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Cerium oxide nanoparticles stimulate proliferation of primary mouse embryonic fibroblasts *in vitro*

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ABSTRACT

The increasing application of cell therapy technologies in the treatment of various diseases requires the development of new effective methods for culturing primary cells. The major limitation for the efficient use of autologous cell material is the low rate of cell proliferation. Successful cell therapy requires sufficient amounts of cell material over a short period of time with the preservation of their differentiation and proliferative potential. In this regard, the development of novel, highly efficient stimulators of proliferative activity in stem cells is a truly urgent task. In this paper we have demonstrated that citrate-stabilized cerium oxide nanoparticles (nanoceria) enhance the proliferative activity of primary mouse embryonic fibroblasts *in vitro*. Cerium oxide nanoparticles stimulate cell proliferation in a wide range of concentrations (10^{-3} M–10^{-9} M) through reduction of intracellular levels of reactive oxygen species (ROS) during the lag phase of cell growth and by modulating the expression level of the major antioxidant enzymes. We found the optimal concentration of nanoceria, which provides the greatest acceleration of cell proliferation *in vitro*, while maintaining the levels of intracellular ROS and mRNA of antioxidant enzymes in the physiological range. Our results confirm that nanocrystalline ceria can be considered as a basis for effective and inexpensive supplements in cell culturing.

1. Introduction

Nanocrystalline cerium oxide has been widely used for a long time in various fields of industry and technology. It is used in solar batteries [1], as a catalyst [2], a fuel additive [3], *etc.* Quite recently the unique bioactivity of nanoceria was discovered. It was demonstrated that cerium oxide nanoparticles have a strong antioxidant effect [4], protect cells from UV [5] and gamma radiation [6] and stimulate proliferation of stem cells in a culture [7]. The ability of nanoceria to stimulate stem cell proliferation in a culture with a preservation of their differentiation potential and native immunophenotype allows considering this material as a promising cultural supplement. However, the mechanisms underlying such highly effective stimulation of proliferation remain poorly investigated. Some authors have speculated that this effect is conditioned by a certain prooxidant activity [8,9], since ROS in nanomolar concentrations can stimulate cell proliferation through activation of redox-sensitive transcription factors. Other authors [10,11] consider its antioxidant activity as the main property, which stimulates the proliferative activity of cells, implying the fact that high level of intracellular ROS can inhibit the proliferation rate during the lag-phase of cell growth [12]. On the other hand, it has been reported [13] that cerium oxide nanoparticles are able to adsorb Ca^{2+}-ions from culture medium and, upon entering the cell cytoplasm, activate calcium-dependent proteins (for example, calpain), thereby affecting intracellular signaling pathways and the overall cell metabolism.

In this regard, elucidation of mechanisms underlying the effect of CeO_2 nanoparticles on the proliferative activity of cultured cells, including embryonic cells, is a relevant task. In the present study we analyzed the influence of nanoceria in a wide range of concentrations (10^{-3} M–10^{-9} M) on the proliferative activity of primary mouse embryonic fibroblasts. It was shown that over the whole range of the analyzed concentrations cerium oxide nanoparticles accelerate the proliferation of embryonic stem cells *in vitro*. Intracellular ROS level was determined, as well as the expression levels of key antioxidant enzymes (SOD1, SOD2, catalase, glutathione peroxidase, glutathione reductase) 24 h after the addition of CeO_2 nanoparticles to the cells. The obtained results showed that nanoceria possess antioxidant properties and are able to modulate the expression of antioxidant enzymes and to reduce the level of intracellular ROS. Our data confirm the possibility of using CeO_2 nanoparticles in cultural supplements, providing effective proliferation of cells in a culture.

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2. Materials and methods

2.1. Preparation and analysis of physicochemical properties of nanoparticles

The aqueous sol of nanocrystalline cerium oxide stabilized by citrate ions was used in the present work. It was obtained by dissolving 0.24 g of citric acid in 25 ml of 0.05 M aqueous solution of cerium (III) nitrate, which was then quickly added with stirring to 100 ml of a 3 M ammonia solution and then kept for 2 h [14]. Transmission electron microscopy testified that the sol consisted of weakly aggregated nearly isotropic CeO_2 particles of 2–3 nm. The concentration of CeO_2 nanoparticles in the sol was 0.01 M. The concentration of the re-suspended ceria sol was checked by standard gravimetric analysis [60]. The pH value of the sol was in the range of 7.2–7.4. Just before biological experiments, the CeO_2 nanoparticles were precipitated by bringing the pH value to 3 *via* the addition of 10% hydrochloric acid, followed by centrifugation at 20 °C, 11,200 g for 10 min. The precipitated nanoparticles were resuspended in distilled water and re-centrifuged under the same conditions. The resulting precipitate was resuspended in culture medium DMEM/F12 + 10% fetal bovine serum (Gibco, USA).

2.2. Cell culture

The culture of primary mouse embryonic fibroblasts was obtained from embryos of SHK white mice on day 13 of pregnancy according to the protocol of [15]. The cells were cultured in DMEM/F12 (1:1) medium, with the addition of 10% fetal calf serum and 100 U/ml penicillin/streptomycin, under 5% CO_2 at 37 °C. All experiments were conducted using cultures taken from passages 1–2.

2.3. MTT assay

Determination of activity of mitochondrial and cytoplasmic dehydrogenases in living cells was performed using the MTT assay, which is based on the reduction of a colorless tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, MTT) [16]. After 1, 3 and 5 days of culturing standard MTT assay was performed.

2.4. Analysis of proliferative activity

Mouse primary fibroblasts were seeded in 96-well plates (Corning Costar, USA) at a density of 10 thousand/cm^2 and cultured for 12 h under 5% CO_2 37 °C, and then various concentrations of nanoceria (10^{-3} M — 10^{-9} M) were introduced by replacing the medium. Each day in the course of 5 days the proliferative activity was analyzed and the cell count was estimated in each well, using a plate reader CloneSelect Imager System Plate Reader (Molecular Devices, USA). Cell micrographs were made using SYTO 9 green fluorescent dye (Thermo Fisher, USA).

2.5. Analysis of ROS level

The level of intracellular ROS *in vitro* was determined using 2′,7′-dichlorofluorescein diacetate, a dye that diffuses through the lipid membrane into the cell and subsequently gets oxidized by intracellular ROS, with the formation of highly fluorescent dichlorofluorescein. Cells were preincubated with cerium oxide nanoparticles in various concentrations for 24 h in a 96-well plate, and after that the culture medium was replaced by Hanks' solution containing 2,7-DCFH-DA (100 μM). The fluorescence was measured using a plate reader Tecan 200 PRO (Tecan Corp., Switzerland) (485 nm/520 nm).

2.6. Confocal microscopy

The cells were seeded on the surface of glass slides at a density of 10 thousand/cm^2. In 24 h after cell seeding 10^{-5} M of cerium oxide nanoparticles was added to the culture medium. Following incubation (24 h) the medium in the plates was replaced with PBS containing DNA-tropic fluorescent dye Hoechst-33,342 (excitation at 345 nm, emission at 487 nm). The morphology analysis of the cells was carried out using an upright confocal microscope LSM-510 with multiphoton excitation of fluorescence and an image analysis system (Germany).

2.7. Transmission electron microscopy

To prepare the samples for transmission electron microscopy analysis, the samples were washed with PBS and fixed for 2 h in 1% solution of osmium tetroxide. Following dehydration of samples in an ascending series of alcohol and acetone, they were embedded in Embed 812 (EMS, USA). Ultrathin sections were prepared using an LKB Ultratome 3 and contrasted by uranyl acetate and lead citrate. The samples were studied using an electron microscope Tesla BS-500 at 10,000-fold magnification.

2.8. Real-time PCR

Total RNA from the sample cells was isolated using an RNA extraction kit (SINTOL, Russia). Reverse transcription reaction was carried out using a kit for RT (SINTOL, Russia). Real-time PCR amplification was realized using a thermocycler ANK-32 (SINTOL, Russia). Primers used for estimation of transcription level for the analyzed gene and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (reference gene) were supplied by SINTOL. A kit containing SYBR Green I intercalating dye was used for real-time PCR. The parameters of the PCR reaction were as follows: 95 °C — 5 min, followed by 40 cycles of: 95 °C — 30 s, 60 °C — 50 s. Then the threshold cycle values obtained in the result of PCR were determined. The sequences of primers used in real-time PCR:

Gpx-1 F: CCACCGTGTATGCCTTCT, R: GAGACGCGACATTCTCAATGA;
Gsr F:AAAGAAGACCCCATCGGGCTCGGA, R:AGAGNAGGCAATCGACATCCGGAA;
Catalase F: GCAGATACCTGTGAACTGTC R: GTAGAATGTCCGCACCTGAG
CuZnSOD F: GTACCAGTGCAGGACCTCATTTT, R: GTCTCCAACATGCCTCTCTTCAT;
MnSOD F:CCACACATTAACGCGCAGAT, R: GGTGGCGTTGAGATTGTTCA;
GAPDH F: ATGTGTCCGTCGTGGATCTG R:CCTGCTTCACCACCTTCTTG

2.9. Flow cytometry

Cells were seeded in Petri dishes (Ibidi, USA) at the density of 10 thousand/cm^2, and after 12 h of incubation the culture medium was replaced with a medium containing ceria nanoparticles in various concentrations (10^{-3} M–10^{-9} M). After 24-hour culturing the cells were removed from the dishes with a mixture of trypsin and versene solutions (1:1), then precipitated by centrifugation for 3 min at 350 g and washed with phosphate buffer saline (PBS). Then the cells were fixed in 100% ethanol on ice. The fixed cells were precipitated by centrifugation, washed with PBS and treated with RNase A solution (20 mg/ml) in the presence of propidium iodide (50 mg/ml) for 15 min at 37 °C. The analysis of cell distribution by the phases of the cell cycle was performed on a flow cytometer Partec III (Germany), at analysis speed of 1000 cells per 1 s. Numerical analysis of histograms (determination of cell fractions corresponding by the amount of DNA to cycle phases G_0/G_1,S and G_2 + M) was performed using Cyflogic software (version 1.2.1). In each case at least 100 thousand cells was analyzed.