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Article

Nanoceria Inhibit the Cell Migration from 3D Tumor Spheroid Formed From 4T1 Human Breast Cancer Cells

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Abstract: The use of various nanoformulations for cancer radiotherapy has great potential due to their increased effectiveness and safety. At the stage of preclinical evaluation of such nanoformulations *in vitro*, they are tested on monolayers of tumor cell cultures, which is an unrealistic representation of tumors *in vivo*, which leads to an inaccurate selection of suitable drugs. To solve this problem, 3D cellular spheroids are becoming an additional tool for confirming the effectiveness of new therapeutic nanoformulations. Here we show the possibility of using citrate-stabilized cerium oxide nanoparticles (nanoceria, CeO₂ NPs) as a nanoradiosensitizer in an experimental 3D model of tumor spheroids formed from 4T1 human breast cancer cells. It has been shown that ultra-small size (about 1-2 nm) CeO₂ NPs have pH-sensitive radiocatalytic activity, increasing the generation of ROS under irradiation conditions. It has been shown that preloading of CeO₂ NPs into a cellular spheroid at a micromolar concentrations does not adversely affect the rate of spheroid formation and its final size. However, such preloading has a pronounced inhibitory effect on the rate of cell migration from the spheroid after X-ray irradiation and also reduces their clonogenic activity. Thus, CeO₂ NPs can be considered as a promising nanoradiosensitizer for radiotherapy of tumors.

Keywords: nanoparticles; 3D tumor spheroid; cerium oxide; irradiation; redox activity

1. Introduction

Functional nanomaterials, due to their physico-chemical properties and unique biological activity, are widely used in various biomedical fields, opening up new therapeutic and diagnostic possibilities for cancer therapy [1]. There is a wide range of metal-containing nanoformulations that have already been introduced into clinical practice, providing both visualization and selective therapy of tumors [2]. For example, hafnium oxide (HfO₂) nanoparticles are used as a radiosensitizer in the framework of radiation therapy of tumors [3], and iron oxide nanoparticles [4] are used to effectively contrast tumor nodes in the brain in combination with their therapy by remote heating of these nanoparticles in a magnetic field. Gadolinium-containing nanoformulations (AGuIX®) are used for imaging and subsequent radiotherapy of brain tumors [5]. These nanoparticles are a 5 nanometer macromolecular polysiloxane complexes containing 15 gadolinium atoms, which provides unique relaxation characteristics (8.9 mM⁻¹s⁻¹ per Gd³⁺ ion). AGuIX® are at the 3rd stage of clinical trials and show high efficacy in combination therapy with glioblastoma and gliosarcoma [6]. Thus, we can confidently say that biomedical nanotechnology is being actively introduced into clinical practice in the field of tumor radiotherapy.

Inorganic nanozymes belong to a new promising class of functional nanomaterials for biomedical purposes [7]. Despite the fact that natural enzymes have unprecedented advantages in

neutralization of reactive oxygen species (ROS), such as high efficiency, specificity and selectivity, at the same time they also have low stability and inability to recover their activity, which, in turn, limits their use. Inorganic nanozymes are considered as the most promising substances for the effective correction of critical pathological conditions associated with oxidative stress [8]. The class of inorganic nanozymes includes nanomaterials based on manganese [9], vanadium [10], gold [11], platinum [12], iron [13], cerium [14] etc. Such compounds are able to mimic the activity of a wide range of endogenous enzymes, such as superoxide dismutase, catalase, peroxidase, phosphatase, etc. [15]. The most prominent representative of the class of inorganic nanozymes is nanocrystalline cerium oxide [14,16,17]. Ce^{3+} and Ce^{4+} ions coexist on the surface of the crystal lattice of cerium oxide nanoparticles, forming oxygen vacancies for the implementation of redox reactions [18]. CeO_2 NPs are able to mimic the activity of enzymes such as superoxide dismutase (SOD) [19], catalase [20], phosphatase [21], peroxidase [22] etc. The non-stoichiometric ratio of $\text{Ce}^{3+}/\text{Ce}^{4+}$ on the surface determines the catalytic characteristics of cerium oxide nanoparticles and strongly depends on the synthesis methods [23], ion microenvironment [24] and pH [25]. CeO_2 NPs can easily change and adjust its electronic configuration by reacting directly to the microenvironment, which allows it to exhibit antioxidant or prooxidant activity. CeO_2 NPs is considered as a promising nanomaterial for use in oncotherapy of various types of tumors [26]. The use of CeO_2 NPs in cancer treatment can be considered from the side of 4 main concepts: (1) Selective protection of normal cells from oxidative stress at physiological pH (antioxidant activity) or induction of oxidative stress in cancer cells by generation of ROS in a slightly acidic microenvironment of tumor tissue (prooxidant activity) [27]. (2) Generation of molecular oxygen to enhance oxygenation of hypoxified tumor sites and sensitization of tumor cells for photodynamic, photothermal and radiation therapy [28,29]. (3) Increased destruction of cancer cells through synergistic combination with other anti-cancer agents that are already used in the clinic [30,31]. (4) Using as a nanocarrier to improve the delivery of anti-cancer drugs [32,33]. Thus, cerium-containing nanoparticles can be considered as promising antitumor agents and radiosensitizers. It is worth noting that cerium oxide nanoparticles can be easily additionally functionalized in order to give them multimodality. In particular, we have previously shown that the doping of CeO_2 NPs with gadolinium ions gives them the properties of MRI contrast. Such nanoparticles have selective cytotoxic activity against MCF-7 tumor cells [34]. Controlled absorption of calcein onto the surface of a CeO_2 NPs ensures the creation of a ROS-sensitive theranostic sensor, which allows for accurate visualization of the inactivation zone of intracellular ROS [35]. Cerium-containing nanoparticles are also used as luminescent nanosensors for analytical chemistry [36]. Given the unique biological activity and high degree of biocompatibility of cerium-containing nanomaterials, it is safe to talk about the prospects for their clinical implementation in the near future.

Cellular spheroids are a unique experimental model that allows to perform primary screening of potential antitumor agents and new promising compounds, including nanoformulations. The structure of the cellular spheroid makes it possible to simulate the conditions of the tumor cellular microenvironment as accurately as possible [37]. The spheroid has a structure close to the structure of the tumor node *in vivo*, modeling all zones: the proliferation zone, the hypoxia zone and the necrosis zone [38]. Spheroids are considered as a relevant model for testing the cytotoxicity [39] and biological activity of nanoparticles [40]. This experimental model makes it possible to conduct accelerated screening of potential radiosensitizers, successfully screening out ineffective and toxic compounds, which further ensures more effective preparation and conduct of preclinical studies on animal tumor models.

Here we demonstrate the radiosensitizing properties of citrate-stabilized CeO_2 nanoparticles in a 3D tumor spheroid model under X-ray irradiation, discussing possible molecular mechanisms of radiation-induced cytotoxic action of cerium-containing nanoparticles.

2. Materials and Methods

2.1. Synthesis and Characterization of CeO₂ NPs

CeO₂ NPs were obtained by hydrothermal method. 5.88 g of Sodium citrate tribasic dihydrate was dissolved in 50 ml of water (solution 1). Separately, 3.73 g of cerium heptahydrate chloride was dissolved in 50 ml of water (solution 2). Solution 1 and solution 2 were mixed with active stirring on a magnetic stirrer. Then, without stopping stirring, 1.5 ml of 25% ammonia solution was added to the resulting solution. Further, the mixing was continued for 24 hours. After obtaining a dark brown sol of nanoparticles, it was dried at 80 °C in a dry oven. The resulting powder formed a colloidal sol well when dissolved in water. The resulting CeO₂ sol was analyzed by a wide range of methods. CeO₂ NPs size and shape were determined using transmission electron microscopy on a Zeiss Leo912 AB Omega electron microscope at an accelerating voltage of 100 kV. A diffraction pattern reflecting the diffraction of electrons on nanoparticles in the sample was also obtained. The concentration of the CeO₂ NPs sols was determined gravimetrically. A DS-11FX spectrophotometer (Denovix, USA) was used to measure CeO₂ NPs sol absorbance in the UV-visible range. Measurements were carried out in the wavelength range from 220 to 600 nm in 0.1 nm increments. The size distribution of CeO₂ NPs in deionized water, Hank's Balanced Salt Solution (HBSS) (PanEco, Russia), cell culture medium DMEM/F-12 (1:1) (PanEco, Russia), containing 2 mM of L-glutamine, 100 U/mL of penicillin and 100 µg/mL of streptomycin (PanEco, Russia) with and without 10% of Fetal Bovine Serum (FBS) (HyClone, USA) were measured by Dynamic Light Scattering (DLS) at 25 °C using a BeNano 90 Zeta particle analyzer (BetterSize, China).

2.2. Detection of ROS Generation after X-Ray Irradiation of CeO₂ NPs

2',7'-Dichlorodihydrofluorescein diacetate (H₂DCF-DA) dye was used as the probe for ROS level detection under X-ray irradiation. For this aim, 2 mM solution of H₂DCF-DA in PBS was deacetylated to form H₂DCF in 10 mM of NaOH water solution for 30 min at 4 °C. The H₂DCF solution was stored on the ice and in the dark for further use. Oxidation of this dye by ROS leads to formation of fluorescent compound, 2',7'-dichlorofluorescein (DCF). CeO₂ NPs at 0-100 µg/mL concentration were mixed with cold solution of 8 µM H₂DCF in 0.1 M Tris-HCl. Then, samples were placed on the ice and irradiated by X-ray irradiation for 1 min at doses of 2 and 4 Gy. Then, CeO₂ NPs were eliminated by centrifugation at 20 000 g for 20 min at 4 °C using D1524R microcentrifuge with refrigerator (DLab Scientific, China). Fluorescence intensity of the solutions was measured on a INNO S plate reader (LTEK, Korea) using FITC filter (Ex=467-498 nm, Em=513-556 nm). Then, four experimental groups were analyzed to measure the production of ROS: (1) non-treated H₂DCF solution, (2) non-irradiated H₂DCF solution treated with CeO₂ NPs NPs, (3) H₂DCF solution irradiated under X-ray, and (4) H₂DCF solution both treated with CeO₂ NPs NPs and irradiated under X-ray. ROS generation effectivity was determined as:

$$ROS = \frac{S_{X-ray\ with\ NPs} - S_{NPs}}{S_{X-ray} - S_{no\ treatment}},$$

where S_x – mean fluorescence signal from the corresponding DCF solutions.

2.3. Cell Culture

4T1 human breast cancer cell line was obtained from European Collection of Authenticated Cell Cultures (ECACC). 4T1 cells were cultivated in DMEM/F-12 medium (1:1) (PanEco, Russia), containing 2 mM of L-glutamine, 100 U/mL of penicillin and 100 µg/mL of streptomycin (PanEco, Russia) and 10% of Fetal Bovine Serum (FBS) (HyClone, USA). The cells were cultivated in incubator at 37 °C in humidified atmosphere containing 5% CO₂. As the cells grew and reached subconfluent state, they were treated with a 0.25% trypsin-EDTA (PanEco, Russia) solution and passed into new T25 or T75 cell culture flasks (SPL Life Sciences, Korea) at a ratio of 1:4.

2.4. Formation of Spheroids

3D tumor spheroids were formed from 4T1 cells by the hanging drop method for 72 hours. The seeding density of cells was 1000 cells per 1 spheroid. The diameter of the formed spheroids was about 350-370 μm .

2.5. Irradiation of Spheroids

X-ray irradiation of spheroids was carried out at the RUT-15 device (ITEB RAS, Pushchino) with a dose rate of 1 Gy/min. The spheroids were placed in a 2 ml tube in HBSS for the period of irradiation.

2.6. Analysis of Cell Migration from Irradiated Spheroid

After X-ray irradiation, the spheroids were transferred (1 piece per well) into the wells of a 24-well plate (SPL Life Sciences, Korea), photographed on a Clone Select Imager (Molecular devices, USA) at $\times 6$ magnification. The migration of cells from the spheroid was observed for 3 days. The analysis of the area of the cell migration zone from the spheroid was carried out using the ImageJ software.

2.7. Colony Assay

After X-ray irradiation, the spheroids were broken down into suspension of individual cells by incubation in a solution of 0.25% trypsin-EDTA for 1 hour. Then cells were seeded in 6-well plates (SPL Life Sciences, Korea) at concentration of 1000 cells per well in DMEM/F12 with 10% FBS culture medium and cultured in incubator at 37°C in humidified atmosphere containing 5% CO₂. Colony formation was monitored daily using a CloneSelect Imager plate reader (Molecular Devices, USA). After the completion of colony formation in the control group (9 days), the cells were washed 3 times with phosphate buffered saline and fixed in 4% paraformaldehyde solution (Sigma Aldrich, USA), and then stained with 0.1% crystal violet (PanEko, Russia). Cell aggregations of more than 50 cells were considered as 1 colony. Colonies were counted manually using a magnifying glass.

2.8. Statistical Analysis

The experimental data are presented as the Mean $\pm$ Standard Deviation (SD). The statistical significance of differences between the values in experimental groups was determined using the Welch's t-test in GraphPad Prism. Differences were considered statistically significant at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.005$ (***), $p < 0.001$ (****).

3. Results

Physico-Chemical Properties of CeO₂ NPs

CeO₂ NPs were synthesized in a one-step manner as shown in Figure 1a. The analysis of transmission electron microscopy data confirms the ultra-small size of nanoparticles about 1-2 nm, which are partially aggregated into larger nanoparticles (apparently, this is due to sample preparation) (Figure 1b). The proposed synthesis method makes it possible to obtain CeO₂ NPs stable colloid sol by dissolution of dried powder in water without the formation of aggregates or sedimentation as shown in Figure 1c. The UV spectrum corresponds to the composition of cerium oxide nanoparticles (Figure 1d). It is shown that the hydrodynamic size distribution of CeO₂ NPs in deionized water was about 1-2 nm (Figure 1e). However, hydrodynamic size distributions of CeO₂ NPs in various physiological media increases almost tenfold: size=110.6 $\pm$ 21 nm and PDI=0.211 in HBSS and size=83.14 $\pm$ 14.28 nm and PDI=0.296 in DMEM/F-12 culture medium without FBS. At the same time, the use of a DMEM/F-12 containing 10% FBS ensures maintaining CeO₂ NPs size at the level of 4.85 $\pm$ 1.25 nm (PDI: 0.580), which indicates the effective opsonization of CeO₂ nanoparticles and the important role of the protein corona in their stabilization in physiological environment.

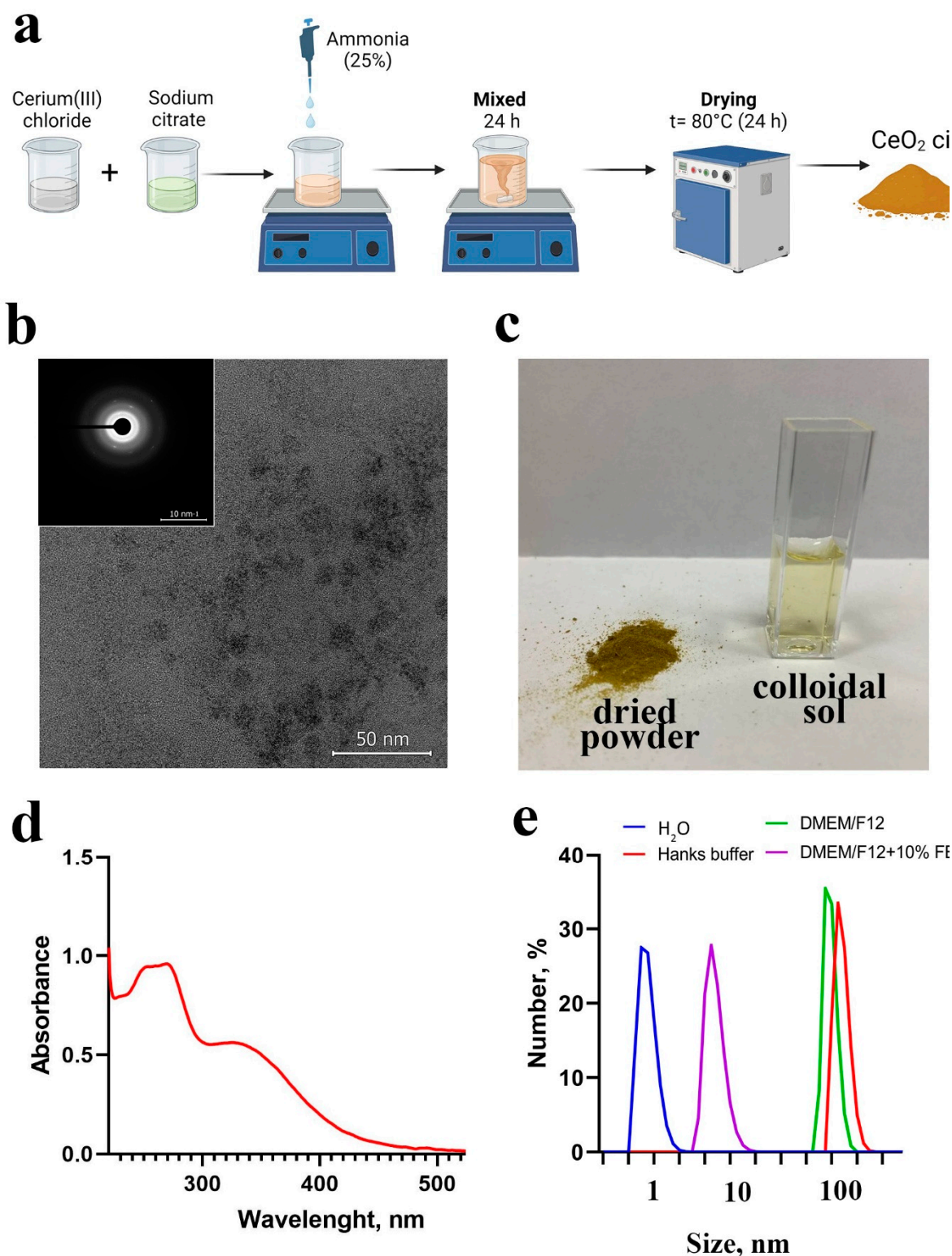


Figure 1. a) Schematic representation of the synthesis process b) TEM images of CeO_2 NPs and electron diffraction pattern of CeO_2 NPs c) Visual appearance of dried CeO_2 NPs and the corresponding colloidal solution; d) UV spectra of CeO_2 NPs; e) Size distribution of CeO_2 NPs by dynamic light scattering (DLS) in deionized water, HBSS and cell culture medium DMEM/F-12 with and without 10% FBS.

It is well known that cerium-containing nanoparticles exhibit pH-dependent redox activity [41]. CeO_2 NPs are characterized by a combination of fully oxidized Ce^{4+} and fully reduced Ce^{3+} . The ratio of $\text{Ce}^{3+}/\text{Ce}^{4+}$ plays an important role in determining the catalytic properties of these nanoparticles and

determines their action in a pro- or antioxidant ways. To identify the radiocatalytic activity of CeO_2 NPs, we analyzed the generation of ROS in solutions containing different concentrations of nanoparticles, which were irradiated under X-ray at doses of 2 and 4 Gy (Figure 2). It has been shown that at a pH of 6.5, CeO_2 NPs increase the level of ROS in solution, thereby confirming a significant level of their prooxidant activity. On the contrary, under neutral conditions (pH 7.4), CeO_2 NPs do not induce an increase in ROS levels. Thus, it can be concluded that the synthesized nanoparticles have pH-dependent radiation-induced redox activity.

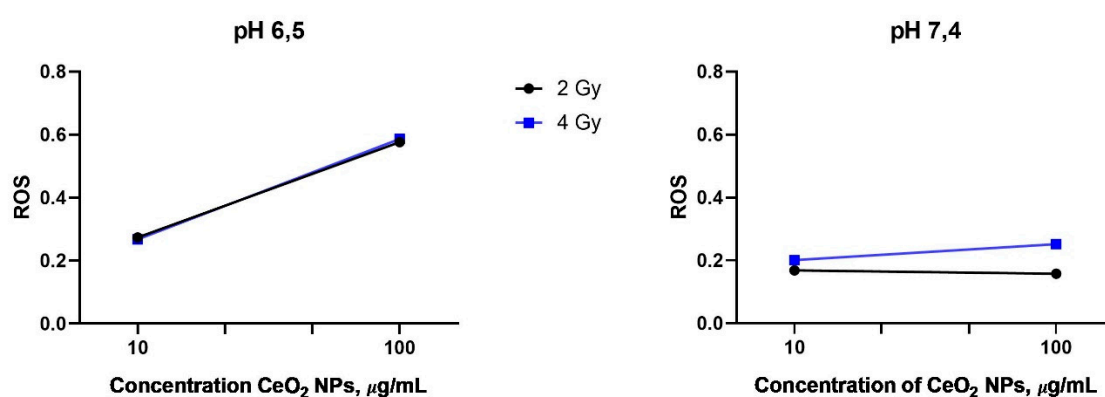


Figure 2. ROS generation by CeO_2 NPs in solutions with slightly acidic (6.5) and physiological (7.4) pH under X-ray irradiation at doses of 2 and 4 Gy.

Testing of cytotoxicity and biological activity of various nanoformulations on 3D cellular spheroids is carried out by cocubation of the spheroid at various time intervals with nanoparticles at different concentrations. Fluorescent labels are often used to visualize the penetration of nanoparticles into various layers to analyze their accumulation and localization [42]. At the same time, the diffusion of nanoparticles deep into the structure of the spheroid can be severely limited and largely depends on the size, shape, charge and stabilizer of the nanoparticles [43]. Here we have proposed a different technique for loading CeO_2 NPs into a cellular spheroid, which consisted in the initial accumulation of nanoparticles in a 2D monolayer of cells and subsequent formation of a spheroid from these cells (Figure 3). This approach makes it possible to ensure an even distribution of nanoparticles in all structures of the spheroid and confidently say that the identified biological effects during irradiation of spheroids are ensured by influencing all structural layers of the spheroid.

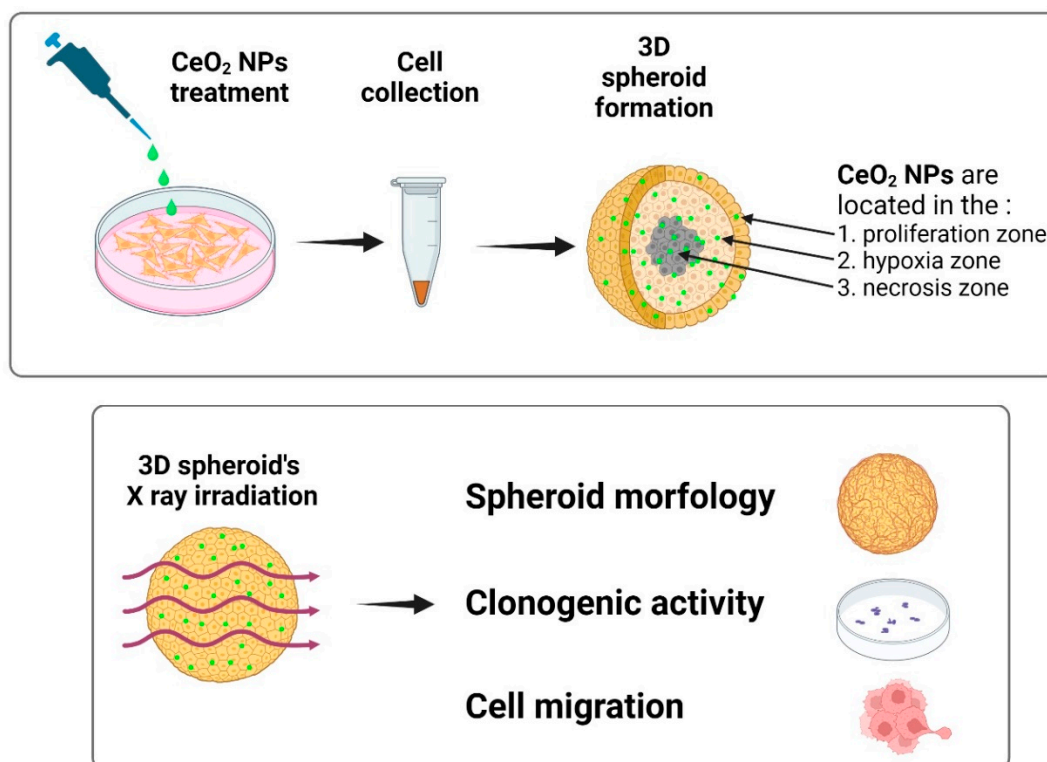


Figure 3. Principal experiment scheme: loading of CeO₂ NPs into tumor cells followed by the formation of 3D spheroids from the cells. Subsequently, the obtained spheroids are irradiated under X-ray and then analyzed for their morphology, cell migration and clonogenic activity.

We assume that loading of tumor cells with CeO₂ NPs leads to uniform distribution of the nanoparticles in all cells in monolayer. Consequently, CeO₂ NPs will be evenly distributed over all layers of the 3D tumor spheroid formed from the loaded cells. Thus, as the spheroid grows, CeO₂ NPs appear in all structures of the spheroid: in the necrosis zone, in the hypoxia zone and in the proliferation zone. Considering the sensitivity of cerium oxide nanoparticles to the microenvironment, we assume that the redox activity of CeO₂ NPs will provide various biological effects depending on their localization. To confirm this assumption, an analysis of cell migration activity was carried out by quantifying the area of cell spreading after transfer of the spheroid to an adhesive plastic (Figure 4a). We found that CeO₂ NPs without X-ray irradiation reduce the migration activity of tumor cells compared to the untreated control (Figure 4b). Without CeO₂ NPs, irradiation at a dose of 6 Gy led to a twofold decrease in the spreading zone. However, there is no statistically significant difference between this irradiated group and irradiated group with CeO₂ NPs. Meanwhile, we observed a tendency to decrease the spreading area in irradiated cells in the presence of the CeO₂ NPs.

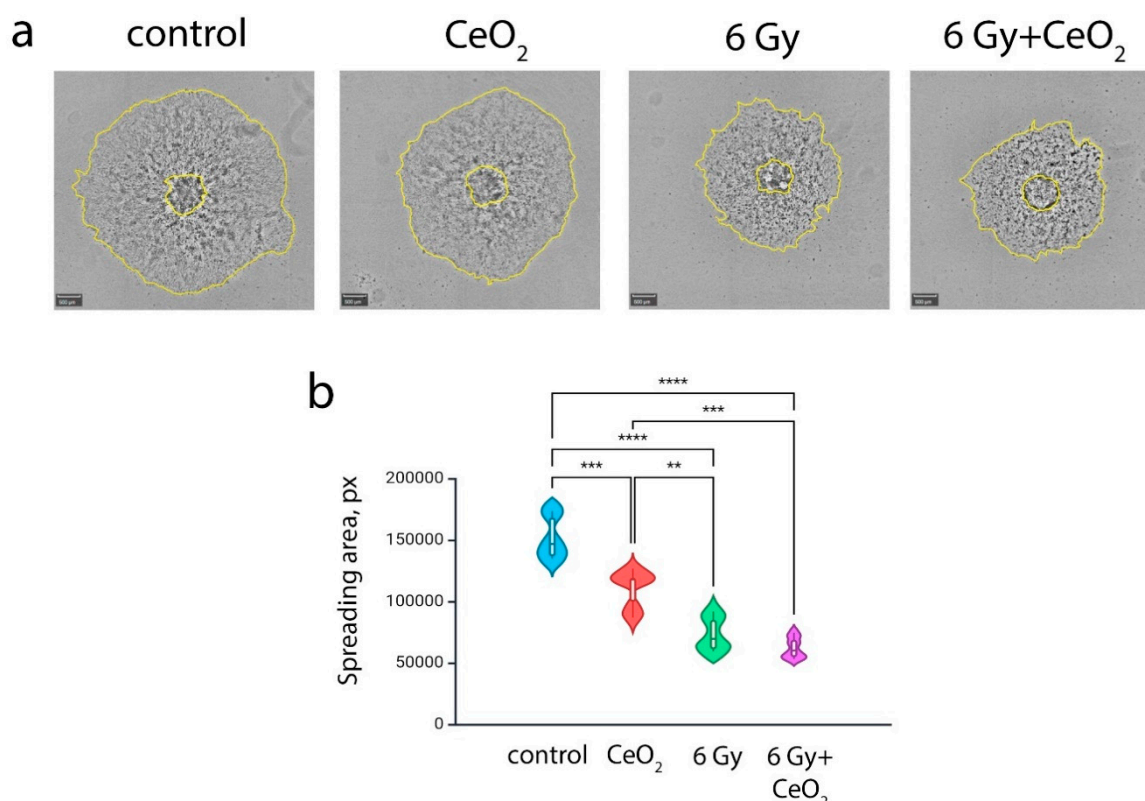


Figure 4. Migration activity of cells from a 3D cellular spheroid 5 days after irradiation at a dose of 6 Gy in the presence CeO₂ NPs (0.172 µg/mL). The data is presented as the violin plot with Mean ± Standard Deviation (SD). The statistical significance of differences between the values in experimental groups was determined using the Welch's t-test. Differences were considered statistically significant at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.005$ (***), $p < 0.001$ (****).

The analysis of clonogenic activity is one of the «gold standards» for determining the proliferative activity of cells after exposure to toxic agents or different types of radiation [44]. We analyzed the clonogenic activity of cells obtained from irradiated spheroids (Figure 5). A decrease in the clonogenic activity of cells for the group with nanoparticles (0.172 µg/mL) was revealed by 25% relative to the untreated control. Irradiation of spheroids at a dose of 6 Gy resulted in a 90-95% fold decrease in clonogenic activity. The combined effect of CeO₂ NPs and X-ray irradiation almost completely suppressed the clonogenic activity of cells. The inhibitory effect of CeO₂ NPs may be related with their redox activity, which is realized both in the necrosis zone and in the hypoxia zone due to the used spheroid formation scheme.

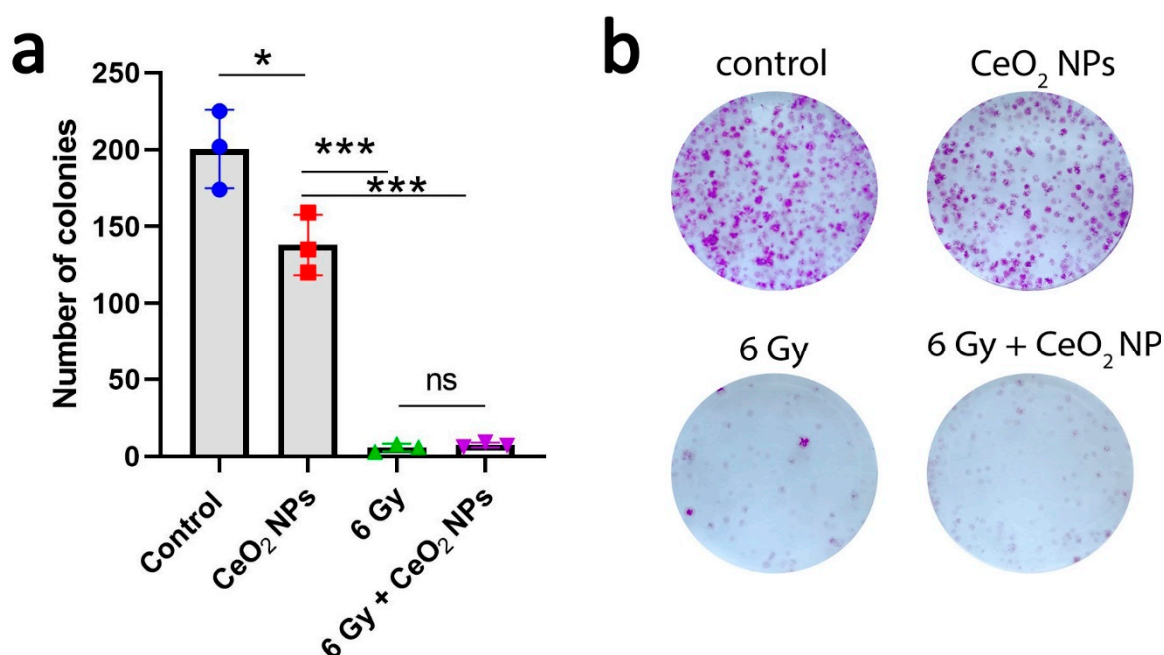


Figure 5. Clonogenic assay after X-ray irradiation of 3D spheroids in the presence of CeO₂ NPs: quantitative assessment (a) and photographs of colonies (b). The data is presented as the Mean $\pm$ Standard Deviation (SD) with experimental points. The statistical significance of differences between the values in experimental groups was determined using the Welch's t-test. Differences were considered statistically significant at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.005$ (***), $p < 0.001$ (****).

Discussion

Cerium-containing nanoparticles are considered as a promising antitumor agent with selective cytotoxic activity against tumor cells and a high degree of biocompatibility with respect to normal cells. A key feature of cerium-containing nanomaterials, that allows them to be used in anticancer therapy, is their sensitivity to pH of the microenvironment, which makes it possible to damage tumor cells while maintaining a safe environment for normal cells under irradiation conditions. The chemical mechanism of controlled catalytic activity of cerium oxide nanoparticles is based on the ability of cerium ions located in the near-surface layer to easily and reversibly change their degree of oxidation from Ce³⁺ to Ce⁴⁺ and back. Using the reaction of CeO₂ nanoparticle with hydrogen peroxide as an example, this phenomenon can be explained as follows: under neutral conditions, a redox reaction is initiated between the Ce⁴⁺ and the hydrogen peroxide molecule, adsorbed on the surface of nanoparticle. Then products of the hydrogen peroxide decomposition are released, exposing the surface of the nanoparticle again, and Ce³⁺ is oxidized to Ce⁴⁺ for the next antioxidant catalytic cycle. Under acidic conditions, an excess of protons can inhibit the transition from Ce⁴⁺ to Ce³⁺, which, in turn, disrupts the reactivity of catalytic centers and blocks the antioxidant cycles of the nanoparticle [41]. Thus, the microenvironment of cerium oxide can determine the direction of its bioactivity. The cellular spheroid, like the tumor node *in vivo*, has different zones, which differ not only in the level of ATP, the degree of oxygenation, but also in the pH level. Considering that all approved clinical nanoradiosensitizers are administered to patients intratumorally, a very important aspect determining the effectiveness of radiotherapy is the control of their localization and accumulation directly in the tumor node. In this work, it is shown that the preloading of cerium oxide nanoparticles into a spheroid ensures their uniform distribution in all zones of the spheroid, which ensures prooxidant activity in the zone of necrosis and hypoxia. We conducted a study of radiation-induced ROS generation in various microenvironment conditions (pH 6.5 and 7.4), which showed a prooxidant effect in slightly acidic conditions. Despite the fact that we have not experimentally confirmed the generation of ROS directly in spheroid cells, we can assume the probability of

prooxidant activity of CeO₂ NPs in acidified spheroid structures, which leads to a decrease in the migration activity of cells from irradiated spheroids. We confirmed that the proposed approach for preloading cells with nanoparticles ensures their effective accumulation in all zones of the spheroid, which provides a new opportunity to study the radiosensitizing effect of nanoparticles *in vivo* tumor models.

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