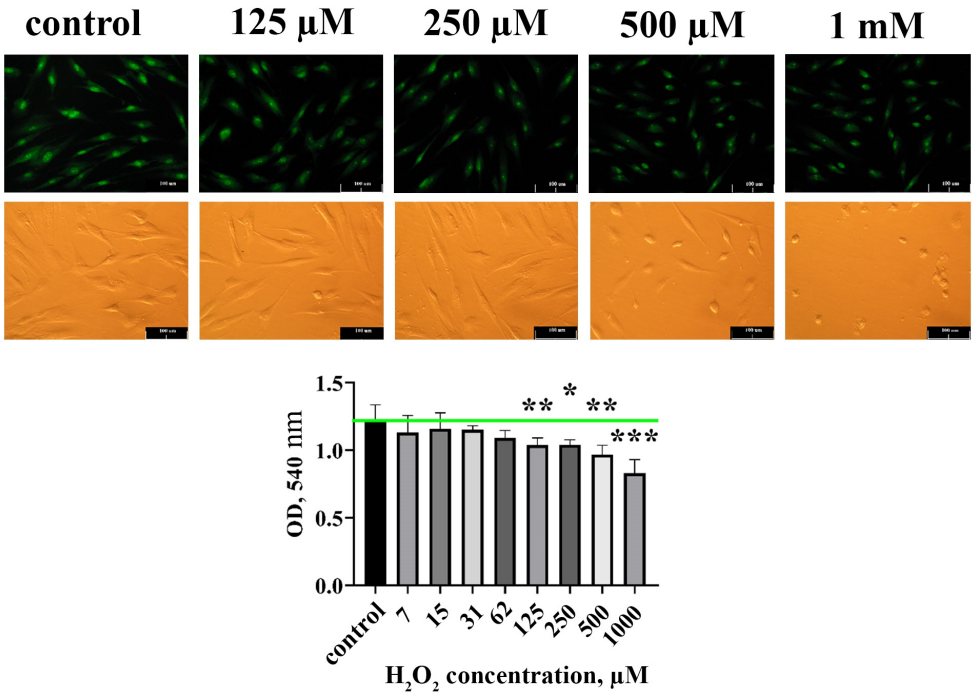

FIG. 4. Validation of the model of oxidative stress induced by the injection of exogenous hydrogen peroxide to human MSCs. Top panel: micrographs recorded in phase contrast and fluorescence modes. Bottom panel: a graph of the optical density of formazane according to the MTT assay 24 hours after the treatment of the cells with hydrogen peroxide

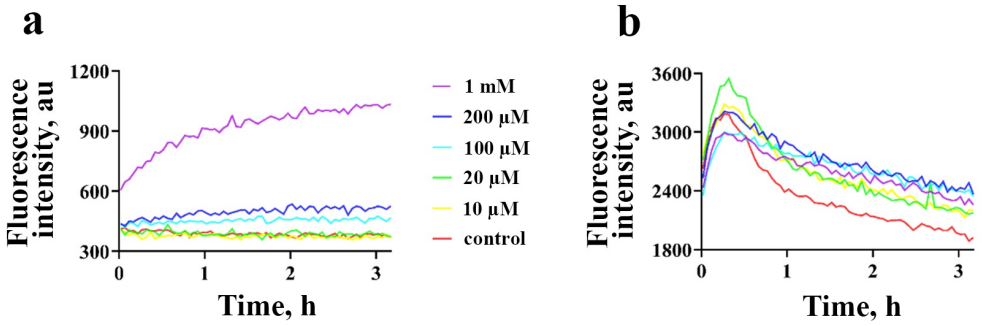

FIG. 5. Dynamics of fluorescence intensity change of CeO_2-calcein nanoconjugate in human MSCs after 24 hours of their incubation and further H_2O_2-induced oxidative stress for 3 hours. The excitation wavelength is 485 nm, the emission wavelength is 528 nm (a). Dynamics of MMP change (fluorescence of TMRE dye) in human MSCs after 24 hours of their incubation and further H_2O_2-induced oxidative stress for 3 hours. The excitation wavelength is 552 nm, the emission wavelength is 580 nm (b)

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Conflict of interest: the authors declare no conflict of interest.